Toll-like receptor stimulation in splenic marginal zone lymphoma can modulate cell signaling, activation and proliferation

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ABSTRACT

Recent studies on splenic marginal zone lymphoma identified distinct mutations in genes belonging to the B-cell receptor and Toll-like receptor signaling pathways, thus pointing to their potential implication in the biology of the disease. However, limited data is available regarding the exact role of TLRs. We aimed at characterizing the expression pattern of TLRs in splenic marginal zone lymphoma cells and their functional impact on the activation, proliferation and viability of malignant cells *in vitro*. Cells expressed significant levels of TLR1, TLR6, TLR7, TLR8, TLR9 and TLR10 mRNA; TLR2 and TLR4 showed a low, variable pattern of expression among patients whereas TLR3 and TLR5 mRNAs were undetectable; mRNA specific for TLR signaling molecules and adapters was also expressed. At the protein level, TLR1, TLR6, TLR7, TLR9 and TLR10 were detected. Stimulation of TLR1/2, TLR2/6 and TLR9 with their respective ligands triggered the activation of IRAK kinases, MAPK and NF-κB signaling pathways, and the induction of CD86 and CD25 activation molecules, although in a heterogeneous manner among different patient samples. TLR-induced activation and cell viability were also inhibited by a specific IRAK1/4 inhibitor, thus strongly supporting the specific role of TLR signaling in these processes. Furthermore, TLR2/6 and TLR9 stimulation also significantly increased cell proliferation. In conclusion, we demonstrate that splenic marginal zone lymphoma cells are equipped with functional TLR and signaling molecules and that the stimulation of TLR1/2, TLR2/6 and TLR9 may play a role in regulating disease pathobiology, likely promoting the expansion of the neoplastic clone.

Introduction

Splenic marginal zone lymphoma (SMZL) is a distinct mature B-cell neoplasm which was included in the 2008 WHO classification of tumors of hematopoietic and lymphoid tissues.[1] SMZL is characterized by the accumulation of small and medium-sized clonal lymphocytes into the spleen and splenic hilar lymph nodes, with frequent involvement of the bone marrow and the peripheral blood.[1,2] The clinical behavior of SMZL is generally indolent; however, in a proportion of patients SMZL may evolve into an aggressive Diffuse Large B Cell lymphoma.[2]

Recent studies have reported mutations affecting the NF-κB signaling pathway in a proportion of SMZL cases.[3-5] Such mutations have been identified in both the B-cell Receptor (BCR) and Toll-Like Receptor (TLR) signaling pathways e.g. *CARD11* and *MYD88* gene mutations, respectively,[3,5-9] implicating immune signaling in the natural history of selected SMZL cases. This claim is also supported by the particularly skewed immunoglobulin heavy variable (IGHV) gene repertoire in SMZL, where a substantial proportion of cases (from 20% to more than 30%, depending on the series) express a single IGHV gene allele, namely IGHV1-2*04, suggesting (super)antigenic pressure on the malignant clone involving inflammatory/infectious agents.[10-12] Indeed, existing evidence shows that SMZL is associated with viral infections, e.g. with the hepatitis C virus (HCV), as suggested by a number of complete remissions after viral eradication.[13] Interestingly, antigen reactivity profiling studies have shown that the recombinant mAbs from SMZL clones expressing IGHV1-2*04 BcR IGs are poly- and self-reactive. They bind to nuclear, cytoplasmic and membrane antigens expressed by human cells and also react against human serum, implying derivation from natural antibody-producing B cells of MZ origin.[10]

Therefore, SMZL can be considered a prototypic infection/inflammation associated tumor. Nevertheless, while several studies focused on the role of the BcR in SMZL, information is still lacking on the potential role played by TLR in disease onset and progression, which is relevant to investigate given that TLRs are implicated in the immune recognition of inflammatory/infectious elements in both normal and malignant B-cell clones.[14,15] Briefly, TLRs recognize distinct molecular patterns belonging to danger signals or microbes, and trigger an inflammatory signaling pathway inside the cells.[16] The

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adaptor molecule MyD88 connects distinct TLRs to proximal IRAK kinases which then trigger a cascade of phosphorylation events, eventually leading to MAPK and IKK activation and the induction of specific transcriptional programs including that of the NF-κB complex.[16]

With all the above in mind, we herein aimed at characterizing TLR expression and function in SMZL, in order to assess the potential impact of TLR signaling on the biology of this disease.

Methods

Cell purification

Blood and tissue samples were obtained from SMZL patients after informed consent as part of a study (ViVi-NHL) approved by the institutional ethics committee. SMZL cells were negatively selected and purified using a B-cell enrichment kit (RosetteSep; StemCell Technologies) following the manufacturer's instructions. Normal B cells were purified by negative selection (EasySep; StemCell Technologies) from buffy coats. Preparations were virtually devoid of NK cells, T-lymphocytes and monocytes.

In 19 SMZL cases, total peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation without further purification (the percentage of $CD19^+$ cells in these cases is reported in *Online Supplementary Table S1*). In all cases, subsequent flow cytometric analyses were performed after gating on CD19+ cells.

TLR expression analysis

SMZL cells were analyzed either at the time of blood withdrawal or after thawing. The analysis was performed by flow cytometry using the following antibodies: anti-TLR1 and anti-TLR7 (AbCam); anti-TLR2 (Invitrogen); anti-TLR6 and anti-TLR10 (IMGENEX); anti-TLR8 (DENDRITICS), and anti-TLR9 (e-Biosciences). The BD Cytofix/Cytoperm reagent was used for the intracellular staining of TLR7, TLR8 and TLR9. Antibodies against IgD, IgM, CD38 and CD19 were used to detect normal MZ B cells in spleen samples, as previously described.[18] 7-Amino-Actinomycin D (7-AAD, BD Pharmingen™) was used for the exclusion of nonviable cells in flow cytometric assays. All flow cytometric analyses were performed on a FC-500 (Beckman Coulter) or on a FACSAria II (Becton Dickinson).

Cell culture and functional studies

SMZL cells were either unstimulated or stimulated with specific TLR ligands (*Online Supplementary Methods*). Collection was performed either after 24 hours for CD86 (BD Pharmingen) and CD25 (Beckman Coulter) flow cytometry analysis, or after 48 hours to assess cell proliferation (Ki67 staining, BD Biosciences), apoptosis (Annexin V and Propidium Iodide, Bender Med Systems) and viability (CellTiter-Glo Luminescent Cell Viability Assay, Promega). The IRAK1/4 inhibitor I (Sigma) was used at the concentration of 3 μM 30 minutes before TLR stimulation. The MyD88 inhibitory peptide and control peptide (Novus Biologicals) were used at the concentration of 100 μM.

Molecular studies

i. Immunogenetic analysis.

PCR amplification and sequence analysis of IGHV-IGHD-IGHJ gene rearrangements was performed as previously described.[20]

ii. Qualitative assessment of *TLR1-10* and *TIR8* expression by Reverse transcription-polymerase chain reaction (RT-PCR) with appropriate primers (*Online Supplementary Table S2*).

iii. Gene expression profiling

Total RNA was isolated with the RNeasy Mini kit (QIAGEN). RNA was converted by means of reverse transcription to cDNA using the RT2 First Strand Kit (SABiosciences). Gene expression profiling of the TLR signaling pathway was performed by Real-time RT-PCR on cDNA arrays using the RT² Profiler™ PCR Array kit (PAHS-018 array, SABiosciences). Data analysis was performed as previously reported.[21] We assigned a "High expression level" to mRNAs showing an average $\Delta Ct \leq 6.6$; "Intermediate expression level" to mRNAs showing an average $\Delta Ct > 6.6$ and ≤ 9.9; "Low expression level" to mRNAs showing an average $\Delta Ct > 9.9$ and ≤ 13.2 and "Negative expression" to mRNAs showing an average $\Delta Ct > 13.2$.

Western blot analysis

Antibodies used for Western blot analysis were: Phospho-ERK (Cell Signaling Technology), ERK (Santa Cruz Biotechnology, CA), Phospho-IKK (Cell Signaling Technology), IKK (Santa Cruz Biotechnology, CA), Phospho-p38 (Cell Signaling Technology), p38 (Santa Cruz Biotechnology, CA) and anti-β-Actin HRP-conjugated (Sigma).

Results

TLR expression profiling in splenic marginal zone lymphoma

We examined peripheral blood $CD19^+$ malignant lymphocytes from patients with SMZL (*Online Supplementary Table S1*) for the expression pattern of TLRs. First, we analyzed the mRNA expression of *TLR1-10* and *TIR8* (also known as *SIGIRR*) by RT-PCR and we identified mRNA transcripts from only *TLR1, TLR2, TLR6, TLR7, TLR8, TLR9* and *TLR10* (Figure 1A). We also analyzed normal mononuclear cells as technical positive controls which, as expected, expressed all analyzed mRNA transcripts (Figure 1A).

We then evaluated the protein levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9 and TLR10 by flow cytometry (Figure 1B,C) and observed a robust correlation with the mRNA levels. In particular, we observed a high percentage of cells expressing TLR1, TLR7 and TLR9, intermediate expression of TLR2, TLR6, TLR8 and TLR10 and low to undetectable expression of TLR4 (*data not shown*), as expected by the RT-PCR data.

We focused our attention on TLR10 because it is "B-cell restricted",[22,23] and its function and expression patterns are ill-defined in the different B-cell subsets. We compared TLR10 expression between SMZL cells from peripheral blood or spleen and normal circulating B lymphocytes from buffy coat. We observed that the percentage of TLR10 positive cells was higher in SMZL samples from both spleen and peripheral blood ($P<0.01$; n=6 and n=18 respectively) compared to normal B cell samples from buffy coat (n=10); in contrast, no significant difference was observed between SMZL cells from spleen versus peripheral blood (Figure 1D). We then identified, by six-color flow cytometry staining, the 7-AAD-/$CD19^+$/$CD38^-$/$IgD^{neg/low}$/IgM^+ MZ B cell population in 5 normal spleen samples and further screened these MZ cells for TLR10 expression. We found a similar level of expression between MZ B cells from spleen and total B cells from peripheral blood, which resulted as being lower compared to SMZL spleen and PB samples (Figure 1D).

To assess the possibility that SMZL cells were also equipped with TLR signaling mediators, we quantified the mRNA expression of 84 genes relevant to the TLR signal-

ing pathways and to the other inflammatory pathways such as *NFKB1* (see *Online Supplementary Table S3* for the list of genes analyzed) using a Real-Time RT-PCR array in 11 SMZL samples. We observed detectable levels for most downstream TLR signaling molecules with the highest levels for *FOS, JUN, MAPK* and *NFKB1* family members (Figure 2); medium to high levels for the key proximal TLR signaling molecules including *MYD88, TRAF6, IRAK* and *BTK*, and low to undetectable levels for *TLR3* and TLR5, the latter confirming the RT-PCR data.

TLR differentially trigger SMZL cell signaling and activation

In order to understand the functional role of TLR in SMZL, we stimulated *in vitro* lymphoma cells with specific ligands for the TLR1/2 heterodimer, TLR2/6 heterodimer and TLR9 receptors (Pam3CSK4, MALP-2 and CpG-ODN2006, respectively). We analyzed the activation of the TLR signaling pathway by monitoring the phosphorylation status of relevant kinases i.e. IKK, p38 and ERK. We found that all tested TLR ligands were able to induce activation of these kinases, with the strongest effect observed after CpG-ODN2006 treatment (two representative patients are shown in Figure 3A).

The functional impact of TLR triggering on SMZL cell behavior was also confirmed by the flow cytometric analysis of CD86 and CD25 expression after 24 hours of exposure to TLR ligands on SMZL cells from 45 patients. In more detail, Pam3CSK4, MALP-2 and CpG-ODN2006 increased the percentage of CD86 and CD25 positive cells, that was higher in the case of CpG-ODN2006 (TLR9 stimulation), in accordance with the biochemical results (Figure 3B,C and *Online Supplementary Table S4*).

We then evaluated the potential synergistic or antagonistic effect of concurrent BCR and TLR activation. For this purpose, we stimulated cells with soluble anti-IgM alone or in combination with CpG. We did not observe any significant differences in the numbers of CD86 and

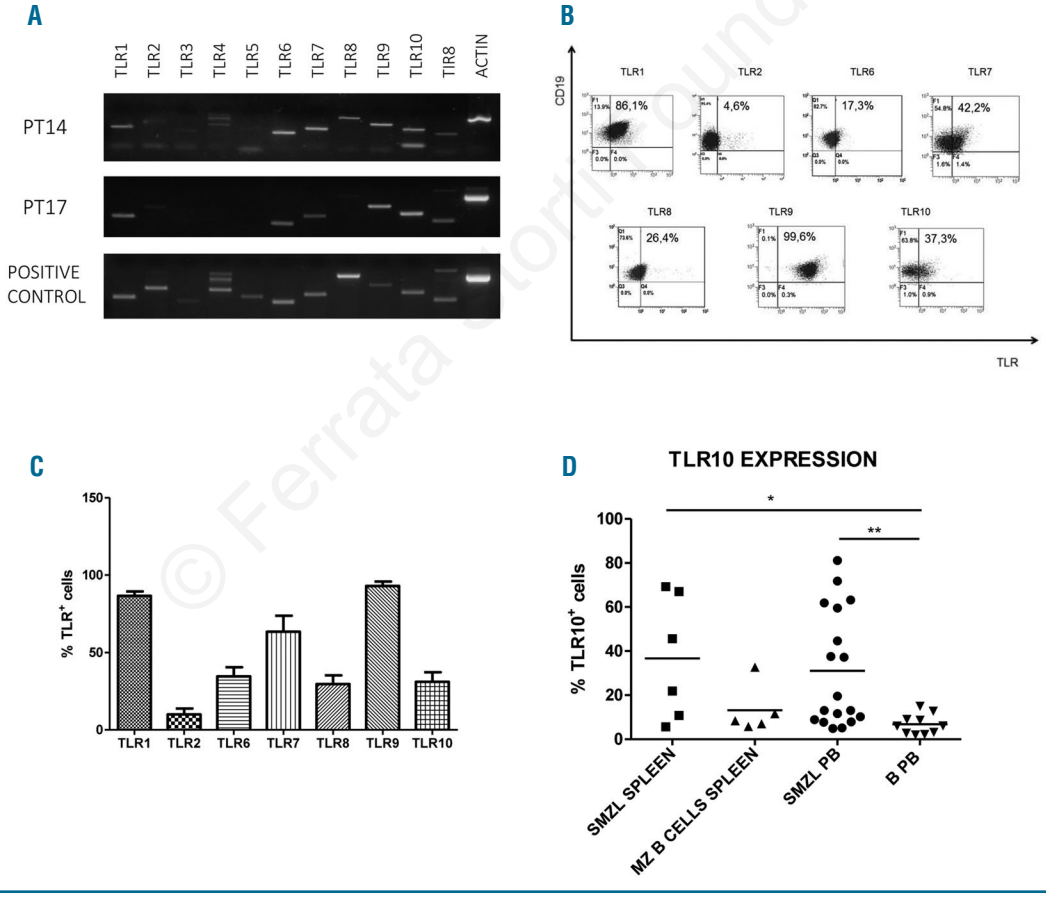

Figure 1. TLR expression in SMZL cells. (A) RT-PCR was performed to analyze the expression of Toll-like receptors 1-10 mRNAs (lanes 1-10) and of SIGIRR/TIR8 mRNA (lane 11). Actin mRNA was analyzed as internal control (lane 12). Two representative SMZL samples (out of 5 analyzed) are shown in the upper part of the panel (Patient 14 and Patient 17). Normal Human Peripheral Blood Mononuclear Cells (PBMC) were analyzed as technical control and are shown in the lower part of the panel. (B) A representative flow cytometry analysis for each TLR is reported; $CD19^+$ SMZL cells were analyzed for TLR1, TLR2, TLR6, TLR7, TLR8, TLR9 and TLR10 (sample 24, 20, 19, 9, 28, 19 and 8 respectively of *Online Supplementary Table S1*). (C) TLR expression was calculated as percentage of TLR-positive cells among $CD19^+$ SMZL cells (mean +/- SEM). 13 patient samples were analyzed for TLR1, TLR6 and TLR9; 19 for TLR2; 15 for TLR7 and TLR8; 10 for TLR10. (D) Flow cytometry analysis of TLR10. Percentages of TLR10 positive cells were analyzed in B cells from splenectomy specimens obtained from 6 SMZL patients, in normal marginal zone B cells from 5 spleen samples, in B cells from peripheral blood of 18 SMZL patients and in B cells from peripheral blood of 10 healthy donors. The significance of the observed differences was assessed with the Mann-Whitney test. *indicates a *P* value <0,05; **indicates a *P* value < 0,01.

CD25 positive cells when SMZL samples were cultured with anti-IgM alone (Figure 3D,E). The combination of the two stimuli induced similar effects to those observed with CpG stimulation alone.

According to recently published data, SMZL patients can be classified into three distinct subgroups with a different somatic hypermutation (SHM) status in their clonotypic IGHV genes:[11] significantly mutated (<97% identity with the IGHV gene germline sequence), minimally mutated (identity, 97-99.9%) or truly unmutated (identity, 100%). In our cohort, 22 out of 43 cases (51%) were classified as significantly mutated (SM), 14 out of 43 (33%) as minimally mutated (MM) and 7 out of 43 (16%) as truly unmutated (TU) (*Online Supplementary Table S1*). We observed a trend towards lower CD86 and CD25 expression after TLR9 ligation in cells as the SHM load decreases. This was particularly evident for patients carrying truly unmutated IGHV genes, though statistical significance was not reached, probably due to small sample numbers (Figure 3F,G).

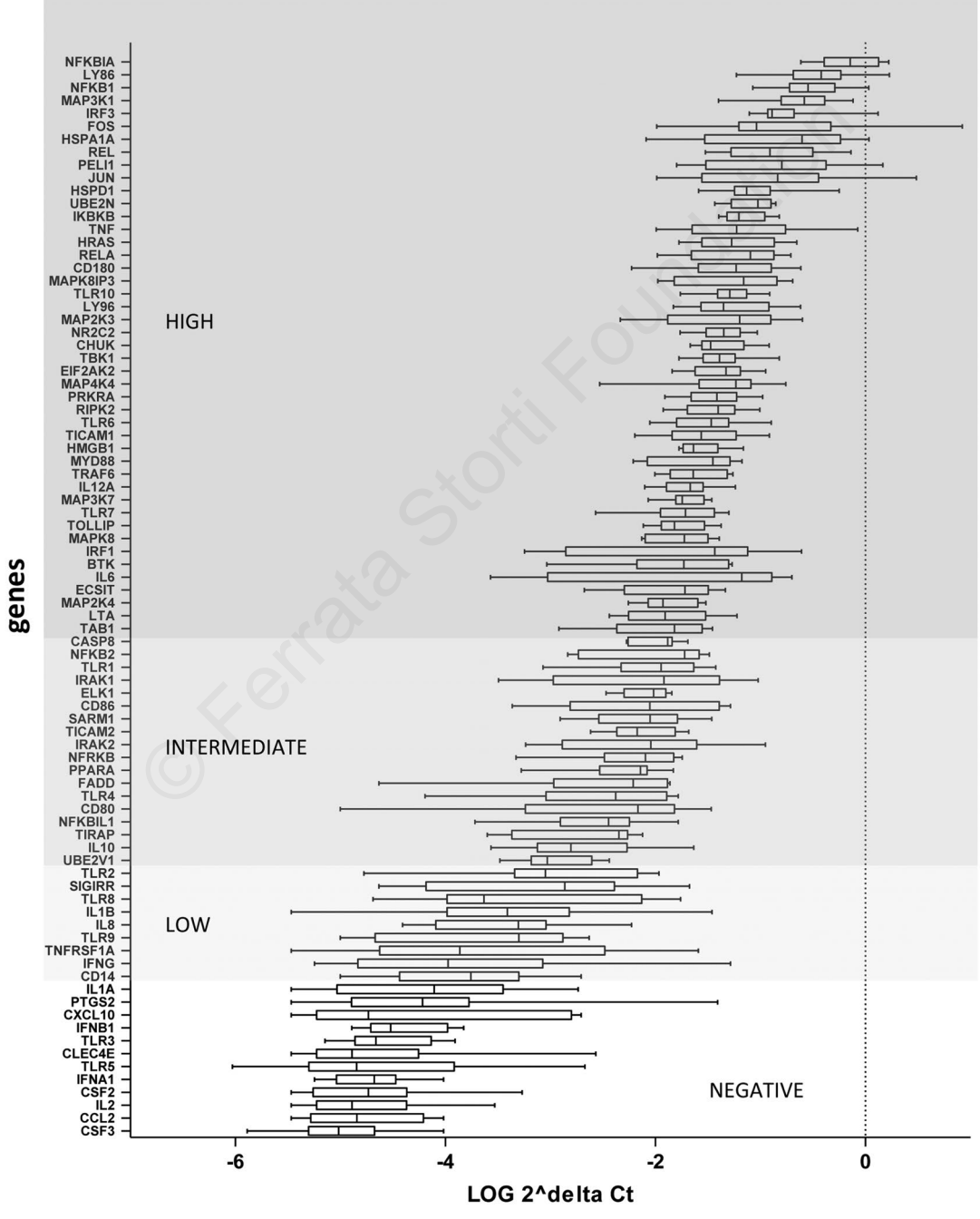

Figure 2. Gene expression profile of 84 genes belonging to the TLR signaling pathway was performed in SMZL cells from 11 patient samples by Real Time RT-PCR array. Relative expression of each indicated RNA is reported as 2^-ΔCt value by a box and whiskers plot.

TLR2/6 and TLR9 stimulation induces cell proliferation

We monitored the viability of lymphoma cells cultured *in vitro* with the TLR ligands Pam3CSK4, MALP-2 and CpG-ODN2006 for 48 hours by measuring the levels of ATP that correlate with cell metabolic activity. We observed that the relative number of metabolically active SMZL cells was very heterogeneous among different patient samples. Nevertheless, the Wilcoxon matched-pairs signed-rank test showed a significant increase in cell viability when cells were cultured with TLR9 ligand (P<0.01) (Figure 4A). The apoptosis rate was not significantly different among samples which were either stimulated or not stimulated through the TLR, as measured by Annexin V staining (Figure 4B). When we analyzed the induction of proliferation by Ki67 staining on up to 33 patient samples (depending on the particular TLR analyzed), we observed a significant increase of Ki67$^+$ cells after the addition of MALP-2 or CpG, suggesting that the cell viability increase observed in panel A was likely due to proliferation rather than apoptosis (Figure 4C,D).

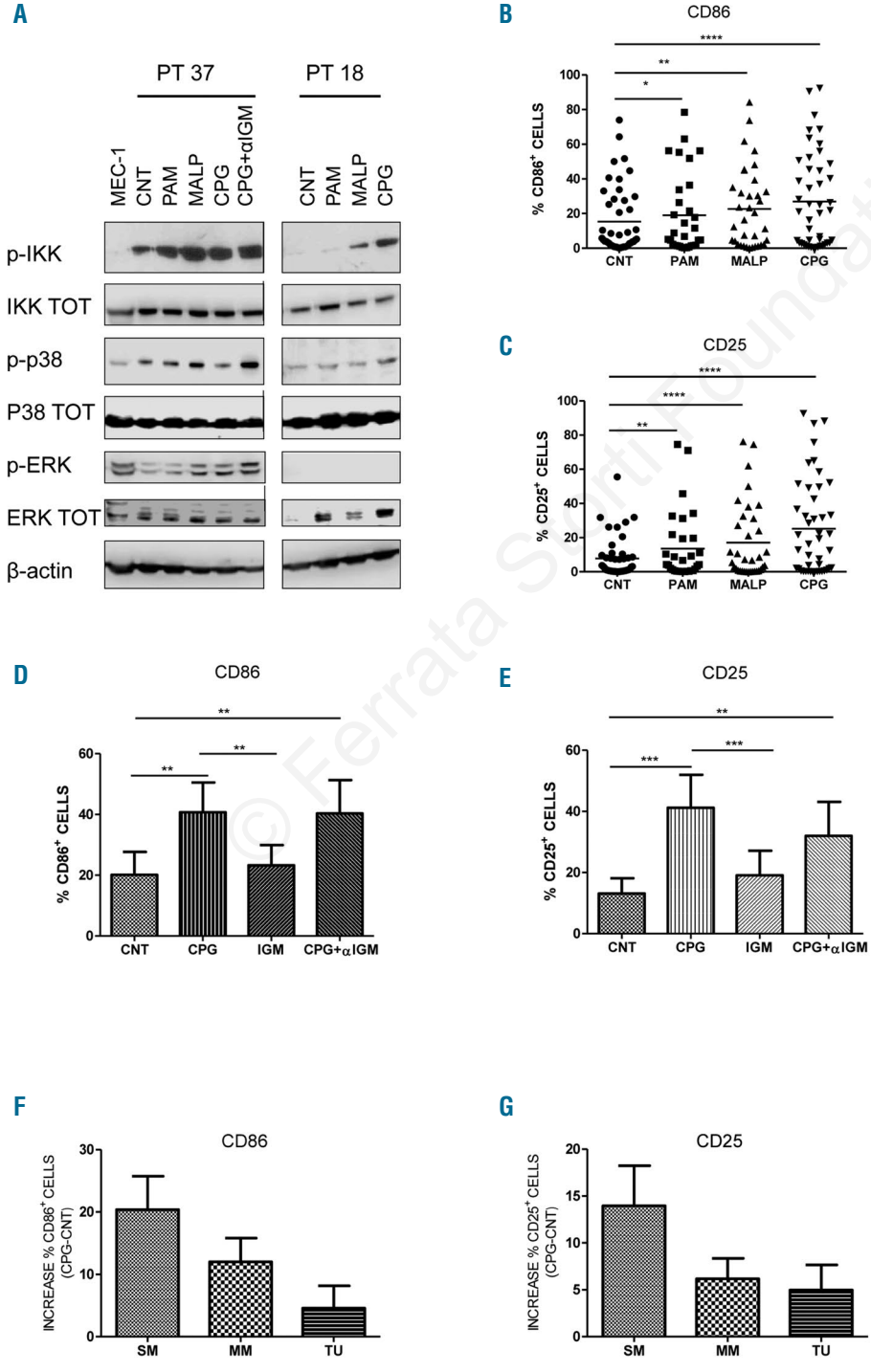

Figure 3. ***In vitro*** **activation of SMZL cells after TLR stimulation. (A) Western blot analysis of lysates of SMZL cells stimulated for 1 hour with distinct TLR ligands (Pam3CSK4 for TLR1/2; MALP-2 for TLR2/6 and CpG-ODN2006) or with the combination of CpG and anti-IgM was performed to detect the phosphorylation status of the IKK, p38 and ERK molecules. Total protein levels of the corresponding kinases, as well as β-actin levels, were analyzed as internal controls. MEC-1 cell lysate was used as internal control. (B-C) SMZL cells were incubated with Pam3CSK4 (n=31), MALP-2 (n=35) and CpG-ODN2006 (n=45). After 24 hours, cells were analyzed by flow cytometry for the expression of CD25 and CD86. The percentage of CD86$^+$ and CD25$^+$ cells (in graph B and C, respectively) was calculated considering only CD19$^+$ cells. The mean percentage of CD86$^+$ cells increased to 19.03±22.99 when stimulated with Pam3CSK4 (P<0.05); 22.59±22.88 in the case of MALP-2 stimulated cells (P<0.001) and 26.97± 27.05 for CpG-stimulated cells (P<0.0001). Regarding the percentages of CD25$^+$ cells, the mean percentage for Pam3CSK4-stimulated cells was 13.56±19.95 with a P value <0.01; for MALP-2 stimulated cells it was 17,01±22,16; and for CpG-stimulated cells it was 25,22±27,71 with a P value < 0.0001 for both conditions. (D-E) Patient samples were analyzed for CD86$^+$ and CD25$^+$ cells upon 24 hours of cell culture with or without the addition of the indicated stimuli. 12 cases were tested for unstimulated (CNT), CpG-ODN2006 and anti-IgM conditions; 10 cases were analyzed after concomitant stimulation with CpG-ODN2006 and anti-IgM antibodies. The Wilcoxon matched-pairs signed rank test was used for the statistical analysis. *indicates a P value <0,05; ** indicates a P value < 0,01; *** indicates a P value < 0,001; **** indicates a P value < 0,0001. (F-G) The increase in the percentage of CD86$^+$ and CD25$^+$cells upon CpG stimulation over untreated cells was analyzed in different groups of patient samples with different somatic hypermutation status of the IGHV genes (SM: significantly mutated; MM: minimally mutated; TU: truly unmutated).**

Accordingly, after CpG treatment we observed a decrease in the levels of the p27 protein, an inhibitor of the cell cycle entry that is downregulated during cell cycle progression (Figure 4E).

IRAK1/4 inhibitor blocks TLR-induced signaling pathway, activation and cell viability

In order to confirm the specificity of the TLR-induced effects, we treated SMZL cells with a specific IRAK1/4 inhibitor and monitored the activation status of the cells with or without the different TLR ligands. We found significantly reduced expression of both CD25 and CD86 upon the IRAK inhibitor pre-treatment and following CpG stimulation as compared to untreated cells (Figure 5A,B). We then measured cell viability in the presence or absence of IRAK1/4 inhibitor of cells stimulated or not with different TLR ligands. We found that the increase in cell viability induced by TLR9 stimulation was blocked by IRAK1/4 inhibitor (Figure 5C). Finally we performed Western Blot analysis of the TLR signaling molecules previously analyzed, and we observed a marked reduction in the phosphorylation of IKK and a trend for the inhibition of phosphorylation of p38 and ERK upon IRAK1/4 inhibitor treatment (Figure 5D).

We also used another TLR inhibitor, namely MYD88 inhibitory peptide, and analyzed 4 SMZL samples for CpG-induced activation, noting a trend for reduced CD25 expression (*P* value=0.053); (*Online Supplementary Figure S1A,B*).

Finally, in order to gain a more comprehensive insight into TLR signaling in SMZL, in addition to circulating SMZL cells, we also analyzed available SMZL spleen samples. Specifically, we cultured the cells *in vitro* and assessed CpG-induced activation and cell viability after 24 hour incubation. As shown in *Online Supplementary Figure S2*, a trend for the induction of CD25 was observed after TLR9 triggering. Moreover, exposure to the IRAK1/4 inhibitor reduced both CpG-mediated activation and cell viability (*Online Supplementary Figure S2C,D*).

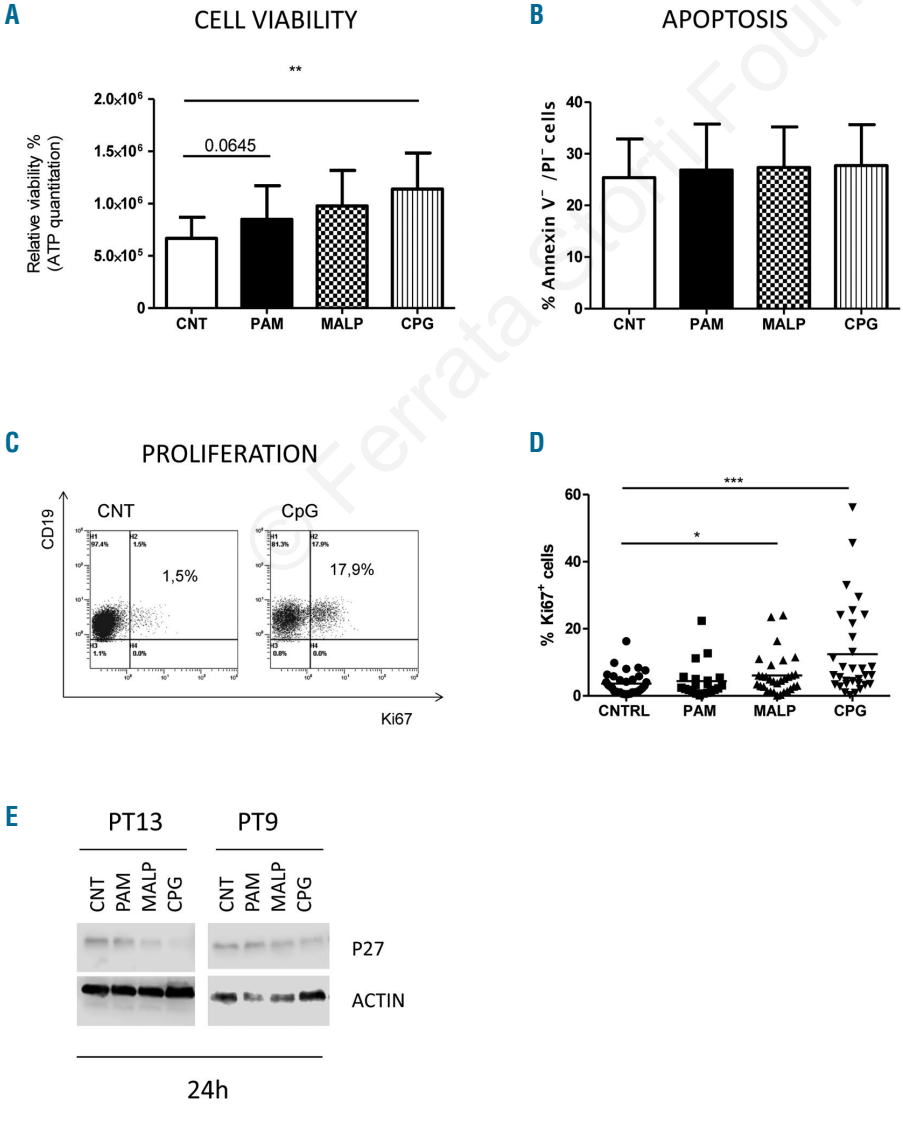

Figure 4. MALP-2 and CpG addition to cell culture medium increases the viability and proliferation of SMZL cells *in vitro*. (A) SMZL cells stimulated for 48 hours with specific TLR ligands as indicated, show an increase of relative viability as measured by ATP concentration. The Wilcoxon matched-pairs test was performed on 15 SMZL patient samples analyzed for CpG, 14 for MALP-2, and 10 for Pam3CSK4. Mean ± SEM of counts per second is reported. (B) The effect of *in vitro* TLR stimulation on spontaneous apoptosis of SMZL cells was evaluated by Annexin V/PI staining and flow cytometry analysis. 9 patient samples were analyzed for Pam3CSK4, 12 for MALP-2 and 13 for CpG. Mean ± SEM of counts per second is reported. (C-D) Ki67 staining was performed and the percentage of Ki67⁺ cells was calculated by flow cytometry analysis (considering Ki67⁺/CD19⁺ cells). A representative example is shown in panel C. The Wilcoxon matched-pairs signed rank test was used for the statistical analysis of data from 23 patient samples treated with Pam3CSK4, 32 with MALP-2 and 33 with CpG (panel D). indicates a *P* value <0,05; ** indicates a *P* value < 0,01; *** indicates a *P* value < 0,001. (E) Western blot analysis of the cell cycle regulator protein p27. Analysis of cell lysates from two patient samples cultured for 24 hours with Pam3CSK4, MALP-2 and CpG.

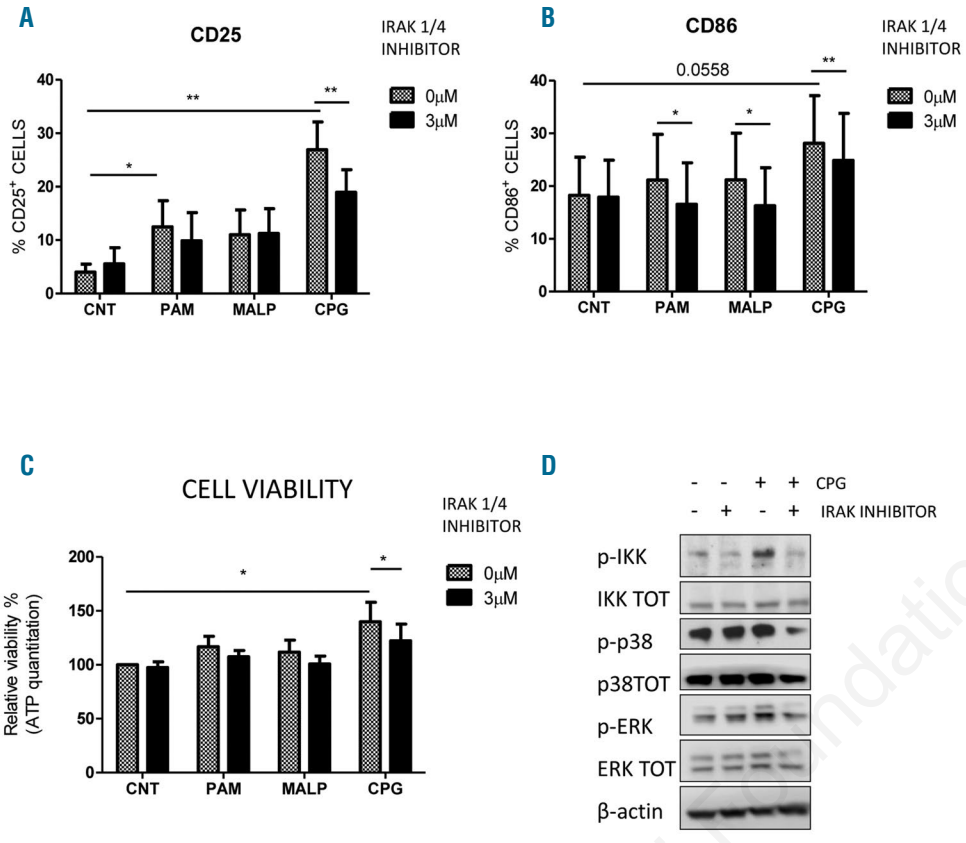

Figure 5. The IRAK1/4 inhibitor reduced the up-regulation of CD25 and CD86 expression induced by TLR stimulation. (A-B) SMZL cells from 9 samples were treated with 3 μm of IRAK inhibitor for 30 minutes and then stimulated with TLR ligands for 24h. CD25 and CD86 markers were then analyzed by flow cytometry and (C) cell viability was measured by ATP concentration. For the cell viability assay, data were normalized with each control (unstimulated untreated cells were considered as 100%). *indicates a *P* value <0,05; **indicates a *P* value < 0,01. (D) Lymphoma cells were treated with IRAK1/4 inhibitor and then stimulated for 1h with CpG (TLR9 ligand). Western Blot analysis was performed to detect the phosphorylation status of IKK, p38 and ERK molecules (one representative patient sample out of 3).

Discussion

TLRs are key modulators of innate immunity through the recognition of microbe-associated molecular patterns (MAMP).[16] In addition, they play a role in tissue repair, in the pathogenesis of autoimmune diseases as well as in the regulation of tumor microenvironment.[24] The involvement of TLRs in diseases associated with chronic inflammation can also be mediated by their ability to recognize danger-associated molecular patterns (DAMP), including endogenous proteins and components of apoptotic cells.[25] TLR ligands are also currently being explored as immunomodulators in oncology.[26,27] Nevertheless, emerging evidence suggests that TLR may also exert a direct effect on tumor cells and may regulate tumor progression.[28,29] For example, TLR ligands induce CLL cell viability and chemoresistance in a proportion of cases.[19] Furthermore, the unabated stimulation through TLR and/or IL-1R pathways accelerates leukemia progression in a mouse model of CLL.[19,30,31]

Recent studies suggest a role for microenvironmental interactions in the natural history of SMZL. In addition to distinct mutations in genes of the BcR, TLR and other signaling pathways, most notably the Notch pathway, SMZL is associated with HCV infection and autoimmunity. All these findings implicate antigen stimulation in SMZL ontogeny and, perhaps, evolution.[13,17,32,33] This claim is also supported by the remarkably skewed IG heavy and light chain gene repertoire,[20] as well as the antigen reactivity profile of SMZL BcR IG.[10]

Prompted by these observations, we explored the precise role of TLR triggering in SMZL, especially since published evidence on this issue is scarce and essentially derives from the analysis of archival material, thus hindering firm conclusions. In order to obtain a more comprehensive insight into the role of TLR signaling in SMZL immunopathogenesis, we performed *ex vivo* studies in a sizable series of well-characterized patients with SMZL, consolidated within the context of an international collaboration. We found that, similarly to normal circulating B cells, SMZL cells express high levels of TLR1, TLR7 and TLR9.[34] We also characterized the expression of TLR10, an orphan receptor that has been suggested to participate in a functional TLR signaling complex together with TLR1 and TLR2.[22] TLR10 has been deemed "B-cell specific", nevertheless, its functional impact on B cells remains obscure. Our studies showed that MZ B cells residing in the normal spleen exhibit TLR10 expression similar to peripheral blood B cells. Interestingly, in SMZL cells from either spleen or peripheral blood, TLR10 was significantly higher compared to normal circulating B cells. A trend for higher TLR10 expression in SMZL cells than in normal splenic MZ B cells was also noted, however, additional experiments are required before reaching definitive conclusions. Altogether, our finding of high levels of TLR10 in SMZL cells from both spleen and peripheral blood samples prompts further studies on the functional role of TLR10 in normal and malignant MZ cells.

We also provide functional evidence that, similar to TLR activation of normal B-cells, specific TLR ligands can activate SMZL cells, as suggested by the up-regulation of CD86 and CD25 on the cell surface, and the activation of kinases regulating classic TLR signaling pathways.[35,36] In order to confirm the specificity of the TLR-mediated activation, we treated SMZL cells with an inhibitor specific

for IRAK1/4, two key upstream kinases of the TLR signaling pathway. We observed a reduction of both the activation markers (CD25, CD86) while no significant effect was observed without TLR stimulation. Interestingly the TLR-mediated pro-survival effect was blocked using the IRAK1/4 inhibitor, suggesting that this pathway may represent a putative therapeutic target in specific subsets of cases.

The heterogeneity in the activation reflected, at least in part, the somatic hypermutation (SHM) status of the clonotypic IGHV genes, in that patients with truly unmutated IGHV genes tended to show lower cell activation after TLR stimulation compared to those bearing somatically hypermutated IGHV genes. This result may be taken to indicate that different modes of BcR and TLR synergism and/or antagonism may exist in SMZL depending on the IGHV SHM status of the malignant clones, similar to what we recently reported in CLL.[37] Admittedly, definitive conclusions regarding the precise biological relevance of these findings cannot be drawn before further validation in a larger cohort of patients, including a higher proportion of cases bearing IGHV1-2*04 IG receptors.

In terms of the functional consequences upon TLR ligation, we observed that TLR stimulation increased the proliferation of lymphoma cells *in vitro*, and that the CpG-ODN2006 induced the strongest signal among the analyzed TLR ligands. This finding was further confirmed by Western blot analysis of a key inhibitor of cell cycle, namely p27-Kip1, that was downregulated after TLR stimulation.

Overall, SMZL cells appear to be rather sensitive to TLR ligands, and this may reflect their similarity to normal MZ cells. Indeed, MZ B-lymphocytes are readily responsive to TLR ligands and they have the unique capacity to rapidly react to distinct bacterial antigens in a T-independent manner,[38] thus serving as "innate-like" lymphocytes acting at the forefront against bacterial antigens, proliferating and producing low-affinity, polyreactive antibodies. Likewise, SMZL cells may retain the capacity to rapidly respond to microbial antigens through activation and proliferation.

In conclusion, our study demonstrates that SMZL lymphoma cells are equipped with functional TLR and signaling molecules, and suggests that the stimulation of TLR1/2, TLR2/6 and TLR9 may play a role in regulating SMZL pathobiology *in vivo*, likely promoting the expansion of the neoplastic clone.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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