Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenic significance in specific subsets of patients

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ABSTRACT

Background
Signaling through the B-cell receptor appears to be a major contributor to the pathogenesis of chronic lymphocytic leukemia. Toll-like receptors bridge the innate and adaptive immune responses by acting as co-stimulatory signals for B cells. The available data on the expression of Toll-like receptors in chronic lymphocytic leukemia are limited and derive from small series of patients.

Design and Methods
We profiled the expression of genes associated with Toll-like receptor signaling pathways in 192 cases of chronic lymphocytic leukemia and explored potential associations with molecular features of the clonotypic B-cell receptors.

Results
Chronic lymphocytic leukemia cells express all Toll-like receptors expressed by normal activated B cells, with high expression of TLR7 and CD180, intermediate expression of TLR1, TLR6, TLR10 and low expression of TLR2 and TLR9. The vast majority of adaptors, effectors and members of the NFKB, JNK/p38, NF/IL6 and IRF pathways are intermediately-to-highly expressed, while inhibitors of Toll-like receptor activity are generally low-to-undetectable, indicating that the Toll-like receptor-signaling framework is competent in chronic lymphocytic leukemia. Significant differences were identified for selected genes between cases carrying mutated or unmutated *IGHV* genes or assigned to different subsets with stereotyped B-cell receptors. The differentially expressed molecules include receptors, NFκB/MAPK signaling molecules and final targets of the cascade.

Conclusions
The observed variations are suggestive of distinctive activation patterns of the Toll-like receptor signaling pathway in subgroups of cases of chronic lymphocytic leukemia defined by the molecular features of B-cell receptors. Additionally, they indicate that different or concomitant signals acting through receptors other than the B-cell receptor can affect the behavior of the malignant clone.

Key words: Toll-like receptor, signaling pathway, chronic lymphocytic leukemia, gene expression profiling.

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The online version of this article has a Supplementary Appendix.

Introduction

A role for antigen in the development of chronic lymphocytic leukemia (CLL) is strongly suggested by the biased immunoglobulin heavy variable (*IGHV*) gene repertoire of the malignant clones, the prognostic implications of *IGHV* gene mutational status and the identification of subsets of patients with almost identical, stereotyped B-cell receptors (BcR), who can also exhibit restricted demographic, biological and clinical features.[1-3]

The structural homology of the BcR indicates a selection pressure exerted by common antigenic elements or classes of structurally similar epitopes which may trigger and/or facilitate the onset and evolution of at least some CLL clones.[4] The nature of the selecting antigens, the mechanistic aspects of their recognition by the clonotypic BcR and the functional impact of antigenic stimulation through the BcR remain largely unknown. Furthermore, the role of additional and concomitant ways of activating CLL cells through "non-specific" innate immune receptors[5] should also be considered, as these receptors concur with BcR stimulation to provide full activation of B lymphoid cells.

The prototypic class of innate immune receptors includes the Toll-like receptors (TLR)[6] which recognize molecular structures that are specific and evolutionarily conserved between pathogens. The central feature of microbe recognition by TLR is the triggering of signaling pathways important for the activation of antigen-presenting cells (APC), including B cells.[7] In this respect, given the role of APC in the activation of T cells, TLR may be considered as a "link" between innate and adaptive immunity.[8,9]

In recent years, the role of TLR in the physiology of B cells has received increasing attention as critical antigen-triggered B-cell differentiation steps have been shown to be influenced by TLR-dependent signals, acting in concert with or superimposed on signals originating from the BcR.[10] The expression of TLR in normal naïve and memory B cells has been mapped: naïve B cells express low levels of TLR1, TLR6, TLR7, TLR8, TLR9 and TLR10, and memory B cells expresses high levels of TLR1, TLR6, TLR7, TLR9 and TLR10 along with low levels of TLR2, TLR4 and TLR8.[11-14]

The stimulation of surface or endosomal TLR leads to the activation of NF-κB and the induction of activation-induced cytidine deaminase, which, in combination with cytokines, induces class switch recombination to specific isotypes.[15-17] This depends on correct intracellular trafficking and localization of the engaged TLR and on the presence of other signals, such as those emanating from the BcR.[10,18-20] The activation of B cells by TLR engagement may lead to a more efficient interaction with T cells and dendritic cells due to up-regulation of the co-stimulatory CD80 and MHCII molecules.[21,22] Finally, TLR-dependent signals may be implicated in the regulation of B-cell immune responses, either by inducing TLR tolerance or by subverting the mechanisms that ensure the silencing of autoreactive B cells, thus promoting autoreactivity.[23]

Several TLR agonists have been used in clinical trials of CLL patients as adjuvants to improve the efficacy of chemotherapy.[24] The data available on TLR expression in CLL are still limited[25-27] but have essentially shown that TLR7 and TLR9 are virtually always expressed. We recently reported that, in addition to TLR7 and TLR9, CLL cells can also express TLR1, TLR2, TLR6 and TLR10.[27] However, most studies, have analyzed small series of patients, thus precluding sound conclusions with regard to the exact TLR expression profile in CLL and preventing possible correlations with various clinico-biological features.

We performed a systematic gene expression profiling of the TLR signaling pathway in a series of 192 patients with CLL. As TLR have a co-stimulatory effect on the BcR, we sought for differences in gene expression profiles among subgroups of cases defined by BcR molecular features, such as the repertoire and mutational status of the *IGHV* genes or the expression of stereotyped BcR. Significant variations indicative of distinctive activation patterns of the TLR signaling pathway were identified, especially among cases assigned to subsets with stereotyped BcR. These findings suggest that different or concomitant signals acting through receptors other than BcR can affect the behavior of the malignant clone with implications for future functional studies that may eventually define the role of TLR signaling in the pathogenesis and evolution of CLL.

Design and Methods

Patients

Peripheral blood samples were collected from 192 patients with typical CLL, all meeting the recently revised diagnostic criteria of the National Cancer Institute Working Group.[28] The patients' demographic, clinical and biological data are shown in *Online Supplementary Table S1*. Patients were mostly untreated (n=155) or off therapy for a median of 24 months before study inclusion (range, 6-192 months). The study was approved by the local Ethics Review Committee of each participating Institution.

Isolation of B cells

$CD19^+$ B cells were negatively selected from peripheral blood samples using the Human B-cell enrichment cocktail kit (RosetteSep; StemCell Technologies, Vancouver, BC, Canada) following the manufacturer's instructions. The desired cells were collected as a highly enriched population by centrifugation on a Ficoll-hypaque gradient. The purity of the isolated cell populations ($CD19^+$ cells) was assessed with the use of flow cytometry of the cell suspension and was always found to exceed 97%.

RNA extraction and cDNA preparation

Total cellular RNA was isolated with the Qiagen RNAeasy mini kit (QIAGEN, Hilden, Germany). The isolation procedure included an additional incubation step with DNase (QIAGEN, Hilden, Germany) to ensure that the final product was devoid of genomic DNA. One microgram of RNA was reversed transcribed to cDNA using the RT² First Strand Kit (SABiosciences, USA).

Polymerase chain reaction amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements

Reverse transcriptase-polymerase chain reaction (RT-PCR) of *IGHV-IGHD-IGHJ* rearrangements was performed using IGHV leader primers along with appropriate *IGHJ* genes, as previously described.[29] Purified PCR amplicons were subjected to direct sequencing on both strands. Sequence data were analyzed using the IMGT® databases and the IMGT/V-QUEST tool (*http://www.imgt.org*).[30,31]

Gene expression profiling of the Toll-like receptor signaling pathway

Gene expression profiling of the TLR signaling pathway in CLL

was performed by real-time quantitative PCR (RQ-PCR) on cDNA arrays using the RT² *Profiler*™ PCR Array kit (PAHS-018A array, SABiosciences). The method combines the advantages of RQ-PCR using SYBR Green I with the potential to analyze the expression of multiple genes at once. Each RQ-PCR product was further validated by running a melting curve program immediately after the cycling program; only PCR products with one peak in temperatures above 80°C were further evaluated. In addition, the hot start polymerase used in all experiments ensured accurate results both by preventing the amplification of primer dimers and other non-specific products and by providing high amplification efficiencies even for those genes that are more difficult to amplify.

The array consisted of a panel of 96 primer sets used for the amplification of 84 genes relevant to the TLR pathway (*Online Supplementary Table S2*) plus five housekeeping genes (*B2M, HPRT1, RPL13A, GAPDH and ACTB*), a genomic DNA control, three reverse transcription and three PCR quality controls. Only samples passing the PCR array run quality control, assessing the absence of genomic DNA contamination and proper amplification of the reverse transcription controls and the positive PCR controls, were further evaluated.

Data were obtained as threshold cycle (Ct) values. The threshold value was set at 0.01 for all experiments. According to the manufacturer's instructions, Ct values greater than 35 were indicative of no expression and further considered equal to 35 for mathematical reasons. If a gene showed an erratic curve in a particular run, the corresponding results were not further evaluated. Four of the five housekeeping genes (*B2M, RPL13A, GAPDH* and *ACTB*) had stable mRNA levels, evidenced by the lack of significant differences in Ct values across the samples, and their average Ct value was used for ΔCt measurement; *HPRT1* showed significant inter-patient variability and was excluded from the analysis. The C_t value consistency for the housekeeping genes indicated a proper normalization method and was used for ΔCt measurements. The difference between the Ct value of each gene of interest and the average Ct value of housekeeping genes in each sample (ΔCt) was then measured. Based on the ΔCt value, which indicates the expression level for each TLR pathway-associated gene in relation to the reference (i.e. the average expression of the housekeeping genes), cases were assigned to four different expression levels: high (median ΔCt value ≤6.6), intermediate (median ΔCt value >6.6 and ≤9.9), low (median ΔCt value >9.9 and ≤13.2) and negative (ΔCt value >13.2).

Fold differences in gene expression between different subgroups of patients were determined using the $2^{-\Delta\Delta Ct}$ algorithm.[32] The difference in expression of a certain gene between two subgroups was considered significant only if: (i) the fold difference in average $2^{-\Delta\Delta Ct}$ values was greater than 2 or less than -2 (indicative of up-regulation or down-regulation, respectively); and, (ii) the difference in ΔCt values was statistically significant ($P<0.05$) according to the t-test.

Western blot analysis

Total cellular protein was isolated from purified B cells. Cells were washed twice with ice-cold phosphate-buffered saline and lysed with lysis buffer (0.5M Tris-HCl, 5M NaCl, 0.5M EDTA pH 7.4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and a cocktail of protease inhibitors containing leupeptin and PMSF). Twenty to forty micrograms of protein were run on a 10% NuPAGE Bis-Tris gel (Invitrogen, Paisley, UK) under denaturing and reducing conditions. Proteins were transferred to PVDF membranes (BioRad, USA). Non-specific binding of antibody to the membrane was blocked by incubation for 1 h with WesternDot blocking buffer. Immunoblot analysis was performed using goat anti-TLR1 (1:200 dilution, R&D Systems, Minneapolis, USA), goat anti-TLR2 (1:200 dilution, R&D Systems), mouse anti-TLR8 (1:200 dilution, Santa Cruz Biotechnology, CA, USA) and mouse anti-TLR9 (1:200 dilution, Santa Cruz Biotechnology). Mouse anti-β-actin (1:5000 dilution, Invitrogen) was used as a protein marker for the quantification of the protein bands and MagicMark™ XP as a protein standard for molecular weight estimation. The immunodetection of proteins was performed with the use of the WesternDot™ 625 Western Blot Kit (Invitrogen). The detection step relies on a biotinylated secondary antibody, goat anti-mouse or rabbit anti-goat respectively, and an interaction with a QdotR 625 streptavidin conjugate. Given that the QdotR 625 nanocrystal has a high extinction in the UV and blue wavelengths, the protein was detected using a MiniBIS Pro UV detection system (DNR Bio-Imaging Systems, Jerusalem, Israel) according to the manufacturer's protocol. Ratios of TLR protein band intensity relative to β-actin band intensity were calculated for each sample using the QelQuant software provided with the UV detection system.

Flow cytometry

$CD19^+$ cells were collected and washed twice in phosphate-buffered saline. The cells were stained with anti-TLR1 (AbCam, Cambridge, UK), anti-TLR2 (Caltag, Buckingham, UK), anti-TLR4 (AbCam) and anti-TLR6 (AbCam) for 15 minutes. Intracellular staining was performed for TLR7 (AbCam), TLR8 (Dendritics; Lyon, France) and TLR9 (AbCam) using the BD Cytofix/Cytoperm kit (BD Cytofix/Cytoperm™ Plus Fixation Kit; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) following the manufacturer's instructions. In all cases, the analysis was performed by triple staining with anti-CD19 and 7-amino-actinomycin D (7-AAD) vital dye (Beckman Coulter; Brea, CA) to exclude dead cells. Appropriate isotype controls were also used for each tested TLR. Details and concentrations of the reagents used in these experiments are given in *Online Supplementary Table S3*. Data were acquired on a BD FACS CANTO flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The analysis was performed using the BD FACS DIVA software. Only 7-AAD negative (viable cells) were analyzed for TLR expression.

Statistical analysis

Descriptive statistics for discrete parameters included counts and frequency distributions. For quantitative variables, statistical measures included means, medians, standard deviation and minimum–maximum values. The statistical significance of bivariate relationships between factors was assessed using χ^2 tests and t-tests. Progression-free survival was measured from diagnosis to disease progression, and overall survival was measured from diagnosis to death or last follow-up. Survival curves were plotted using the Kaplan-Meier method. Bivariate differences in survival distributions were studied using the log-rank test. Multivariate Cox regression models were implemented for the study of the simultaneous effect of factors on survival outcomes taking into account the relative effect of remaining parameters. Hazard ratios and 95% confidence intervals of outcomes under study were calculated for each parameter estimate. All analyses were performed with the statistical package SPSS 17.0, taking the level of statistical significance as 5%.

Results

IGHV gene repertoire and mutational status

IGHV-IGHD-IGHJ sequences were available for all cases in the study except one. According to the 98% cut-

off value of identity to germline, 124/191 sequences (64.9%) were characterized as mutated, whereas the remainder (67/191 sequences, 35.1%) were characterized as unmutated; 52/67 unmutated sequences had 100% identity to germline. Detailed information on *IGHV* gene repertoire and mutational status is provided in *Online Supplementary Table S4*. Following previously described criteria, 30/191 cases expressed stereotyped BcR assigned to seven different subsets (*Online Supplementary Table S5*). Within this group, ten cases each expressed IGHV1/5/7-IGKV1(D)-39 BcR (subset #1) or IGHV4-34/IGKV2-30 BcR (subset #4), respectively, while four cases expressed IGHV4-39/IGKV1(D)-39 BcR (subset #8). Subset #1 and #8 cases were uniformly unmutated, whereas subset #4 cases were uniformly mutated.

Gene expression profiling of the Toll-like receptor signaling pathway: analysis at cohort level

Eighty-four genes relevant to the TLR signaling pathway were evaluated in the present study (*Online Supplementary Table S2*). The cDNA array included receptors, adaptors and proteins that interact with TLR to form the signaling complex, plus effectors of the TLR signaling pathway. Members of the NF-κB, JNK/p38 and IRF signaling pathways which are activated by TLR signaling complex were also included. Finally, the array included cytokines and co-stimulatory molecules induced by TLR through activation of the NF-κB and JNK/p38 signaling pathways.

Eighty-three of the 84 genes showed normal fluorescence curves in the great majority of runs. In contrast, the *PTGS2* gene showed an erratic fluorescence curve in most runs and was not further analyzed. As detailed in the *Design and Methods* section, for each of the 83 TLR pathway-associated genes finally evaluated, cases were assigned to four different mRNA expression levels (high, intermediate, low, negative) based on ΔCt values, which indicate the expression level for each gene of interest in relation to the reference (i.e. the average expression of four housekeeping genes, all with stable expression in all analyzed samples). A graphic summary of the results obtained at the cohort level is given in Figure 1. Detailed results from the RQ-PCR experiments are listed in Table 1 and *Online Supplementary Table S6*.

Overall, 12 receptors were analyzed (TLR1-10, and the TLR-associated CD180 and SIGIRR). The highest mRNA expression levels were recorded for *TLR7* and *CD180*. Intermediate expression was found for *TLR1*, *TLR6* and *TLR10*, while *TLR2* and *TLR9* were generally characterized by low expression. The expression of *TLR4* and *TLR8* was low to undetectable, with significant variation between the low positive cases. The great majority of cases were negative for *TLR3*, *TLR5* and *SIGIRR/TIR8* (Figure 1, Table 1 and *Online Supplementary Table S6*).

Almost all the adaptors and the TLR interacting proteins were highly expressed. In particular, among the adaptors, high mRNA expression was recorded for (i) MyD88, the central signaling molecule shared by all TLR except TLR3, and (ii) TICAM1, which is responsible for mediating signaling from TLR3 and TLR4. The expression of the bridging adaptors TICAM2 and TIRAP, required for MyD88 and TICAM1 signaling, was low. The expression of TOLLIP, which inhibits subsequent events required for signaling was intermediate. Intermediate expression was also found for (i) the IL-1 receptor-associated kinase-1 and -2 (IRAK1, IRAK2) which interact with the adapters to form the signaling complex and (ii) TRAF6, which is associated with the IRAK family members to mediate signaling (Figure 1, Table 1 and *Online Supplementary Table S6*).

Several molecules that modulate the function of the TLR pathway ("effectors") were found to be expressed in CLL, though variably. High to intermediate expression was recorded for all the genes involved in NF-κB and JNK/p38 pathways except for *CLEC4E*, which is also not expressed by normal B lymphocytes. Significant variability was identified for *MAP4K4* and the transcriptional factors *JUN* and *FOS*, (Figure 1, Table 1 and *Online Supplementary Table S6*).

Tumor necrosis factor (*TNF*) and lymphotoxin alpha (*LTA*, also known as *TNFB*) exhibited intermediate expression. Among interleukins (IL), low-to-undetectable mRNA levels were recorded for *IL1B*, *IL6*, *IL8* and *IL10* with significant variability among positive cases, while *IL1A* and *IL2* were not expressed. The expression of *CD80* and *CD86* was low and intermediate, respectively, with significant inter-patient variability (Figure 1, Table 1 and *Online Supplementary Table S6*).

Since TLR can also induce type I interferons through the activation of interferon regulatory factors (IRF), we also evaluated the expression of several members of the IRF pathway and found high expression for *IRF1* and *IRF3*. In addition, the expression of *IFNG* was low with significant variation between different CLL cases (Figure 1, Table 1 and *Online Supplementary Table S6*).

Toll-like receptor protein expression in chronic lymphocytic leukemiaL

In order to determine whether the observed mRNA expression patterns reflect the actual proteins expressed, at least for selected TLR pathway-associated genes, flow

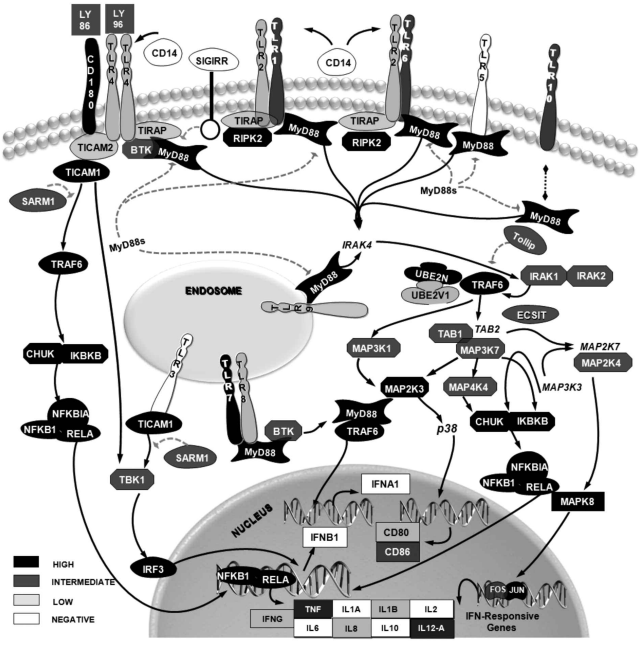

Figure 1. Expression patterns of the TLR signaling pathway in CLL. For reasons of clarity only the major molecules involved in TLR signaling are shown in the figure. Detailed results about the mRNA expression levels of all the molecules evaluated in this study are given in *Online Supplementary Table S6*. Gene names are those approved by the HUGO Gene Nomenclature Committee.

Table 1. mRNA expression levels for the 83 genes analyzed in the present study.

Gene	Expression Level	Gene	Expression Level
Receptors		**NF-κB pathway /JNK/p38 pathway**	
CD180	High	*CHUK*	High
TLR7	High	*IKBKB*	High
TLR1	Intermediate	*JUN**	High
TLR6	Intermediate	*MAP2K3*	High
TLR10	Intermediate	*MAP3K1*	High
TLR2	Low	*MAPK8*	High
*TLR4**	Low	*NFKB1*	High
*TLR8**	Low	*NFKBIA*	High
TLR9	Low	*REL*	High
SIGIRR	Negative	*RELA*	High
TLR3	Negative	*ELK1*	Intermediate
TLR5	Negative	*FOS**	Intermediate
Signaling complex		*MAP2K4*	Intermediate
HMGB1	High	*MAP4K4**	Intermediate
HRAS	High	*NFKB2*	Intermediate
HSPA1A	High	*NFKBIL1*	Intermediate
HSPD1	High	*NFRKB*	Intermediate
LY86	High	*CLEC4E*	Negative
MAPK8IP3	High	**Cytokines and co-stimulatory molecules**	
MYD88	High	*CD86**	Intermediate
PELI1	High	*LTA*	Intermediate
RIPK2	High	*TNF*	Intermediate
TICAM1	High	*IL12A*	Intermediate
TRAF6	High	*CD80**	Low
BTK	Intermediate	*IL1B**	Low
IRAK1	Intermediate	*IL8**	Low
IRAK2	Intermediate	*CCL2*	Negative
LY96	Intermediate	*CSF2*	Negative
SARM1	Intermediate	*CSF3*	Negative
TOLLIP	Intermediate	*IL10**	Negative
TICAM2	Low	*IL1A*	Negative
TIRAP	Low	*IL2*	Negative
CD14	Negative	*IL6**	Negative
Effectors		*TNFRSF1A*	Negative
EIF2AK2	High	***IRF* pathway**	
NR2C2	High	*IRF1*	High
PRKRA	High	*IRF3*	High
UBE2N	High	*TBK1*	Intermediate
CASP8	Intermediate	*IFNG**	Low
FADD	Intermediate	*CXCL10*	Negative
MAP3K7	Intermediate	*IFNA1*	Negative
PPARA	Intermediate	*IFNB1*	Negative
TAB1	Intermediate		
ECSIT	Intermediate		
UBE2V1	Low		

Genes with significant variation in this particular gene expression among different samples. Gene names are those approved by the HUGO Nomenclature Committee. Additional information is given in Online Supplementary Table S2.

cytometry (FACS) and/or western blot analysis were carried out on the TLR that had been found to be expressed at the mRNA level (TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9 and TLR10) plus the CD80 and CD86 molecules in 30 and 59 cases, respectively, with available material. Detailed results from these experiments are given in *Online Supplementary Tables S7 and S8.*

With a 5% cut-off value for positivity in FACS analysis, TLR1, TLR7, TLR10 and CD86 proteins were expressed in almost all CLL cases, in accordance with their mRNA levels, while CD80 was negative in all cases. TLR9 expression was detected in 8/30 (26.6%) cases tested; however, only 3/30 cases carried more than 10% positive cells. TLR6 protein was expressed in 20/30 cases. Despite high mRNA levels for TLR6, in most cases (17/20) this protein was expressed by a minority of CLL cells (5-10%). In contrast, TLR2 and TLR8 proteins were expressed by almost all cases despite low mRNA expression.

The FACS expression patterns of TLR1, TLR2, TLR8 and TLR9 were further confirmed by western blotting (Figure 2, *Online Supplementary Table S8*). In particular, TLR1 and TLR2 were positive in all cases tested, TLR8 was positive in 43/59 cases (72.7%), while TLR9 was positive in 18/59 (30.5%) cases. The ratio of TLR9 relative to β-actin protein band intensity was low in most positive cases.

Gene expression profiles in relation to the molecular features of B-cell receptors

Based on the fact that TLR are considered to have a co-stimulatory effect on the BcR, we sought for differences in expression profiles for TLR signaling pathway-associated genes in subgroups of cases defined by BcR molecular features, such as the repertoire and mutational status of the

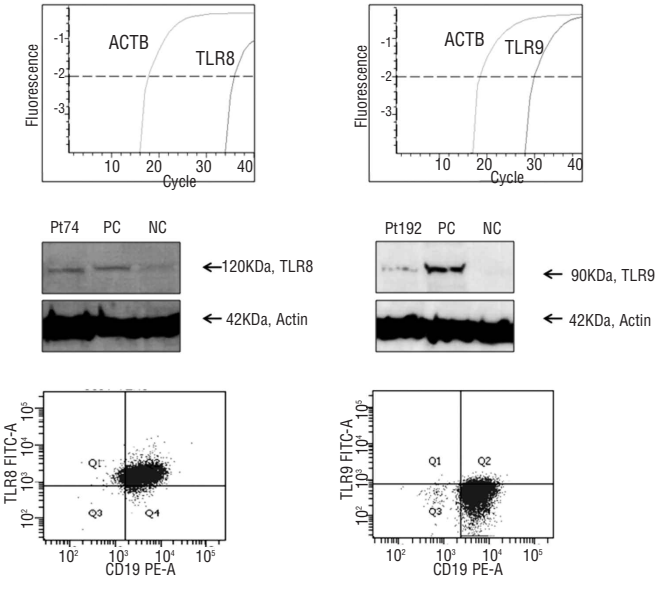

Figure 2. TLR8 and TLR9 mRNA and protein expression in CLL. RQ-PCR (upper diagrams), western blotting (middle diagrams) and flow cytometry results (lower diagrams) are shown for two representative cases. Overall, both mRNA and protein levels for TLR9 were low, whereas TLR8 protein levels were relatively high despite generally low mRNA levels, indicating possible post-transcriptional regulation of TLR8 expression. ACTB: β-actin.

IGHV genes or the expression of stereotyped BcR. All comparisons were performed for the complete gene set profiled in the present study. However, in the following paragraphs, specific reference is made only to those genes showing statistically different expression between the subgroups compared.

I. IGHV gene mutational status

Comparison in subgroups of cases carrying mutated or unmutated *IGHV* genes (124 and 67 cases, respectively) revealed significant up-regulation of *CD80*, *CD86*, *IL6*, *IFNG* and *TLR4* and down-regulation of *TLR8* and *NFKBIL1* (coding for an IkB-like protein) in the mutated subgroup, with the greatest difference recorded for CD86. The results of this comparison are presented graphically in Figure 3 and also detailed in *Online Supplementary Table S9*. Differences in CD86 protein expression were also found by FACS analysis: in particular, the median percentage of positive cells in the mutated and unmutated subgroups was 42% (range, 9.9-94.2%) and 18% (range, 5.3-51.1%), respectively ($P<0.01$).

II. IGHV gene usage and B-cell receptor stereotypy

We analyzed the gene expression profiles of cases expressing stereotyped BcR utilizing certain *IGHV* genes by comparing subset #4 (mutated IGHV4-34/1GKV2-30 BcR, 10 cases) *versus* subset #1 (unmutated IGHV1/5/7-IGKV1(D)-39 BcR, 10 cases) *versus* subset #8 (unmutated IGHV4-39/IGKV1(D)-39 BcR, 4 cases). Significant differences ($P<0.05$) were identified for: (i) up-regulation of *TLR7* and *NFKBIA* (also known as IkBalpha) and down-regulation of *CD86* and *TLR4* in subset #1 *versus* subset #4 cases, respectively; (ii) up-regulation of *TLR4* and *MAP4K4*, which is considered to activate IKBKB, and down-regulation of *NFKBIA* and *RIPK2* (a component of TLR signaling complex) in subset #8 *versus* subset #1 cases, respectively; and, finally, (iii) up-regulation of *LY96* (associates with the extracellular domain of TLR4 and TLR2 and enhances their responses to the respective ligands) and down-regulation of *RIPK2* and *CD86* in subset #8 *versus* subset #4 cases, respectively. These differences are shown graphically in Figure 4 and also listed in full in *Online Supplementary Table S9*.

In order to investigate whether these distinctive, "subset-biased" profiles were independent of *IGHV* gene usage or mutational status, we focused on subset #4, which we compared to: (i) all other mutated cases; and (ii) non-subset #4 cases with IGHV4-34 BcR. In both comparisons, subset #4 cases expressed significantly higher levels of CD86 ($P<0.05$) and, *vice versa*, significantly lower levels of *IL10* ($P<0.05$). Additionally, subset #4 cases exhibited significantly lower expression of *IFNG* ($P<0.05$) compared to all other mutated cases and lower expression of *NFKBIA* ($P<0.05$) compared to non-subset #4 IGHV4-34 cases *(Online Supplementary Table S9)*.

Clinical correlations

With a median follow-up of 53 months (range: 4-278 months), the median progression-free and overall survival times in the entire cohort were 72 and 202 months, respectively (95% CI: 44.2-99.8 for progression-free survival and 123-281 for overall survival). Genes with significant variation in expression level were further evaluated for possible correlations with survival. On univariate analysis, significant parameters ($P<0.05$) for both progression-free and overall survival were Binet clinical stage at diagnosis, *IGHV* gene mutational status and CD38 expression; up-regulation of *CD86*, *IL6* and down-regulation of *NFKBIL1* were correlated only with longer progression-free survival (Table 2). Multivariate Cox regression analysis (including all factors with significant associations) revealed that only clinical stage at diagnosis and *IGHV* mutational status retained statistical significance for both progression-free survival and overall survival.

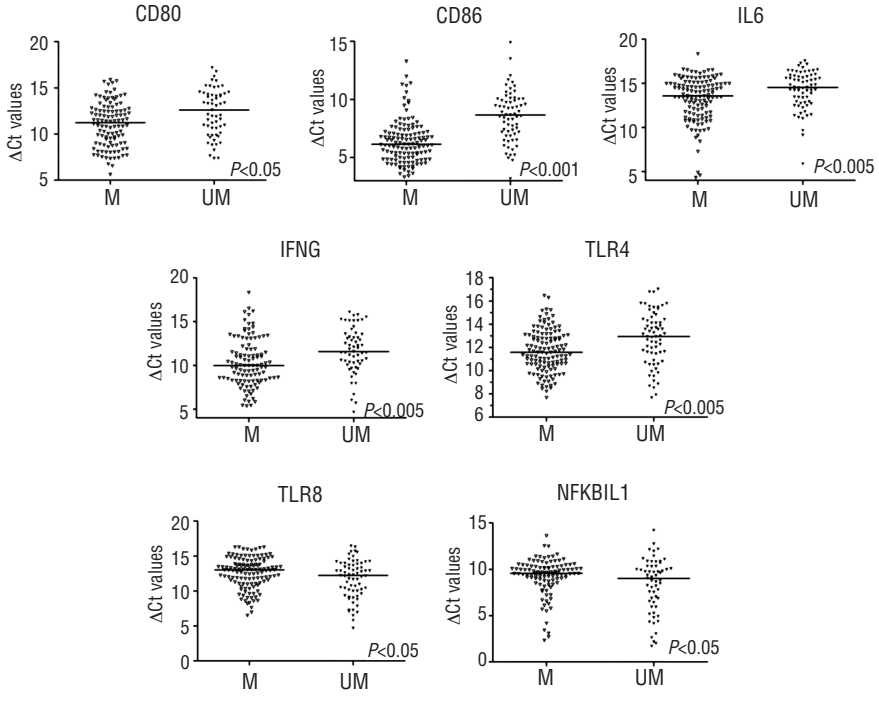

Figure 3. Differential expression of TLR pathway-associated genes in CLL cases with mutated (M) or unmutated (UM) IG receptors. IL6: interleukin 6; IFNG: γ-interferon; NFKBIL1: nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor-like 1. Note: lower values on the y axis correspond to higher expression levels, given that the ΔCt of each sample is determined as the difference between the Ct value of the gene of interest and the average Ct value of the housekeeping genes; for additional details, see the *Design and Methods* section. The graphs were created using the GraphPad Prism 5 software (La Jolla, CA, USA).

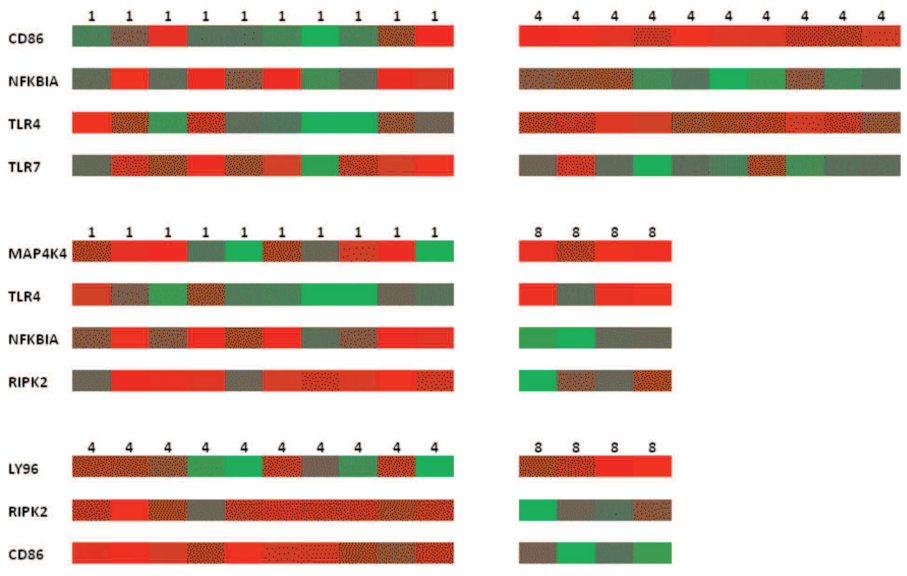

Figure 4. Differential expression profiles in subsets of CLL cases with stereotyped BcR. Each column concerns a different case, while each cell depicts graphically the actual results (ΔCt values) obtained for a given case. A two-color scale formating scheme was utilized for the conditional formating of the cells, ranging from red (high) to green (low). 1: subset #1 (IGHV1/5/7-IGKV1(D)-39); 4: subset #4 (IGHV4-34/IGKV2-30); 8: subset #8 (IGHV4-39/IGKV1(D)-39); subset numbering follows Stamatopoulos *et al.*[29]

Discussion

Molecular, functional and epidemiological findings indicate that both auto- and exogenous antigens expressed by common pathogens might be involved in the initiation and/or progression of CLL by selecting and stimulating leukemic B cells endowed with the appropriate antigen receptors.[4] A large body of data has emphasized the role of adaptive immune receptors (BcR); however, other possibilities must be taken into account, including stimulation via innate immune receptors such as TLR which have co-stimulatory activity in adaptive immune responses. We here report a comprehensive gene expression profiling of the TLR signaling pathway in a series of 192 CLL patients showing that CLL cells are molecularly competent for TLR signaling pathways with expression profiles indicative of antigen-activated B cells and also suggesting that TLR-mediated stimulation may be relevant to CLL development and evolution.

TLR9 along with TLR7 are the most studied members of the TLR family in CLL. Stimulation of CLL cells with CpG oligonucleotides, the natural ligand of TLR9, up-regulates the expression of co-stimulatory molecules, thereby potentially inducing the immunogenicity of CLL cells, and also has variable effects on proliferation and apoptosis.[33,34] Most studies have focused only on the functional outcome after stimulation and relatively little is known about the precise expression patterns of TLR9 in CLL. Our finding of low TLR9 mRNA and protein levels in most cases, with only a minority exhibiting intermediate levels, are in keeping with the results of a previous study in which variable mRNA expression levels and low protein levels were found in most CLL cases.[35] The effects of CpG stimulation, widely used to obtain metaphases in classic cytogenetic analysis, should not, therefore, be attributed exclusively to TLR9-mediated signaling. Furthermore, the fluctuations of TLR9 expression by cells under different experimental conditions and/or in a different activation status must be taken into account.

The highest expression among the receptors was recorded for TLR7, in agreement with literature data.[27]

Table 2. Clinical correlations: results from univariate analysis.

Parameter	Overall survival Log Rank test	Progression-free survival Log Rank test
IGHV gene mutational status	<0.005	<0.005
CD38 positivity (cutoff: 7%)	0.006	<0.005
Binet stage (A *versus* B+C)	<0.005	<0.005
Upregulation of *CD86*	0.445	<0.005
Upregulation of *IL6*	0.819	0.052
Downregulation of *NFKBIL1*	0.959	<0.005

This observation underlines the importance of stimulation via TLR7, as also shown by the treatment of CLL cells with TLR7 agonists, indicating that this receptor regulates a number of immunogenic properties[36,37] and is possibly involved in resistance to apoptosis.[38] High expression was also recorded for CD180, in line with previous reports that CD180 may promote the activation of both CLL and activated B cells.[39]

Interestingly, significant discrepancies were identified between mRNA and protein levels for certain TLR (TLR2, TLR6 and TLR8), as a high number of mRNA transcripts did not always correspond to strong protein expression and *vice versa*. Several factors could account for these discrepancies such as cellular intraclonal heterogeneity, differential activation status of malignant cells, different cell viability in different samples, etc. However, these results might also be taken as evidence that post-transcriptional regulatory mechanisms might modulate TLR expression in CLL, in keeping with previous studies on other surface-membrane antigens (including nitric oxide synthase and CD71).[40,41]

The observed inter-patient variability in the expression patterns of some TLR and downstream molecules prompted us to investigate potential associations with other features related to molecular pathways that distinguish various subgroups of CLL patients. Given that each TLR rec-

ognizes distinct pathogen molecular patterns and that extensive "cross-talk" occurs between TLR- and BcR-mediated signals, it is reasonable to suggest that the observed variability might reflect distinctive antigen encounters. Along this line of reasoning, we explored potential differences in the TLR signaling pathway among CLL cases carrying BcR with different molecular characteristics, in view of emerging evidence that the functional antigen reactivity profile endowed by the BcR likely underlies the biological behavior of the CLL clone, eventually determining clinical outcome.[2,4,42]

First, we compared cases with mutated or unmutated immunoglobulin receptors and uncovered few differences, in keeping with the well-established uniform gene expression profile of CLL regardless of *IGHV* gene mutational status.[43,44] That notwithstanding, among the few genes differentially expressed, those for the co-stimulatory molecules CD80 and CD86 were significantly up-regulated in mutated cases. This finding is in agreement with previous reports showing that stimulation of CLL cells through TLR as well as CD40 induces CD80 and CD86 expression and increases cell immunogenicity.[33,45] A plausible interpretation is that mutated cases expressing higher levels of CD80 and CD86, being potentially more immunogenic, are more susceptible to microenvironmental control, which would explain, at least in part, their more indolent clinical behavior.

CLL subgroups defined by *IGHV* gene mutational status are not homogeneous. Rather, within each mutational category, cases assigned to subsets expressing distinct stereotyped BcR have been shown to share distinctive, subset-biased genomic aberrations, gene expression profiles and, very likely, clinical presentation and outcome,[40,47] leading to the concept that the clinical behavior of CLL might reflect the antigen reactivity profile of the leukemic clones.[42,48,49] On this basis, we narrowed down our comparisons to cases assigned to different subsets with stereotyped BcR with a special focus on subsets #1 and #4. This choice was partly guided by practical considerations: individually, each subset accounts for only a small fraction of a given CLL cohort and sample availability is, therefore, a limiting factor. At the same time, subsets #1 and #4 are the most populated subsets in the unmutated and mutated category, respectively, with increasing evidence that they may be considered as prototypes of "bad prognosis" and "good prognosis" subsets.

Comparison of the two subsets suggests a TLR7-tolerized state for CLL clones assigned to subset #4. As recently reported, CLL B cells can become TLR7-tolerized after exposure to TLR7 ligands with the tolerant state being recognized by the down-regulated *TLR7* mRNA levels and the expression of high levels of co-stimulatory molecules.[37] Our finding of significant down-regulation of *TLR7* and up-regulation of *CD86* in subset #4 is in line with this scenario. Notably, we observed "subset #4-biased" profiles when we compared stereotyped subset #4 IGHV4-34 cases to: (i) cases utilizing "non-subset #4" IGHV4-34 BcR; and, (ii) cases with mutated BcR utilizing other *IGHV* genes. "Subset-biased" profiles of the TLR signaling pathway, independently of *IGHV* gene usage or mutational status, were also obtained when comparing unmutated cases belonging to subsets #1 and #8.

In conclusion, the main findings of our study can be summarized as follows. First, all the TLR expressed in activated B cells were also expressed (though variably) in CLL, further supporting the notion that CLL B cells are antigen-experienced. Second, the TLR-signaling framework is competent in CLL cells, since several TLR are expressed together with their cognate signaling mediators. Finally, variability of expression for specific TLR and related molecules was observed within different subsets of patients with stereotyped BcR. This last finding suggests that CLL clones with distinctive antigen reactivity are able to respond in a distinct fashion also to different members of the TLR family, alluding to subset-biased recognition of and selection by the respective ligands.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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