CHAPTER 3

Expression and Function of Toll Like Receptors in Classical Hodgkin Lymphoma

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Submitted.
Abstract

Toll like receptors (TLRs) are implicated in the pathogenesis of hematological malignancies by modulating the immune response and by providing tumor cell survival signals. In classical Hodgkin lymphoma (cHL), which is characterized by an extensive reactive infiltrate, the role of TLRs has not been studied yet. TLR4, TLR7 and TLR9 were found to be expressed in Hodgkin Reed-Sternberg (HRS) cells in a variable percentage of cHL cases, whereas TLR2 was consistently negative. No correlation was found between expression of TLR4, TLR7 and TLR9 and the presence of regulatory T cells or of $T_{H\cdot}17$ cells and also not with expression of HLA class I and HLA class II in HRS cells. Ligation of TLR4, TLR7 and TLR9 did not induce of IL-1$\beta$, IL-6 or IL-10 in cHL cell lines. Ligation of TLR4 and TLR7 promoted cell growth in some cHL cell lines with the most pronounced effect observed upon ligation of TLR7 in KMH2 cells, also associated with upregulation of p-JNK1/2 and p-Erk1/2. Triggering of TLR9 suppressed cell growth in some cHL cell lines. In conclusion, we found expression of TLR4, TLR7 and TLR9 in HRS cells and a cell physiological effect upon stimulation of TLRs in cHL cell lines.
3.1 Introduction

Classical Hodgkin lymphoma (cHL) is characterized by a minority of neoplastic cells, the Hodgkin and Reed Sternberg (HRS) cells, which are surrounded by an extensive infiltrate of reactive cells. The HRS cells are derived from pre-apoptotic germinal center (GC) B-cells that acquired crippling immunoglobulin gene mutations and/or lost their capacity to express a high affinity B-cell receptor (BCR) [1]. Epstein-Barr virus (EBV) infection can rescue HRS precursor cells from apoptosis by mimicking B cell receptor (BCR) signaling [2]. Constitutive activation of nuclear factor κB (NF-κB) also contributes to the survival of HRS cells [1]. Furthermore, HRS cells orchestrate a microenvironment that favors their growth and suppresses an effective anti-tumor immune response, by attracting T helper (T\textsubscript{H})-2 and regulatory T (Treg) cells and suppressing T\textsubscript{H}-1 cells, CD8 cytotoxic T cells and natural killer (NK) cells [3-5]. More recent, interleukin (IL)-17 positive cells were identified in the cHL microenvironment indicating the presence of T\textsubscript{H}-17 cells [6].

Toll like receptors (TLRs) are transmembrane proteins that are expressed mainly on antigen-presenting cells (APC) such as dendritic cells (DC), macrophages and B cells. TLRs respond to microorganisms by recognition of pathogen associated molecular patterns (PAMPs). Stimulation of TLR signaling pathways results in activation of NF-κB and mitogen-activated protein kinase (MAPK) and induction of a variety of downstream target cytokines that initiate innate immunity and modulate adaptive immunity [7]. Human B cells are characterized by high expression of TLR1, 6, 7, 9, and 10, low expression of TLR2 and TLR4, and no expression of TLR3, 5 and 8 [8].

Expression and function of TLRs have been studied in several B cell malignancies. TLR2 expression was detected in B-cell acute lymphoblastic leukemia (B-ALL) [9] and chronic lymphocytic leukemia (CLL) [10]. Ligation of TLR2 in B-ALL cells significantly enhanced the ability of the tumor cells to stimulate allogeneic T cells as shown by a strong en-
hancement of interferon-γ production [9]. In CLL, ligation of TLR2 induced CD25 and CD86 expression, activation of the NF-κB signaling pathway and protection against induction of apoptosis [10]. TLR4 protein was detected in MALT lymphoma but not in CLL or mantle cell lymphoma (MCL) [11]. Single nucleotide polymorphism (SNP) analysis showed that the Asp299Gly variant of the TLR4 gene is associated with a higher incidence of mucosa-associated lymphoid tissue (MALT) lymphoma and cHL [12]. TLR7 and TLR9 were shown to be expressed in multiple myeloma (MM) [13-14] and B cell non-Hodgkin lymphoma (NHL) [15-16]. Ligation of TLR7 in CLL resulted in increased expression levels of costimulatory molecules (CD80, CD86, CD83, CD54 and CD40) and inflammatory cytokines, and increased sensitivity to killing by cytotoxic T cells (CTL) [16]. In MM, TLR7 and TLR9 stimulation promoted cell proliferation mediated by autocrine IL-6 secretion [13-14]. In small lymphocytic lymphoma (SLL), follicular lymphoma (FL), CLL, marginal zone lymphoma (MZL), MCL and large cell lymphoma (LCL) TLR9 triggering induced increased expression of costimulatory and antigen-presenting molecules CD20, CD40, CD54, CD80, CD86, MHC I and MHC II, and enhanced proliferation [15]. In this study, we analyzed the expression and function of TLR2, TLR4, TLR7 and TLR9 in cHL cell lines and cHL patient tissues.

3.2 Materials and Methods

3.2.1 Patient samples and cell lines

Eight frozen and 19 formalin fixed paraffin embedded tissue samples of cHL patients were obtained from the files of the department of Pathology and Medical Biology, University Medical Centre Groningen, the Netherlands. All frozen cHL samples were of nodular sclerosis (NS) subtype. The paraffin embedded samples consisted of cHL subtypes: 15 NS, two mixed cellularity (MC), one lymphocyte rich (LR) and one not otherwise specified (NOS) cHL. The protocols for obtaining and
studying human tissues were approved by the institution’s review board for human subject research. The cHL cell lines L428 [17], KMH2 [17], L1236 [17] and U-HO1 [18] were cultured in RPMI-1640 medium (Lonza Walkersville, Walkersville, MD USA) supplemented with ultraglutamine-1, 100 U/ml penicillin/streptomycin, 10% fetal calf serum (FCS) (5% for L428) (Lonza Walkersville). Peripheral blood mononuclear cells (PBMCs) were isolated from three healthy donors by Ficoll–Isopaque density gradient centrifugation.

PBMCs (1 × 10^6 cells/ml) and cHL cell lines (1 × 10^6 cells/ml) were cultured with LPS (TLR4 ligand) (Sigma Aldrich, St Louis, USA) (100ng/ml), R837 (TLR7 ligand) (Invivogen, San Diego, USA) (5µg/ml), ODN-2006 (TLR9 ligand) (Invivogen) (1µM) and ODN2006 control (TLR9 ligand control) (Invivogen) (1µM). Supernatants were collected after 72 hours for PBMC culture and after 24 hours for cHL cell culture (ELISA and proliferation) or ten minutes (Western blot).

### 3.2.2 Immunohistochemistry

Immunostaining was performed with a monoclonal antibody against TLR2 (CD282, Hycult Biotechnology, Uden, The Netherlands) (1:50) on frozen cHL tissue sections and cytopsins of cHL cell lines, and polyclonal antibodies against TLR4 (Abcam, Cambridge, UK) (1:20), TLR7 (Abcam) (1:20) and TLR9 (26C593, Acris) (1:100) on paraffin embedded cHL tissue sections and cytopsins of cHL cell lines. Monoclonal antibodies for Foxp3 (236A/E7, Abcam) (1:100) and HLA class II (CR3/43, DAKO, Glostrup, Denmark) (1:500), and polyclonal antibodies for IL-17 (R&D systems, Minneapolis, USA) (1:100) and β2-microglobulin (DAKO) (1:200) were used on paraffin embedded cHL tissue sections. Positive staining was visualized using an HRP-labeled second step and 3-amino-9-ethylcarbazole (AEC) for frozen cHL tissue sections and cytopsins, and 3, 3’-diaminobenzidine (DAB) for paraffin embedded cHL tissue sections. Cases were defined as positive when more than 50% of HRS cells showed a clear staining.
3.2.3 ELISA

Cytokines IL-1β, IL-6 and IL-10 protein levels were measured in cell culture supernatant from cultured cHL cell lines and PBMCs by ELISA (R&D Systems) following the protocol provided by the manufacturer.

3.2.4 Proliferation assay

Alamar blue (Invitrogen, Breda, The Netherlands) was added to the cells following the protocol provided by the manufacturer. Fluorescence was measured every 24 hours at 560/590 nm.

3.2.5 Western blot

Cells were lysed in 1x SDS Sample Buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Cell lysates were separated on polyacrylamide gels and electroblotted onto nitrocellulose membranes using standard laboratory protocols. Blots were blocked in blocking buffer (TBS with 0.05% Tween 20, pH 7.6 with 5% skimmed milk), washed and incubated with primary antibodies at 4°C overnight. The antibodies against phosphorylated (p)-SAPK/JNK (Tyr183/185) (81E11), p-p44/42 MAPK (Thr202/Tyr204) (20G11), p-Akt (Ser473) (D9E) were purchased from Cell Signaling Technology (CST, Boston, USA). Immunostaining was amplified by incubation with HRP-conjugated antibodies and chemiluminescence was detected with ECL (Pierce, Rockford, USA).
3.2.6 Statistical analysis

SPSS statistical software version 16.0 (SPSS Inc., Chicago, IL) was used. To analyze the association between the expression of TLRs in HRS cells and the presence of Treg and Th-17 cells, student’s t-test was used. Correlation between the expression of TLRs and HLA class I and HLA class II was analyzed using Fisher’s exact test. Cytokine induction in PBMCs and cHL cell supernatants and cell growth were analyzed using paired student’s t-test.

3.3 Results

3.3.1 Expression of TLRs

No TLR2 staining was found in KMH2, L428, L1236 and U-HO1 cytospins. TLR4 showed a moderate staining in KMH2, L1236 and U-HO1 and a weak staining in L428. Staining for TLR7 was strong in KMH2 and L1236 and weak in L428 and U-HO1. TLR9 staining was strong in KMH2 and U-HO1 and weak in L428 and L1236. Representative staining results are shown in Figure 3.1. A-D.

Consistent with the lack of staining in cHL cell lines, TLR2 was also undetectable in HRS cells of eight cHL frozen tissue sections. Dendritic cells present in the reactive infiltrate showed a strong staining for TLR2 in all cHL cases (Figure 3.1. E). For TLR4, positive staining was observed in the majority of HRS cells in nine out of 19 cHL cases and in a minority of HRS cells (5 and 30%) in two cases (Figure 3.1. F, Table 3.1). TLR7 expression was observed in 5-20% of HRS cells in four cHL cases (Figure 3.1. G, Table 3.1). TLR9 stained positive in more than 50% of HRS cells in nine out of the 19 cHL cases and in approximately 10% of HRS cells in three cases (Figure 3.1. H, Table 3.1). TLR2, TLR4, TLR7...
Figure 3.1: Immunohistochemistry for TLRs in cHL cell lines and cHL tissue sections. A-D: TLR2 (A) is negative in cHL cell lines (L428 is shown as a representative), whereas TLR4 (B), TLR7 (C) and TLR9 (D) are positive in all four cHL cell lines (KMH2 is shown as a representative example of the cHL cell lines). E-H: TLR2 (E) is positive in the reactive cells but not in HRS cells in cHL tissues, whereas positive staining of TLR4 (F), TLR7 (G) and TLR9 (H) is observed in HRS cells in cHL tissues.
and TLR9 stained positive in (part of) the reactive background cells in all cHL cases. Representative staining results of TLR2, TLR4, TLR7 and TLR9 are shown in Figure 3.1.

Table 3.1: Expression of TLR4, TLR7 and TLR9 in HL cases, presence of \( T_{H-17} \) and FoxP3+ Treg cells, HLA-I and II expression.

<table>
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<th>EBV subtype</th>
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<th>TLR9</th>
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<th>Foxp3/HPF</th>
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“+” indicates more than 50% positive HRS cells;
“-/+” indicates the presence of positive HRS cells but less than 50%;
“-” indicates no positive tumor cell observed;
N.E: not evaluable;
N.D: not determined.

3.3.2 Capacity of TLR to modulate the immune response in cHL

Stimulation of TLRs (i.e. TLR4, TLR7 and TLR9) has been implicated in the differentiation of Treg and \( T_{H-17} \) cells by inducing production of cytokines, that support differentiation into these cell types and regulate their function [19-20]. To assess a possible relation between TLR expression and Treg or \( T_{H-17} \) cells we identified Treg cells by Foxp3
and T<sub>H</sub>-17 cells by IL-17 staining. The mean number of Foxp3+ lymphocytes in the close vicinity of HRS cells was 117/HPF (range 2-400/HPF) (Table 3.1). The number of IL-17 positive lymphocytes in the vicinity of HRS cells was 0-2/HPF (Table 3.1). A variable percentage of IL-17 positive neutrophils was observed in most cHL cases. No correlation was observed between expression of TLR4, TLR7 or TLR9 in HRS cells and presence of Treg or T<sub>H</sub>-17 cells in cHL tissues.

We further investigated the correlation between the expression of TLRs and HLA class I and HLA class II in HRS cells in cHL tissue. Cell surface β2M staining was considered representative for cell surface HLA class I expression [21] and was found in seven out of 14 cHL cases. HLA class II expression was observed in nine of 16 cases (Table 3.1). No correlation was found between the expression of TLR4, TLR7 and TLR9 and HLA class I and HLA class II positivity in HRS cells in cHL tissue.

### 3.3.3 Functional effects of TLR ligation in cHL cell lines

To determine the effect of TLR stimulation on cHL cell lines, we studied induction of cytokines, proliferation and phosphorylation of downstream signaling components. IL-1β was undetectable in cHL cell lines with or without stimulation of the TLRs (data not shown). IL-6 and IL-10 were expressed in all cHL cell lines, but no induction was found upon TLR ligation (Supplementary Table 3.1). PBMCs used as a positive control for TLR stimulation [19, 22] indeed showed a pronounced induction of IL-6 and IL-10 by ligation of TLR4, TLR7 and TLR9 and of IL-1β by ligation of TLR4 and TLR7 consistent with the findings reported in the literature.

Cell growth analysis after three days of TLR ligation in cHL cell lines revealed a slightly enhanced proliferation in L428 upon TLR4 activation and in L1236 cells upon TLR4 and TLR7 activation, (Figure 3.2A), and a more pronounced induction of proliferation in KMH2 cells upon TLR7
Figure 3.2: Cell growth effect by TLR ligation in cHL cell lines. A: TLR promoted cell growth in L428 and L1236 cells. B: TLR7 promoted cell growth in KMH2 and L1236 cells. C: TLR9 suppresses cell growth in L428, L1236 and U-HO1 cells.
Figure 3.3: Effect on downstream targets of TLRs in cHL cell lines. A: TLR7 induced p-JNK1/2 and p-Erk1/2 in KMH2 cells. B: No induction on downstream targets was observed in L428 cells.
triggering (Figure 3.2B). Triggering of TLR9 showed a slight suppression of cell growth in L428, L1236 and U-HO1 (Figure 3.2C).

No effect on p-JNK1/2 and p-ERK1/2 was observed in L428, L1236 and U-HO1 upon ligation of TLR4, TLR7 and TLR9 (Figure 3.3 and data not shown). In KMH2 cells, a prominent induction of p-JNK1/2 and p-Erk1/2 was observed upon TLR7 ligation (Figure 3.3), whereas ligation of the other TLRs had no effect.

3.4 Discussion

Expression and function of TLRs have been previously studied extensively in leukemia and MM and showed a marked impact on immune response and tumor cell growth. In contrast, there is only limited data on expression and functionality of TLRs in common B cell lymphomas such as diffuse large B cell lymphoma, follicular lymphoma, and Burkitt lymphoma. In the present study we showed expression of TLR4 and TLR9 in HRS cells in half of the cHL patients and expression of TLR7 in a small proportion of HRS cells in a minority of the patients. TLR2 expression was not detectable in cHL cases and cell lines.

TLR2 has been shown to be expressed in B-ALL and CLL, and its ligation revealed activation of NF-κB amongst others. In HRS cells, which in general are characterized by constitutional activation of the NF-κB pathway, no expression of TLR2 was found in tissue biopsies of cHL patients and cytospins of the cHL cell lines. Low TLR2 expression in GC B cells enables formation of functional heterodimers with TLR1 and TLR6 [23]. TLR2 senses bacterial lipopeptides and triggers an immune response by heterodimerization with TLR1 or TLR6. The absence of TLR2 suggests that this pathway is not functional in HRS cells in cHL.

TLR4 expression has been observed in MALT lymphoma, but not in CLL and MCL [11]. In cHL, TLR4 was detected in more than 50% of
HRS cells in approximately half of the cHL cases and in all four cHL cell lines. In GC B cells, a low expression of TLR4 has been observed. These findings might indicate that HRS cells have acquired expression of TLR4 during the malignant transformation process. Besides LPS, also some endogenous ligands that are released in the context of tissue damage, cellular stress, or cell death are thought to activate TLR4 [23]. HSP60, one of the endogenous ligands for TLR4 is abundantly expressed in HRS cells [24] and this may result in activation of the TLR4 signaling pathway.

TLR7 expression is found in B-ALL, B-CLL and MM [23]. Unlike the high expression of TLR7 in GC B cells [8], TLR7 was only detected in a low percentage (5-20%) of HRS cells in a minority of the cHL patients. In contrast, TLR7 was present in all four cHL cell lines suggesting that the downregulation of TLR7 in vivo might be induced by the interaction with the infiltrating inflammatory cells. Although natural ligands of TLR7 remain unknown, the function of TLR7, to some extent, was revealed using synthetic ligands. In response to imidazoquinoline, activation of immune cells and synthesis of interferon-α and other cytokines was found, depended on the presence of TLR7 and Myeloid differentiation primary response gene 88 (MyD88) [25]. The downregulation of TLR7 in HRS cells may be associated with immune suppression in cHL, since stimulation of TLR7 would both activate the T cells in the background and increase the immunogenicity of the tumor cells, resulting in an effective antitumor immunity [26].

TLR9 expression was detected in several NHL like CLL/SLL, MCL, FL, LCL, MZL and MM [23]. Expression of TLR9 was observed in more than 50% of HRS cells in half of the cHL cases and also in the cHL cell lines. In B cells, expression of TLR9 was found especially on memory and GC B cells. Viral and bacterial unmethylated CpG DNA ligates with TLR9 and initiates the production of pro-inflammatory cytokines. Three out of five EBV+ cases showed TLR9 positivity. In cHL, the TLR9 signaling pathway is possibly activated through the presence of EBV in HRS cells.
Many of the cytokines produced after ligation of the TLRs play a role in the induction of T<sub>H</sub>-17 and Treg cells. IL-6 is necessary for the induction of T<sub>H</sub>-17 cells [27] and will inhibit Treg cells [27], IL-10 plays a role in the induction of Treg cells [28], TGF<sub>β</sub> is important for the induction of both cell types [27, 29] and IL-1β [27] takes part in T<sub>H</sub>-17 differentiation. To study functionality or relevance of TLRs in cHL tissue we studied presence of Foxp3+ Treg cells and T<sub>H</sub>-17 cells in the vicinity of HRS cells. No relation was found between expression of TLR4, TLR7 or TLR9 in HRS cells and presence of Treg and T<sub>H</sub>-17 cells. These findings are supported by the lack of cytokine induction upon TLR ligation in the cHL cell lines. Moreover, expression of HLA class I and HLA class II in HRS cells in cHL cases was also not associated with expression of TLRs. Consistent with these findings, no induction of cellular surface markers (HLA class I, HLA class II, CD20, CD40, CD56 and CD86) was observed by ligation of TLRs (data not shown). In NHL a marked induction of these surface markers has been reported [15-16], indicating that the responsiveness of the TLRs in cHL is very low. A significant but limited effect was observed on cell growth by ligation of TLR4, TLR7 and TLR9 in cHL cell lines. Only triggering of TLR7 in KMH2 cells induced cell growth and upregulation of p-JNK1/2 and p-Erk1/2. In contrast TLR ligation in MM cell lines induced proliferation and survival, this difference is probably caused by the increase in IL-6, a potent growth factor in MM [15].

The overall hyporesponsiveness of TLR4, TLR7 and TLR9 in cHL may be caused by several mechanisms. First, somatic mutations or SNPs of TLRs or their downstream components could result in the impairment of TLR signaling pathway. This has been shown for co-segregating missense mutations (Asp299Gly and Thr399Ile) that affect the extracellular domain of TLR4 [30]. Intriguingly, the TLR4 Asp299Gly variant has been shown to be a risk factor for HL. More recently, a SNP variant of MyD88 adapter-like (Mal)/TIRAP, which acts as a bridging adapter between TLRs and its downstream adaptor MyD88, was shown to result in loss of MyD88 binding and reduced TLR2/TLR4 signaling [31]. SNP analysis revealed that the 1237C and 2848A variants of the TLR9 gene were risk factors for HL [32]. Second, in contrast to memory B
cells, naïve B cells and GC B cells are more difficult to activate and less sensitive to TLR stimulations [23]. Accordingly, it might be speculated that the hyposensitivity of GC B cell derived HRS cells to activation of TLR is an intrinsic characteristic of GC B cells, which is not related to the pathogenesis. Consistent with this, a marked induction of proliferation, and expression of antigen presenting and costimulatory molecules has been observed upon TLR9 ligation in memory B cell derived NHL in comparison to GC B cell derived NHL [15]. In plasma cell derived MM, ligation of TLRs showed a prominent effect on cell growth [13-14]. Third, cell lines may have acquired irreversible changes that make cells resistant or insensitive to stimulation by TLR ligands. Although MM cells [13-14] show an increase in proliferation after TLR ligation, MM cell lines are resistant to TLR stimulation in terms of proliferation and differentiation [33].

In summary, we show that TLR4, TLR7 and TLR9 are expressed by HRS cells in cHL, whereas TLR2 is not expressed. The responsiveness to ligation of these TLRs is limited with no effect on cytokine production and only a slight effect on cell growth in cHL cell lines and no association with presence of Treg and T<sub>H</sub>-17 cells or expression of HLA. These findings indicate that the TLRs are hyporesponsive in cHL.

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References


Supplementary Table 3.1: Cytokines of IL-6 and IL-10 induction after TLRs stimulation.

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<th>R837(TLR7 ligand)</th>
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</tr>
</tbody>
</table>

SD: standard deviation; 0: below detection limits; NA: not available.