Toll-like Receptor Interference in Myeloid Dendritic Cells through Head and Neck Cancer

HENNING FRENZEL[1*], BETTINA HOFFMANN[1*], CARSTEN BROCKS[1], PETER SCHLENKE[2], RALPH PRIES[1] and BARBARA WOLLENBERG[1]

[1]*Department of Otorhinolaryngology, University of Schleswig-Holstein Campus Lübeck, 23538 Lübeck;*
[2]*Institute of Immunology and Transfusion Medicine, University of Lübeck, 23538 Lübeck, Germany*

Abstract. *Background: In head and neck squamous cell carcinoma (HNSCC) the secretion of various immunosuppressive mediators contributes to large-scale effects on the immune functions. The influence of HNSCC on the Toll-like receptor (TLR) expression profiles of human myeloid dendritic cells (MDCs), which have been identified in human solid tumor tissue of the head and neck, was analysed. Materials and Methods: MDCs were isolated from peripheral blood by 'magnetic bead separation' and subsequently incubated with supernatants of HNSCC permanent cell lines. TLR expression profiles were investigated using flow cytometry and SDS-PAGE. Results: Human MDC from peripheral blood were found to express all human TLRs except TLR4 and TLR9. Incubation of MDC with supernatants of HNSCC resulted in an increased expression of TLR7. Conclusion: The data suggest an interference of TLR7 expression through HNSCC and provide novel evidence of TLR alterations as a potential tumor promoting event in head and neck cancer.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common tumor, occurring almost exclusively among middle-aged tobacco and alcohol abusers (1). Despite innovative surgery, new radiation strategies and the development of novel chemotherapeutic drugs, the overall 5-year survival rate has remained poor and virtually unchanged during the past few decades (2, 3).

Previous studies have reported that the cells of head and neck cancer produce various immunosuppressive mediators which contribute to large-scale effects on the immune functions and allow an escape from efficient antitumor immune responses (3-5).

The innate immune system builds up the host defence against a huge diversity of pathogens which are recognized by the so-called pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) (6). TLRs are proteins located on a variety of immune cells and stromal cells that traverse cell membranes. In humans, 10 different TLRs have been discovered which recognize and bind specific molecules on foreign pathogens which can be subdivided into different groups with respect to their phylogenetic relations (Figure 1) (7). Of special interest are those TLRs located on innate immune cells, such as macrophages and dendritic cells (DCs), which trigger the release of pro-inflammatory cytokines that attract additional immune cells from the blood. Thus, TLRs represent a critical link between innate and adaptive immune responses (8, 9). This complex network requires a well tuned regulation as malfunctions are thought to be involved in distinct aspects of oncogenesis, as well as in HNSCC (10).

Initiation of primary immune responses requires proper function of DCs, which are CD34+ bone marrow-derived leukocytes and most potent among antigen-presenting cells (11-13). DCs, as well as other types of immune cells, are known to infiltrate human solid tumor tissues of HNSCC, but their cellular functions are strongly affected. It has been shown that tumor cells down-regulate the antigen-processing machinery in DCs generated from peripheral blood monocytes (14-16).

The influence of HNSCC on TLR expression profiles of human myeloid dendritic cells (MDCs) was analyzed. Since monocytes-derived MDCs show significant deviations compared to *in vivo* maturated MDCs, the cells were freshly isolated from peripheral blood of healthy donors (17, 18).

The aim of this work was to analyze the TLR protein levels of MDC from peripheral blood, as well as the influence of HNSCC on these expression profiles.

*Both authors contributed equally to this work.

Correspondence to: Barbara Wollenberg, UK-SH, Klinik für HNO, Ratzeburger Allee 160, 23538 Lübeck, Germany. Tel: +49 451 500 2241, Fax: +49 451 500 2249, e-mail: Barbara.Wollenberg@hno.uni-luebeck.de

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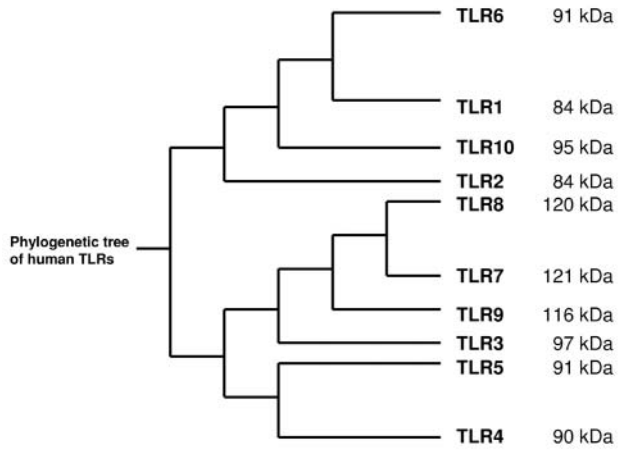

Figure 1. *Phylogenetic tree of human Toll-like receptors (TLRs). The diagram shows the protein sizes of the different TLRs and illustrates their phylogenetic relations with respect to protein homologies.*

Materials and Methods

Isolation of MDC. MDCs were isolated from human peripheral blood (PBMC, buffy coats) provided by the blood bank of the University Hospital Lübeck, Germany. Blood donors (range 18-65 years of age) were healthy donors who were tested negative for allergies. Additional exclusion criteria were manifest infections during the previous 4 weeks, fever, or medication of any kind. PBMCs were obtained from buffy coats by Ficoll-Hypaque density gradient centrifugation, as described previously (19). MDCs were isolated by magnetic bead separation using magnetic labeled anti-BDCA-1 antibodies (Miltenyi, Bergisch Gladbach, Germany). Isolated cells were characterized by flow cytometry, as described previously (20).

Cell culture and preparation of supernatants. Permanent HNSCC cell lines BHY (DSMZ, Germany, (21)) and PCI-1 (Hypopharyngeal cancer, Pittsburgh Cancer Institute) were cultured in DMEM (Dublecco's Modified Eagle Medium; Gibco, MD, USA) supplemented with 10% fetal calf serum (FCS), 1 mM glutamine and 0.1 mM sodium pyruvate. Cell-free supernatants were collected by centrifugation and filtration after 48 h of cell cultivation.

Flow cytometry. Surface antigen staining was performed, as described previously (22). Cells were stained with fluorescein-5-isothiocyanate (FITC-), phycoerythrin- (PE), peridinin-chlorophyll-protein- (PerCP) conjugated antibodies by incubation on ice for 15 min followed by washing with phosphate-buffered saline (PBS). Fluorescence-labeled monoclonal antibodies against CD11c, Lin-markers and HLA-DR were purchased from BD Biosciences (Becton Dickinson, Heidelberg, Germany). Fluorescence-labeled, as well as purified antibodies against human TLR1-10 were purchased from Biomol, Germany, and eBioscience, USA, respectively.

Samples were analyzed on a FACSCanto (BD Biosciences) and data acquisition was performed using the FACS DIVA software (BD Biosciences). The vital dye propidium iodide (PI) was used in conjunction with Annexin V-FITC staining to identify dead cells or cells which are in an early apoptotic stage (Annexin-V-FITC positive, PI negative).

Protein analysis. Cell extracts were prepared and solubilized, and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) with bovine serum albumin (BSA) as a standard. Aliquots of protein extracts (20 μg) were run on 10% acrylamide SDS-PAGE and then transferred to a nitrocellulose membrane. Specific antibodies against human TLR1-10 were purchased from Biomol, Germany, and eBioscience, USA.

Results

Isolation and immunophenotyping of MDCs. Human MDCs make up about 0.5 to 1.0% of the total cells in human peripheral blood and show a monocytoid morphology. MDCs express CD11c, HLA-DR and CD1c (human blood dendritic cell antigen-1, BDCA-1) and are negative for lineage markers (CD3, CD16, CD19, CD20, CD56).

The scale of molecular investigations on human myeloid dendritic cells is restricted by the limited proportion of this cell type and, therefore, requires efficient isolation procedures (23). MDCs from human peripheral blood of healthy donors were isolated by magnetic bead separation using magnetic labeled anti-BDCA-1 antibodies. Apart from MDCs, CD1c (BDCA-1) is also expressed on a subpopulation of CD19+ small resting B lymphocytes. Therefore, CD19 MicroBeads were used for the depletion of B cells before enriching BDCA-1+ MDCs. Light-microscopy was used to determine cell numbers, as well as to analyze the viability of the isolated cells by trypan-blue staining of dead cells. After isolation the cells were examined by flow cytometry using forward-scatter (FSC) and sideward-scatter (SSC) properties and the expression of characteristic surface antigens was analyzed. MDCs were identified by their lack of lineage (lin) markers, as well as their expression of HLA-DR and CD11c, as described previously (20, 24) (Figure 2).

TLR expression by human MDCs. Most investigations examining the TLR expression profiles on DC subsets have been done using RT-PCR approaches, due to the lack of available TLR antibodies. Since suitable antibodies are now available, the protein expression levels for TLR1-10 in human myeloid dendritic cells were analyzed using western-hybridization experiments, as well as flow cytometry. Our investigations revealed detectable expression levels of all human TLRs except TLR4 and TLR9 (summarized in Table I). No significant expression levels of these two TLRs could be found. These data corroborate previous RT-PCR based studies, although the expression of TLR7 has been controversially discussed. In some studies, TLR7 expression was detected on both plasmacytoid (PDC), as well as myeloid (MDC) dendritic cells, whereas others found TLR7 exclusively on PDCs (25-28).

Influence of HNSCC on TLR expression. In order to analyze the influence of the HNSCC microenvironment on TLR expression profiles of human MDCs, isolated myeloid

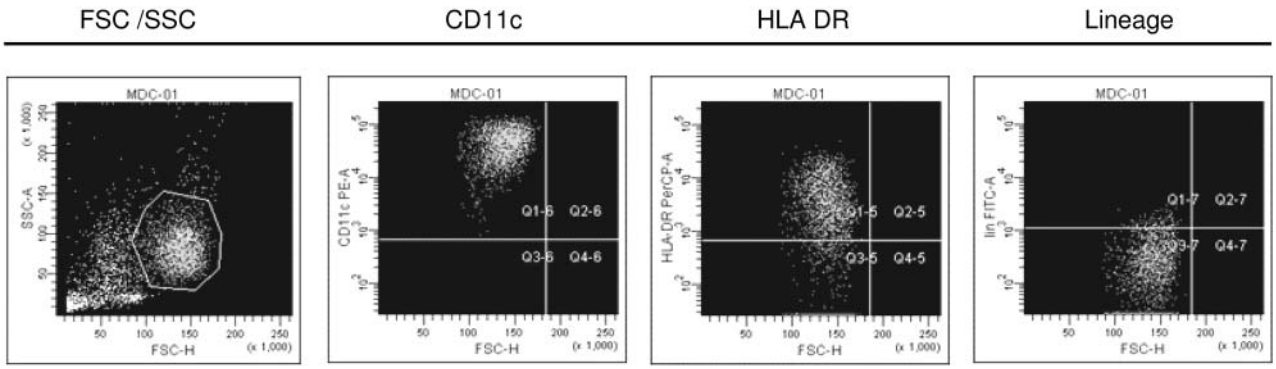

Figure 2. *Flow cytometric identification of isolated myeloid dendritic cells (MDC). MDCs are characterized as a population of lineage-negative, as well as HLA-DR- and CD11c-positive cells.*

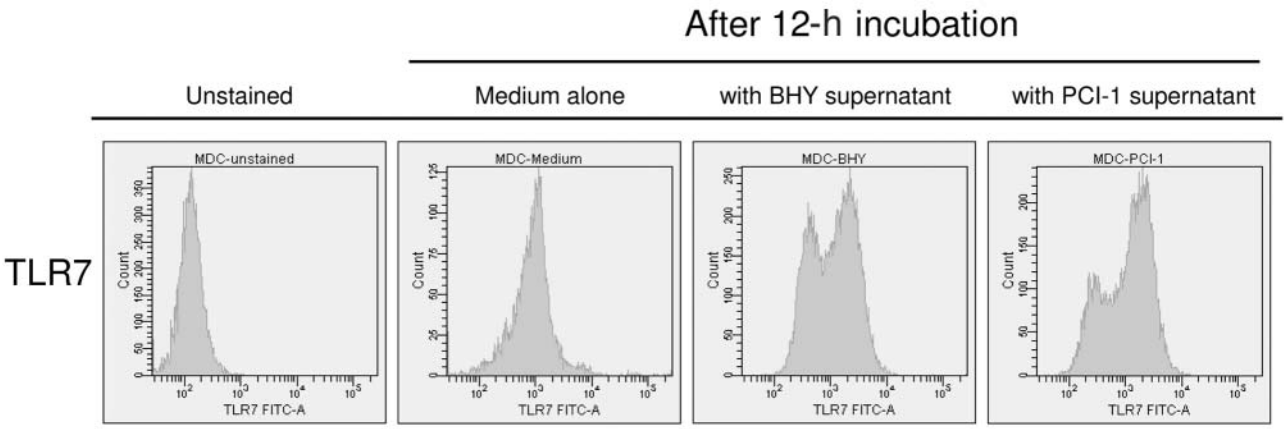

Figure 3. *Flow cytometric analyses of TLR7 expression in human MDC in the absence and presence of supernatants from HNSCC permanent cell lines BHY and PCI-1, respectively.*

Table I. *Toll-like receptor (TLR) expression profile by human MDC from peripheral blood.*

TLR1	TLR2	TLR3	TLR4	TLR5
+	+	+	–	+
TLR6	TLR7	TLR8	TLR9	TLR10
+	+	+	–	+

dendritic cells were incubated with supernatants of the permanent HNSCC cell lines BHY and PCI-1 for 12 h at 37˚C. Flow cytometric analysis revealed an increased expression of TLR7 on MDCs in response to HNSCC supernatants (Figure 3). No significant expression alterations of TLR1-6 and TLR8-10 could be detected. Our results demonstrate an increased TLR7 expression on the majority of analyzed MDC (Figure 3).

In addition to flow cytometric analyzes, western-hybridization experiments were carried out to investigate the overall TLR expression levels of human MDCs. Our results confirm the flow cytometrically observed interference of TLR7 expression through HNSCC supernatants. As shown in Figure 4, increased TLR7 protein expression could be detected after 12 h of incubation with supernatants of the HNSCC permanent cell lines BHY and PCI-1, respectively. At least three independent measurements were carried out for each condition, resulting in an average increase of TLR7 expression of approximately 30% of the overall protein (Figure 4).

Discussion

Human solid tumor tissues of HNSCC are known to be infiltrated by various kinds of immune cells, whereas the HNSCC microenvironment leads to massively impaired immune responses (14-16). Alterations in immune, inflammatory, as well as angiogenic responses within the

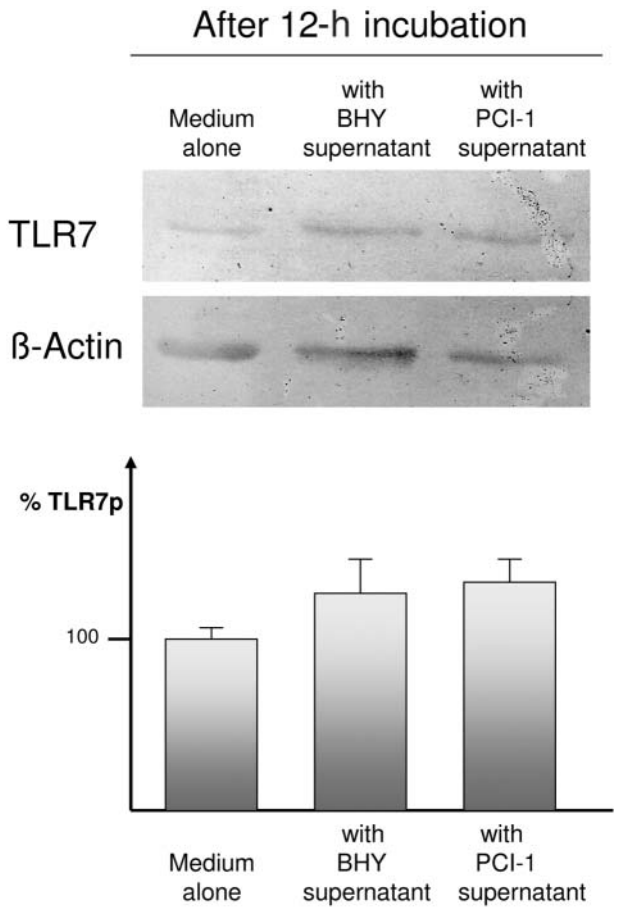

Figure 4. *Expression of Toll-like receptor (TLR) 7 in protein extracts of human MDC in response to 12 h of incubation with supernatants from HNSCC permanent cell lines BHY and PCI-1, respectively. Results expressed as mean % compared to control (medium alone).*

HNSCC microenvironment are considered to play a critical role in tumor aggressiveness and its influence on the cellular immune system.

Recent data suggest a partial Th2 cytokine bias within the HNSCC microenvironment, as well as a more aberrant expression of Th2 cytokines, such as IL-4, IL-6 and IL-10 in the plasma of patients with a more advanced disease (5, 24, 29).

DCs are central to T-lymphocyte activation and differentiation into T-helper (Th1) cells, Th2 cells, as well as cytotoxic T-lymphocytes (CTL) (30). For PDC, it has been shown that they are able to induce Th1 differentiation, although they were originally shown to trigger Th2 cytokine secretion by naïve CD4+ T-lymphocytes (31, 32). Distinct cellular functions of dendritic cells, such as phagocytosis, presentation of antigens, chemokine receptor expression or cytokine secretion are regulated through the recognition of pathogens by TLRs (33).

Therefore, our aim was to investigate the influence of the HNSCC microenvironment on TLR expression in human MDCs. It was demonstrated that human MDCs from peripheral blood revealed an increased expression of TLR7 in response to HNSCC supernatants, whereas other TLRs were not affected.

Previous studies on tumor tissue homogenates of HNSCC resulted in an overall detection of cytokine IL-1, which seems to play a crucial role in the regulation of cytokine production by tumor and resident tissue cells (34). IL-1 is produced by monocytes, macrophages and DCs and has been demonstrated to modestly induce the production of gelatines and, thus, contribute to tumor invasion and metastasis (35, 36). Surprisingly, no significant levels of IL-1 could be measured in supernatants of short-term primary cultures of HNSCC in the absence of immune cells (34). These data suggest that cells of HNSCC not only impair proper immune functions, but also trigger local immune cells, such as DCs to secrete tumor-promoting cytokines, most likely by modulating cellular receptor profiles and distinct biosynthesis pathways.

In this context, our data indicate that soluble immunomodulatory mediators within the HNSCC microenvironment alter the profile of TLR7 expression in MDCs, and suggest that this TLR interference participates in tumor promoting events.

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