## Expression of functional toll-like receptors by B-chronic lymphocytic leukemia cells

**This study reports that B-chronic lymphocytic leukemia (B-CLL) cells display the same pattern of toll-like receptors (TLRs) proteins expression as normal B-cells, yet with overexpression of TLR9. Furthermore, TLR7 and TLR9 appear to be functional and liable to respond to specific ligands, respectively imidazoquinolines and CpG-ODN thus potentially opening new therapeutic approaches.**

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Toll-like receptors (TLRs) are major agents of innate immunity and initiators of adaptive immunity, involved in the activation of normal B-lymphocytes. TLRs are pattern recognition receptors (PRP) recognizing pathogens via pathogen-specific molecular patterns (PAMPs).[1] Although mRNAs derived from TLR genes have been reported in B-cells from lymphoproliferative disorders,[2] the expression and functionality of TLR proteins on these cells has seldom been investigated. However, such structures could provide an interesting therapeutic target if they are functional and their triggering induces relevant modifications of tumoral cells. Such approaches could be proposed for second-line therapy in CLL patients who harbour minimal residual disease after achieving complete remission.

Using flow cytometry, we examined the expression of TLRs proteins in normal and CLL B-cells. We then evaluated the effect of TLRs ligands directed to two of the TLRs expressed on B-CLL cells in terms of costimulary immunophenotype, proliferation and apoptosis.

In a first series, nineteen B-CLL patients with a Matutes score of 4 or 5 and absence of CD38 expression were evaluated. Three out of 19 were treated at the time of the study. The mean level of $CD5^+CD19^+$ peripheral B-cells was 91.6±3.8%. The same expression pattern of TLRs was noted on B-cells from 17 healthy donors and B-CLL patients, i.e. absence of TLR3 and TLR6, low expression of TLR2 and TLR4, and strong expression of TLR7, TLR9 and TLR10. TLR9 appeared significantly overexpressed on B-CLL cells. In a second series of 11 B-CLL patients and 11 normal controls, the expression of TLR7, TLR9 and TLR10 was further investigated separately on $CD19^+/CD5^-$ and $CD19^+/CD5^+$ cells since these compartments are respectively predominant and scarce in normal subjects, while the opposite is true for B-CLL patients. Normal $CD19^+/CD5^-$ cells from both populations displayed strictly identical expression of the three TLRs tested (MFI ratios of respectively 4.9±1.1 in B-CLL patients vs. 4.2±1 in controls for TLR7, 9.11±5.3 vs. 11.2+4.4 for TLR9 and

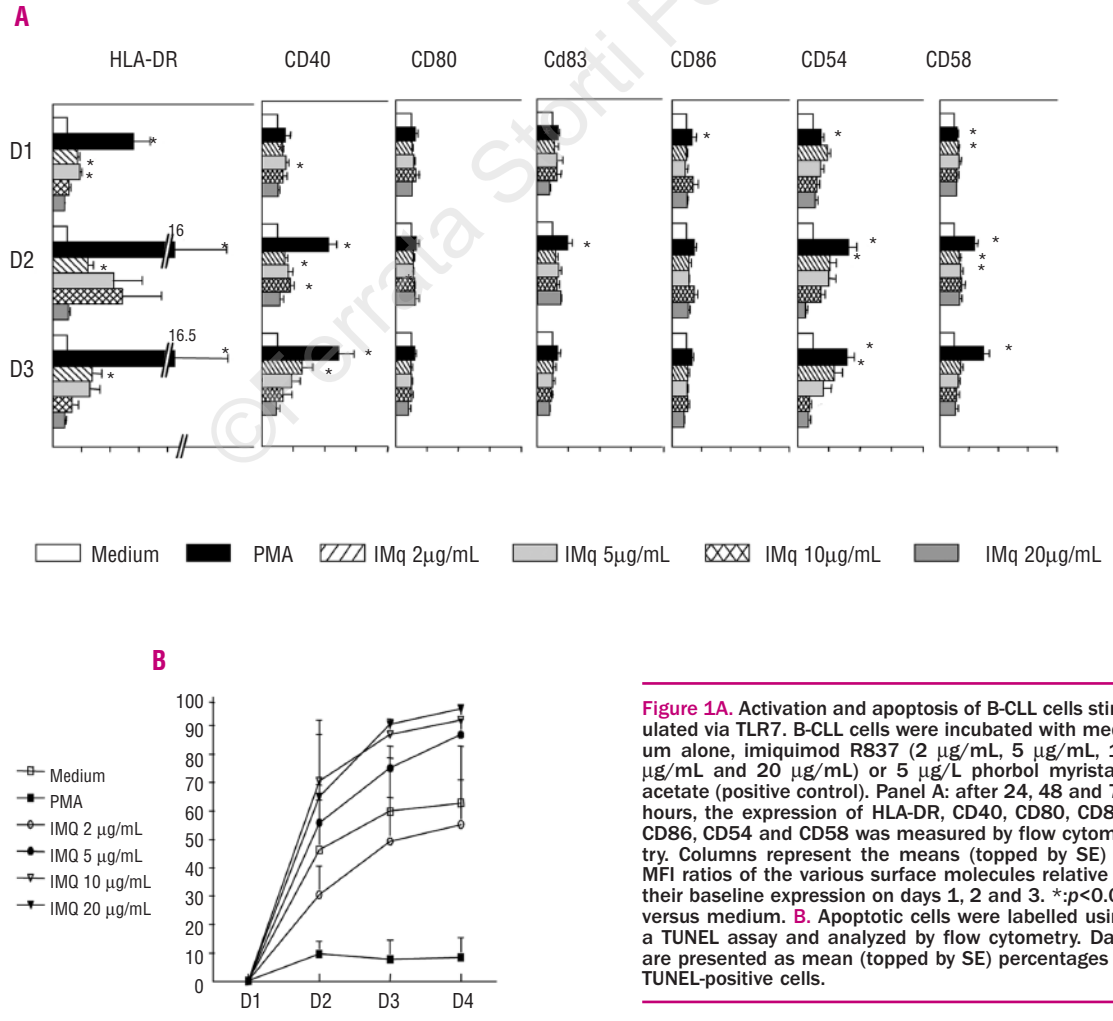


**Figure 1A.** Activation and apoptosis of B-CLL cells stimulated via TLR7. B-CLL cells were incubated with medium alone, imiquimod R837 (2 μg/mL, 5 μg/mL, 10 μg/mL and 20 μg/mL) or 5 μg/L phorbol myristate acetate (positive control). Panel A: after 24, 48 and 72 hours, the expression of HLA-DR, CD40, CD80, CD83, CD86, CD54 and CD58 was measured by flow cytometry. Columns represent the means (topped by SE) of MFI ratios of the various surface molecules relative to their baseline expression on days 1, 2 and 3. *:$p<0.05$ versus medium. B. Apoptotic cells were labelled using a TUNEL assay and analyzed by flow cytometry. Data are presented as mean (topped by SE) percentages of TUNEL-positive cells.

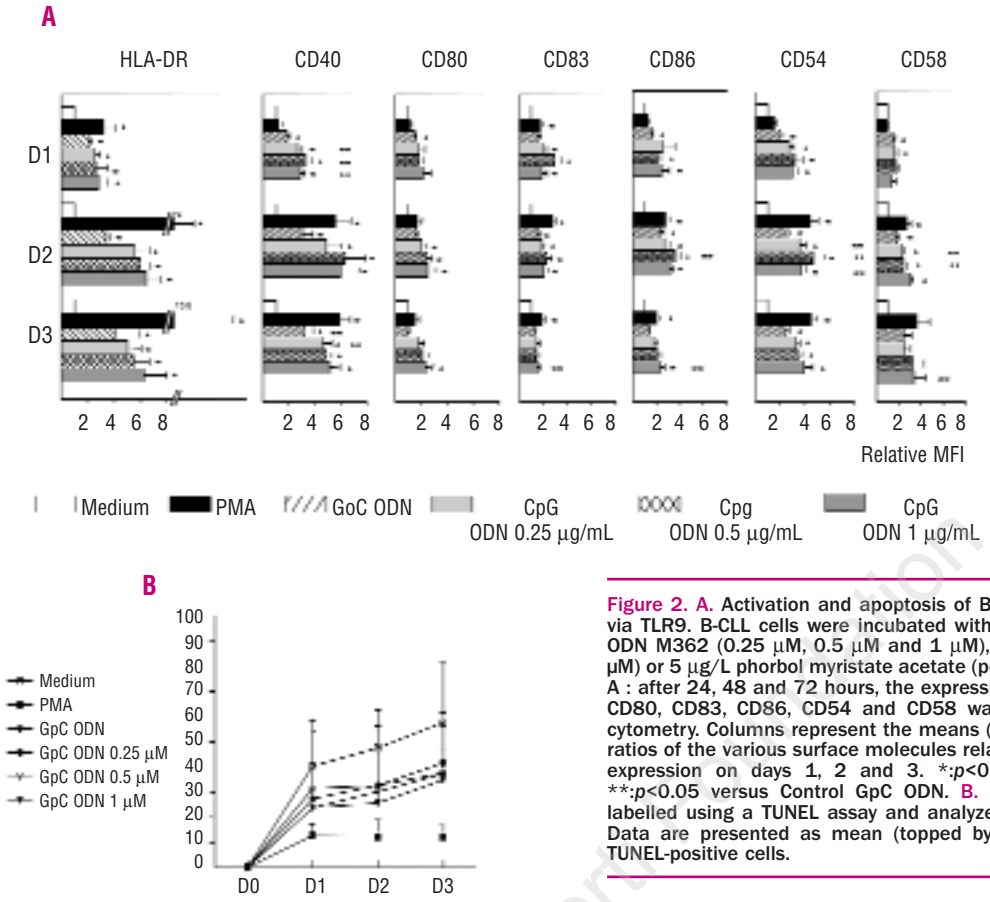


**Figure 2. A.** Activation and apoptosis of B-CLL cells stimulated via TLR9. B-CLL cells were incubated with medium alone, CpG ODN M362 (0.25 µM, 0.5 µM and 1 µM), Control GpC ODN (1 µM) or 5 µg/L phorbol myristate acetate (positive control). Panel A : after 24, 48 and 72 hours, the expression of HLA-DR, CD40, CD80, CD83, CD86, CD54 and CD58 was measured by flow cytometry. Columns represent the means (topped by SE) of MFI ratios of the various surface molecules relative to their baseline expression on days 1, 2 and 3. *:$p<0.05$ versus Medium; **:$p<0.05$ versus Control GpC ODN. **B.** Apoptotic cells were labelled using a TUNEL assay and analyzed by flow cytometry. Data are presented as mean (topped by SE) percentages of TUNEL-positive cells.

2.8±1.4 vs. 2.2±0.17 for TLR10). By contrast, $CD19^+/CD5^+$ cells from B-CLL patients significantly overexpressed TLR7 and TLR9 compared with normal $CD19^+/CD5^+$ cells (MFI ratios of respectively 5.7+1.9 in B-CLL patients vs. 4.1+1.4 in controls for TLR7 ($p$=0.02), 17.7+7.3 vs. 9.6+3.1 for TLR9 ($p$=0.002)) while there was no difference in TLR10 expression.

Since imidazoquinolines have been described in the literature as TLR7 ligands,[3] the imidazoquinoline-like molecule, imiquimod R837 was used to engage TLR7 on B-CLL cells. This induced almost no variation in the expression of CD80, CD83, CD86, CD54 or CD58. By contrast, it induced a significantly increased expression of HLA-DR, and CD40 on all B-CLL cells in all patients (Figure 1A). Imiquimod R837 at 5 µg/mL or higher was concomitantly responsible for a rapid and major induction of apoptosis as shown by scatter changes of the cells in flow cytometry and as measured by the TUNEL method detecting endonucleases-related DNA breaks and repair by TdT. While spontaneous apoptosis was impaired by PMA and 2 µg/mL of imiquimod R837, apoptosis reached 70% by day 1 with the highest dose of imiquimod R837 and almost 100% on day 3 (Figure 1B). This is consistent with data reported by Spaner et al.[4] using another imidazoquinoline, S28690, at a lower concentration together with PMA. These authors observed a slightly higher expression of activation and costimulatory markers compared with our results. However, they reported that B-CLL cells treated with S28690 alone could not induce T-cell proliferation in an MLR assay. Interestingly, the lowest concentration of imiquimod R837 used here, which is nearest to that used by Spaner *et al.*,[4] appeared to protect B-CLL cells from apoptosis, while the proapoptotic effect was observed from 5 µg/mL. These data suggest that imidazoquinolines, already used as topic in humans,[5] could be used as complementary chemotherapy for CLL clearance, as reported in lymphoma.[6,7]

TLR9 is a natural receptor for CpG of bacterial origin. Several synthetic oligonucleotides with CpG motifs have been reported to be efficient activators of cells displaying mRNA expression of TLR9.[8] The effect of B-CLL cells' TLR9 engagement was tested with a synthetic CpG-ODN M362 containing both A and B type immunostimulatory sequences.[9] CpG-ODN M362 induced a significant upregulation of the expression of all costimulatory molecules, and especially HLA-DR and CD40 (Figure 2A). Opposite to imiquimod R837, CpG-ODN M352 was responsible for a significant decrease of spontaneous apoptosis whatever concentration was used (Figure 2B). These data are consistent with results reported for B-cells from healthy donors or from patients with B-cell malignancies[10] where B-CLL cells showed the strongest activation on stimulation from different CpG ODN, leading to B-CLL cell proliferation followed by apoptosis. The strong and fast induction of co-activation molecules that we observed could also enhance the antigen-presenting cell properties of CLL B-cells on ODN stimulation. This could lead to the generation of TH1 signals, as in normal B-cells. If this is the

case, then provided that their anergy can be overcome, this approach could be highly efficient to trigger tumor-specific cytotoxic T-cells. This could be considered for CLL patients in remission who only harbor residual disease.

*Cindy Grandjenette,*+ Anne Kennel,*+ Gilbert C. Faure,* Marie C. Béné,* Pierre Feugier°*

*Laboratoire d'Immunologie, EA3441-IFR 111, Faculté de Médecine Nancy Université and CHU de Nancy, 9 Avenue de la Forêt de Haye, BP 184, 54500 Vandoeuvre les Nancy, France; °Service d'Hématologie Adulte, CHU de Nancy, Allée du Morvan, 54500 Vandoeuvre les Nancy, France

+These authors contributed equally.

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*Correspondence: Pierre Feugier, Hématologie Adulte, Hôpitaux de Brabois, Allée du Morvan, 54 500 Vandoeuvre les Nancy, France. Phone: international +33.383153282. Fax: international +33.383153558. E-mail: p.feugier@chu-nancy.fr*

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