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PATTERN-RECOGNITION RECEPTORS AND NEUTROPHILS IN CANCER INFLAMMATION

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TABLE OF CONTENT

ABSTRACT

Chronic inflammation, induced by the use of tobacco and alcohol, or caused by infections has long been suggested to constitute a risk factor for head and neck squamous cell carcinoma (HNSCC). The innate immunity is the first line of defense against pathogens, comprising physical and chemical barriers, anti-microbial peptides, pattern-recognition receptors (PRRs) as well as different kinds of cells, including neutrophils. Among the PRRs, Toll-like receptors (TLRs) and Nucleotide oligomerization domain (Nod)-like receptors (NLRs) have gained much attention. They both recognize viruses and bacteria. In addition to their protective role against infections, accumulating evidence suggests a role for these receptors in cancer. Neutrophils are among the first cells to migrate into an inflamed tissue, and their role in various infections is well described. Several studies have demonstrated anti-tumor activities of these cells, but they are also believed to have a tumor-promoting role. Recent data indicate the existence of three distinct neutrophil subsets, CD16^{dim} CD62L^{high}, CD16^{high} CD62L^{high}, and CD16^{high} CD62L^{dim} cells, with diverse roles in infection, inflammation and cancer. The overall aim with this thesis was to investigate the potential role of PRRs and neutrophils in HNSCC.

The thesis demonstrated that the HNSCC cells exhibited high levels of TLR2, TLR3, and TLR5, and a diverse NLR expression. Stimulation of TLR2, TLR3, TLR5, and Nod1 induced a robust inflammatory response and cell death in HNSCC cells that differed from what was seen in corresponding healthy epithelial cells. Following PRR stimulation the cancer cells up-regulated their expression of ICAM-1, and TLR activation increased the secretion of IL-1β, IL-6, and IL-8. In contrast, Nod1 enhanced the production of G-CSF and GM-CSF in HNSCC cells. In addition, the TLRs also affected the survival of the malignant cells. Altogether, this strengthens the suggestion that PRRs might mediate receptor specific tumor effects that can be either anti- or pro-tumorigenic. In the present study of HNSCC, TLRs induced an anti-tumorigenic response, whereas Nod1 activation caused pro-tumorigenic effects.

Generally, HNSCC patients had a higher level of leukocytes and specifically more neutrophils in blood than healthy controls. Consequently, the neutrophil/lymphocyte ratio was high in the cancer patients, and a high ratio predicted worse prognosis. The three different neutrophil subsets mentioned above were found in the circulation of patients with HNSCC. The cancer patients exhibited a higher percentage of CD16^{high} CD62L^{dim} cells than the healthy controls. Among the HNSCC patients, individuals with a high percentage of CD16^{high} CD62L^{dim} neutrophils had a better outcome. In addition, the CD16^{high} CD62L^{dim} cells represented the most active neutrophil phenotype. Hence, it might be that these activated neutrophils have anti-tumorigenic properties, and therefore are more favorable for the survival of the HNSCC patients. Altogether this emphasizes

the beneficence of having an ongoing process of neutrophil recruitment and activation in patients with HNSCC.

Patients with allergic rhinitis (AR) and HNSCC were found to exhibit distinct immunological reactions. The allergic patients exhibited enhanced serum levels of both Th1 and Th2 cytokines. The same increase was also seen in supernatants from their cultured PBMC. In contrast, HNSCC patients had an increase in serum level of cytokines reflecting an innate immune reaction. PMN isolated from these patients showed a generally increased basal activation, and responded strongly to TLR stimulation. Further, tumor biopsies from HNSCC patients displayed a higher Nod2 mRNA expression than nasal biopsies from healthy controls and AR patients outside and during pollen season. All in all, the immune reaction among the allergic patients had an adaptive character with an enhanced T cell activity, whereas the immune reaction of the HNSCC patients was dominated by an innate immune response with suppressed T cells. It is therefore tempting to propose that the enhanced systemic adaptive immune response seen among patients with AR might protect against development of HNSCC.

In summary, this thesis demonstrates a receptor specific expression and function of PRRs in HNSCC. It also reveals that the inflammation in HNSCC is dominated by innate immune activities, and that recruitment and activation of neutrophils is important for the survival of these patients. Consequently, the ability to muster a proper inflammatory reaction might be vital for the defense and survival in patient with HNSCC.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Huvud- halscancer är den sjätte vanligaste cancerformen i världen och består huvudsakligen av tumörer i de övre luftvägarna. Kronisk inflammation framkallad av tobak, alkohol och infektioner anses vara en bidragande orsak till dess uppkomst och utveckling. Målet med föreliggande avhandling är att undersöka betydelsen av så kallade patogen-igenkännande receptorer (PRRs) och neutrofiler i detta utvecklingsförlopp.

Immunförsvaret kan förenklat delas in i ett adaptivt och ett medfött immunförsvar. Det medfödda försvaret är snabbt och ospecifikt och utgörs bland annat av PRRs och neutrofiler. PRRs känner igen invaderande bakterier och virus och består till exempel av toll-lika (TLRs) och nod-lika receptorer (NLRs). När de känner igen en främmande mikroorganism framkallar dessa receptorer ett inflammatoriskt svar vars primära roll är att bekämpa inkräktaren och därmed skydda mot infektioner. Det har dock visat sig att felaktig eller långdragen kontinuerlig aktivering samt mutationer i PRR gener kan ge upphov till cancer

I avhandlingens första del undersöktes TLRs och NLRs uttryck och funktion i huvud-halscancer. Uttrycket av TLRs och NLRs i cancercellerna skiljde sig påtagligt från uttrycket i de friska cellerna. Aktivering av TLRs och NLRs gav i cancercellerna upphov till ett starkt inflammatoriskt svar, medan nästan ingen effekt sågs hos de friska kontrollcellerna. Dessutom förkortade TLR stimulering cancercellernas livslängd. Kortfattat kan man säga att TLR aktivering gav en antitumerogen effekt, medan NLR framkallade ett inflammatoriskt svar som närmast främjade cancerns utveckling. Följaktligen uppvisar PRRs en receptorspecifik påverkan på huvud- halstumörer.

Neutrofiler är viktiga för att bekämpa och ta död på inkräktande mikrober. De migrerar tidigt ut i inflammerad vävnad med målet att ta död på bakterier. På senare tid har neutrofiler även visat sig ha andra funktioner. Bland annat tycks de ha en betydande roll vid utvecklingen av tumörer, men har även visats motverka cancerns utveckling. Kanske kan en del av förklaringen till dessa motstridiga funktioner ha sin grund i att man nyligen upptäckt olika subpopulationer av neutrofiler som uppför sig på skilda sätt vid infektion och inflammation. Man talar om tre olika grupper $CD16^{dim}$ $CD62L^{high}$, $CD16^{high}$ $CD62L^{high}$ och $CD16^{high}$ $CD62L^{dim}$ celler.

Avhandlingens andra del fokuserar på neutrofilernas roll vid huvud- halscancer. Cancerpatienterna visades ha en högre andel neutrofiler i blodet än friska kontrollpatienter och de tre neutrofilpopulationer som beskrevs ovan återfanns i blodet från cancerpatienterna. Cancerpatienter med hög andel $CD16^{high}$ $CD62L^{dim}$ celler hade en klart bättre överlevnad. Dessutom uppvisade de $CD16^{high}$ $CD62L^{dim}$ neutrofilerna en aktiverad profil. Dessa resultat tyder på att en ökad andel $CD16^{high}$ $CD62L^{dim}$ celler kan

tolkas som ett tecken på en ökad neutrofil aktivering som troligen bidrar till att hålla cancern i schack.

Under flera år har det debatterats huruvida det finns något samband mellan allergi och cancer. Diskussionen har främst baserats på epidemiologiska studier och slutsatserna skiljer sig åt. I avhandlingens avslutande arbete studerades likheter och skillnader i det immunologiska svaret vid huvud- halscancer och pågående pollenallergi. Tillstånden kan ses som representanter för var sin form av kronisk inflammation inom huvudhalsområdet. Den allergiska reaktionen karakteriserades av ett adaptivt svar, medan det medfödda försvaret dominerade vid tumörsjukdomen. Dessa resultat stödjer den tidigare framförda tanken om att ett överaktivt adaptivt försvar hos patienter med allergisk rinit kan skydda mot utvecklingen av huvud- halscancer.

Sammantaget belyser denna avhandling betydelsen av PRRs och dess förändrade uttryck och funktion i huvud- halscancer. Dessutom åskådliggörs vikten av neutrofiler och neutrofil framkallad inflammation hos patienter med huvud- halscancer. Avhandlingen betonar betydelsen av det medfödda immunförsvaret som ett skydd mot cancer.

ABBREVIATIONS

ANXV   Annexin V

AR   Allergic rhinitis

CD   Cluster of differentiation

CT   Cycle treshold

ELISA   Enzyme-linked immunosorbent assay

FSc   Forward scatter

G-CSF   Granulocyte-colony stimulating factor

GM-CSF   Granulocyte monocyte-colony stimulating factor

HNEC   Human nasal epithelial cells

HNSCC   Head and neck squamous cell carcinoma

HPV   Human papillomavirus

ICAM-1   Intercellular adhesion molecule-1

iE-DAP   γ-D-glutamyl-meso-diaminopimelic acid

IFN   Interferon

Ig   Immunoglobulin

IL   Interleukin

LPS   Lipopolysaccharide

MCP-1   Monocyte chemotactic protein-1

MIP-1β   Macrophage inflammatory protein-1β

Naip   Neuronal apoptosis inhibitor protein

Nlrp   NACHT domain-, leucine rick repeat-, and pyrine domain containing protein

Nod   Nucleotide oligomerization domain

NLR   Nod-like receptor

PAMP   Pathogen-associated molecular pattern

PBMC   Peripheral blood mononuclear cells

PCR   Polymerase chain reaction

PI   Propidium iodide

PMN   Polymorphonuclear leukocytes

PRR   Pattern-recognition receptor

RLR   Rig-like receptor

SSc   Side scatter

Th   T helper

Th1   Th type 1

Th2   Th type 2

TLR   Toll-like receptor

TNF-α   Tumor necrosis factor-α

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-V). The papers are appended at the end of this thesis.

I.   **Rydberg C,** Månsson A, Uddman R, Riesbeck K, Cardell LO.
      Toll-like receptor agonists induce inflammation and cell death in a model of head and neck squamous cell carcinomas.
      Immunology. 2009 Sep;128(1 Suppl):e600-11.

II.   **Millrud CR**, Kvarnhammar AM, Tajti J, Munck-Wikland E, Uddman R, Cardell LO.
      Nod-like receptors in head and neck squamous cell carcinoma.
      Accepted Acta Oto-Laryngologica.

III. **Millrud CR**, Kvarnhammar AM, Uddman R, Björnsson S, Riesbeck K, Cardell LO.
      The activation pattern of blood leukocytes in head and neck squamous cell carcinoma is correlated to survival.
      PLoS One. 2012;7(12):e51120.

IV.   **Millrud CR**, Kågedal Å, Winqvist O, Uddman R, Razavi R, Munck-Wikland E, Cardell LO.
      $CD16^{high}CD62L^{dim}$ neutrophils predict improved survival in head and neck squamous cell carcinoma.
      Manuscript.

V.   **Millrud CR**, Hylander T, Kumlien Georén S, Kågedal Å, Winqvist O, Cardell LO.
      Inverse immunological responses induced by allergic rhinitis and head and neck squamous cell carcinoma.
      Cond Accepted PLoS One.

Change of family name from Rydberg to Rydberg Millrud (2012)

AIMS

The overall aim with this thesis was to investigate the importance of pattern-recognition receptors and neutrophils in cancer inflammation. More specific the aims were to:

- Examine the expression profile and functional importance of toll-like receptors in head and neck squamous cell carcinoma.

- Characterize the expression, function and inflammatory role of nod-like receptors in head and neck squamous cell carcinoma.

- Investigate the phenotype of peripheral leukocytes, and to evaluate the prognostic value of the different leukocytes and their markers in head and neck squamous cell carcinoma.

- Characterize different neutrophil subsets and their prognostic role in patients with head and neck squamous cell carcinoma.

- Experimentally compare immune responses induced by allergic rhinitis and head and neck squamous cell carcinoma.

INTRODUCTION

HEAD AND NECK SQUAMOUS CELL CARCINOMA

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Despite its origin in the aerodigestive tract it is a heterogeneous group of tumors in the oral cavity, oropharynx, hypopharynx, and larynx (Figure 1). Chronic inflammation is suggested to constitute a risk factor for the development of cancer. A large proportion of HNSCC is induced by tobacco and alcohol consumption, but human papillomavirus (HPV) infection has been recognized as an increasingly important risk factor for HNSCC, especially for oropharyngeal tumors. The survival of patients with HPV positive tumors is better than for patients with HPV negative tumors. Although advances in multimodality therapies the overall survival rate has only to a minor degree increased the past two decades (1-6).
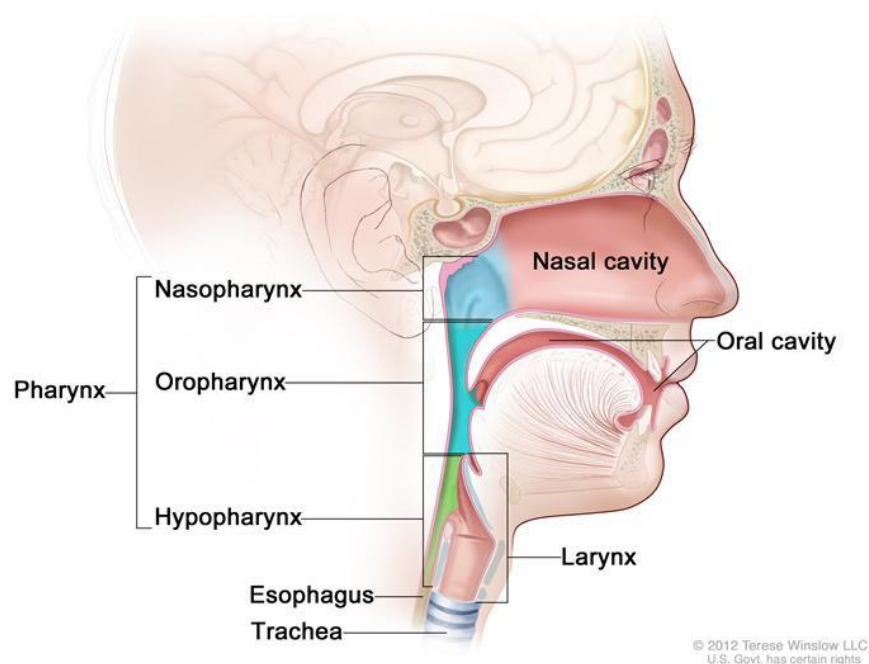

Figure 1. Schematic overview of the upper airway (http://www.cancer.gov).

ALLERGIC RHINITIS

Allergic rhinitis (AR) is a common chronic inflammatory disease induced by an IgE mediated reaction to normally harmless antigens called allergens. The reason why some people develop AR is unknown, but several theories have been put forward. There seems to be a consensus around the idea that AR is the result of the combined influence of

genetic and environmental factors, and that respiratory pathogens might play a role. Allergy affects millions of people around the world and the prevalence is increasing particular in countries with a western life style. AR is characterized by symptoms such as sneezing, rhinorrhea, nasal congestion, nasal pruritus, and ocular pruritus/irritation upon allergen exposure. It also affects the quality of life, such as work performance, daily activity, and sleep (7, 8).

The existence of an association between allergy and cancer development has been debated for several years. The discussion is entirely based on information derived from epidemiological studies with diverse outcomes (9-13). Three different perspectives currently dominate the discussion; the antigenic stimulation hypothesis, the immune surveillance theory, and the prophylaxis postulate. The antigenic stimulation hypothesis states that atopic inflammation causes oxidative damage that activates mutations in tumor suppressor genes, post-translational modifications in proteins involved in DNA repair, or apoptotic control. Altogether, this is said to enhance the development of cancer. The immune surveillance theory proposes that atopy is a consequence of a generalized enhanced immune responsiveness that can detect and eradicate dysregulated cells. This would decrease the risk for the development of cancer. The prophylaxis postulate is based on the assumption that patients with allergy tend to avoid environments with increased risk for allergen exposure, and such behavior also prevents exposure to microorganisms, toxins, and environment contaminants known to promote cancer development (13-15).

THE IMMUNE SYSTEM

THE INNATE IMMUNE SYSTEM

The immune system is traditionally divided into an adaptive and an innate branch. The innate part of the system is the first line of defense against pathogens. It is thought to be fast and non-specific, and comprises physical and chemical barriers, anti-microbial peptides, pattern-recognition receptors (PRRs) as well as different kinds of cells, including neutrophils and eosinophils (16, 17).

PATTERN-RECOGNITION RECEPTORS

PRRs are an important part of the innate immune system that recognizes conserved molecular motifs of microbial origin called pathogen-associated molecular patterns (PAMPs). To date, the PRRs consist of at least three receptor families; Toll-like receptors (TLRs), Nucleotide oligomerization domain (Nod)-like receptors (NLRs), and Rig-like receptors (RLRs). To ensure an effective detection and clearance the different receptor families recognize different classes of pathogens. TLRs sense bacteria, viruses, protozoa, and fungi, NLRs identify bacteria, and RLRs recognize viruses. In addition, the different

receptors are located at various cellular compartments; TLRs are positioned at the cell surface and in the endosomes, whereas the NLRs and RLRs are located in the cytosol (Figure 2). Despite the protective effect of PRRs against infections evidence suggests a role for these receptors in the pathogenesis of various diseases.
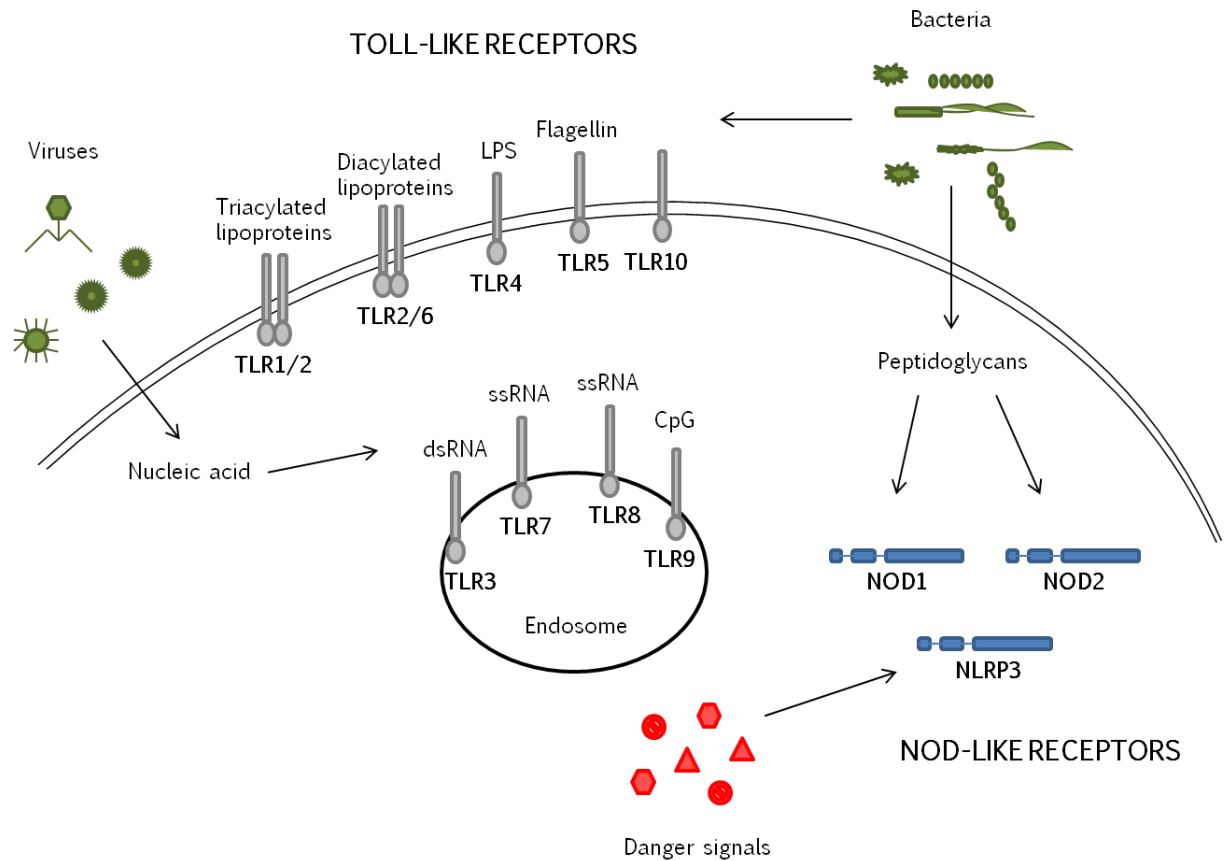

Figure 2. Outline of the TLR and NLR family and their cognate ligands. The surface TLRs recognize structures from mainly bacteria, whereas the endosomal members sense nucleic acid from primarily viruses. The NLRs located in the cytosol identify danger signals and peptidoglycans from bacteria.

TOLL-LIKE RECEPTORS

TLRs were the first recognized PRRs, and have since then been extensively examined. 10 members have been demonstrated in humans both at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and intracellulary in the endosomes (TLR3, TLR7, TLR8, and TLR9). The TLRs positioned at the cell surface predominantly identify bacterial structures, whereas those receptors that are located in the endosomes sense viral components. Each TLR recognizes specific PAMPs. TLR2 acts in a heterodimer in concert with TLR1 or TLR6 to respond to diacyl (TLR1/2) and triacyl (TLR2/6) lipopeptides, lipoteichoic acids, peptidoglycan, and zymosan. TLR3 is involved in the recognition of double stranded RNA from viruses, TLR4 senses lipopolysaccharide (LPS), TLR5 identifies the bacterial component flagellin, TLR7 and TLR8 mediate responses to single stranded viral RNA, and TLR9 has been demonstrated to sense

bacterial and viral DNA containing unmethylated CpG motifs. No specific ligand has so far been identified for TLR10 (18-21).

TLRs have been demonstrated in various cells and tissues including epithelial cells, neutrophils, dendritic cells, lymphocytes, nasal mucosa, and tonsils (22-25). Tumor cells have also been shown to express TLRs, often with levels that differ from what is seen in normal tissues (26, 27). The importance of TLRs in host responses to tumors became evident as polymorphism in TLR genes was found to be associated with susceptibility to cancer (28-31). Increasing evidence suggests that TLRs might play a dual role in cancer progression. TLR3 has been demonstrated to directly inhibit tumor growth by inducing apoptosis and to decrease proliferation in human breast, melanoma, and prostate cancer cells (32-34), whereas TLR4 seems to promote tumor growth in human ovarian cancers and HNSCC (26, 35). It also appears as the same TLRs can induce both anti-tumorigenic and pro-tumorigenic effects all depending of the types of tumors and tissues involved. For instance, TLR9 has been demonstrated to induce cell proliferation and to increase invasiveness in breast cancer cells (36), whereas it inhibits proliferation and causes cell death in neuroblastoma cells (37)

The TLR7/8 agonist Imiquidmod is used clinically for treating basal cell carcinoma. Today there are many TLR ligands in clinical trials both as adjuvants in cancer immunotherapies and as monotherapy. In particular, TLR7/8 and TLR9 agonists have attracted attention due to their leukocyte mediated and tumor induced anti-tumor functions (38).

NOD-LIKE RECEPTORS

The NLR family consists of more than 20 known members in humans. Based on the nature of the N-terminal, the NLRs can be divided into three subfamilies; caspase recruitment domain (CARD)-containing NODs, NACHT-, leucine rich repeat-, and pyrine domain containing proteins (Nlrps), and baculovirus inhibitor repeat containing neuronal apoptosis inhibitor proteins (Naips) (39-41). The NLRs are expressed by a variety of cells of both the innate and the adaptive systems. Presence of Nod1 and Nod2 has for instance been demonstrated in airway epithelial cells, lymphocytes, and neutrophils (42-46). The NLRs are located in the cell cytosol where they respond to bacterial proteins and danger signals. So far, specific ligands have been identified for Nod1, Nod2, and Nlrp3. Nod1 and Nod2 recognize the major component of the bacterial cell wall, peptidoglycan. More specifically, Nod1 detects γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), specific for Gram-negative bacteria, and Nod2 senses muramyldipeptide, a component of all types of bacterial peptidoglycans (39-41). In contrast, Nlrp3 recognizes microbial products and danger signals released by injured or/and dying cells. It has also been shown to respond to aluminium adjuvants used in many vaccines (47, 48).

NLRs have been associated with cancer, but their role in tumor development is still far from understood. Most data indicate that NLRs might have a protective role. For instance, Nod1 has been demonstrated to have a suppressive effect against colon cancer and estrogen sensitive breast cancer (49, 50), and Nlrp3 has shown protective effects against the development of colon cancer (51, 52). Conversely, genetic variations in Nod2 have been associated with cancer in some studies, but not linked to cancers in others (53-56).

NEUTROPHILS

Neutrophils are essential for the innate immune response. They have a key role in eliminating invading pathogens and in promoting tissue repair. Their half-life in blood is normally about 6 to 8 h, but it can be significantly extended upon migration into inflamed tissue (57). Neutrophils respond quickly to intruding pathogens by migrating into inflamed tissue where they phagocytose and kill bacteria. This is followed by rapid apoptosis and clearance by resident macrophages (Figure 3). Lately, neutrophils have been demonstrated to have functions beyond their role in the acute inflammation. They are for instance able to migrate to and reside in lymph nodes as well as to migrate back to the peripheral blood (57, 58).
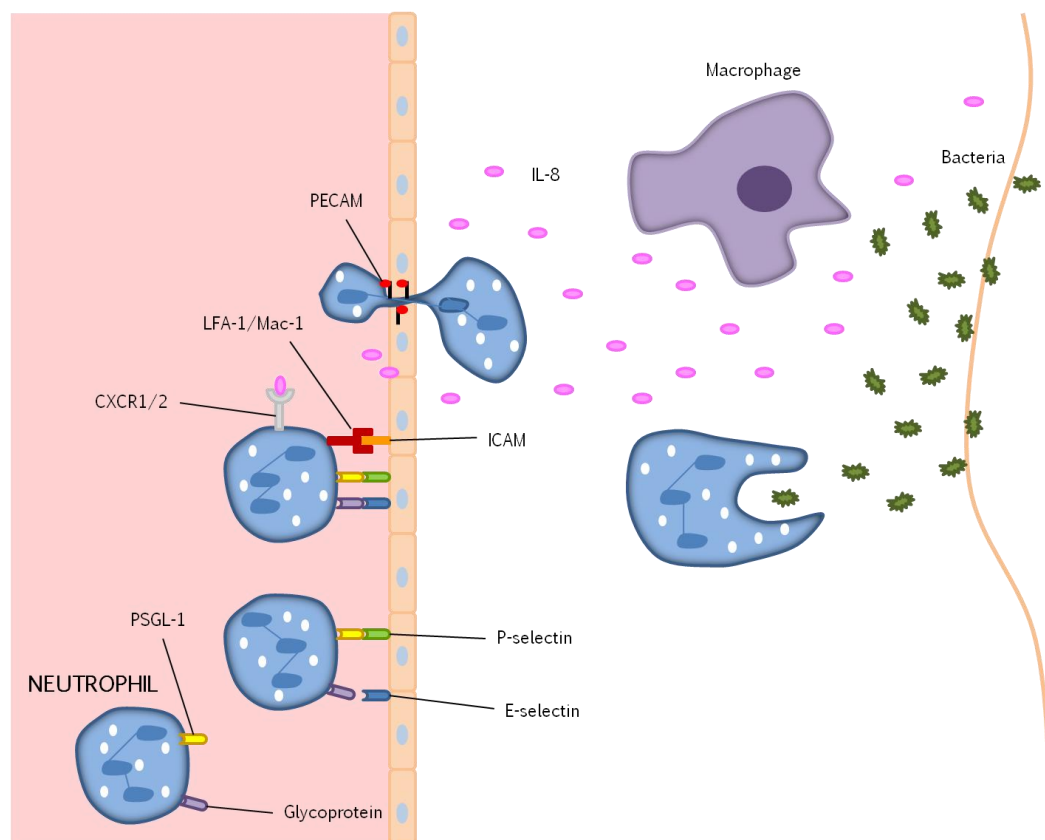

Figure 3. Schematic overview of the recruitment of neutrophils into inflamed tissue. CXCR 1/2 – CXC chemokine receptor 1/2; ICAM-1 – Intercellular adhesion molecule-1; IL-8 – Interleukin-8; LFA-1 – Leukocyte function associated antigen-1; Mac-1 – Macrophage-1 antigen; PECAM – Platelet endothelial cell adhesion molecule; PSGL-1 – P-selectin glycoprotein ligand-1.

Neutrophils are thought to be involved in the pathophysiology of cancer and tumor progression (59). It has been demonstrated that high levels of tumor-infiltrating neutrophils and blood neutrophils are associated with poor clinical outcome (60-62). Nonetheless, neutrophils have been attributed both pro- and anti-tumor functions. The best characterized pro-tumorigenic effect of neutrophils is related to the inducement and regulation of angiogenesis (58, 59, 63, 64). Neutrophils are also able to directly modulate the biology of tumor cells by secretion of pro-inflammatory factors that promote motility and migration. In addition, neutrophils have been attributed the capacity to promote tumor cell invasion and immune suppression by inhibiting anti-tumor effector cells (58, 59, 63, 65). Despite the evidence for a tumor promoting role of neutrophils there are also convincing results that support an anti-tumorigenic activity of these cells. The release of antimicrobial and cytotoxic granule contents by neutrophils has the potential to eliminate malignant cells, and the secretion of cytokines and chemokines may activate other anti-tumor effector cells (58, 59, 64).

The pro- and anti-tumor functions of neutrophils illustrate the plasticity and dichotomy of this cell type. Until recently, neutrophils were thought to consist of one population, but accumulating evidence proposes that there are distinct neutrophil subsets with diverse roles in infection, inflammation and cancer (66-68). Pillay *et al* recently identified three distinct neutrophil subsets in humans based on the expression of CD16 and CD62L. The CD16^{dim} CD62L^{high} subset showed a banded nuclear morphology characteristic of neutrophils derived from the bone marrow, the CD16^{high} CD62L^{high} cells had the phenotype of normal mature neutrophils, and the CD16^{high} CD62L^{dim} population demonstrated a hypersegmented nucleus (67).

MATERIALS AND METHODS

STUDY POPULATIONS

Fresh human materials were used in all studies. The studies were approved by the ethics committee at Lund University and/or Karolinska Institutet and an informed consent was obtained from all participants.

- In **PAPER I**, three HNSCC biopsies were collected and primary human nasal epithelial cells (HNEC) were obtained from six healthy control patients.

- In **PAPER II**, six nasal biopsies from healthy controls and four HNSCC biopsies were used. In addition, HNEC were isolated from seven healthy donors.

- In **PAPER III**, blood was acquired from 20 newly diagnosed still untreated HNSCC patients and from 20 healthy controls.

- In **PAPER IV**, blood from 31 newly diagnosed still untreated HNSCC patients and 19 healthy controls was obtained.

- In **PAPER V**, blood was obtained from ten newly diagnosed still untreated patients with HNSCC, 13 patients with AR sampled during pollen season, and 10 healthy controls.

METHODS

The studies were performed at Skåne University Hospital Malmö (**PAPERS I-V**), Karolinska Institutet, and Karolinska University Hospital (**PAPERS IV-V**).

CELL CULTURE

In **PAPERS I-II,** primary HNEC were isolated from healthy non-smoking individuals by nasal brushing of the inferior turbinates of both nostrils. After brushing, the cell mixture was centrifuged and grown on collagen coated tissue culture flasks. The cells were cultured in airway epithelial cell growth medium supplemented with 0.4 % bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 6.7 ng/ml triiodothyronine, 10 μg/ml transferrin, 0.1 ng/ml retinoic acid, 100 U/ml pencillin, and 100 μg/ml streptomycin. Primary epithelial cells in passage 1-4 were used in the experiments.

The human pharyngeal carcinoma cell lines Detroit-562 (**PAPERS I-II**) and FaDu (**PAPER II**) from ATCC were used as a model for HNSCC. These cells were cultured in minimum essential medium (MEM) with Earl's salt and 2 mM L-glutamine, and supplemented with FBS. The complete medium for Detroit-562 also contained 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 µg/ml gentamicin, and 0.25 µg/ml fungizone, whereas only 100 U/ml pencillin and 100 µg/ml streptomycin were added to the medium for FaDu.

The non-tumorigenic cell line NL-20 (ATCC) derived from a normal bronchus was used as control cells to the HNSCC cell lines. The cells were cultured in Ham's F12 medium supplemented with 2.7 g/l glucose, 5 µg/ml insulin, 10 ng/ml epidermal growth factor, 1 µg/ml transferrin, 500 ng/ml hydrocortisone, 2mM L-glutamine, 0.1 mM non-essential amino acids, 50 µg/ml gentamicin, and 4% FBS.

All cells were cultured at 37°C in humidified 5 % CO_2. Before each experiments, epithelial cells were plated on 24-well culture plates at a concentration of 250 000 cells/ml and incubated over night.

CELL ISOLATION

Ficoll-Paque™ was used to separate polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) in blood. PMN and erythrocytes with high density sedimented to the bottom of the tube, and PBMC with low density could be found at the interface of plasma (above) and Ficoll-Paque™ (below). To recover pure PMN the erythrocytes have to be lysed. This was done with ammonium chloride buffer. The cells were then cultured in RPMI-1640 supplemented with 0.3 g/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 % autologous plasma to a concentration of 1 $\times 10^6$ PBMC and 4 $\times 10^6$ PMN at 37°C in humidified 5 % CO_2 .

PMN was isolated from 11 HNSCC patients (**PAPERS IV**), whereas both PMN and PBMC were recovered from 13 patients with AR, ten HNSCC patients, and ten healthy controls (**PAPER V**).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is an antibody-based method to identify proteins in tissues and cells. The antibody-protein conjugation is detected with an enzyme-labeled polymer conjugated secondary antibody. In our studies, horseradish peroxidase (HRP) was used as enzyme. After incubation with a substrate, a positive immunoreactivity occurs. If DAB is used as a substrate, a brown color can be seen. To provide contrast to the sections and to visualize the nuclei, the slides are usually counterstained with haematoxylin. To rule out unspecific background staining, negative controls for mouse and/or rabbit primary antibodies are used.

Immunohistochemistry was used in **PAPERS I-II** to detect the expression of TLRs and NLRs in the epithelium of nasal biopsies and in HNSCC biopsies.

REAL-TIME RT-PCR

Real-time PCR is used to quantitate the gene expression based on mRNA manifestation in cells and tissues. Before real-time PCR can be performed, the total RNA has to be extracted from the cells of interest followed by reverse transcriptase of RNA into cDNA. The obtained cDNA can be used for real-time PCR where a cyclic heating and cooling procedure denatures the double-stranded cDNA, enables attachment of sequence specific oligonucleotide primers or probes, and promotes extension of new DNA strands. This then starts over for another set of cycles. Real-time PCR can be performed with either sequence specific primers or probes. Primers are used in the presence of fluorescent dyes such as SYBR® green, which emits a fluorescent signal when binding to double stranded DNA. Probes, on the other hand, are labeled with a reporter fluorophore and a quencher fluorophore, and during DNA amplification these two fluorophores are separated, which enables the reporter fluorophore to emit a fluorescent signal. When the level of fluorescence reaches a predetermined value a cycle threshold (CT) value can be determined. The relative amount of mRNA is determined by subtracting the CT value of the investigated gene with the CT value of the housekeeping gene, and expressed in relation to 100 000 mRNA molecules of the housekeeping gene ($100\ 000 \times 2^{-\Delta CT}$) (69). β-actin was used as housekeeping gene.

In this thesis, RNA was extracted from isolated cells with RNeasy mini kit from Qiagen, and the RNA quality and concentration was determined by spectrophotometry based on the wavelength absorption ratio (260/280 nm), all samples in the range 1.7-2.1. Subsequently, RNA was reversely transcribed into cDNA using Omniscript reverse transcriptase kit (Qiagen) with oligo-dT primer.

Real-time PCR was performed either on a Smart cycler II (Cepheid; **PAPERS I**) or a Stratagene Mx300 (Aglient Technologies; **PAPER II**). To detect TLR probes (**PAPER I**) TaqMan Universal PCR brilliant II QPCR Master Mix, No AmpErase UNG and assay-on-Demand gene expression products (Applied Biosystems) were used, whereas Stratagene Brilliant® QPCR Mastermix (Aglient Technologies) was utilized to detect NLR probes in **PAPER II**.

FLOW CYTOMETRY

Flow cytometry is a method that analyzes the physical and chemical properties of individual cells based on how they scatter light from a laser beam. Through different detectors flow cytometry gives information about cell size (displayed by forward scatter; FSc), granularity (displayed by side scatter; SSc), and fluorescence intensity of

fluorochrome conjugated antibodies against extra- or intracellular antigens to provide information about cell phenotype. By gating on FSc and SSc lymphocytes, monocytes, and granulocytes can be distinguished (Figure 4). Analyzes were made on a Coulter Epics XL, FC500, Navios (Beckman Coulter), or a BD LSRFortessa (BD Bioscience). Data were analyzed with Expo32 ADC software, CXP analysis software (Applied cytometry software), or FlowJo software (Tree Star Inc.).
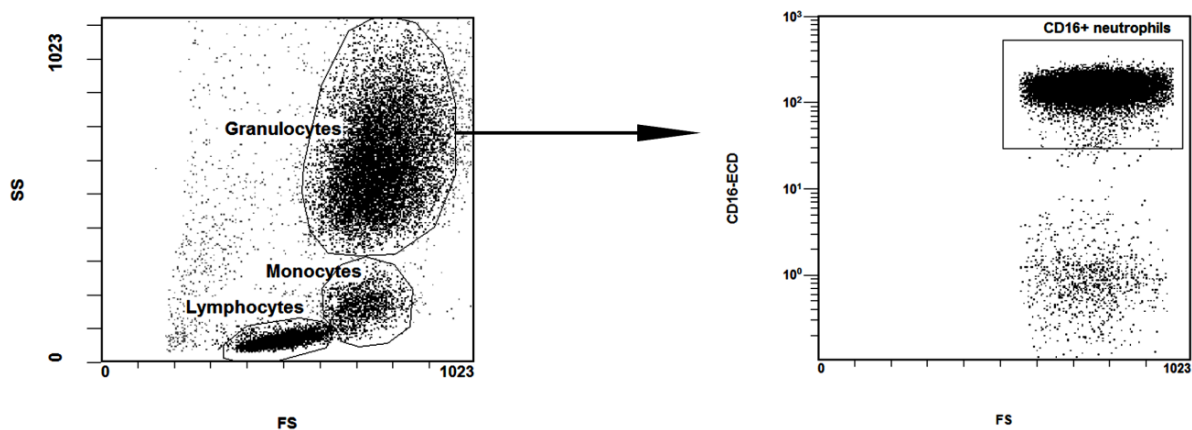

Figure 4. Flow cytometry identifications of lymphocytes, monocytes, and granulocytes in peripheral blood based on FSc and SSc properties. By further plotting CD16 versus FSc from granulocytes neutrophils can be distinguished.

In **PAPERS I-II**, flow cytometry was used to determine the expression of TLRs and NLRs in epithelial cells, and to identify the effects of TLR and NLR agonists on the expression of the epithelial cell activation marker intercellular adhesion molecule (ICAM)-1. In addition, the stimulatory effects on viability and apoptosis were established with Annexin V (ANXV) and propidium iodide (PI). ANXV binds to phosphatidylserine that is translocated to the plasma membrane during apoptosis, whereas PI is a nucleic acid binding dye used to discriminate between apoptotic and dead cells (70). The leukocyte phenotypes were assessed in patients with HNSCC and healthy controls with flow cytometry (**PAPER III**). In **PAPERS IV-V**, neutrophil subsets were characterized based on the expression of CD16 and CD62L, neutrophils were identified as CD16$^+$ granulocytes and Th cells as CD4$^+$ lymphocytes. Flow cytometry was also used to characterize the neutrophil and the Th cell responses to TLR agonists by measuring the activation markers CD11b, CD25, CD69, and CD98.

ELISA

ELISA is a specific method for quantification of antigens and/or antibodies in for instance cell culture supernatants. The ELISAs used were of sandwich type where a microplate is pre-coated with antibodies against the antigen of interest. When standards

(with known concentration) or samples are added, the antigen binds to the immobilized antibody. For detection of antigens, levels of antigen specific enzyme-linked polyclonal antibodies are added. As a substrate solution is added the enzyme will be converted into a detectable color that is proportional to the amount of antigen. By measuring the color intensity with a microplate reader and comparing it to the standards the levels of antigen in the sample can be determined.

In **PAPERS I-II**, commercial ELISA kits from R&D systems were used to determine the concentration of interleukin (IL)-1β, IL-6, IL-8, granulocyte-colony stimulating factor (G-CSF), and granulocyte monocyte-colony stimulating factor (GM-CSF) in epithelial cell culture supernatants. In **PAPER IV**, PMN cell culture supernatants were assessed for levels of IL-8 and IL-6 with ELISA plates from eBisoscience.

LUMINEX MULTIPLEX IMMUNOASSAY

Luminex multiplex immunoassay is a method that quantifies multiplex proteins or peptides at the same time in one sample of for instance serum or cell culture supernatants. The assay principle is similar to that of a sandwich ELISA, but with the exception that the antibodies directed against the antigen of interest are covalently coupled to magnetic beads dyed with fluorescent dyes. The fluorescently dyed beads each have a distinct color code that permits discrimination of individual antigens. When the standards (with known concentration) or samples are assessed, the antigen binds to the antibody-bead complex. For detection, the biotinylated detection antibody is added followed by addition of streptavidin-phycoerythrin conjugate. Phycoerythrin serves as a fluorescent indicator/reporter. The median fluorescent intensity is then measured with for instance the Bio-Plex system from Bio-Rad Laboratories, and the level of antigens in the samples is determined by comparison to the standards.

In **PAPER V**, the cytokine profile in serum and supernatants from TLR stimulated PBMC from patients with AR, HNSCC patients, and healthy individuals were detected with the Bio-Plex Pro Human Cytokine 17-plex assay from Bio-Rad Laboratories.

STATISTICAL ANALYSES

Statistical analysis was performed using GraphPad Prism 5. In **PAPERS I-II AND IV-V**, data were presented as mean ± standard error of the mean (SEM), whereas individual values and a horizontal line representing the mean were displayed in **PAPERS III-IV**. A p-value ≤ 0.05 was considered statistically significant, and n is equal to the number of independent donors or experiments performed.

Distribution of data was assessed using D'Agostino and Pearson omnibus normality test. Normally distributed data were analyzed with parametric tests, whereas non-parametric tests were used to analyze not normally distributed data.

When two sets of normally distributed paired data were compared paired t-tests were used, and for comparison of more than two sets of normally distributed paired data with a control one-way repeated measures analysis of variance (ANOVA) with Dunett's post-test was utilized. For two sets of normally distributed unpaired data, student's t-test with Welch correction if the variance was non-homogenous was used. ANOVA with Tukey's post test was used to analyze more than two sets of normally distributed paired data with each other. The survival function from life-time data was estimated using Kaplan-Meier analysis, and a log rank test was utilized to examine the significance of the different survival distribution between the two groups. The nonparametric Mann-Whitney test was used to determine the statistical difference between different groups, and for paired data the nonparametric Wilcoxon signed rank test was utilized.

RESULTS AND COMMENTS

TOLL-LIKE AND NOD-LIKE RECEPTORS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (PAPERS I-II)

RESULTS

TLRs have previously been demonstrated to have anti-tumorigenic properties, and Nod1 has been attributed a protective role against colon cancer and estrogen-sensitive tumors (32, 49, 50, 71). However, the role of TLRs and NLRs in HNSCC is far from understood. The present studies were designed to characterize the TLR and NLR expression and function in HNSCC. To this end, the HNSCC cell lines Detroit-562 and FaDu were used as a model of HNSCC. In **PAPER I**, Detroit-562 was compared to the healthy bronchial cell line NL-20 and primary HNEC. In **PAPER II**, Detroit-562 and FaDu were compared to HNEC.

High mRNA levels of TLR2, TLR3, and TLR5 were seen in Detroit-562. HNEC showed varied expression levels of TLR1-5, whereas low mRNA levels of TLR3 and TLR4 were present in NL-20. The expression of TLR2, TLR3, and TLR5 in Detroit-562 was confirmed with flow cytometric protein analyzes. NL-20 showed presence of both TLR2 and TLR3 proteins. Nod1 and Naip were consistently expressed in the two cancer cell lines as demonstrated by both mRNA and protein studies. HNEC showed a broader NLR profile with presence of all NLRs investigated, i.e. Nod1, Nod2, Nlrp1, Nlrp3, and Naip at both mRNA and protein level (Figure 5).

The expression of TLR2, TLR3, TLR5, Nod1, and Naip was corroborated in HNSCC biopsies with immunohistochemistry. These biopsies also showed an inconsistent expression of Nod2 and Nlrp3 (Figure 6).
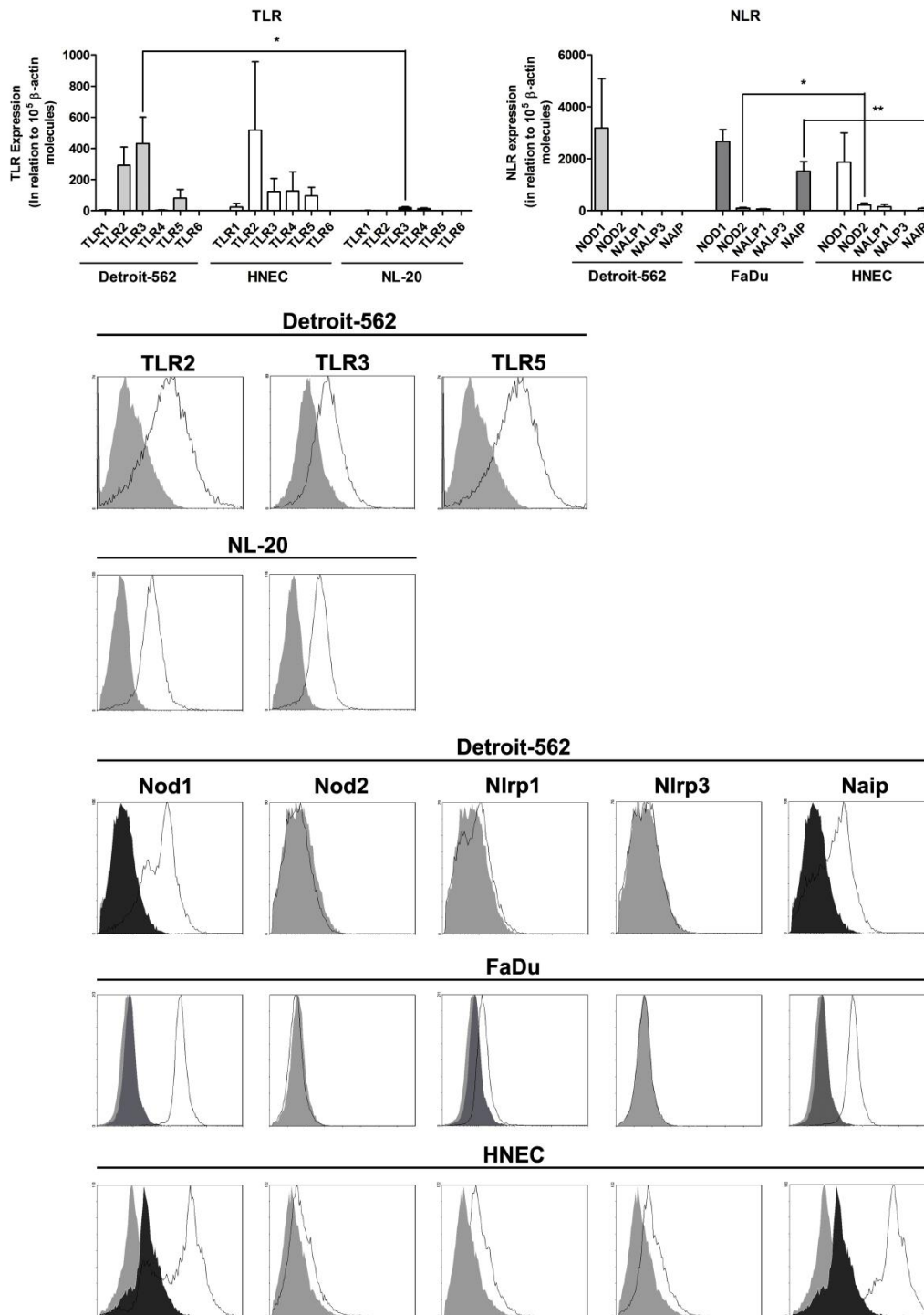
Figure 5. The TLR and NLR expression profiles in Detroit-562, FaDu, NL-20, and HNEC. The mRNA expression of TLR1-6, Nod1, Nod2, Nlrp1, Nlrp3, and Naip was investigated with real-time RT-PCR. Values are depicted in relation to the housekeeping gene β-actin $100\ 000 \times 2^{-\Delta CT}$, and presented as mean ± SEM. This was followed by protein analyses with flow cytometry. Open histogram represent antibodies against TLR2, TLR3, TLR5, Nod1, Nod2, Nlrp1, Nlrp3, and Naip, light grey is denoting the isotype control, and dark gray a secondary antibody used as an additional control for Nod1 and Naip. * $p \leq 0.05$; ** $p \leq 0.01$.
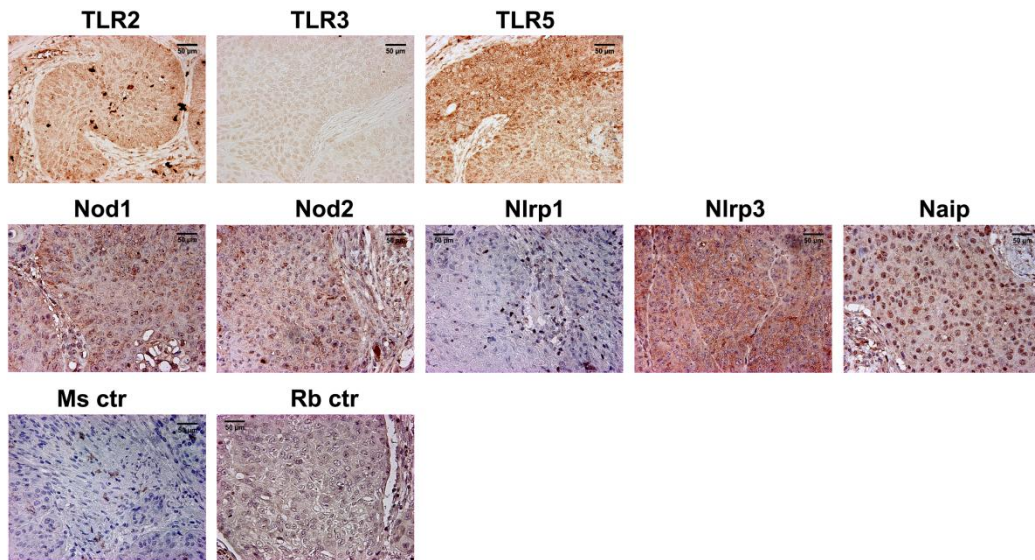
Figure 6. TLR and NLR expression in HNSCC biopsies. The protein expression of TLR2, TLR3, TLR5, Nod1, Nod2, Nlrp1, Nlrp3, and Naip was confirmed in HNSCC biopsies with immunohistochemistry. The NLR stained and control (ctr) sections were counterstained with haematoxylin (magnification 200X).

Specific NLR ligands exist for Nod1, Nod2, and Nlrp3, but since only Nod1 was found to be consistently expressed by the HNSCC cell lines the functional part of the NLR studies was focused on Nod1. Since TLR2, TLR3, and TLR5 were expressed by Detroit-562, their function was also examined. To this end, the cells were incubated with or without their cognate ligands Pam$_3$CSK$_4$, poly(I:C), flagellin, and iE-DAP. Tumor necrosis factor (TNF)-α was used as positive control. All ligands activated the HNSCC cell lines. They up-regulated the expression of ICAM-1 (Figure 7), and gave rise to specific cytokine profiles. Pam$_3$CSK$_4$, poly(I:C), and flagellin increased the secretion of IL-6 and IL-8. Poly(I:C) also induced the release of IL-1β. Nod1 activation generated an increased production of G-CSF and GM-CSF (Figure 8).
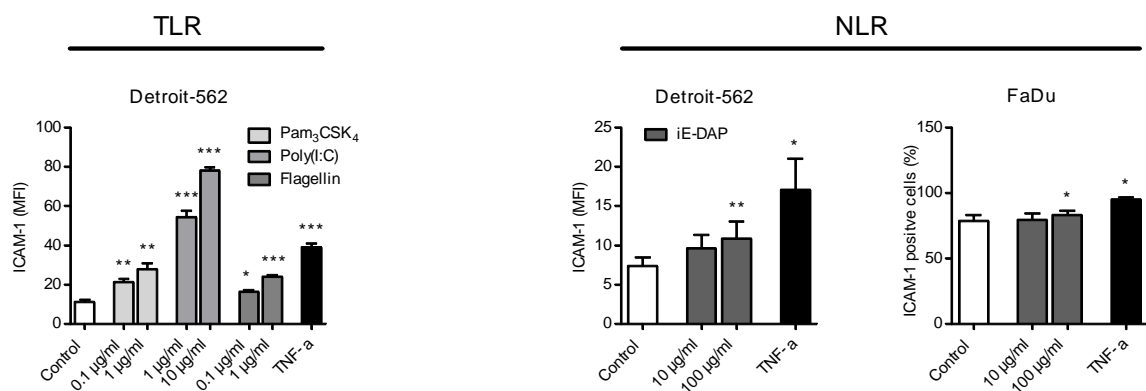

Figure 7. TLR2, 3, 5, and Nod1 stimulation induced the expression of ICAM-1 in the HNSCC cell lines. Detroit-562 was incubated with the TLR2, 3, 5, and Nod1 agonists, Pam$_3$CSK$_4$, poly(I:C), flagellin, and iE-DAP, respectively, and FaDu with iE-DAP for 24 h. The effects on ICAM-1 expression were examined with flow cytometry. MFI=mean fluorescence intensity; * p≤0.05; ** p≤0.01; *** p≤0.001.
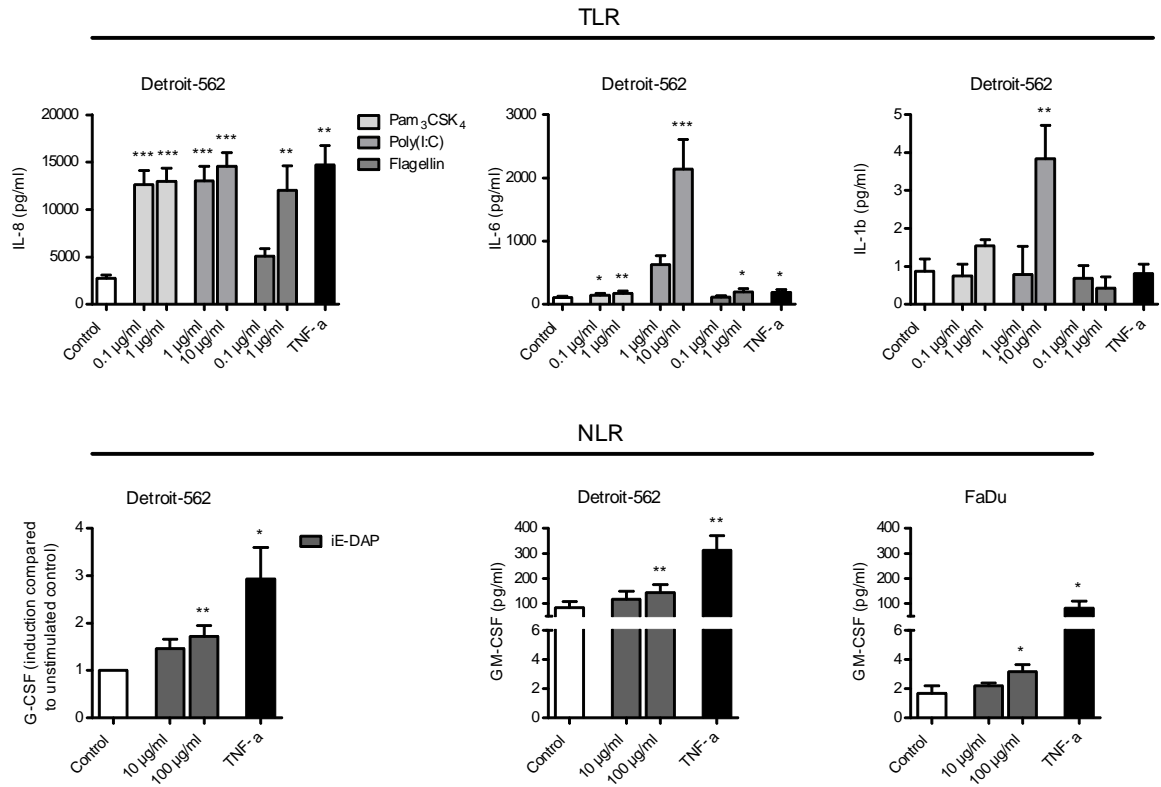
Figure 8. TLR2, 3, 5, and Nod1 stimulation induced specific cytokine secretion profiles in HNSCC. Detroit-562 was stimulated with Pam$_3$CSK$_4$, poly(I:C), and flagellin for 24 h, and Detroit-562 and FaDu were stimulated with iE-DAP for 24h. The cell culture free supernatants were then analyzed for the secretion of IL-8, IL-6, IL-1β, G-CSF, and GM-CSF with ELISA. * p≤0.05; ** p≤0.01; *** p≤0.001.

Poly(I:C) induced the strongest response with an enhancement of IL-1β, IL-6, and IL-8 secretion in HNEC, and IL-6 and IL-8 in NL-20 (Figure 9). The secretion of IL-6 was also increased in HNEC after stimulation with flagellin. No effects were observed after iE-DAP stimulation in the healthy cells.

TLR2, TLR3, and TLR5 activation decreased the viability of Detroit-562 cells, and accordingly a high amount of apoptotic and dead cells was seen. Corresponding effects were not seen in the healthy cells (Figure 10).
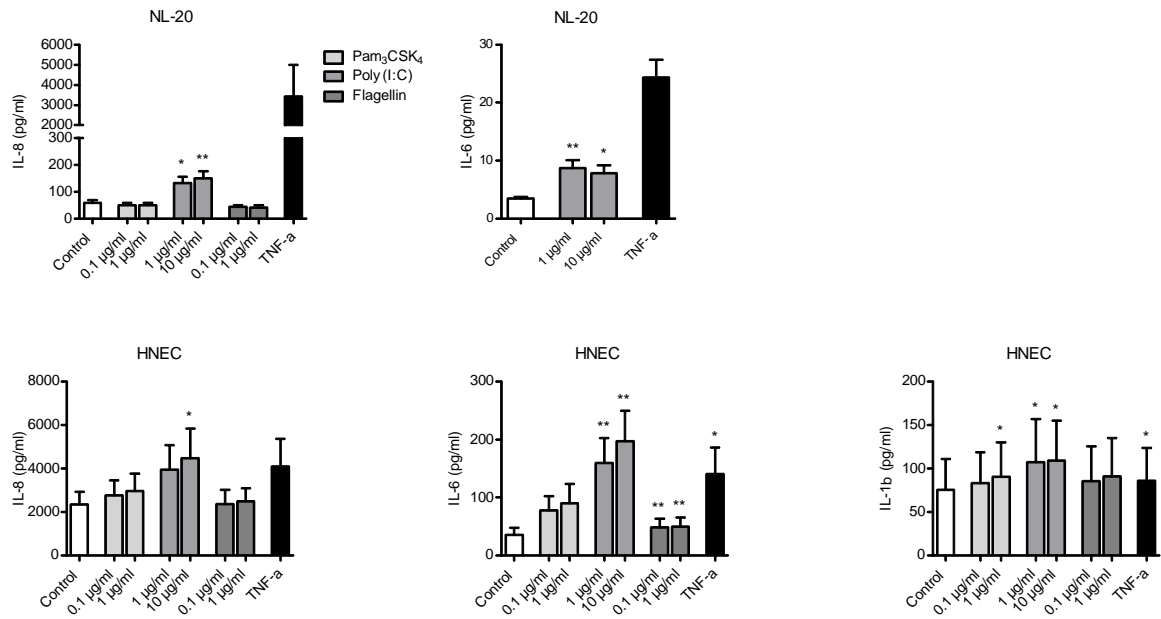
Figure 9. TLR induced cytokine secretion in healthy control cells. NL-20 and HNEC were incubated with Pam$_3$CSK$_4$, poly(I:C), and flagellin for 24 h, and the cell culture supernatants were analyzed for levels of IL-8, IL-6, and IL-1β. * p≤0.05; ** p≤0.01.
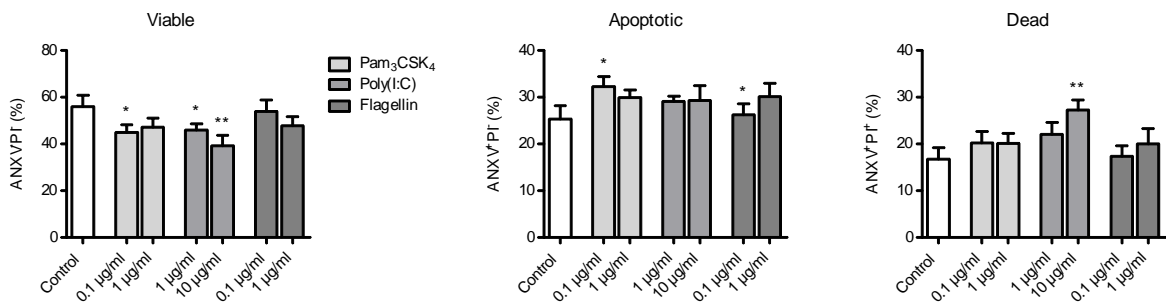

Figure 10. Decreased viability in Detroit-562 after TLR activation. Detroit-562 was incubated with Pam$_3$CSK$_4$, poly(I:C), and flagellin for 24 h, and analyzed for the percentage of viable (ANXV$^-$PI$^-$), apoptotic (ANXV$^-$PI$^+$), and dead cells (ANXV$^+$PI$^+$) with flow cytometry. * p≤0.05; ** p≤0.01.

COMMENTS

The present studies demonstrated the presence of TLR2, TLR3, TLR5, Nod1, and Naip in HNSCC. Ligand stimulation of TLR2, TLR3, TLR5, and Nod1 activated the HNSCC cells in a way that differed from what was seen in healthy cells. In addition, TLRs and Nod1 stimulation exhibited separate activation patterns.

The response to Nod1 activation in HNSCC cells was relatively modest, whereas the reaction to a corresponding TLR stimulation was more marked. Hence, the biological significance of the former stimulation could be questioned. However, even though the response of Nod1 to iE-DAP stimulation in HNSCC in terms of ICAM-1 expression and GM-CSF production was somewhat limited it was consistent in two different squamous

cell carcinoma cell lines, Detroit-562 and FaDu. This consistency strengthens the idea of Nod1 as an enhancer of the inflammatory response in tumorigenic cells.

The present studies further highlight the importance of TLR and NLR activation in cancer. TLR stimulation in HNSCC induced a robust inflammatory response in combination with a decreased survival of the tumor cells, thus showing anti-tumorigenic properties. NLR stimulation, on the other hand, induced a more pro-tumorigenic kind of inflammation. Different PRRs have been shown to induce anti-tumorigenic effects or pro-tumorigenic responses in the same tumor type (38, 72). TLR2, TLR3, and TLR5 appear to have mainly anti-tumorigenic properties in HNSCC, whereas TLR4 seems to promote the development of HNSCC (26). In addition, the same PRR receptor can exhibit different characteristics depending on the tumor type. For instance, we showed that Nod1 induces an inflammation that seems to have pro-tumorigenic effects, whereas Chen *et al* and da Silva Correia *et al* reported that Nod1 displays a protective role against development of colon cancer and estrogen-sensitive tumors (49, 50).

The immune system is important in the defense against tumors, but it may also have pro-tumorigenic properties. Depending on the activation and induction of the immune cells they will attain an anti- or pro-tumorigenic profile (73, 74). In the present investigation TLR induced a pro-inflammatory response in HNSCC with a potential to mobilize leukocytes, especially neutrophils, by the induction of IL-8. In **PAPERS III-IV**, we demonstrated that increased activation of neutrophils predicts better prognosis, and that an increased infiltration of CD16^{high} CD62L^{dim} neutrophils might account for this. Therefore, the TLR induced inflammation seen might be anti-tumorigenic in nature. IL-8 has been demonstrated to promote angiogenesis, which is important for the development of cancer (75, 76). In this case angiogenesis would further increase the amount of infiltrating neutrophils, especially the CD16^{high} CD62L^{dim} cells, supporting the theory of a TLR induced anti-tumorigenic response.

In contrast to the TLRs, Nod1 induced an inflammation characterized by an increase in G-CSF and GM-CSF, which are hematopoetic growth factors with angiogenic functions. The ability of these mediators to attract, and stimulate proliferation and maturation of granulocytes and macrophages are often used to ameliorate cancer therapy side effects (77, 78). However, GM-CSF has also been demonstrated to trigger the mobilization of immune suppressive CD34$^+$ cells that have the ability to impair the anti-tumor immune response. GM-CSF and G-CSF have also been associated with a poor HNSCC prognosis and with an ability to promote proliferation and migration of tumor cells (79-81). Hence, the Nod1 induced inflammation might be regarded as anti-tumorigenic.

This part of the result section demonstrates that HNSCC have altered their TLR and NLR expression and their functional responses to the corresponding ligands. This gives an impression that the HNSCC cells have exploited TLRs and NLRs with a somewhat contradictory outcome.

NEUTROPHIL INFLAMMATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (PAPERS III-IV)

RESULTS

HNSCC is known to cause immune suppression, but how the immune system is affected is not fully established. Patients with HNSCC have an altered neutrophil activation (82, 83), and accumulating evidence has indicated the existence of distinct neutrophil subsets with specific roles in cancer inflammation (67, 84). The presented investigations were designed to characterize different neutrophil subsets, and to evaluate their prognostic role in HNSCC.

HNSCC patients were found to have higher levels of leukocytes and neutrophils in blood than healthy controls. Consequently, the neutrophil/lymphocyte ratio was higher in the cancer patients compared to the control individuals, and a high ratio predicted worse prognosis (Figure 11).
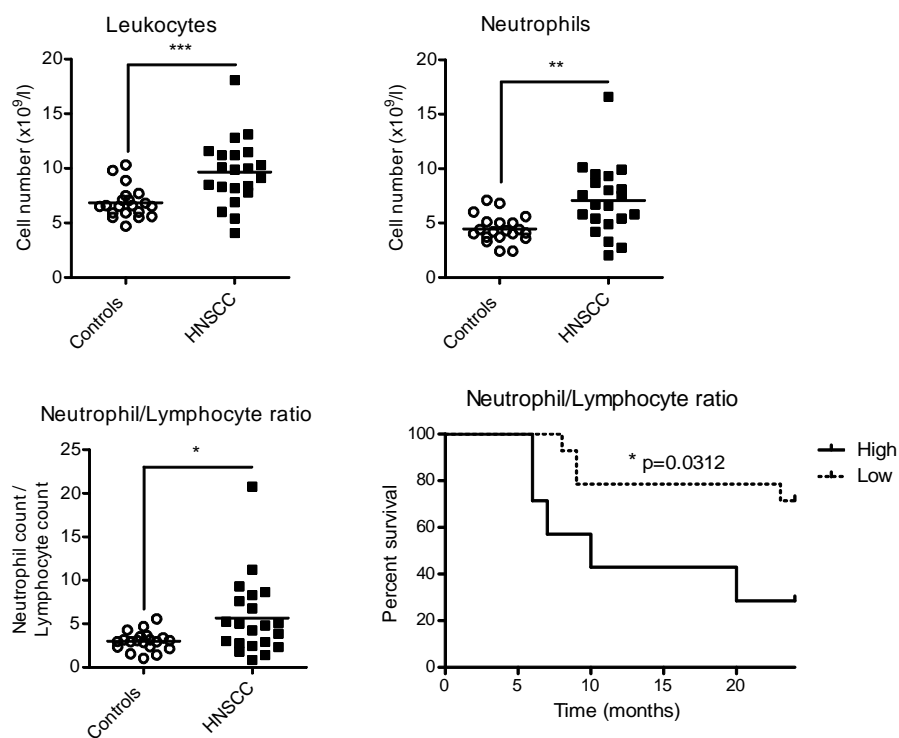

Figure 11. The number of total leukocytes, neutrophils, and the neutrophil/lymfocyte ratio in blood from HNSCC patients and healthy control individuals was determined with leukocyte differential count analysis. A Kaplan-Meier survival analysis following diagnosis of HNSCC patients, before start of treatment, was also performed. * p≤0.05; ** p≤0.01; *** p≤0.001.

Three different neutrophil subsets; CD16^{dim} CD62L^{high}, CD16^{high} CD62L^{high}, and CD16^{high} CD62L^{dim}, were found in the circulation of these patients. In comparison to the healthy controls, the HNSCC patients had a higher percentage of CD16^{high} CD62L^{dim} cells. The patients could be divided into two distinct groups; those with a high level and those with a more "normal" percentage of CD16^{high} CD62L^{dim} cells. The patients with a high percentage of CD16^{high} CD62L^{dim} neutrophils had a better prognosis than the patients with a more "normal" amount (Figure 12).
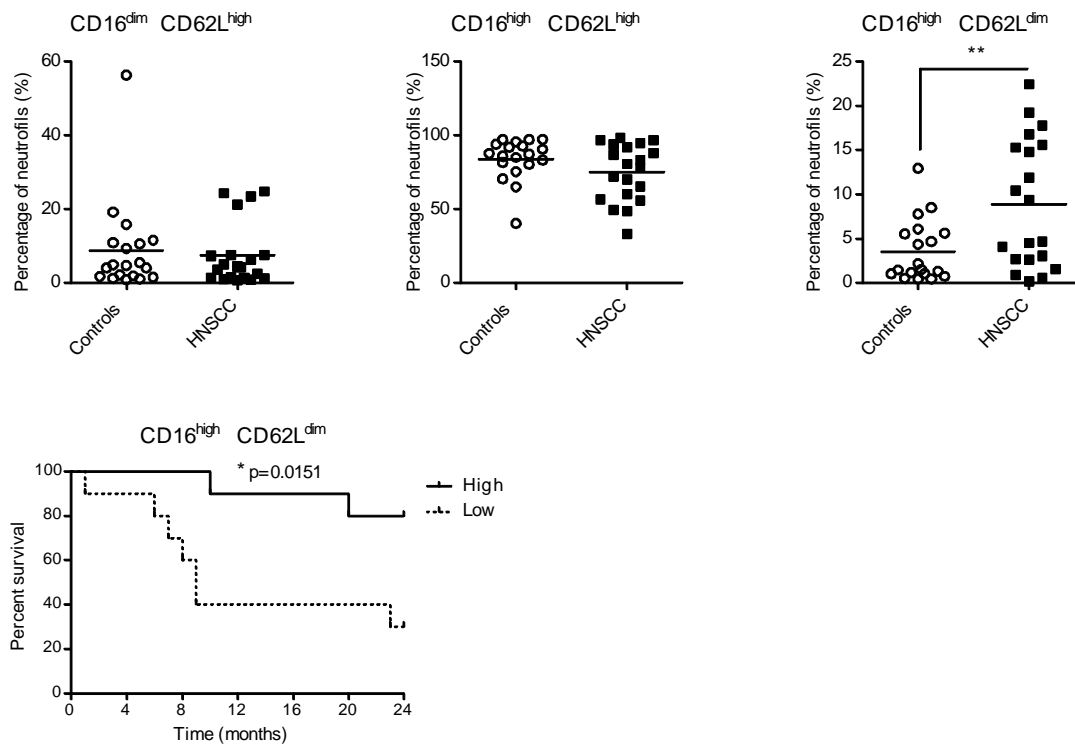

Figure 12. Increased CD16^{high} CD62L^{dim} neutrophils predict better prognosis in patients with HNSCC. The neutrophil subsets; CD16^{dim} CD62L^{high}, CD16^{high} CD62L^{high}, and CD16^{high} CD62L^{dim} cells, were identified with flow cytometry in blood from HNSCC patients and controls. A Kaplan-Meier survival analysis following diagnosis of HNSCC patients, before start of treatment, was also performed. * p≤0.05; ** p≤0.01.

It is also worth noticing that CD16^{high} CD62L^{dim} neutrophils had a higher CD11b and CD18 expression, and a decreased IL-8 level as compared to the CD16^{dim} CD62L^{high} and CD16^{high} CD62L^{high} cells (Figure 13).
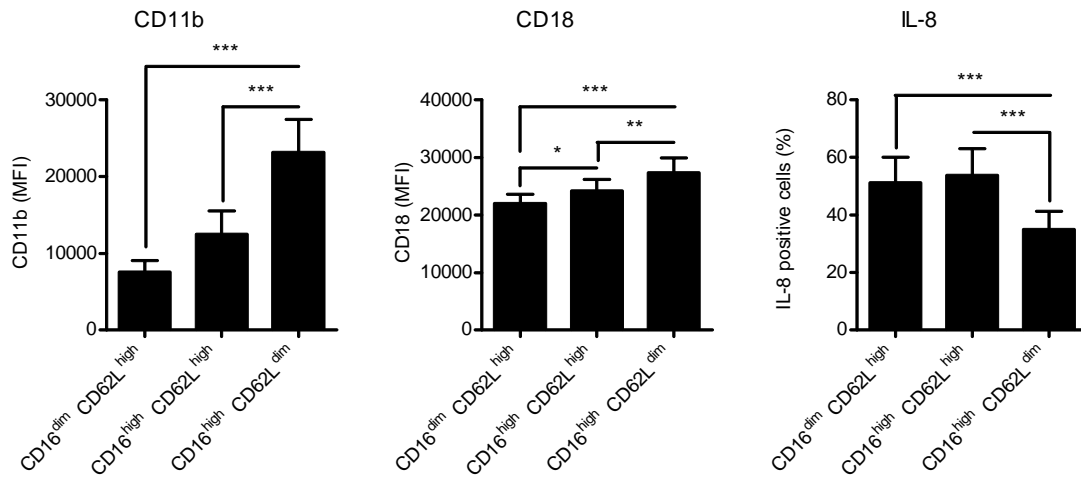
Figure 13. CD16^{high} CD62L^{dim} neutrophils displayed an active phenotype. The three different neutrophil subsets were characterized with flow cytometry upon the expression of CD11b, CD18, and IL-8 in blood obtained from HNSCC patients. * p≤0.05; ** p≤0.01; *** p≤0.001.

COMMENTS

The second part of this thesis focuses on the three different neutrophil subsets that were recently identified by Pillay *et al* (67). The increased number of leukocytes, neutrophils, and the high neutrophil/lymphocyte ratio indicate that there is an ongoing inflammation in patients with HNSCC. The neutrophil/lymphocyte ratio has been suggested to reflect an ongoing systemic inflammation (85). The increased inflammatory activity among the cancer patients seems to be related to an increase in neutrophil activation. However, it is widely debated whether the neutrophil inflammation is beneficial or of disadvantage for the patient. Regardless, inflammation is considered to be an important factor in the defense against tumors. T cells and the adaptive branch of the immune system have the ability to identify and destroy malignantly transformed cells.

The neutrophil/lymphocyte ratio has been suggested to be a prognostic factor for various cancers. The ratio *per se* reflects an elevated number of neutrophils, and consequently an increase in neutrophils seems to be related to a poor prognosis (86-89). Generally the elevated neutrophil numbers is thought to reflect an increase in the amount of immature neutrophils (90). This may not be true for HNSCC. In the present study, an elevated percentage of activated neutrophils, CD16^{high} CD62L^{dim} cells, were detected in the cancer patients and correlated to a better prognosis. Pillay *et al* suggested that the CD16^{high} CD62L^{dim} subset causes immunosuppression by suppressing T cell proliferation (67). There are clinical studies demonstrating that high numbers of tumor infiltrating and blood neutrophils is associated with poor prognosis (60, 61). At the same time, there is evidence that neutrophils have anti-tumorigenic properties (91-93). It has been proposed that activated neutrophils can elicit anti-tumorigenic activity. This agrees with our studies showing that activated CD16^{high} CD62L^{dim} neutrophils, demonstrated by a decreased CD62L expression, a reduced IL-8 level, and an increased Mac-1 complex, were

35

associated with an increased survival of HNSCC patients. In addition, the Mac-1 complex, i.e. CD11b and CD18, has previously been demonstrated to be of importance for the tumor cytolytic function of neutrophils (92).

Altogether, this emphasizes the importance of neutrophils and neutrophil inflammation in patients with HNSCC. Activated neutrophils seem to be beneficial for the survival, perhaps as a reflection of their anti-tumorigenic properties. This also signifies the tight link between the immune phenotype, the anti-tumorigenic immune response, and the survival of the patients.

IMMUNE REACTIONS IN ALLERGIC RHINITIS VERSUS HEAD AND NECK SQUAMOUS CELL CARCINOMA (PAPER V)

RESULTS

The information about immunologic differences between allergy and cancer is scarce. Several epidemiological studies have investigated the relation between these two diseases with contradicting results. The present study was designed to investigate and compare immune responses induced by AR and HNSCC.

Sera from patients with HNSCC exhibited a slight increase, although not significant, of innate related cytokines like IL-1β, IL-17, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β as compared to sera from AR patients and healthy controls. In addition, IL-7 was increased in sera from HNSCC patients compared to sera from healthy individuals. As expected, sera from allergic patients displayed elevated levels of Th2 cytokines like IL-5 and IL-13 compared to cancer patients and controls. Sera from AR patients also showed lower levels of IL-1β compared to healthy controls. Increased levels of the T cell related cytokine IL-7 as well as a tendency towards an elevated amount of Th1 cytokines like IL-12 and IFN-γ were also seen among the allergic patients (Figure 14).
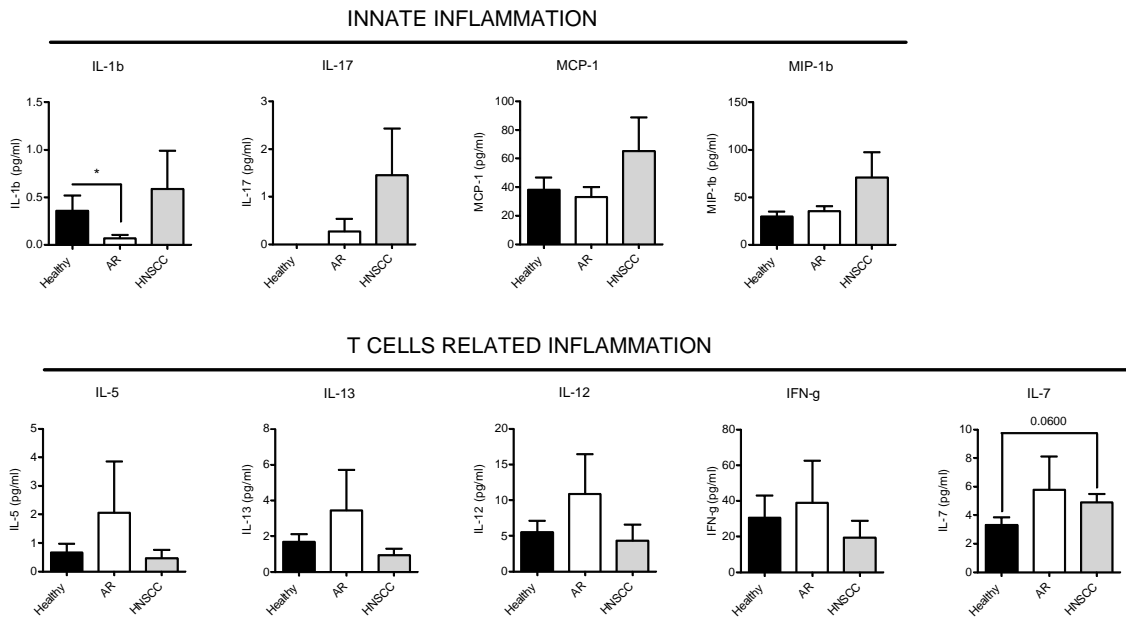
Figure 14. Cytokine profiles in sera from HNSCC patients, AR patients, and controls. Sera was obtained from patients with HNSCC, AR, and healthy controls, and analyzed with Luminex Multiplex Immunoassay for cytokine levels. * p≤0.05.

Isolated PMN from AR, HNSCC, and healthy control patients were stimulated with or without the TLR2, TLR4, TLR7, and TLR9 ligands, Pam3CSK4, LPS, R837, and CpG, respectively. PMN from HNSCC patients displayed a higher basal secretion of IL-8 and generally responded stronger to TLR stimulation, demonstrated by an enhanced expression of CD11b and CD69, than PMN from both allergic patients and controls (Figure 15).
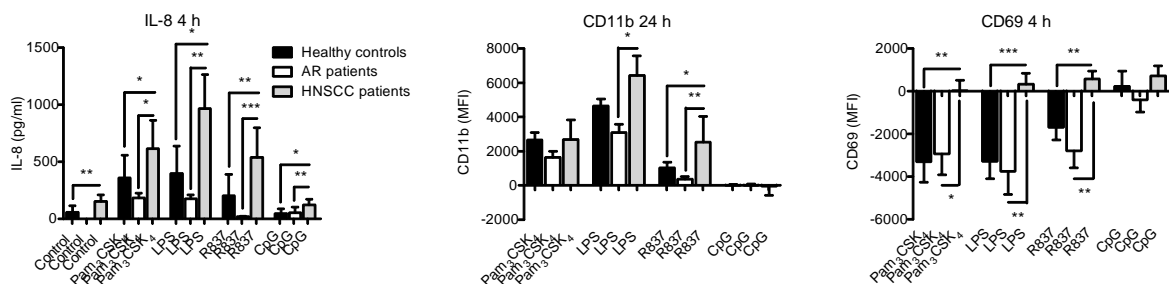
Figure 15. Increased PMN activation in patients with HNSCC. PMN were isolated from blood obtained from patients with HNSCC, AR, and controls, and stimulated with or without Pam3CSK4 (1 μg/ml), LPS (1 μg/ml), R837 (5 μg/ml), or CpG (0.3 μM) for 4 and 24 h. The cell culture supernatants were analyzed for the release of IL-8 with ELISA, whereas the cells were investigated for the expression of CD11b and CD69 with flow cytometry. MFI=mean fluorescence intensity; * p≤0.05; ** p≤0.01; *** p≤0.001.

When PBMC were stimulated with the same ligands as PMN, PBMC from HNSCC patients demonstrated a lower T cell activation than PBMC from AR patients and controls. This was seen as a diminished expression of CD25 and CD98 in combination with a reduced secretion of cytokines like IL-4, IL-7, and IL-12. In contrast, PBMC from allergic patients showed a high secretion of the same T cells related cytokines. A tendency towards an increased basal secretion of these cytokines was also revealed among the rhinitis patients (Figure 16).
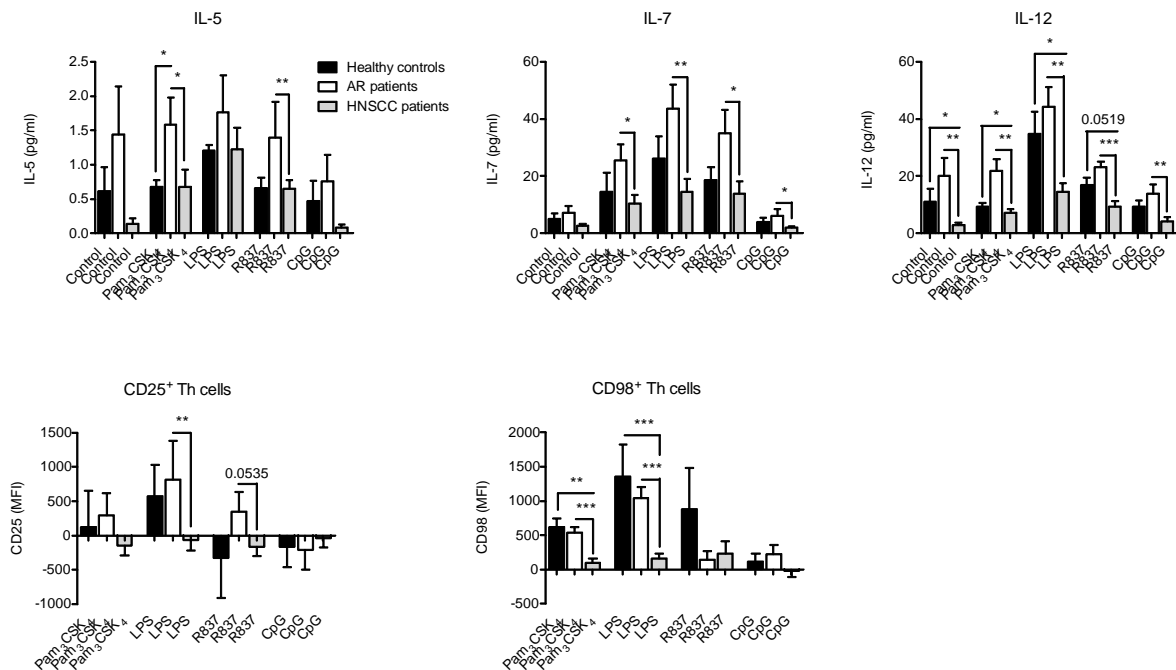
Figure 16. Decreased PBMC activation in HNSCC patients. PBMC were isolated from blood collected from patients with HNSCC, AR, and healthy individuals, and cultured with Pam_3CSK_4 (1 μg/ml), LPS (1 μg/ml), R837 (5 μg/ml), or CpG (0.3 μM) for 24 and 72 h. The Th cells were examined for the expression of CD25 and CD98 with flow cytometry, and the secreted cytokine profile was investigated in cell culture supernatants with Luminex Multiplex Immunoassay. MFI=mean fluorescence intensity; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

HNSCC biopsies and nasal biopsies from healthy individuals and AR patients, outside and during pollen season, were collected and investigated for the expression of Nod2 mRNA. Cancer biopsies showed a higher Nod2 mRNA expression than nasal biopsies from controls, and allergic patients both outside and during pollen season. No differences were found between AR patients and healthy controls (Figure 17).

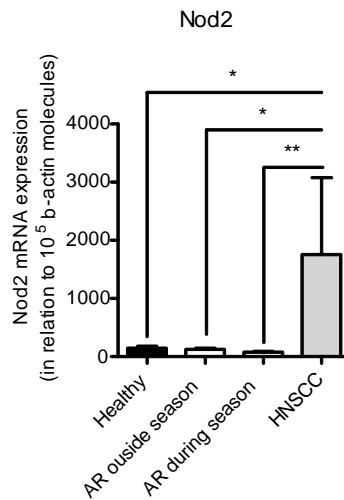**Figure 17.** High Nod2 expression in HNSCC biopsies. Nod2 mRNA expression in HNSCC biopsies and nasal biopsies from healthy controls and AR patients outside and during pollen season. The Nod2 mRNA expression was investigated with real-time RT-PCR. Values are depicted in relation to the housekeeping gene β-actin 100 000 × $2^{-\Delta CT}$, and presented as mean ± SEM. * p≤0.05; ** p≤0.01.

COMMENTS

AR and HNSCC patients were characterized by distinct immunological reactions. HNSCC exhibited a dominant innate immune response with suppressed T cells, whereas the AR immune reactions were of an adaptive character with enhanced T cell activity.

Previous reports regarding the association between allergy and cancer are inconsistent. This might partly be related to variations between tissues. Allergies occur primarily at body surfaces and for that reason the relation to cancer should be made to tumors with a corresponding location (14, 15). AR and HNSCC are both diseases with a systemic inflammatory component that affect the upper airway. In the present study HNSCC patients were sampled upon detection of their disease prior to initiation of treatment. This is often when the tumor disease is most active. For the same reason, the allergic individuals were sampled during pollen season.

AR is a chronic lymphocyte mediated inflammatory condition traditionally characterized by increased Th2 activity (94). Generally, chronic inflammation is thought to constitute a risk factor for the development of cancer (73, 74), and cancer is also thought to have a dominating Th2 component that suppresses the anti-tumorigenic response of the adaptive immune system (95, 96). Therefore, it was interesting to find that HNSCC is characterized by an innate immune reaction with suppressed T cells, while allergy was confirmed to be a lymphocyte mediated inflammatory disease. However, the finding of both Th1 and Th2 cytokines in sera and PBMC culture supernatants of AR patients supports recent ideas that allergy is a complex disease, and not only characterized by an imbalance in the Th1 and Th2 activity (97). Therefore, the perspective that the Th2 dominated reaction in allergy would suppress the Th1 response, and thereby inhibit the

anti-tumor immunity does not seem to hold true. A strong adaptive response is a prerequisite for a proper defense against tumors. Consequently, the enhanced adaptive immune response that was found in the allergic individuals might rather have a protective role against development of HNSCC. This also further emphasized that the nature of the immune response evoked will determine the efficacy of the tumor defense.

In addition to the differences in the immune responses of circulating leukocytes, disparity was also found for the Nod2 expression between nasal epithelial cells from AR patients and HNSCC cells. We have previously reported that a reduced expression of Nod1 was detected in nasal biopsies from patients with ongoing allergy compared to controls (98), whereas we showed that there is a tendency towards an increased Nod1 expression in HNSCC biopsies compared to nasal biopsies from healthy controls (**PAPER II**). However, no real comparison can be made between these two studies since mRNA was assed with primers in the AR study and probes in the HNSCC investigation. Taken together, this suggests a distinguishing role of NLRs in airway allergy and cancer. It also further stresses that there may not only be systemic differences between these two diseases, but local disparities as well.

SUMMARY AND CONCLUSIONS

- HNSCC cells exhibited a more marked expression of TLR2, TLR3, and TLR5 than normal cells. Stimulation of Detroit-562, an epithelium derived cancer cell line, with TLR2, TLR3, and TLR5 agonists resulted in an up-regulation of ICAM-1, increased secretion of IL-1β, IL-6 and IL-8, and decreased viability. In addition, the activation of TLR3 also affected the migratory behavior of the cancer cells. The non-tumorigenic cell line NL-20 and the primary HNEC did not display the same robust inflammatory response and cell death as the cancer cells did. This suggests a dual action of the TLR agonists in HNSCC – as immune stimulators and as apoptosis inducers. It also emphasizes the TLR system as an important target in future anti-tumor immunotherapy.

- The NLR expression in HNSCC cells differed from what was seen in healthy nasal epithelial cells. Nod1 and Naip were the only investigated NLRs that were consistently expressed in the airway epithelial cells, and since there are no ligands available for Naip, only Nod1 could be functionally assessed. Like the expression, the functional response to Nod1 stimulation in HNSCC was found to differ from the reactions obtained in normal epithelial cells. Nod1 increased the production of β-defensin 2, GM-CSF, and G-CSF, and up-regulated ICAM-1 in malignant cells, with no corresponding response in normal cells. This indicates that Nod1 has the ability to enhance the migration of immunosuppressive myeloid cells into the tumor. If so, it implies that airway bacterial infections might enhance the tumor-induced inflammation that in turn may have immunosuppressive properties.

- HNSCC patients displayed an increased amount of total leukocytes, neutrophils, and monocytes, and a higher neutrophil/lymphocyte ratio than control subjects. An enhanced percentage of activated T cell subsets and NK cells, as determined by an elevated CD69, CD71, and CD98 expression was also observed among the cancer patients. In addition, CD14^{high} CD16^{+} monocytes, and neutrophils from HNSCC patients displayed a low expression of CD62L. A high activation frequency of total T cells, Th cells, NK cells, and monocyte populations could be correlated with a more severe disease. Further, the neutrophil/lymphocyte ratio and the activation state of neutrophils, CD14^{high} CD16^{+} monocytes, and Th cells at the time of cancer diagnosis could be linked to the life expectancy. The increased systemic inflammation seen among HNSCC patients signifies a connection between the immune phenotype, the anti-tumor immune response, and the survival of the patient.

- Three different neutrophil subsets characterized as CD16^{dim} CD62L^{high}, CD16^{high} CD62L^{high}, and CD16^{high} CD62L^{dim}, were identified in the blood from HNSCC patients. The number of CD16^{dim} CD62L^{high} cells was more pronounced among the cancer patients than the controls, and the cancer patients could be divided into two groups. Patients with a high percentage of CD16^{high} CD62L^{dim} neutrophils seemed to have a better survival than those with a more normal level. It is also worth noticing that CD16^{high} CD62L^{dim} neutrophils constitute a specific phenotype characterized by high CD11b and CD18 expression, and low IL-8 production. The CD16^{high} CD62L^{dim} cells were also found to be more prone to migrate than the other neutrophils, and IL-8 appeared to be involved in the transformation of neutrophils into CD16^{high} CD62L^{dim} cells. The three neutrophil subgroups appeared to correlate to specific variations in the cellular activation. Patients with a high amount of CD16^{high} CD62L^{dim} cells in the circulation also seemed to have more neutrophils migrating into the tumor, and thereby benefiting from a more marked anti-tumorigenic immune response.

- Patients with AR showed a general trend towards an increase in Th1 and Th2 cytokines in serum. The same phenomenon was seen in supernatants from cultured PBMC. This signifies an adaptive immune reaction. The corresponding trend in HNSCC patients was dominated by innate immune cytokines like IL-1β, IL-17, MCP-1, MIP-1β, and G-CSF. PMN isolated from cancer patients showed a generally increased basal activation. These PMN also responded strongly to TLR stimulation by up-regulating the expression of CD11b, CD69, and increasing the secretion of IL-8. Hence, upper airway allergy and cancer seems to depict two distinct immunological events. The tumorigenic immune response is dominated by an innate immune reaction, and by suppressed T cells, whereas the allergic immune reaction is of a more adaptive nature characterized by an enhanced T cell activation. It is therefore tempting to propose that the enhanced systemic adaptive immune response seen among patients with AR might protect against development of HNSCC

FUTURE PERSPECTIVES

TLRs are located at the cell surface and in endosomes, whereas NLRs are found in the cytosol. The different locations make it tempting to assume that these receptors complement each other to ensure an effective clearance of pathogens. In this thesis, we show that TLRs and NLRs cause separate immunological reactions in HNSCC. In addition, concomitant activation of these two receptor families did not induce any synergistic response in HNSCC cells (unpublished observation).

In **PAPER I**, it was described that TLR stimulation can induce apoptosis of HNSCC cells and increase inflammation by secretion of cytokines like IL-8. In **PAPER IV,** it was shown that an elevated amount of CD16^{high} CD62L^{dim} neutrophils can predict better survival of patients with HNSCC. Altogether the presented data makes it tempting to speculate in a connection between TLR induced increase in IL-8 and an enhancement of CD16^{high} CD62L^{dim} neutrophils in blood and tumors. If such a relation exists it would strongly support the idea of anti-tumorigenic effects of TLR stimulation.

TLRs have been shown to possess an ability to generate anti-tumorigenic activities in many forms of cancer. Ligands of this receptor family are presently undergoing clinical investigations for their immunostimulatory effects as adjuvants in vaccines (38, 99). The wide spread activation associated with these receptors, including their potential effect on tumor cells, makes it necessary to proceed with caution when implementing these drugs for therapeutic use. Hence, activation of the TLR system in cancer cells can cause both anti- and pro-tumorigenic responses (26, 100).

The role of neutrophil migration and activation in cancer inflammation was evaluated in **PAPERS III-V**. We found that the tumor influences the activation of neutrophils (**PAPER V**). This finding was not surprising since HNSCC tumors are known to dictate the immune reaction in its favor (101). Nevertheless, in **PAPER IV** we showed that a high percentage of activated CD16^{high} CD62L^{dim} neutrophils correlated with a better survival. If a favorable neutrophil phenotype is the result of an inborn ability to muster an innate immune response by the host or if this relates to the characteristics of the tumor per se remains to be evaluated.

It is well known that the adaptive branch of the immune system is needed to launch a correct anti-tumorigenic immune reaction. However, the present studies show that innate immune responses seem to be equally important for the host, by causing neutrophil activation and migration. Even though the innate immunity is dominant in HNSCC, it is important to acknowledge the complexity of the immune system. The immune system should not be looked upon as two different compartments in the immunological reaction,

but rather be regarded as an interlaced system. The right proportion of activation of the different parts of the immune system is probably the best defense against tumors.

Tumor biopsies from HNSCC patients displayed a higher Nod2 mRNA expression than nasal biopsies from both healthy controls and rhinitis patients outside and during pollen season. This suggests a strong local component of the PRR system in cancer to add to the more extensively evaluated systemic differences between allergic and cancer inflammation. Further, this raises questions about what other local inflammatory differences could be found in the mucosa of AR patients and HNSCC tumors.

The thesis highlights the importance of a more thorough immunologic characterization of HNSCC. Such endeavors would not only increase the prognostic capacity, but also open up for individualized interventions in order to create a more favorable form of cancer inflammation. In this context it would be especially interesting to explore the anti-tumorigenic effects of CD16^{high} CD62L^{dim} neutrophils, and its benefits for the survival of patients with HNSCC.

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APPENDICES