In Vitro Sensitivity of CLL Cells to Fludarabine May Be Modulated by the Stimulation of Toll-like Receptors

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Abstract

Purpose: The emerging role of Toll-like receptors (TLR) in the pathogenesis of chronic lymphocytic leukemia (CLL) led us to ask whether TLR stimulation may protect CLL cells from drug-induced apoptosis.

Experimental Design: We cultured in vitro malignant B cells freshly isolated from 44 patients with CLLs in the presence or the absence of different concentrations of fludarabine before or after 24-hour TLR stimulation with specific ligands and evaluated cell viability, apoptosis, and molecular pathways involved.

Results: Heterogeneity was observed among samples. In leukemic cells from patients bearing adverse prognostic factors, TLR stimulation caused a significant increase of protection to fludarabine treatment, whereas this did not occur in the cells from patients with good prognosis. To identify novel molecular mechanisms accounting for the dichotomy of response between the two groups of patients, we conducted an apoptosis gene expression profile on leukemic cells either unstimulated or stimulated with TLR9 ligand. Strikingly, TLR9 stimulation specifically upregulated the expression of lymphotoxin-alpha in cells where an increased protection to fludarabine treatment was observed. Also, the expression of miR-155-3p was significantly increased after stimulation of distinct TLR in cells where fludarabine treatment was less effective.

Conclusions: These results suggest that at least in a proportion of patients, in vitro sensitivity to fludarabine may be modulated by the stimulation of TLR, likely mimicking microenvironmental signals occurring in vivo. Clin Cancer Res; 19(2); 367–79. ©2012 AACR.

Introduction

Chronic lymphocytic leukemia (CLL) is heterogeneous in its clinical course. Some patients have an indolent disease, whereas others have an aggressive clinical outcome. Poor prognosis patients tend to express negative prognostic factors, including the expression of ZAP70 or CD38, the presence of deleterious cytogenetic abnormalities (17p and/or 11q deletion), and the absence of somatic mutations in the immunoglobulin heavy chain variable (IGHV) genes (1). Despite significant progress in the treatment modalities, CLL remains incurable, and drug resistance is a major cause of treatment failure. Resistance to purine analogues is a challenging issue in clinical practice; patients who are or become refractory to fludarabine-based regimens have low response rates to salvage therapy and poor survival (2, 3). In many instances, such resistance is associated with the presence of 17p deletions and/or p53 mutations (4–6); more recently, distinct mutations in SF3B1 splicing factor or BIRC3 signaling molecules were associated with fludarabine refractoriness (7, 8). Nevertheless, other so far unknown biologic factors may contribute, an example being CD40-induced chemoresistance (9, 10). Within this context, we concentrated our attention upon Toll-like receptors (TLR). TLR are key players in host defense from infection; they recognize a set of different molecular patterns found in microbial components or autoantigens and, upon stimulation trigger NF-κB signaling pathway, induce the production of inflammatory cytokines and the expression of costimulatory molecules (11, 12).

Previous reports (mainly focused onto TLR9) showed that CpG immunostimulatory oligonucleotides induce proliferation, cytokine production, and shape an immunogenic phenotype in CLL cells (13, 14). However, it was also reported that TLR9 signaling by CpG-B oligonucleotides induces an apoptotic pathway in CLLs (15, 16). Furthermore, several groups described a heterogeneous response to TLR9 stimulation in terms of proliferation and apoptosis within different groups of patients (15, 17); in detail, it was reported that CpG induced apoptosis in mutated and...
proliferation in unmutated patient samples (18, 19). These complex results indicate that additional molecular characterization of the functional role of different TLR recognizing different microbial components is needed. We recently showed that TLR1/2 and TLR2/6 heterodimers can activate and protect leukemic cells from spontaneous apoptosis in vitro (20, 21). Again, heterogeneity was observed between patients samples and apoptosis induction was reported in a proportion of cases (15); nevertheless, we recently showed that distinct innate immunity pathways can be activated in subgroups of CLL with distinct immunoglobulin receptors (21).

In addition, we reported that in mouse models, the lack of the inhibitory receptor TIR8, which allows an unabated TLR-mediated stimulation, triggers CLL progression in vitro (22). Considering the potential role of TLR in CLL natural history, we here tested the hypothesis that signals through the TLR might also have a role in inducing drug resistance in leukemic cells. This study shows that distinct TLR ligands induce, at least in a proportion of patients, a unique antiapoptotic program that eventually leads to protection of leukemic cells from fludarabine-induced apoptosis and that is marked by upregulation of lymphotoxin-α and miR-155-3p. Therefore, these molecules could be exploited as novel potential biomarkers of fludarabine resistance to be validated in future studies involving large cohorts of refractory patients.

Materials and Methods

Reagents

Antibodies used for Western blot analysis were Bcl-2 (Millipore), Mcl-1 (Santa Cruz), PARP (Cell Signaling Technology), Bcl-xL, (Cell Signaling Technology), XIAP (Cell Signaling Technology), LTA/TNF-β (Genetex), cleaved caspase-3 (Cell Signaling Technology), and anti-β-Actin horse-radish peroxidase (HRP)-conjugated (Sigma). Reagents used for flow cytometry were anti-LTA/TNF-β Fluorescein (R&D systems), AnnexinV-FLTC, and propidium iodide-PE (PI-PE) (BenderMed System). For intracellular flow cytometric analysis of LTA/TNF-β, cells were treated with Bre-feldin for 4 hours, stained with anti-LTA/TNF-β, and analyzed. Concentrations of TLR ligands were chosen as previously published, as following manufacturer’s indications (20). In details, Pam3CysSerLys4 (Pam3CSK4; Invivogen), a synthetic tripalmitoylated lipopeptide specific for TLR1/TLR2 heterodimer, was used at the concentration of 1 µg/mL. Macrophage-activating lipopeptide-2 (MALP-2; Axcor Platform), a specific ligand for TLR2/TLR6 heterodimer, was used at the concentration of 0.2 µg/mL. CpG oligonucleotide type B-Human (ODN 2006; Invivogen), a specific ligand for TLR9, was used at the concentration of 2.5 µg/mL. Human recombinant TNF-β (Peprotech), was used at the concentration of 10 ng/mL. Fludarabine (fludarabine phosphate) was from TEVA.

Tissue samples and cell purification

Leukemic lymphocytes were obtained from peripheral blood of patients with CLLs diagnosed according to the recent IWCLL/NCI guidelines (23) and analyzed in different experiments. In parallel, the following parameters were analyzed for each patient: age, sex, disease stage at diagnosis, CD38 expression, IGHV gene mutational status, and ZAP70 expression. Patients included into this study were representative of the whole cohort about the distribution of parameters listed in Supplementary Table S1.

All patients were either untreated or off therapy for at least 6 months before the study.

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Cell culture and analysis

Purified leukemic cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L l-glutamine, and 15 µg/mL gentamicin (Euroclone) at a concentration of 3 × 10^6 cells/mL. Cells were either left unstimulated or stimulated with TLR ligands for 24 hours; we then added increasing concentrations of fludarabine (10 nmol/L–10 µmol/L) for additional 24 to 48 hours (without removing the TLR ligands). At each time point, cells were collected and analyzed for viability by using CellTiter-Glo Luminescent Cell Viability Assay as according to manufacturer’s instructions and/or MACS-beads for negative selection (Miltenyi Biotec). Purity of all CD19^+ CD5^− preparations was always more than 98% as checked by flow cytometry (FC500; Beckman Coulter). Preparations were virtually devoid of natural killer (NK), T lymphocytes, and monocytes.

All tissue samples were obtained with the approval of the Institutional Ethics Committee of San Raffaele Scientific Institute (Milan, Italy), after informed consent.

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Western blot analysis

Total cell lysates were resolved by SDS-PAGE, and proteins from gel were electron-transferred onto nitrocellulose membranes and incubated overnight at 4°C with indicated antibodies. Immunoreactivity was revealed by incubation with HRP-anti-rabbit Ig or HRP-anti-mouse Ig (GE-Healthcare), followed by ECL reaction (Pierce) and film exposures.

Gene expression profiling

After 24-hour culture, cells stimulated or not with CpG were collected, washed twice with PBS, and total cellular RNA was isolated with Qiagen RNAeasy mini kit (Qiagen). One microgram of RNA was reversed transcribed to cDNA using the RT First Strand Kit (SABiosciences). Gene expression profiling of the apoptosis pathway in stimulated and unstimulated CLL cells was conducted by real-time quantitative PCR using the Human Apoptosis RT² Profiler PCR Array kit (PAHS-012A, SABiosciences) according to manufacturer’s instructions. The Human Apoptosis Array profiles the expression of 84 key genes involved in programmed cell death. The array consists of a panel of 96 primer sets used for the amplification of 84 genes relevant to the apoptosis pathway (Supplementary Table S2) plus 5 housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB), a gDNA control, 3 reverse transcription, and 3 PCR quality controls. 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. The difference between the Ct value of each gene of interest (GOI) and the average Ct value of housekeeping genes in each sample (ΔCt) was then measured. Fold differences (FD) in gene expression between different subgroups of patients were measured by the 2-ΔΔCt algorithm (24). The difference in expression of a certain gene between 2 subgroups was reported only if: (i) the fold difference in average 2-ΔΔCt values was >4 or < -4 (indicative of up- or downregulation, respectively); and (ii) the difference in ΔCt values was found statistically significant (P<0.05) by the t test method. In addition, multiple comparisons analysis was conducted (ANOVA and Bonferroni test).

Real-time PCR

RNA was purified and the reverse transcription was conducted as previously described (see above, gene expression profiling). Expression of LT-α (TNF-β) mRNA was analyzed by using a specific TaqMan gene expression assay (Applied Biosystems). The experiments were carried out in triplicate, and they were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression for each condition. Data are expressed as relative expression (2^-ΔCt).

miRNA expression

RNA was purified by TRIzol. Reverse transcription and real-time PCR for mature microRNA were carried out by using hsa-miR-155-3p TaqMan MicroRNA assay (Applied Biosystems). The results are the average of triplicate, data were normalized on the expression of RNU6B and expressed as relative expression (2^-ΔCt).

ELISA

Supernatants were collected after 24 hours of TLR stimulation, and the amount of soluble LT-α (TNF-β) cytokine was quantified by using a specific ELISA assay following the manufacturer’s instructions (Human TNF beta ELISA, eBioscience).

Statistical analysis

The datasets of viability, apoptosis, activation, and PCR were analyzed with a Wilcoxon matched pairs test or Mann–Whitney unpaired test or Spearman correlation analysis or Student t test or Dunnett multiple comparison test as indicated (GraphPad Prism 5.01 software).

All statistical computations on clinical data were done using GraphPad Prism 5.01 software. All P values were two-sided and were regarded as statistically significant if <0.05.

Results

TLR stimulation modulates viability of CLL cells treated with fludarabine

To test the hypothesis that TLR stimulation might affect fludarabine sensitivity, we cultured in vitro fresh purified leukemic cells isolated from an unselected group of 44 patients with CLL (Supplementary Table S1) in the presence of the absence of distinct TLR ligands (Pam3CSK4 for TLR1/2, MALP-2 for TLR2/6, or CpG ODN2006 for TLR9). Twenty-four hours later, cells were either left untreated or treated with fludarabine at increasing concentrations (without removing TLR ligands), incubated for additional 24 hours, and analyzed with a specific chemiluminescent assay to measure relative viability (Fig. 1A for a representative experiment). As expected, after 48 hours of in vitro culture, untreated samples showed a heterogeneous index of viability (Supplementary Fig. S2A–S2B). At cohort level, relative viability increased after MALP and CpG treatment (Fig. 1B and Supplementary Fig. S1A–S1C). For each condition of drug exposition (1, 3, and 10 μmol/L fludarabine), the relative viability of CLL cells as compared with untreated cells was calculated after fludarabine treatment with or without TLR stimulation. As shown in Fig. 1B, all the TLR ligands analyzed increased relative viability of CLL cells at cohort level. TLR9 stimulation emerged as inducing significant protective effect in all the conditions tested (Supplementary Fig. S1). Time course analysis showed that a single TLR9 stimulation protected cells up to 48 hours, irrespective of the concentrations of fludarabine tested; as shown in Fig. 1C and Supplementary Fig. S1D and S1E, whereas untreated cells were virtually all dead after long fludarabine treatment, TLR-stimulated samples contained a significant proportion of viable cells.

TLR stimulation induces fludarabine protection in a proportion of CLL cases

Response heterogeneity was observed between samples (Supplementary Fig. S2A and S2B); specifically, in 29 of 44
Figure 1. Pam3CSK4, MALP-2, and CpG prestimulation induces fludarabine resistance in CLL cells. A, a representative experiment is reported (Pt 4); CLL cells, prestimulated for 24 hours with specific TLR ligands (Pam3CSK4 for TLR1/2, MALP-2 for TLR2/6, and CpG ODN2006 for TLR9), are protected from death induced by treatment with increasing fludarabine concentrations. Mean ± SD of technical triplicate is reported. B, Wilcoxon matched pairs test confirms the statistically significant TLR-induced resistance effect at a cohort level (44 patients with CLL analyzed for CpG, 33 for MALP-2, and 29 for Pam3CSK4; 3 μmol/L fludarabine). Mean CLL cells viability ± SEM is reported for 1, 3, or 10 μmol/L fludarabine treatment as indicated. C, the protective effect of TLR prestimulation was maintained after 48-hour treatment with fludarabine (10 patients were analyzed for all the ligands, namely: 1, 2, 4, 6, 9, 10, 13, 19, 33, 39). Mean CLL cells viability ± SEM is reported for 3 or 10 μmol/L fludarabine treatment as indicated. D, patients with a cellular viability increase more than 10% after CpG stimulation are arbitrarily considered as “resistant,” whereas patients with a cellular viability increase less than 10% after CpG stimulation are considered “sensitive.” E, relative viability was calculated for each sample in fludarabine-treated versus untreated cells. Fold change was calculated for each sample as follows: Fold change CTRL = (relative viability with fludarabine)/(relative viability without fludarabine). Fold change CpG = (relative viability with CpG and fludarabine)/(relative viability with CpG and without fludarabine). CTRL indicates samples without TLR addition; PAM-3, MALP-2, and CpG indicate samples with the addition of TLR ligands. A total of 23 “resistant” samples were analyzed for Pam3CSK4, 23 for MALP-2, and 29 for CpG (patient number 1–29); mean fold change ± SEM is reported in the graph. F–H, Spearman correlation analysis of TLR-induced cell viability differences of samples treated with 3 μmol/L fludarabine. F, CpG versus Pam3CSK4. G, CpG versus MALP. H, Pam3CSK4 versus MALP (P < 0.0001 for all panels). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
cases analyzed, TLR stimulation induced a robust protective effect, as determined by a TLR9-mediated increase of relative cellular viability 10% or more as compared with untreated cells (mean percentage of viable cells was 37.1 ± 11.8% for control and 106.1 ± 21.7% for CpG; 3 μmol/L fludarabine for 24 hours after TLR pretreatment for 24 hours). In the remaining 15 patients with CLLs, the effect of TLR was less than 10% (n = 6) or negative (n = 9; mean percentage of viable cells was 31.6 ± 17.1% for control and 26.6 ± 19.7% for CpG; Supplementary Table S1). For clarity purposes, samples with a cellular viability increase more than 10% after CpG stimulation will be defined as “resistant,” whereas samples with a cellular viability increase less than 10% after CpG stimulation will be considered “sensitive” (Fig. 1D for a schematic representation).

To further evaluate the intrinsic sensitivity of CLL cells to fludarabine before and after TLR stimulation and to analyze whether the observed effects are specific for that stimulation and are independent of the cell viability without treatment, a fold change was calculated for each sample. In details, fold change of control “resistant” sample was calculated as (relative viability with fludarabine)/(relative viability without fludarabine); fold change for TLR-treated “resistant” samples was calculated as (relative viability with TLR ligand and fludarabine)/(relative viability with TLR ligand and without fludarabine).

At 3 μmol/L of fludarabine treatment (24 hours of TLR prestimulation and 24 hours of fludarabine), unstimulated cells showed a significantly lower mean ratio of viability than TLR-stimulated cells (Fig. 1E).

Moreover, analysis of spontaneous cellular viability index showed no significant difference between the “resistant” and the “sensitive” group (Supplementary Fig. S2C and S2D). Therefore, triggering TLR on leukemic cells induced, in a proportion of cases, intrinsic fludarabine resistance.

The response to TLR stimulation in terms of relative viability of the cells was heterogeneous among the patients; that notwithstanding, significant associations were noted between the pattern of response (in terms of protective effect) after stimulation with the different ligands. Data analysis showed that the differences in viability of CLL cells stimulated with CpG correlated with the differences in viability of cells stimulated with Pam3CSK4 (Fig. 1F); similarly, CpG and MALP-2 or MALP-2 and Pam3CSK4 significantly correlated (Fig. 1G and H, respectively).

**TLR stimulation preferentially protects cells from patients with adverse prognostic factors**

Given the observed response heterogeneity among patients (Supplementary Table S1), we asked whether patients with different clinical course and prognostic factors might have a differential response to CpG in terms of protection from fludarabine treatment (24 hours of TLR prestimulation and 24 hours of fludarabine; Fig. 2A–E). We observed a significant difference in the change of cell viability after treatment with CpG in samples with: (i) stable versus progressive disease (Fig. 2A); (ii) low Rai stage versus intermediate Rai stage (ref. 25; Fig. 2B), (iii) mutated versus unmutated IGHV genes (Fig. 2C); (iv) >30% CD38 versus <30% CD38 positive cells (Fig. 2D); (v) favorable (13q deletion and/or trisomy 12 or normal FISH karyotype) versus unfavorable (17p deletion and/or 11q deletion) cytogenetic aberrations (Fig. 2E and Supplementary Fig. S1). Leukemic cells from patients with adverse prognostic factors (unmutated IGHV genes and/or CD38-positive and/ or unfavorable cytogenetic carriers) had significantly higher cell viability increase after TLR stimulation than those with favorable prognosis (mutated IGHV genes and/or CD38-negative and/or favorable cytogenetic carriers). Overall, these data show that the functional in vitro response to CpG and fludarabine reflects a different clinical behavior in patients with CLLs.

**TLR stimulation protects CLL cells from fludarabine-induced apoptosis**

To understand the mechanism of the observed TLR-induced protection from fludarabine treatment, we calculated the percentage of viable, apoptotic, and necrotic cells after incubation of CLL cells with different concentration of fludarabine (3 and 10 μmol/L). Thirteen patients were analyzed for the response to Pam3CSK4 (TLR1/2), 17 for the response to MALP-2 (TLR2/6), and 22 for the response to CpG (TLR9). As expected from previous work from different groups including ours, heterogeneous response was observed after treatment of the cells with different TLR ligands for 3 days; overall, at cohort level, no significant differences were observed at this time point (Fig. 3A; ref. 21). As depicted in Fig. 3A, at cohort levels, the percentage of viable, nonapoptotic cells significantly increased when cells were pretreated with TLR2/6 and TLR9 ligands and following treated with increasing concentrations of fludarabine. In details, mean percentage value of AnnexinV–PI− cells was 17.18 ± 6.01 (SD) for control and 33.43 ± 28.2 for CpG at a dose of 3 μmol/L fludarabine and 13.4 ± 11.7 for control and 32.9 ± 26.2 for CpG at a dose of 10 μmol/L fludarabine. In the same experiments, similar results were obtained with TLR2/6 ligand (mean percentage value of AnnexinV–PI− cells was 24.6 ± 21.64 for MALP-2 at a dose of 3 μmol/L fludarabine and 22.4 ± 13.6 at a dose of 10 μmol/L fludarabine) and with TLR1/2 ligand (mean percentage value of AnnexinV–PI− cells was 27.4 ± 17.4 for Pam3CSK4 at a dose of 3 μmol/L fludarabine and 20.5 ± 11.7 for Pam3CSK4 at a dose of 10 μmol/L fludarabine).

If we consider the group of “resistant” samples only (Fig. 3B), a highly significant difference was observed between unstimulated and TLR-stimulated samples before and after fludarabine treatment (see Fig. 3C for a representative experiment). To investigate whether the effects observed after treatment with fludarabine/TLR stimulation are specific for that stimulation and are independent of the spontaneous cell apoptosis, we calculated the percentage of apoptotic cells (without any treatment) in both the “resistant” and the “sensitive” samples, and we observed no significant difference between the 2 groups (Supplementary Fig. S2D). These findings are in line with the results obtained with the viability assay (Fig. 1) and suggest that...
TLR stimulation specifically protected CLL cells from fludarabine-induced apoptosis. This possibility was confirmed by Western blot analysis which showed that TLR ligation prevented the apoptotic cleavage of PARP and caspase-3 (Fig. 3D). Of note, the TLR-mediated antiapoptotic activity was evident only in combination with fludarabine further supporting the concept of TLR-induced protection.

**TLR stimulation upregulates activation and anti-apoptotic molecules in both “sensitive” and “resistant” samples**

To investigate the molecular mechanisms on the basis of the observed heterogeneity between patients, we asked whether it was reflected by a differential TLR-mediated cellular activation. We analyzed expression of CD86 and CD25 activation molecules before and after TLR1/2, TLR2/6, and TLR9 stimulation in both the “resistant” and the “sensitive” group; we observed no significant difference between the 2 groups of patients analyzed; nevertheless, a trend of lower activation in the “sensitive” group was reported (Fig. 4A and B for CD25 and CD86, respectively).

Next, we analyzed by Western blotting, the expression of key antiapoptotic proteins known to play an important role in CLL such as Mcl-1, XIAP, Bcl-2, and Bcl-xL. As expected (26, 27), CLL cells expressed high basal levels of Bcl-2; no further evident increase of the corresponding band was observed after TLR stimulation. XIAP protein band was only modulated by fludarabine and CpG treatment. Bands corresponding to Mcl-1 and Bcl-xL antiapoptotic molecules were not evident in untreated samples while becoming strongly evident after TLR stimulation in all cases analyzed (n = 11 and n = 5, respectively), including both “resistant” and “sensitive” samples (Fig. 4C and D, respectively). Importantly, the increased levels of Mcl-1 and Bcl-xL were maintained also after treatment of leukemic cells with high doses of fludarabine (10 μmol/L, Fig. 4C and D).
To examine whether TLR engagement affected the mRNA expression levels of additional genes specifically involved in the apoptotic process, we studied a large number of genes using a specific gene expression array (see Supplementary Table S2 for a complete gene list). Cells were cultured for 24 hours in the presence or the absence of CpG (TLR9 ligand); gene expression analysis was conducted in leukemic cells isolated from 4 "resistant" CLL samples. The data showed that 3 genes were upregulated (>4-fold difference; LTA/TNF-β, NOL3, CD40) and 3 genes were downregulated (<4-fold difference; PYCARD, CD27, TNFSF10; Fig. 4E). However, only LTA, CD40, and CD27 upregulations are statistically...
significant after applying correction test for multiple comparisons (ANOVA and Bonferroni test).

**TLRs selectively upregulate LTA/TNF-β in "resistant" samples**

We were particularly intrigued by the important upregulation of LTA/TNF-β (>20-fold regulation), and we set up a real-time PCR analysis that revealed significant upregulation of LTA/TNF-β mRNA after CpG treatment (Fig. 5A).

Interestingly, a trend for lower expression of LTA/TNF-β mRNA was apparent, after CpG treatment, in the "sensitive" group as compared with the "resistant" group (Fig. 5B). Next, we conducted Western blot analysis to detect LTA/TNF-β protein which was evident only in the "resistant" samples (Fig. 5C). Intracellular staining and flow cytometric analysis of leukemic cells (CD5⁺ CD19⁺) allowed to identify CLL cells as the cells of origin of LTA/TNF-β (Fig. 5D and E). Finally, we measured, by specific ELISA assay, the
amount of cytokine released by CpG-treated cells; as shown in Fig. 5F, CpG only significantly increased the production and release of LTA/TNF-β by CLL cells. Remarkably, when we compared the total amount of LTA/TNF-β released, we observed a significant difference between the "resistant" and "sensitive" samples (mean amount of LTA/TNF-β was 364 and 160 pg/mL, respectively; Fig. 5G).

As a proof of principle, we then pretreated leukemic cells from 7 patients with LTA/TNF-β, and we observed a modest but significant increase of cellular viability (Supplementary Fig. S3A and S3B), and protection from apoptosis (Supplementary Fig. S3C and S3D); this effect was specific for "resistant" samples (Supplementary Fig. S3B and S3D), further supporting a specific role of LTA/TNF-β in mediating CpG-induced resistance in a selected group of patients.

**TLR-induced protection from fludarabine is marked by upregulation of miR-155-3p**

To analyze the molecular differences between the 2 groups of patients and in an effort to explain the resistance effect induced by CpG, we analyzed the expression of microRNA-155-3p. We chose this miRNA starting from the observation that CpG was previously shown to upregulate miR-155 in normal leukocytes (28–30); moreover, it was shown that lack of bic/miR-155 in mouse models resulted in impaired production of lymphotoxin-α by B cells (30). It was also recently shown that mature miR-155**
(miR-155-3p) is strikingly upregulated by CpG treatment of CLL cells with unmutated IGHV (31). We asked whether CpG could upregulate this microRNA in CLL cells before and after fludarabine treatment. CpG, and to a lesser extent MALP-2, significantly increased miR-155-3p expression levels (Fig. 6A); higher levels of miR-155-3p were maintained also in the presence of fludarabine (Fig. 6C). Strikingly, higher levels of CpG-induced miR-155-3p were present in the "resistant" samples as compared with the "sensitive" samples (Fig. 6B) and this difference was evident also in the presence of fludarabine (Fig. 6D).

Discussion
Specific combination of cell types and soluble factors present in microenvironment niches protect CLL cells from cell death. Microenvironmental interactions also influence drug resistance, an example being CD40-induced chemoresistance which is mediated by NF-kB activation and increased levels of Bcl-xL and Mcl-1 (32, 33). In this work, we tested the hypothesis that TLR triggering may influence drug resistance of CLL cells. Our results suggest that reduced sensitivity to fludarabine treatment may be ascribed, at least in a proportion of patients, to the stimulation operated by microenvironment TLR ligands. Recent reports have shown that distinct TLR may act as modulators of chemoresistance in solid tumors. TLR7 and TLR8 triggering induce cell survival and resistance to different drugs in human lung cancer cells (34), and TLR4 signaling promotes tumor growth and paclitaxel resistance in ovarian cancer (35).

In our study, several parameters were analyzed including activation, cell viability, and apoptosis. At cohort level, despite a heterogeneous response, we observed a general protective effect to fludarabine treatment when CLL cells were prestimulated with TLR1/2, TLR2/6, and TLR9 ligands. Interestingly, patients with progressive disease and bearing different unfavorable prognosis markers (unmutated IGHV genes, surface CD38, cytogenetic abnormalities) were significantly enriched for those in whom TLR stimulation led to protection from drug activity. In line with these observations, different studies showed that CpG induces proliferation mainly in CLL B cells from patients with progressive disease and unmutated IGHV genes, whereas cell-cycle arrest and apoptosis are induced in leukemic B cells from stable/IGHV-mutated CLL (15, 18, 19, 36), further supporting a dichotomy of response between mutated and unmutated cases. Importantly, our study shows that a single TLR prestimulation (24 hours) was sufficient to trigger an anti-apoptotic program which resulted in drug protection in leukemic cells isolated from patients bearing adverse prognosis markers. Time course analysis showed that TLR9 prestimulation (24 hours) protected cells up to 48 hours later; however, prosurvival effect diminished overtime, suggesting that a "chronic stimulation" may be required to sustain the effect. Recently, it was reported that combined CD40/TLR9 triggering differentially affects NF-kB signaling and chemoresistance. However, in vitro TLR9 prestimulation (for 3–5 days) did not protect cells nor augmented the CD40-mediated chemoprotective effect even in unmutated CLL cases (19). This apparent discrepancy with our results
may be due to the different experimental conditions used and kinetics. It will be interesting to analyze whether the continuous addition of TLR ligands (somehow mimicking a chronic inflammatory and/or infectious condition) may impact on the kinetic of drug resistance.

When we specifically analyzed apoptosis, we observed that TLR ligation protected from drug-induced apoptosis; however, antiapoptotic effect was less significant than general cellular viability. As the viability assay is based on the measurement of ATP concentration, these results suggest that, in addition to apoptosis, either energy metabolism and/or proliferation may be regulated by TLR ligation as previously described (18, 36).

We previously showed that TLR stimulation induces activation of the NF-κB pathway as determined by phosphorilation of IKK (20); we herein show the induction of specific antiapoptotic proteins including Bcl-xL and Mcl-1. In addition, the increase of antiapoptotic proteins was maintained also when cells were subsequently treated with fludarabine, thus supporting the hypothesis that TLR-induced Bcl-xL and Mcl-1 may participate in the observed fludarabine protection; however, as these molecules were upregulated also in “sensitive” samples, we searched for additional molecular mechanisms involved.

Searching for novel molecules specifically modulated by TLR and possibly involved into the observed protective effect, we identified several genes up- or downregulated after CpG (TLR9 ligand) treatment in “resistant” samples. Different TNF family members (LTA/TNF-β, CD40) and antiapoptotic molecules (NOL3) were strikingly upregulated.

TNF family members were described as key regulators of CLL cell biology. LTA/TNF-β can support the proliferation of in vitro activated CLL cells (37, 38), suggesting that this pathway may also contribute to the observed TLR-induced protection from drug-induced cell death. Additional cytokines and antiapoptotic molecules may converge to the final chemoresistance effect. Our observation that TLR stimulation increases CD40 transcripts suggests that this pathway may also play a relevant role in TLR-induced chemoresistance. NOL3 gene encodes an antiapoptotic protein that inhibits the enzymatic activities of caspases and of tumor protein p53.

Among the genes specifically downregulated by CpG, we detected distinct proapoptotic proteins including a proapoptotic TNF receptor family member (CD27; ref. 39) and a proapoptotic TNF family member (TNFSF10 also known as TRAIL; ref. 40); and PYCARD is an adapter molecule regulating inflammatory and apoptotic pathways (41).

Overall, the gene expression analysis supports the hypothesis that TLR induces a potent antiapoptotic transcriptional program in CLL cells. Therefore, it is tempting to propose LTA/TNF-β as novel potential biomarkers of fludarabine resistance to be validated in future studies involving large cohorts of refractory patients.

miRNAs are an abundant class of small noncoding RNAs that modulate the expression of target mRNAs at posttranscriptional level. Many miRNAs have been shown to play a role in different tumors including CLLs. We previously showed that the miR-221/222 cluster is upregulated in CLL cells by CpG treatment and modulates the expression of p27kip1 protein (42). Of note, miR-221 and miR-222 were recently shown to play a key role in chemoresistance of solid tumors (43) and to be upregulated in vivo in patients with CLLs refractory to fludarabine treatment (44). Our previous data (42) suggest that the observed TLR-mediated drug resistance in CLL may also be influenced by upregulation of miR221/222 cluster.

In our current work, we focused our attention on miR-155, a key regulator of inflammation, immunity, and cancer (30, 45–47); miR-155 was previously shown to be upregulated by different TLR ligands in normal leukocytes (28–30) and to be highly expressed in CLLs (29, 45, 48). Recently, it was also reported that CpG can induce several miRNA including miR-155/miR-155-5p and miR-155’/miR-155-3p in CLL cells with unmutated IGHV (31). Interestingly, it was reported that lymphotixin-α production is impaired in bic/miR-155−deficient mice (30). Our novel data showing that CpG specifically induces miR-155-3p/miR155− in “resistant” CLL samples, either untreated or treated with fludarabine, suggest that a specific program of miRNA regulation may play a role also in lymphotixin regulation and chemoresistance.

In conclusion, we show that specific TLR ligands induce, at least in a proportion of patients, a robust prosurvival program which is marked by upregulation of lymphotixin-α and miR-155-3p and eventually leads to protection of leukemic cells from drug-induced cell death. Targeting TLR signaling pathways by specific inhibitors might be suggested as a novel therapeutic strategy to overcome fludarabine refractoriness in CLLs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Fonte, L. Scarfo, P. Ghia, F. Caligaris-Cappio, M. Muzio
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Ranghetti
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