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The role of inflammatory pathways in neuroblastoma tumorigenesis

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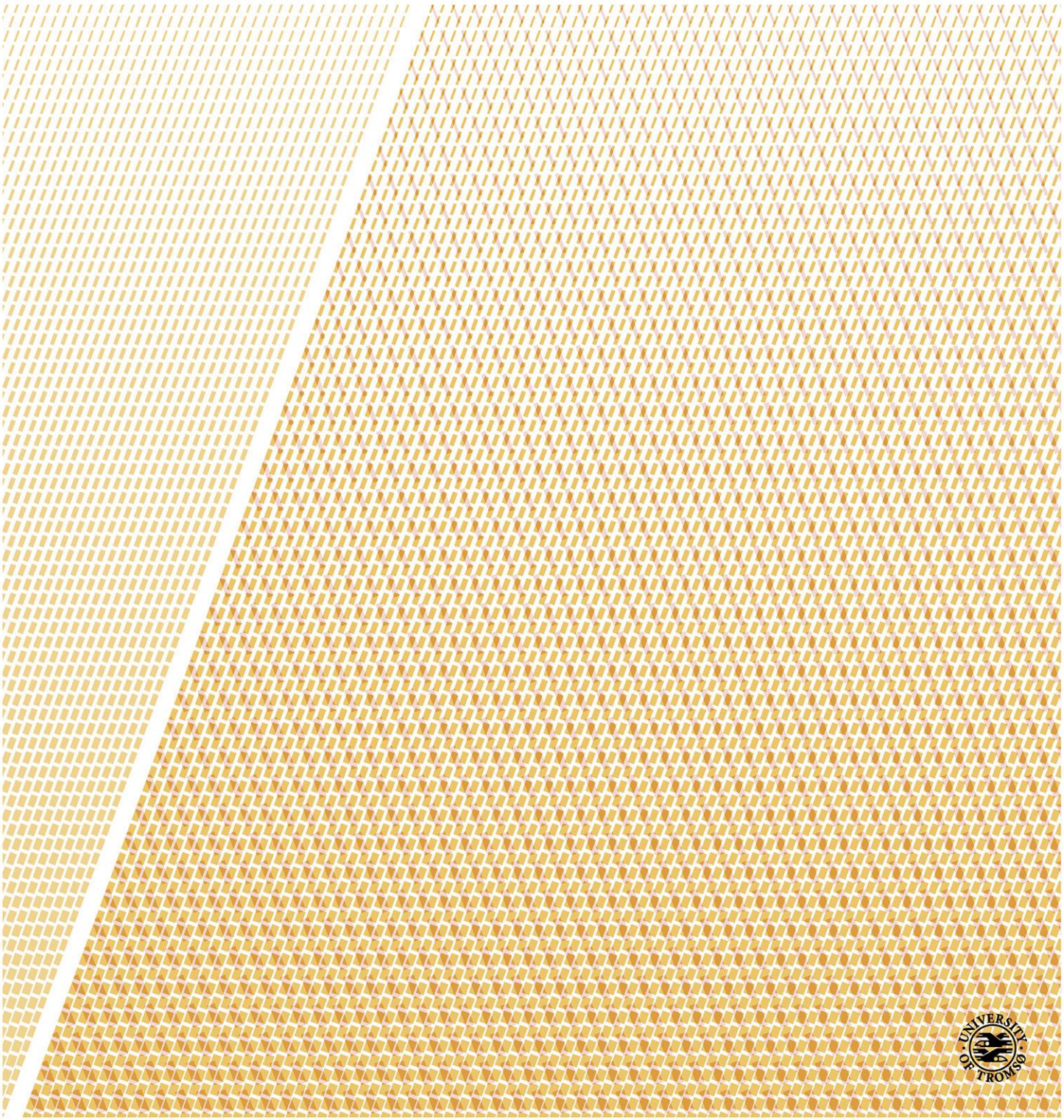

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The role of formyl peptide receptor 1 (FPR1) in neuroblastoma tumorigenesis.

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CMKLR1/chemerin axis in the neuroblastoma microenvironment.

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Cristina Ionica Øie[¶], **Igor Snapkov**[¶], Kjetil Elvevold, Baldur Sveinbjørnsson & Bård Smedsrød

FITC Conjugation Markedly Enhances Hepatic Clearance of N-Formyl Peptides.

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[¶] These authors contributed equally to this work

2. List of abbreviations

ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
CCRL2	C-C chemokine receptor-like 2
CD	Cluster of differentiation
CLRs	C-type Lectin Receptors
CMKLR1	Chemokine-like receptor 1
COX	Cyclooxygenase
CRT	calreticulin
CT	Computed tomography
CyH	Cyclosporin H
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ERK	Extracellular signal–regulated kinases
FITC	Fluorescein isothiocyanate
fMLP	N-formylmethionine-leucyl-phenylalanine
fNLPNTL	N-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tryrosyl-lysine
FPR1	Formyl-peptide receptor 1
GPCRs	G-protein coupled receptors
GPR1	G protein-coupled receptor 1
HIF-1α	Hypoxia-inducible factor 1α
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box 1 protein
HSP	Heat shock proteins
iDCs	Immature dendritic cells
IFN-γ	Interferon gamma
IL	Interleukin
JNK	C-Jun N-terminal protein kinases
kDa	Kilodalton
LSECs	Liver sinusoidal endothelial cells
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
MIBG	Metaiodobenzylguanidine scan

MRI	Magnetic resonance imaging
mTOR	Mammalian (mechanistic) target of rapamycin
MYCN	N-myc proto-oncogene protein
NB	Neuroblastoma
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLRs	NOD-like receptors
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PRRs	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
shRNA	Short (small) hairpin RNA
siRNA	Small interfering RNA
STAT3	Signal transducer and activator of transcription 3
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor beta
TLRs	Toll-like Receptors
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WB	Western blot
α-NETA	2-(α-naphthoyl) ethyltrimethylammonium iodide

3. Introduction

3.1 Cancer and inflammation

The theory about the interplay between inflammation and cancer was suggested by Rudolf Virchow at the end of the 19th century when he observed the presence of immune cells in tumor samples. Virchow supposed that persistent inflammation induces a malignant transformation of the tissues [1]. More than a century later, in 2011, a tumor-promoting inflammation was included as an enabling characteristic in the monumental work of Hanahan and Weinberg "Hallmarks of Cancer: The Next Generation" [2]. Inflammation contributes to tumor angiogenesis, invasiveness and metastatic activity by augmentation of the proliferation and inhibition of death signaling in cancer cells (Figure 1).

In short, the inflammatory process occurs in a tissue as a response to an injury, and its main role is to heal the tissue. Tissue-residing immune cells expressing specific pattern recognition receptors (PRRs), which recognize antigens or danger signals released by damaged cells, produce inflammatory mediators that form a focus of inflammation and induce the migration of various leukocytes from the vessels to the site of the damage. During the sequence of processes, immune cells in cooperation with extracellular matrix forming cells (fibroblasts) eliminate the source of danger signals and resolve the inflammation. In the case of a "normal inflammation", associated with wound healing, these processes are limited and securely controlled by various growth factors including interleukins, TNF-α and TGF-β [3-6]. A loss of regulation in inflammation limiting factors leads to the persistence of focus, and may result in neoplastic formation

Generally speaking, the most potent drivers of the chronic inflammatory process associated with cancer are the sustained presence of inflammatory immune cells and the subversion of inflammatory mediators' production.

The typical cells promoting cancer-related inflammation are tumor-associated macrophages (TAMs) [7, 8]. Depending on the type of polarization, TAMs may play different roles in inflammation. M1 (classical) phenotype macrophages eliminate antigens and cancer cells by the production of IL-12, IL-23, TNF-α and the recruitment of cytotoxic T cells and NK cells. M2 (alternative) phenotype macrophages produce IL-6, IL-10 and TGF-β, thereby resulting in an immune-suppressed response. Additionally, TAMs can produce angiogenic growth factors that contribute to tumor progression [9-15].

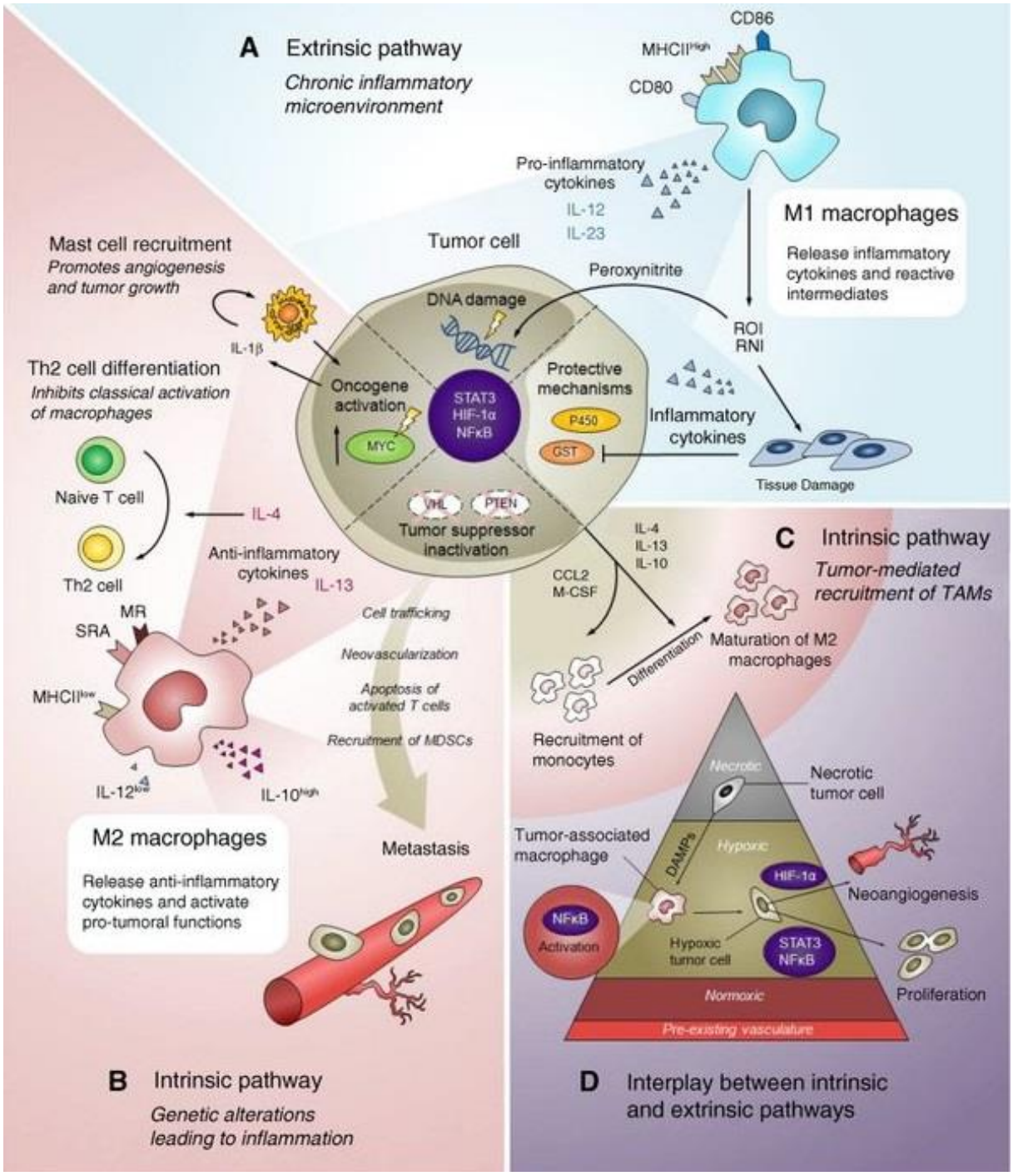

Figure 1. Cancer-related inflammation. There are two pathways driving cancer-related inflammation: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia including the activation of oncogenes, chromosomal aberrations, and the inactivation of tumor-suppressor genes. The extrinsic pathway is driven by inflammatory conditions that predispose to cancer. Together these pathways activate NF-κB, STAT3, and HIF-1α in tumour cells. These transcription factors initiate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of COX-2. These factors recruit various leukocytes. All these events lead to an augmented production of inflammatory mediators and improved generation of cancer microenvironment.

Illustration used with permission, copyright 2016 by the American Thoracic Society (ATS) [16].

Tumor-promoting immune cells are not limited by TAMs, as recent data show that dendritic cells, neutrophils, mast cells and T cells may also contribute to tumor development, releasing chemokines, immunosuppressive cytokines, pro-angiogenic components, ROS and proteases [17-24].

Additional evidence of the significance of inflammation in cancer progression is clearly demonstrated by studies which show that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces colorectal [25], lung [26, 27], breast [28, 29], esophagus [30] and stomach [31] cancer risks. The chemopreventive properties of NSAIDs can be explained by their ability to inhibit cyclooxygenases (COX-1 and -2), while COX-2 stimulates the production of prostaglandins from arachidonic acid [32]. In turn, prostaglandins promote tumor development by apoptosis inhibition, immune suppression, the stimulation of cell proliferation and the activation of pro-survival signaling pathways [33-35].

3.2.1 Pattern recognition receptors and danger signals

The initial discovery of the immune system originated in the BC era. However, the underlying intricate mechanisms of the immune response remained unclear up until the end of the 20th century.

In 1989, Charles A. Janeway, Jr. proposed the pattern recognition concept that revolutionized the field of immunology [36]. Janeway postulated that the initiation of the immune response relies on the set of highly conserved receptors called pattern recognition receptors (PRRs) which recognize specific ligands of microbial origin (e.g. components of the bacterial cell wall, bacterial and viral nucleic acids, etc.) called pathogen-associated molecular patterns (PAMPs). Janeway also emphasized that besides the activation of the innate immunity, PRR-PAMP interactions trigger the adaptive response.

First PRR was described in the middle of the 1990s in a Drosophila model. Lemaitre and co-authors showed that flies with the mutant Toll transmembrane protein lack the antifungal immune response [37]. Further research revealed a number of additional Toll-related proteins and in 1997 Medzhitov and co-authors characterized a human homologue of the Drosophila Toll protein [38]. At present, the PRR superfamily consists of a wide variety of receptors with diverse chemical structures and signaling mechanisms. Among different species, approximately 500 PRRs are currently known [39]. In order to pursue the permanent immune control, PRRs are extensively distributed throughout the body. PRRs can reside in cell membranes (Toll-like receptors, C-type lectin receptors) and cytoplasm (RIG-I-like receptors, NOD-like receptors), or they can be secreted (complement system proteins, pentraxins) (Figure 2). An interaction between PRR and ligands results in the initiation of different

signaling pathways, including NF-κB [40, 41], JNKs [42] and p38 [43], thus leading to the inflammasome formation and production of pro-inflammatory cytokines and interferons.

For a long time, the dogma that the immune system distinguishes between the "self" and "non-self" has dominated the field, though in 1994, Polly Matzinger outlined a theory which seemed to contradict all existing immunology principles [45]. She suggested that the immune response is promoted by so-called "danger signals" (later termed damage-associated molecular patterns - DAMPs), i.e. host-derived substances released by the cells in response to stress, damage, etc. Despite

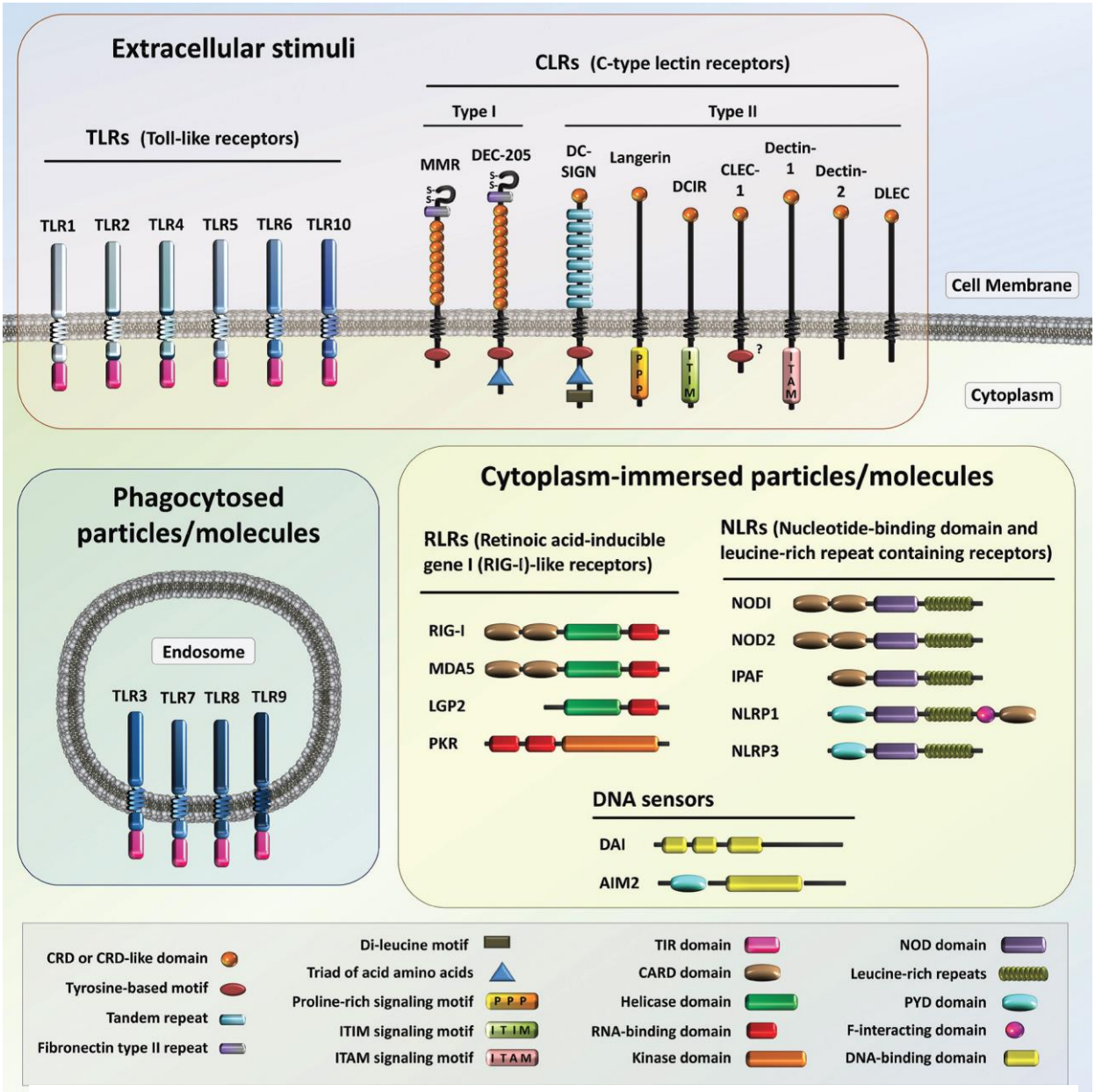

Figure 2. Localization of main classes of PRRs. PRRs can be localized on the cell surface, in the cytoplasm and in the membrane of endosomes.

Illustration used with permission, copyright 2011 by Brazilian Journal of Medical and Biological Research [44].

the controversy, this theory could explain phenomena that previous thoughts have failed to understand.

DAMPs are structurally diverse immunostimulatory molecules that can be released from any compartment of the cell following tissue damage, trauma, inflammation and neoplastic changes. There are several ways to classify DAMPs:

1. Release mechanism
    a. Actively released (calreticulin (CRT), adenosine triphosphate (ATP)) [46]
    b. Passively released (high-mobility group box 1 protein (HMGB1), s100 proteins, etc.) [47]
2. Source
    a. Intracellular DAMPs (mitochondrial formylated peptides, including fMLP; nuclear HMGB1; s100 proteins) [47, 48]
    b. Exosomal DAMPs (heat shock proteins (HSP)) [49]
    c. Extracellular matrix DAMPs (hyaluronic acid) [50]
    d. Plasma components (complement proteins C3a, C4a, C5a) [51]
3. Chemical structure
    a. Proteins (HMGB1, HSP, etc.) [50]
    b. Non-proteins (free nucleic acids, ATP, heparan sulfate, etc.) [50, 52, 53]

Released extracellularly, DAMPs bind to PRRs and exhibit their properties including the stimulation and regulation of dendritic cells (DCs) maturation, which lead to the induction of $CD8^+$ T-cell response [54].

Over the last few decades, it has become apparent that the PRR concept and danger theory are not mutually exclusive, but instead complementary. PRRs work not only as a primitive bacterial recognition machinery, but can also initiate an immune response activated by both foreign antigens (PAMPs) and host substances (DAMPs).

3.2.2 Formyl peptide receptor 1 (FPR1)

The human formyl peptide receptor 1 (FPR1) is a seven transmembrane domain receptor that belongs to the superfamily of G-protein coupled receptors (GPCRs) (Figure 3) [55]. It is expressed at high levels on phagocytic leukocytes, and mediates cellular chemotaxis [56]. Human FPR was identified in the late 1970s on the surface of neutrophils as a specific receptor for bacterial N-formyl peptide formyl-methionine-leucyl-phenylalanine (fMLP) [57, 58]. It has later been shown that the activation

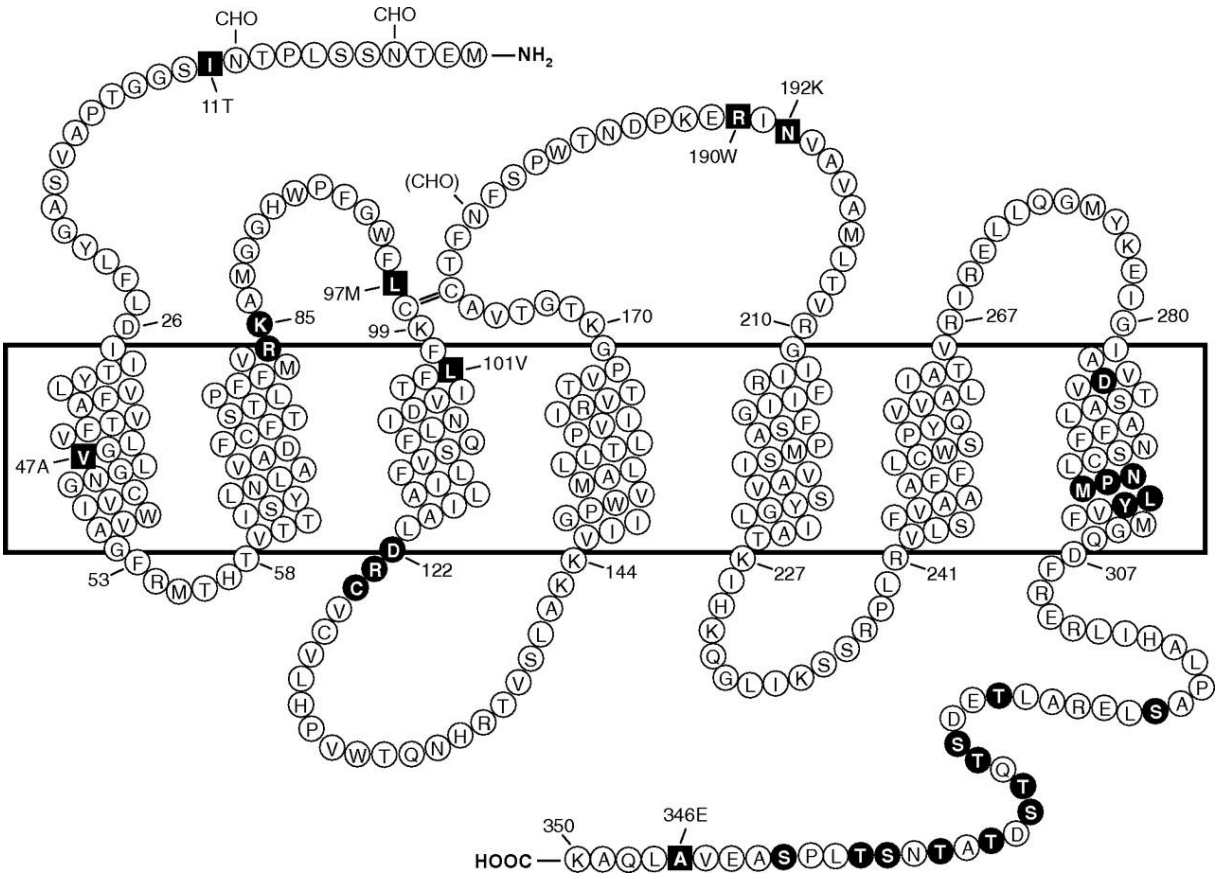

Figure 3. Transmembrane disposition of the human FPR1. One-letter amino acid code is used.

Illustration used with permission, copyright 2009 by The American Society for Pharmacology and Experimental Therapeutics (ASPET) [55].

of FPR1 by picomolar and nanomolar concentrations of fMLP promotes chemotactic cell motility and the mobilization of intracellular Ca^{2+} [59, 60]

Upon binding with an agonist, FPR1 activates heterotrimeric Gi protein, which dissociates into α and βγ subunits, with the subsequent activation of phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K). PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2), resulting in the generation of inositol trisphosphate (IP3), which leads to the release of calcium from endoplasmic reticulum and the activation of protein kinase C isoforms [61-63]. Main intracellular pathways activated by FPR1 are PI3K/Akt, MAPK, STAT3, p38 and Hippo pathways [56, 64-66]. All these intracellular events lead to an increased chemotactic behavior of cells and the development of pro-inflammatory effects. It is known that different types and concentrations of an agonist activate different signaling pathways. As with other GPCRs, FPR1 is subject to homologous desensitization. In the presence of high concentrations of agonists, FPR1 can be internalized and kept in endosomes during long-term agonist exposure, without any signs of degradation [67].

Due to extensive research, the current list of known agonists for FPR1 is significantly broadened. Besides the classic fMLP, it includes:

1. Peptides of bacterial and viral origin: a non-formylated peptide fragment produced by Helicobacter pylori, Hp(2–20); T20, T21 peptides of HIV-1 envelope protein gp41 [68-70]

2. Ligands of endogenous origin: formylated peptides of mitochondria proteins fMMYALF, fMLKLIV and fMFADRW; peptides Ac1-26 and Ac9-25 of Annexin I, a Ca^{2+}-dependent phospholipid binding protein [60, 71]

3. Synthetic peptide library derived agonists: WKYMVm, WKGMVm, WKRMVm [72, 73]

First selective antagonists for FPR1, t-Boc-Met-Leu-Phe (Boc1) and t-Boc-Phe-D-Leu-Phe-DLeu-Phe (Boc2) were synthesized by replacing the formyl group of fMLF with the tertiary butyloxycarbonyl group (t-Boc) [74]. Fungal cyclic peptide Cyclosporin H (CyH), bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) are known FPR1 antagonists [75-78].

Although FPR1 was initially discovered in phagocytic leukocytes, it is widely expressed in other cell types and tissues including dendritic cells, endothelial cells, astrocytes, lens epithelial cells, hepatocytes, Kupffer cells, smooth muscles, etc (Table 1).

Table 1. Distribution of FPR1 in cells and tissues. Modified from [79]. Table used with permission, copyright 2006 by by Elsevier Limited.Therapeutics (ASPET).

Cell types	**Tissues**
Monocytes/macrophages	Thyroid
Neutrophils	Adrenal
Immature DCs	Central nervous system
Endothelial cells	Autonomic nervous system
Platelets	Liver
Hepatocytes	Lung
Astrocytes	Spleen
Microglial cells	Heart
Fibroblasts	Uterus
Vascular smooth muscle cells	Ovary
Lens epithelial cells	Placenta
	Kidney
	Eye
	Stomach
	Bone marrow
	Colon

Accumulating data suggest that FPR1 is involved in a range of diseases and pathologic conditions, such as chronic obstructive pulmonary disease (COPD) [80], inflammatory colitis [81], periodontitis [82, 83] and several cancer types, including gastric cancer [84, 85], astrocytoma [86], melanoma [87], glioblastoma [61, 88], lung alveolar carcinoma [89], hepatocellular carcinoma [90], breast cancer [91] and pancreatic carcinoma [92].

3.3.1 Chemokines

Chemokines are a family of small (8-14 kDa) chemotactic cytokines that induce directed migration of leukocytes. Based on the position of the primary cysteine residues near the N-terminus of these proteins, they are classified into four main subfamilies: C, CC, CXC and CX3C [93]. Chemokines exert their functions by binding to corresponding G-protein coupled receptors on target cells [94]. This binding activates various downstream signals including PTEN/PI3K/Akt, Jak-STAT and MAPK/ERK pathways, hence leading to increased cell motility and proliferation [95-97].

Additionally, chemokines can bind to proteoglycans and glycosaminoglycans. This ability allows them to accumulate on the surface of endothelial cells, or in an extracellular matrix and form a concentration gradient important in migration [98].

According to their functions, chemokines can be classified as inflammatory and homeostatic. The former induce the migration of immune cells to the site of inflammation, while the latter are involved in various stages of organogenesis, stem cell migration and the maintenance of natural leukocyte balance [99, 100].

All immune cells express chemokine receptors, and their migratory potential is dependent on chemokines. In general, C chemokines are necessary for T cell migration to the thymus; CC chemokines promote the chemotaxis of basophils, DCs, macrophages, monocytes, NK cells, T cells, etc.; CXC chemokines attract B- and T- lymphocytes, neutrophils; CX3C chemokines are involved in T cell and NK cell infiltration [93, 101].

Chemokines have been shown to play an essential role in all stages of tumor development. Cancer cells both produce various chemokines and express chemokine receptors. For example, the tumor-derived chemokines CCL2 and CCL22 recruit TAMs (and intensify their M2 polarizaion) and Tregs [102-104]; CXCL12 and CXCL8 upregulate VEGF expression, thus leading to neovascularization [105, 106]; CXCR4 expressed on the cancer cells initiate the migration towards its ligand CXCL12 produced by endothelial cells resulting in epithelial-mesenchymal transition (EMT) and metastasis [107-109].

3.3.2 Chemerin

Chemerin (tazarotene induced gene 2, TIG2; retinoic acid receptor responder 2, RARRES2) is an 18 kDa chemokine-like protein [110]. It is synthesized as a 163-amino acid inactive isoform that is cleaved to 143-amino acid prochemerin and subsequently processed by a variety of extracellular inflammation-associated proteases, which remove a C-terminal hexapeptide to liberate the 157- and 156- amino acid active forms or 154- and 155- inactive forms [111]. First, it was described as a protein involved in the regulation of adipogenesis and overexpressed in psoriatic lesions [112-114].

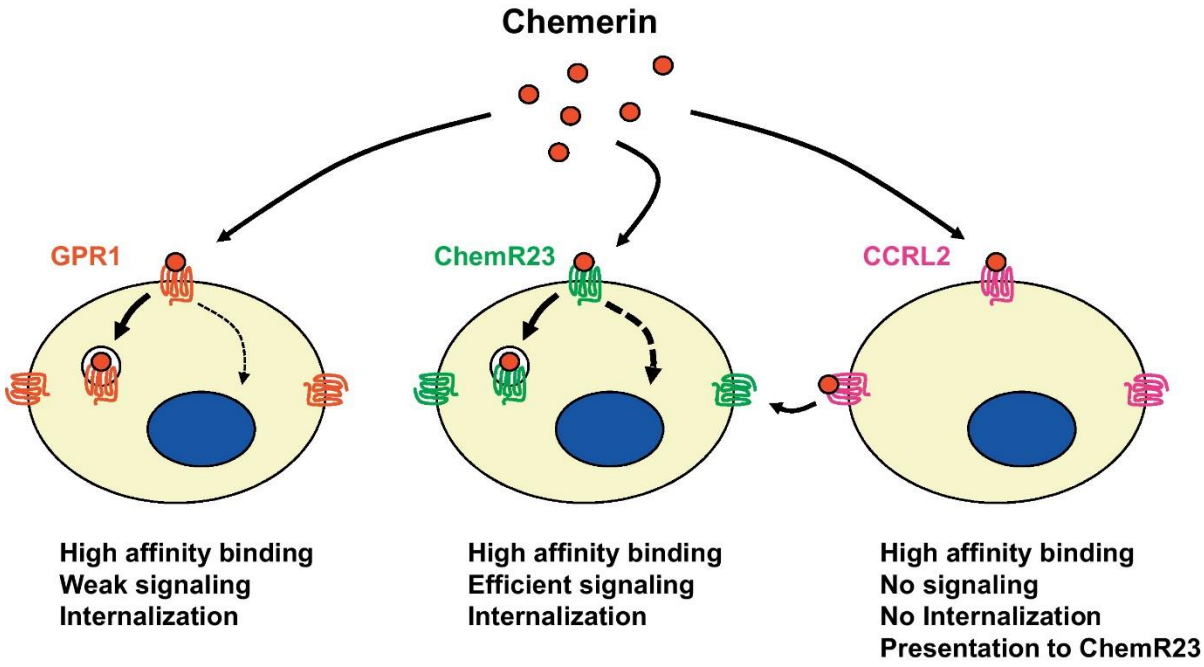

Figure 4. Overview of the three receptors for chemerin.

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Structurally, chemerin is distinct from chemokines, but in binding its receptors, chemerin acts in a chemokine-like manner, inducing leukocyte chemotaxis (particularly macrophages, NK cells, DCs) and the mobilization of intracellular Ca^{2+} [114, 115]. There are three chemerin receptors described so far: chemokine-like receptor 1 (CMKLR1 or chemR23), chemokine CC motif receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1) (Figure 4). While CMKLR1 promotes the main chemoattractive functions of chemerin, the consequences of binding chemerin to GPR1 are not clearly understood. Interestingly, the CCRL2-chemerin complex does not undergo internalization, and it is believed that the main function of CCRL2 is to concentrate chemerin locally in order to present it to chemR23 more abundantly [110, 111, 114, 115]. There is some evidence demonstrating an

association between chemerin-induced inflammation and cancer (prostate cancer, esophageal cancer, gastric cancer, etc.) [116-118].

3.4 Neuroblastoma

Neuroblastoma (NB) is an embryonal tumor of the peripheral nervous system and is the most common and deadly extracranial tumor of the childhood [119]. NB arises from sympathetic ganglia precursor cells developing from the neural crest, a transient embryonal population of cells that gives rise to the central nervous system, melanocytes, neuroendocrine cells, facial cartilage, etc [120].

The median age at diagnosis is 17-18 months [121, 122]. It has been shown that NB is slightly more common among boys than among girls [123] with the global incidence of NB being approxmately 1 case per 8,000 – 10,000 births [122]. In the Norwegian population, the incidence is equal to 0.92 cases per 100,000 (Wesenberg F, Monge O, Nygård JF, Lie HK, Småstuen M. Årsrapport 2009 Norsk Barnekreftregister). Survival in NB depends on the age of diagnosis and the genetic profile of the disease, but the overall survival is approximately 55% [124].

Anatomically, NB can arise at any part of the sympathetic nervous system, but it predominantly occurs in the adrenal medulla. Clinical manifestation of NB depends on the location of primary tumor. The most common sites of NB metastasis are regional lymph nodes, bones and bone marrow and the liver (Figure 5) [125]. The diagnosis is established by histological findings from tumors or metastases biopsies, various imaging techniques (CT, MRI, MIBG) and biochemical analysis of blood and urine (elevated levels of catecholamines and their metabolites are frequently presented) [119].

The causes of NB are not known but two germline mutations in PHOX2B (paired-like homeobox 2b) and ALK (anaplastic lymphoma kinase) genes have been described to be involved in inherited forms of the disease [126, 127]. Familial NB accounts for 1-2% of newly diagnosed cases [128].

The expression of *MYCN*, a member of the *MYC* transcription factors family, is found in ~25% of NB cases. Immediately after its discovery in the 1980s, it has been confirmed to correlate with poor patient survival [129]. *MYCN* amplification is associated with a number of pro-tumorigenic processes that determine the development of high-risk NB, including increased metastatic activity, augmented angiogenesis, the inhibition of apoptosis and the stimulation of cell proliferation and pluripotency [129-131]. To date, *MYCN* amplification is a biomarker used for risk evaluation in NB patients.

Disease prognosis and risk stratification are based on the following characteristics: age at diagnosis, localization of tumor, presence of metastases, histology of tumor, *MYCN* amplification, DNA ploidy, 1p, 11q, and 17q chromosomal aberrations [120, 124, 125].

Treatment depends on the stage of the disease and includes all the existing modalities of modern cancer management (i.e., surgery, radiation therapy, chemotherapy and immunotherapy).

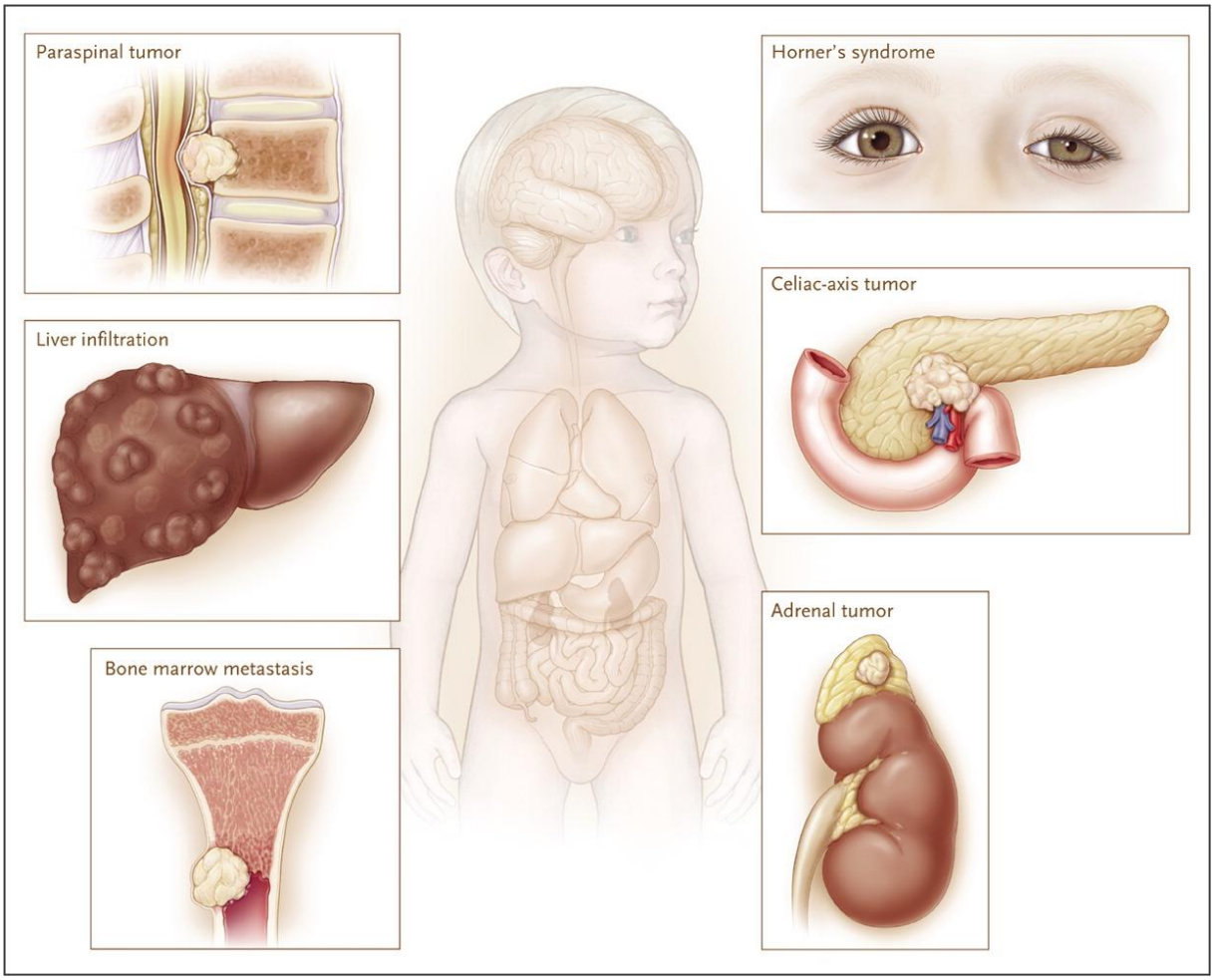

Figure 5. Clinical presentations of neuroblastoma. NB can arise anywhere along the sympathetic nervous system. The most common sites are the adrenal medulla, abdominal sympathetic ganglia, and mediastinum. Primary tumors in the neck or upper chest can cause Horner's syndrome (ptosis, miosis, and anhidrosis). Tumors along the spinal column can cause cord compression, with resulting paralysis. Higher-stage tumors often infiltrate local organ structures, surround critical nerves and vessels such as the celiac axis. NB typically metastasize to regional lymph nodes and to the bone marrow. NB also can metastasize to the liver.

Illustration used with permission, copyright 2010 by Massachusetts Medical Society [125].

The chemokine receptor repertoire of NB is poorly described. There is evidence that CXCR4 expressed by tumor cells promotes non-invasive tumor growth being stimulated by CXCL12 produced within the tumor microenvironment [132]. Airoldi et al. demonstrated that $CXCR5^{+}$ NB cells migrate to the bone marrow in response to CXCL13 synthesis [133]. Additionally, it has been demonstrated that MYCN non-amplified high risk NB tumors can release CCL2, and by doing so attract iNKT cells that kill monocytes involved in tumor elimination processes [134].

3.5 Hepatic clearance of danger signals

The elimination of pathogens and substances produced by damaged cells from the circulation is the essential function of the liver. In order to prevent the unnecessary activation of the entire immune system, the evolution of the liver has developed the capability for the local immune response and the elimination of pathogens. The initiation of innate immune response in the liver is dependent on various subsets of PRRs expressed on the liver-resident immune cells including macrophages (Kupffer cells), hepatic dendritic cells, neutrophils, NK cells and regulatory T cells [135, 136].

Liver sinusoidal endothelial cells (LSECs) are a notable type of liver cells forming the border between the blood and hepatocytes. The main feature of these cells is the formation of fenestrae, multiple pores which provide the opportunity for LSECs to literally filtrate the blood. LSECs are essential cells of liver metabolism. Through fenestrae, they uptake different substances, such as plasma proteins, albumin and lipoproteins [137]. The high endocytic potential of LSECs makes them a very important class of scavenger cells that eliminate the danger signals of both host and non-host origin. The permanent exposure of LSECs to various pathogens determine their involvement in immunity. There are many receptors known to be present on the surface of LSECs (e.g. TLRs, NLRs, RLRs) therefore these cells play a crucial role in antigen-presenting mechanisms and immune response activation [138]. LSECs contain a set of scavenger receptors (SR-A, SR-B, Stabilin-1 (SR-H1) and Stabilin-2 (SR-H2)) which allow these cells to internalize and eliminate a tremendous number of foreign substances from the blood [136, 137]. Importantly, it has been demonstrated that fluorescein-conjugated molecules to a great extent are removed from the blood by scavenger receptors of LSECs [139].

4. Aims of the thesis

The specific aims of this thesis were:

- To study the role of FPR1 in NB development and progression
- To assess the significance of the CMKLR1/chemerin axis in NB tumorigenesis
- To study the function of FPR1 in the liver-mediated clearance of formylated peptides from the circulation
- To reveal the difference in the liver uptake of intravenously injected N-formyl peptide fNLPNTL and its labeled counterpart FITC-fNLPNTL

5. Summary of papers

Paper I

Title: The role of formyl peptide receptor 1 (FPR1) in neuroblastoma tumorigenesis.

In this paper, we studied the role of FPR1, a G protein-coupled receptor with pattern recognition properties in NB tumorigenesis. FPR1 is involved in a broad range of host defense mechanisms and a variety of host-derived agonists of FPR1 have been identified, including formyl peptides released from the disrupted mitochondria of necrotic cells. We demonstrated the expression of FPR1 in seven different neuroblastoma cell lines and in primary tumors. Furthermore, FPR1 is expressed at increased levels in high stage tumors. The addition of the FPR1 agonist N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) to neuroblastoma cells *in vitro* caused an increase of intracellular calcium response and the activation of Akt, p38 and MAPK/ERK signal transduction pathways. All these signal transduction events were abrogated by the use of Cyclosporin H, a specific FPR1 antagonist. To assess the significance of this receptor *in vivo*, a set of neuroblastoma cell clones with different expression levels of FPR1 was generated. Xenograft models showed that cells with an overexpression of the receptor developed tumors significantly faster compared to the control group. Results obtained in this paper suggest that FPR1 may play a significant role in neuroblastoma tumorigenesis, and that the therapeutic intervention of the FPR1 pathway may be an important clinical strategy in neuroblastoma therapy.

Paper II

Title: CMKLR1/chemerin axis in the neuroblastoma microenvironment.

In Paper II, we investigated the impact of chemerin receptor CMKLR1 signaling on NB progression. Chemerin is an adipokine and immunomodulating factor that promotes the chemotaxis of immature DCs, NK cells, macrophages and endothelial cells. Secreted as prochemerin with low activity, it can be C-terminally processed by different proteases expressed by a broad range of cell types and tissues. The resulting isoforms vary in receptor affinity and biological activity and are natural ligands for the G protein-coupled receptors (GPCRs) CMKLR1, GPR1 and CCRL2. To date, the activation of CMKLR1 (Chemokine-like receptor 1) by chemerin and its role in metabolism and metabolic disorders as well as inflammation is best understood.

The screening of microarray databases and the analysis of NB expression data showed a correlation between a high CMKLR1, GPR1 and CCRL2 expression and a reduction in the overall survival

probability. The expression of CMKLR1, GPR1, and chemerin was detected in nine NB cell lines using RT-PCR, Western blots and immunocytochemistry. Furthermore, chemerin and CMKLR1 were detected in NB tumor tissue by immunofluorescence and immunoperoxidase staining. The stimulation of NB cell lines with active chemerin induces calcium mobilization and an increased phosphorylation of MEK1/2 and ERK1/2, thereby indicating an activation of the MAPK pathway. Morover, chemerin stimulation leads to increased NF-κB phosphorylation and translocation to the nucleus. The induction of NF-κB mediated signaling was observed by luciferase reporter assay. Serum, TNFα and IL-1β increased chemerin protein expression and secretion in NB. α-NETA, a small-molecule CMKLR1 inhibitor reduces the clonogenic potential of NB cells *in vitro* and hampers tumor growth in an animal model. Pharmacological interventions that target CMKLR1/chemerin signaling pathway may become an important adjuvant therapy for children with NB but further preclinical *in vivo* studies are warranted.

Paper III

Title: FITC Conjugation Markedly Enhances Hepatic Clearance of N-Formyl Peptides.

In paper III, we demonstrated that the conjugation of an N-formyl peptide N-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fNLPNTL) with FITC significantly increases its uptake in the liver compared to native fNLPNTL. Along with that, we showed that the liver neutralizes circulating N-formyl peptides thus preventing the generalization of inflammation. In this study, anatomical distribution was evaluated by the intravenous injections of FITC-conjugated fNLPNTL and fNLPNTL, both labeled with ^{125}I. The expression of FPR1 was revealed by PCR, WB and immunohistochemistry in both human and murine hepatocytes and LSECs, the unique subsets of liver cells that are capable of removing dangerous substances from the blood. Competitive studies *in vitro* showed that FITC-labeled FPR1 agonist fNLPNTL is taken up in LSECs via both FPR1 and a scavenger receptor. In turn, hepatocytes bind FITC-fNLPNTL and fNLPNTL indistinguishably via FPR1. In this work, we proved that the chromogen conjugation of intravenously injected substances might transform them into ligands for scavenger receptors of the liver. Additionally, we have expanded the knowledge about the role of the liver as an organ that removes strong inflammatory signals from the circulation.

6. Results and discussion

It has been proven that functions of various components of the immune system are not limited to merely the recognition of pathogens. In this thesis, the role of two "inflammatory" receptors FPR1 and CMKLR1 in NB development and progression is described.

Additionally, this work provides two novel findings in the field of liver immunity.

1. FPR1 expressed by liver cells actively removes circulating formyl peptides that might cause systemic inflammatory response syndrome.
2. Fluorescein-labeled molecules are actively taken up from the blood by scavenger receptors of LSECs. Therefore, complicated liver physiology should be taken into account when planning research using the intravenous administration of chromogen-conjugated substances.

6.1 Activation of FPR1 and CMKLR1 induces pro-carcinogenic pathways in NB *in vitro*

FPR1 has previously been described to not only serve as a PRR, but to also participate in a plethora of biological events (Figure 6). Recently, its involvement in carcinogenesis has drawn researchers' attention. Interestingly, the impact of FPR1 on tumor formation seems to be tissue- and organ-specific. The majority of available publications suggest the pro-tumorigenic properties of FPR1 and a negative prognostic significance of highly-expressed FPR1 in tumor tissue [56, 85, 87, 88, 92, 140-143]. However, Prevete and co-authors suggested tumor suppressor functions of FPR1 in gastric cancer [84]. Moreover, a work by E. Vacchelli, Y. Ma et al. reported that FPR1 is necessary for the formation of chemotherapy-induced antitumor immunity [91]. Notably, some studies do not distinguish between FPR1 expressed by tumor cells and the receptor residing on the surface of other cells of the tumor microenvironment; consequently discrepancies in the interpretation of the results may occur.

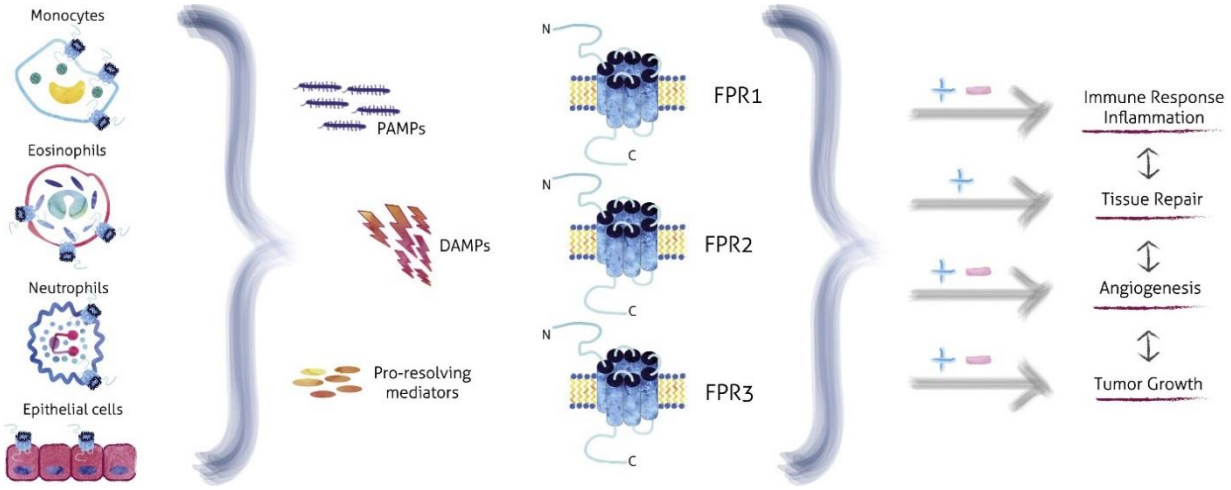

Figure 6. FPRs in humans. In humans, three FPRs are known. These receptors have been described in myeloid cells and later in various tissues and cell types. Depending on the ligand and site of expression, FPRs can initiate different processes.

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In turn, CMKLR1 is expressed by iDCs, macrophages, endothelial cells and NKs, with its main function to recruit immune cells to the site of inflammation in response to the release of chemerin [145]. Besides the immunological functions, chemerin is an essential regulator of adipogenesis, hence contributing to the development of obesity and diabetes type 2 [115, 146]. Additionally, CMKLR1-chemerin interaction has been shown in various conditions, including psoriasis, preeclampsia, atherosclerosis, renal diseases, etc. The functional activity of CMKLR1 is dependent on the cleaved form of chemerin (described in **Introduction 3.3.2**) (Figure 7) [112, 113, 147-149].

The data from the literature describing the role of CMKLR1 in cancer are very limited. Most papers focus on the plasma levels of chemerin, and do not pay attention to the expression and functions of the receptor.

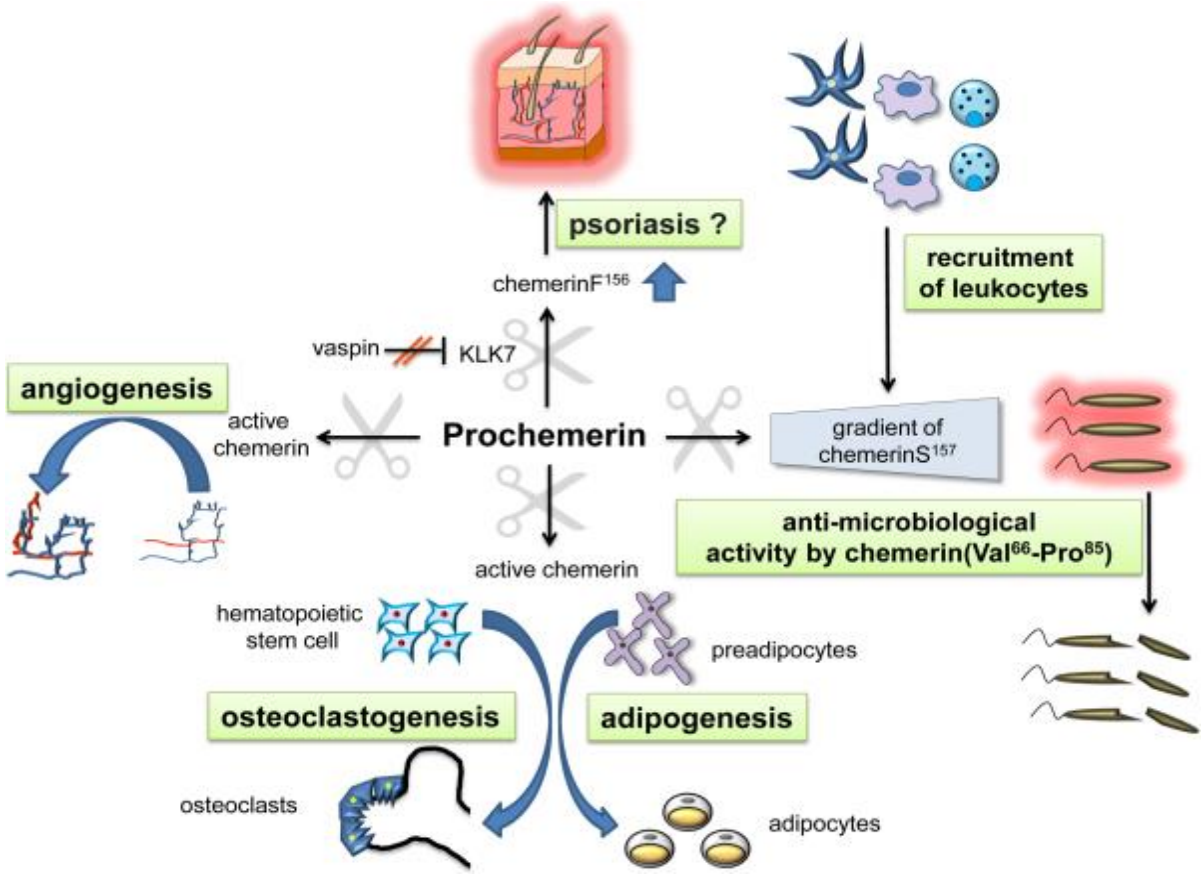

Figure 7. Functions of chemerin and CMKLR1. Depending on the cleavage site, chemerin might be involved in a variety of functions in inflammation, skin, obesity, and cell differentiation.

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In our work we aimed to establish whether FPR1 (Paper I) and CMKLR1 (Paper II) activation influences NB tumorigenesis. As the initial point for these studies, we screened publicly available expression datasets [150], and found that NB patients with tumors expressing high levels of the receptors have a significantly lower survival probability. *In vitro*, we tested seven (Paper I) and 10 (Paper II) NB cell lines, and 27 tumor samples and showed abundant expression of both receptors in all specimens. Using specific agonists, we demonstrated that stimulation of the receptors lead to the enhanced release of Ca^{2+} from intracellular stores and the activation of various signaling pathways including MAPK/ERK, Akt and P38-MAPK for FPR1 and MAPK/ERK, and NF-κB for CMKLR1.

ERK1 and ERK2 are extracellular signal-regulated kinases, members of the family of mitogen-activated protein kinases, transferring diverse extracellular signals to a cell's interior. The active forms of these kinases are detected in approximately one-third of all cancers [151]. Currently, more than 160 ERK 1/2 targets regulating cell growth, motility, survival, differentiation and metabolism have been identified [152]. The main mechanism of ERK 1/2-mediated tumorigenesis is the prevention of apoptosis via the inhibition of BCL-2/MCL-1 complex [153].

The PI3K/Akt/mTOR pathway has been shown to play an important role in the development and progression of NB [154]. Being activated, Akt regulates many oncogenic signaling pathways associated with tumor growth and survival [155]. In NB setting, it has been demonstrated that phosphorylated Akt has a negative prognostic value [156].

NF-κB is a complex proinflammatory transcription factor that comprehensively controls cell survival and the production of cytokines [157]. According to previous studies, its active form is required for the progression and drug resistance of high-risk NB [158]

Intracellular Ca^{2+} is one of the main drivers and regulators of cell migration, and its liberation by GPCRs activation is one of the primary regulatory pathways in cytoplasmic Ca^{2+} balance [159]. Tumor cells can alter Ca^{2+} signaling in order to increase proliferation and metastatic capability [160].

TNFα and IL-1 are inflammatory cytokines that share many biological properties and often work cooperatively in order to maintain inflammation [161, 162]. In paper II, we discovered that TNFα and IL-1β increase the liberation of chemerin by NB cells and tumor microenvironment. Thus, NB-associated inflammation contributes to tumor progression via chemerin-CMKLR1 interaction.

Taken together, our *in vitro* data from Paper I and Paper II indicate that the inflammatory receptors FPR1 and CMKLR1 are functionally expressed by NB cells. Being stimulated by selective agonists, these receptors trigger a variety of cellular responses attributed to augmented tumorigenicity. Speaking of the source of stimulatory signals for the receptors, we assume that formylated peptides are released from the mitochondria of necrotic tumor cells, while chemerin is produced by NB cells or other cells within the tumor microenvironment.

6.2 High expression of FPR1 significantly enhances NB tumorigenesis *in vivo*

In Paper I, in order to confirm our hypothesis *in vivo*, we carried out a xenograft experiment using immunodeficient NMRI nu/nu mice. Despite the growing body of publications on different aspects of FPR1 biology, there are a very limited number of published studies using animal models. Zhou and co-authors in 2005, and Yang and co-authors in 2011, demonstrated that glioblastoma U-87 cells with a siRNA knockdown of FPR1 formed tumors more slowly than control cells, and animals with wild-type cells had died or had to be sacrificed significantly earlier than animals with depleted FPR1 [56, 163]. In contrast, Prevete et al. discovered that shRNA silencing of FPR1 in gastric cancer AGS and MKN45 cell lines led to an accelerated tumor development [84]. They hypothesized that in gastric cancer, FPR1 is a strong inhibitor of angiogenesis; therefore, its silencing induces neovascularization, which results in enhanced tumor growth.

In our work, in addition to the knockdown FPR1 construct, we developed a cell clone with an overexpression of the receptor. The use of doxycycline-inducible constructs allowed us to maintain the constant level of the receptor expression during the entire experiment. We observed that animals injected with NB cells with an increased expression of FPR1 developed tumors and reached a humane endpoint significantly faster compared with other experimental groups. As a result, we demonstrated for the first time that FPR1 augments NB tumorigenesis in experimental animal models.

6.3 A small-molecule CMKLR1 inhibitor α-NETA diminishes the clonogenicity of NB cells *in vitro* and tumor growth *in vivo*

2-(α-naphthoyl) ethyltrimethylammonium iodide (α-NETA) has been described in the 1980s as a selective inhibitor of choline acetyltransferase, the enzyme responsible for the biosynthesis of the neurotransmitter acetylcholine [164, 165]. Recently, α-NETA has been demonstrated to be a potent CMKLR1 antagonist that inhibits the β-arrestin 2 association with CMKLR1 upon chemerin stimulation and decreases chemerin-driven cell migration [166].

In Paper II, we were able to show that the incubation of NB cells with α-NETA reduces proliferation activity and clonogenicity. In order to study the possible effects of CMKLR1 inhibition on NB *in vivo*, we established subcutaneous xenograft tumor model. Animals were divided randomly into three groups: the pre-treatment group, animals receiving s.c. α-NETA injections daily beginning 24 hours after xenografting; the treatment group, animals receiving s.c. α-NETA injections daily when a tumor reached a volume of ≥ 0.15 ml; and the control group, animals receiving vehicle injections. In this experiment, we observed no difference in tumor growth and animals' survival between treatment and control groups, although for the pre-treatment group the delay in tumor formation was statistically significant. Hence, CMKLR1 might exert its tumor promoting effect during the initial steps of NB development, so the blocking of the receptor could represent a therapy option for the treatment of NB.

6.4 Liver is responsible for the removal of formyl peptides from circulation

The liver collects blood from all the organs in the human body, and constantly encounters pathogens and host-released alarmins. Hence, the liver has evolutionary become a major immune organ, broadly involved in both the innate and adaptive immune response [135, 167]. The involvement of the liver in innate immunity is dependent on the broad network of PRRs and scavenger receptors expressed on the surface of hepatocytes and LSECs [137].

Mitochondria-derived formylated peptides released upon massive injury initiate a systemic inflammatory response similar to bacteria-induced inflammation. This could lead to systemic inflammatory response syndrome (SIRS), thus resulting in sepsis [168, 169].

To the best of our knowledge, we found for the first time in Paper III that formyl peptides are actively taken up from the circulatory system by LSECs expressing FPR1. In this work, we revealed that FPR1 is present in the liver cells in both humans and mice, and the incubation of the cells with a FITC-labeled formylated peptide resulting in the internalization of the peptide. *In vivo* experiments, in which we intravenously injected radioactive-labeled formylated peptide into mice, confirmed our *in vitro* data.

Interestingly, in analyzing the results of animal experiments, we observed significant differences in the uptake between FITC-labeled and unconjugated peptide. We confirmed this finding *in vitro* by the preincubation of LSECs and hepatocytes with unlabeled peptide prior to incubation with a FITC-labeled counterpart. As a result, we could not observe the fluorescence in hepatocytes because the original peptide competitively bound all active FPRs. While we observed the fluorescence in LSECs, suggesting that the FITC group converts the protein into a ligand for scavenger receptors apart from FPR1. This raises a fundamental concern on the validity of experiments utilizing significantly modified small peptides for *in vivo* injections.

7. Concluding remarks

Despite the recent scientific breakthroughs and modern advances in therapy, NB remains one of the most unfavorable pediatric cancers. There is a great need for novel prognostic factors and, drug targets in particular. In this thesis, the signaling pathways of the inflammatory receptors FPR1 and CMKLR1 have been studied in terms of their contribution to NB tumorigenesis. We were able to demonstrate that both receptors participate in tumor progression and the development of a highly malignant phenotype. Our experimental results supported by survival data obtained from several cohorts may provide a foundation for future research aimed at specifically targeting of FPR1 and CMKLR1 in NB. Moreover, drugs selectively targeting these receptors could be a novel approach in the treatment of patients with NB.

Additionaly, this thesis expands the knowledge of FPR1 biology, hence revealing the receptor's expression in the liver and identifying its role in hepatic clearance.

Hopefully, the data on the scavenging of fluorescent substances by the liver will help the research community to improve the experimental setup in order to prevent biased results.

8. References

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