Toll-like Receptor-7 Tolerizes Malignant B Cells and Enhances Killing by Cytotoxic Agents

Yonghong Shi,1 Dionne White,1 Liwei He,5 Richard L. Miller,6 and David E. Spaner1,2,3,4

1Division of Molecular and Cellular Biology, Research Institute, Sunnybrook Health Sciences Center; 2Toronto-Sunnybrook Regional Cancer Center; Departments of Medicine, Medical Biophysics, University of Toronto; 3Immunology Platform, Sanofi-Pasteur, Toronto, Canada; and 4Department of Pharmacology, 3M Pharmaceuticals, 3M Center, St. Paul, Minnesota

Abstract

Chronic activation through Toll-like receptors (TLR) occurs in a number of pathologic settings, but has not been studied to the same extent as primary activation. TLR7, expressed by B cells and some dendritic cells, recognizes molecular patterns associated with viruses that can be mimicked by synthetic imidazoquinolines. In response to primary stimulation with the imidazoquinoline, S28690, human mononuclear cells produced tumor necrosis factor-α, but were unable to do so upon restimulation with S28690. This state of “tolerization” lasted at least 5 days. Using chronic lymphocytic leukemia B cells as a model to facilitate biochemical analysis, the tolerized state was found to be associated with altered receptor components, including down-regulated expression of TLR7 mRNA and decreased levels of interleukin-1 receptor-associated kinase 1. Tolerization was characterized by a transcriptionally regulated block in stress-activated protein kinase and nuclear factor κB activation, with relatively preserved activation of extracellular signal-regulated kinase (ERK). Tolerized chronic lymphocytic leukemia cells were found to be more sensitive to cytotoxic chemotherapeutic agents, in part through altered stress-activated protein kinase signaling pathways. This property of the TLR7-tolerized state may potentially be exploited in the treatment of B cell cancers.

Introduction

Toll-like receptors (TLR) regulate innate and adaptive immunity (1), and the identification of these molecules (along with their ligands) suggests new ways to treat infections, autoimmune diseases, and cancer (2). For example, we showed recently that leukemic skin infiltrates in a patient with chronic lymphocytic leukemia (CLL) disappeared after treatment with a TLR7 agonist (3). However, realization of the therapeutic potential of TLRs, particularly TLR7, will require a deeper understanding of their mechanisms of action.

TLR7 is expressed mainly by monocytes, activated B cells, and plasmacytoid dendritic cells (1, 4). The natural ligand for murine TLR7 (ref. 5, and human TLR8, which is related to TLR7; ref. 4) has been identified as single-stranded RNA, whereas oxidized guanosines (6) and imidazoquinolines (7) are synthetic human TLR7 ligands.

Like other TLRs, TLR7 contains a TLR and interleukin-1 (IL1) receptor–related (TIR) domain, which associates with the adaptor protein, MyD88, in the presence of a ligand (8). The TLR7–MyD88 complex recruits the IL1 receptor–associated kinase (IRAK) family members, IRAK1 and IRAK4. IRAK4 phosphorylates and activates IRAK1, which recruits tumor necrosis factor–associated factor (TRAF)–6. IRAK1 and TRAF6 then dissociate from TLR7 and interact with a membrane-associated complex of the mitogen-activated protein kinase kinase kinase kinase (MAP3K) pathways, which in turn activate the mitogen-activated protein kinase kinase (MAP2K) pathways (9). MAP3Ks, such as TAK1, which phosphorylates and activates c-Jun-NH2-kinases (JNK) or p38, respectively. Activated JNK and p38 then translocate to the nucleus where they phosphorylate and activate components of activator protein-1 (AP-1), including c-Jun and c-Fos, which mediates the transcription of genes such as tumor necrosis factor-α (TNFα; ref. 8).

Although these acute signaling events have been studied intensively, the effects of chronic stimulation through TLR7 are not well understood. The two situations may well lead to very different biological outcomes (9). Understanding the effects of repeated stimulation is especially important for the clinical use of TLR7 ligands, where the optimal dose and schedule of injections are not yet known (3). Accordingly, the effects of repeated activation through TLR7 in primary human cells were studied in this paper.

Materials and Methods

Blood samples. Heparinized blood (30–40 mL) was collected from healthy volunteers and consenting CLL patients (with a persistent clonal expansion of CD19+/CD5+/IgM+ lymphocytes; ref. 10), whose clinical characteristics are described in Table 1 and who were untreated for at least 3 months before study. Identification numbers were assigned arbitrarily and maintained throughout the manuscript. Protocols were approved by the Institutional Review Board.

Antibodies and reagents. Phycoerythrin-labeled or FITC-labeled CD19 and TNFα antibodies and blocking Fas antibodies were purchased from PharMingen (San Francisco, CA). Lipopolysaccharide, actinomycin D, cycloheximide, and phorbol di butyrate were from Sigma (St. Louis, MO). Stock solutions of phorbol di butyrate (5 mg/mL) were made in DMSO. Dexamethasone (Pharmascience Inc., Montreal, Quebec), IFNα (Schering Canada Inc., Pointe-Claire, Quebec), Remicade (anti-TNFn antibody; Schering Canada), vincristine sulfate (Faulding Canada Inc., Kirkland, Quebec), doxorubicin (Adriamycin; Pfizer, Canada Inc., Kirkland, Quebec), fludarabine, vincristine, and cetuximab (Erbitux; ImClone Systems, Inc., New York City). Annexin V (PI) (BioSource International, Camarillo, CA) for cytometry was purchased from BD Biosciences (San Jose, CA).

References

Dionne White, David Spaner, Division of Molecular and Cellular Biology, Research Institute, Sunnybrook Health Sciences Center, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5. Phone: 416–480-6100-2510; Fax: 416–480-5737; E-mail: david.spaner@swri.ca. ©2007 American Association for Cancer Research. DOI:10.1158/0008-5472.CAN-06-2381
phosphate (Berlex Canada Inc. Pointe-Claire, Quebec), and cyclosporin A (Novartis Canada Inc., Dorval, Quebec) were purchased from the hospital pharmacy. SP600125 (JNK inhibitor), MG-132 (proteasome inhibitor), and caffeic acid phenylester (CAPE; NF-κB inhibitor) were from Calbiochem (San Diego, CA), and stock solutions (25 mg/mL) were made in DMSO. The TLR7/8 agonist, S28690 (ref. 3; 3M Pharmaceuticals, St. Paul, MN), was dissolved in serum-free AIM-V media (Gibco BRL, Grand Island, NY; with 33% DMSO) at 1.3 mg/mL and stored in the dark at 4°C. PBS was from Wisent Inc. (Saint-Jean-Baptiste de Rouville, Quebec). IRAK4 antibodies were from eBioscience (San Diego, CA). IRAK1 antibodies were a gift from Dr. W.C. Yeh (Ontario Cancer Institute, Toronto, ON, Canada). Antibodies against germinal center kinase, caspase-8 and caspase-9, JNK, p38, p42/p44 extracellular signal-regulated kinase (ERK), IκB, c-Jun, cytochrome d, β-actin, and the serine/threonine-phosphorylated forms of JNK, p38, ERK, IκB, and c-Jun were from Cell Signaling Technology (Beverly, MA). The TNFα converting enzyme inhibitor, TAPI (11), was from Peptides International (Louisville, KY).

**Table 1. Clinical characteristics of CLL patients**

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| 109     | F   | III        | 58     | 1.3 mg/mL and stored in the dark at 4°C. PBS was from Wisent Inc. (Saint-Jean-Baptiste de Rouville, Quebec). IRAK4 antibodies were from eBioscience (San Diego, CA). IRAK1 antibodies were a gift from Dr. W.C. Yeh (Ontario Cancer Institute, Toronto, ON, Canada). Antibodies against germinal center kinase, caspase-8 and caspase-9, JNK, p38, p42/p44 extracellular signal-regulated kinase (ERK), IκB, c-Jun, cytochrome d, β-actin, and the serine/threonine-phosphorylated forms of JNK, p38, ERK, IκB, and c-Jun were from Cell Signaling Technology (Beverly, MA). The TNFα converting enzyme inhibitor, TAPI (11), was from Peptides International (Louisville, KY).

**Primary cell purification.** Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). CLL cells were isolated from blood by negative selection (RosetteSep, StemCell Technologies, Vancouver, BC) as described previously (12).

**TLR7 tolerization of CLL cells.** Purified CLL cells (1.5 × 10^6 cells/mL) were cultured in AIM-V plus 2-mercaptoethanol (Sigma; 5 × 10^-5 mol/L) in 6- or 24-well plates (Becton Dickinson Labware, Franklin Lake, NJ) at 37°C in 5% CO2, S28690 was added at the indicated concentrations. At various times, the cells were collected, washed twice, resuspended at 1.5 × 10^6 cells/mL, and then reactivated with S28690 (1 μg/mL).

**Membrane TNFα detection.** Ten million CLL cells were cultured with or without S28690 in 5-mL polystyrene tubes (Becton Dickinson Labware). TAPI (100 μmol/L) was added to each tube, and CD19-FITC and TNFα-PE antibodies were added 4 h later. Subsequent steps were similar to conventional immunophenotyping (13).

**Analysis of apoptotic and necrotic cell populations.** Cells were washed twice in PBS at room temperature and then resuspended at 10 × 10^6 cells/mL in 0.2 mL of PBS supplemented with 4 μL of Annexin-V-PE (BD PharMingen) and incubated for 15 min at room temperature in the dark. Twenty microliters of 7-amino-actinomycin (7-AAD) solution was then added, and the suspension was incubated for an additional 15 min. Annexin-V labels apoptotic cells, and 7-AAD identifies necrotic cells with

| Abbreviations: C, chlorambucil; P, prednisone; F, fludarabine; R, rituxan; S, splenectomy; CHOP, cyclophosphamide/vincristine/adriamycin/prednisone; T12, trisomy 12; ND, not done. |

| Rai stage: 0, lymphocytosis; I, with adenopathy; I, with hepatosplenomegaly; III, with anemia; IV, with thrombocytopenia (10). |
leaky membranes. The percentages of dead and dying CLL target cells were determined by flow cytometry (14).

Immunophenotyping. Cells were washed once in PBS and then resuspended in PBS plus 1% albumin before flow cytometric analysis. Negative controls were isotype-matched irrelevant antibodies (PharMin- gen). Staining of nucleated cells was determined by gating on forward- and side-scatter properties. Ten thousand viable counts were analyzed with a FACScan flow cytometer and CELLQUEST software (Becton Dickinson, San Jose, CA). Standardization of the flow cytometer was done before each experiment using Spherobeads (Spherotech Inc., Chicago, IL).

Isolation of total RNA and synthesis of cDNA. Total RNA was extracted using the RNasy kit (Qiagen, Mississauga, Ontario) according to the manufacturer’s instructions. To remove contaminating genomic DNA, 10 µg of the total RNA preparation was incubated with 10 units of RNase-free DNase 1 (Promega, Madison, WI) for 30 min at 37°C. Total RNA concentration was determined in a spectrophotometer at 260 nm.

cDNA was made with the Superscript First Strand Synthesis System for reverse transcription-PCR (Invitrogen, Carlsbad, CA) in a 20-µL reaction containing 3 µg of DNase I–treated total RNA, 20 mMol/L Tris-HCl (pH 8.4), 50 mMol/L KCl, 2.5 mMol/L MgCl2, 0.5 mMol/L each of dATP, dGTP, dCTP, and dTTP, and 200 units Superscript II reverse transcriptase. The priming oligonucleotide was annealed to total RNA incubated at 70°C for 5 min and then cooling to 4°C. Reverse transcription was done at 42°C for 2 h, and cDNA was stored at −20°C until PCR analysis.

Real-time PCR amplification. The following primers were used to amplify human TNFα, TLR7, and hypoxanthine phosphoribosyltransferase (HPRT) transcripts: TFNα forward, 5′-ACCTCTCTTAACTACGCC-3′; TFNα reverse, 5′-AGGGACACATGGTTGGAG-3′; TLR7 forward, 5′-CTAAAGACC-CAGCTGTGACCGA-3′; TLR7 reverse, 5′-CGAGCTTCTTCTCTGAGACAT-3′; HPRT forward, 5′-GAGATTTGGAAGGTTGTT-3′; and HPRT reverse, 5′-CAATAGCTTTCACTGCTGA-3′.

PCR was done on a DNA engine Opticon System (MJ Research Inc., Waltham, MA) using SYBR Green I as a double-stranded DNA–specific binding dye. PCR reactions were cycled 40 times after initial denaturation (95°C, 15 min) with the following parameters: denaturation at 95°C for 15 s, annealing of primers at 57°C (TFNα), 54°C (TLR7), and 52°C (HPRT) for 20 s, and extension at 72°C for 20 s. Fluorescent data were acquired during each extension phase. After each PCR reaction, a melting curve analysis of amplification products was done by cooling the samples to 4°C and then increasing the temperature to 95°C at 0.2°C/s. Fast loss of fluorescence is observed uniquely at the denaturing/melting temperature of the amplified DNA fragment. Standard curves were generated with serial 10-fold dilutions of cDNAs obtained using the same primers as for real-time PCR.

Immunoblotting. Protein extracts were made from activated CLL cells as described previously (12). The proteins were resolved in 10% SDS-PAGE gels and transferred onto Immobilon-P transfer membranes (Millipore Corp., Billerica, MA). Western blot analysis was then done according to the manufacturer’s protocols for the specific antibodies. Chemiluminescence signals were assessed with a GS-700 Imaging densitometer and Multi-AnalytSoft (Bio-Rad Laboratories, Hercules, CA).

Cytokine measurement. Cytokines in supernatants of CLL cells cultured for 48 h were measured by a multi-analyte fluorescent bead assay with a Luminex-100 system (Luminex Corp., Austin, TX) as before (12). A kit for human IL10, IL6, and TNFα was used according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Individual cytokine concentrations (average of two separate measurements) were determined from standard curves using Bio-Plex 2.0 software (Bio-Rad, Mississauga, Ontario). The assay was linear between 3 and 10,000 pg/mL for each cytokine.

Statistical analysis. The Student’s t test was used to determine P values for differences between sample means.

Results

Effect of restimulation through TLR7 on TNFα production by PBMCs. Expression of TNFα mRNA by PBMCs from healthy donors increased at least 10-fold within 1 h after primary stimulation by the imidazoquinoline, S28690, a TLR7/8 agonist [for donors 1, 2, and 3 in Fig. 1A, TNFα transcript numbers (relative to HPRT transcripts) before stimulation were 0.03, 0.53, and 0.2 and increased to 9.22, 6.8, and 11.6, respectively, after stimulation]. However, if PBMCs were first activated with S28690 overnight, then washed and stimulated a second time, TNFα mRNA induction was much weaker (Fig. 1A). By analogy with “endotoxin tolerance” (defined by the failure to make TNFα in response to a second stimulation with the TLR4 agonist, lipopolysaccharide; ref. 15), cells that do not make TNFα in response to restimulation with S28690 will subsequently be referred to as being “tolerized.”

TLR7 tolerization was dependent on the strength of the initial stimulation with S28690. The threshold for tolerization was ~0.01 µg/mL because only weak inhibition of TNFα mRNA production in response to a second stimulation with 1 µg/mL of S28690 was seen at this initial dose (see results for donors 1 and 2, Fig. 1A).

To determine the duration of the tolerized state, PBMCs were first exposed to S28690 overnight (to tolerate them) and then cultured for various times before rechallenging them with S28690. Based on the continued inability to transcribe TNFα after restimulation with S28690, tolerization seemed to last at least 5 days (Fig. 1B). Note that primary increases in TNFα transcripts decreased with time (Fig. 1B, columns with diagonal lines), in accordance with the deterioration of normal cells in culture, making it difficult to assess tolerization reversal over a longer time period.

TLR7 tolerization in CLL cells. Because PBMCs are a heterogeneous population of cells that express TLR8, as well as TLR7, the tolerized state caused by S28690 could be mediated through both receptors. As a model to study the effects of restimulating TLR7, primary CLL cells were used because they do not express TLR8 (3) and respond to S28690 by increasing costimulatory molecule expression, NF-κB activation, and cytokine production (including TNFα; refs. 3, 14). Another advantage of this model was that large numbers of monoclonal primary human cells could be obtained easily for immunoblotting experiments.

As with PBMCs, TNFα mRNA transcripts in CLL cells increased after primary stimulation, but not after restimulation with S28690 (data not shown). Tolerization was more transient than in PBMCs, lasting 3 to 5 days (data not shown). However, the results support the use of CLL cells as a model system for characterizing TLR7 tolerization.

Inhibition of TNFα protein production (suggested by impaired mRNA expression) was confirmed in a flow cytometric assay using a tissue inhibitor of metalloproteinase to prevent solubilization of membrane TNFα (mTNFα; ref. 13). As shown in Fig. 1C, mTNFα increased on CLL cells within 4 h of primary stimulation with S28690 and then decreased to baseline levels within 24 h (Fig. 1C, third panel). In accordance with the block in TNFα mRNA production, CLL cells restimulated with S28690 failed to increase mTNFα expression (Fig. 1C, fourth panel). To show that the block in TNFα production was specific to S28690 signaling, TLR7–tolerized CLL cells were restimulated with phorbol dibutyrate (Fig. 1C, fifth panel) and made TNFα, as reported previously (12).

To confirm that the block in TNFα production [indicated by decreased mRNA expression after 1 h (Fig. 1A) and individual cell protein expression after 4 h (Fig. 1C)] was maintained, cytokine levels in culture supernatants were measured 48 h after restimulation with S28690 (Fig. 1D). The Luminex assays again revealed impaired TNFα production by TLR7–tolerized CLL cells that were restimulated with S28690. In contrast to TNFα, IL10 production...
generally increased after restimulation with S28690 (Fig. 1D, Pts. 47, 29, and 21). TLR7-tolerized cells from some patients also made IL6 in response to S28690 (Fig. 1D, Pts. 47, 46, and 29). These observations suggested that some aspects of TLR7 signaling were retained in the tolerized cells.

**TLR7 expression and signal transduction in tolerized cells.**

Decreased expression of TLR7 caused by the initial encounter with S28690 could explain the failure of TLR7-tolerized cells to make TNFα upon restimulation with S28690. We were unable to detect TLR7 proteins by immunoblotting with commercially available antibodies, but using quantitative real-time PCR, TLR7 transcript levels were found to decrease in tolerized CLL cells and PBMCs (Fig. 2A). TLR7 message was generally higher in CLL cells than in PBMCs, which contain many T cells that do not express TLR7.

Despite this apparent decreased receptor expression, the production of IL6 and IL10 by restimulated CLL samples (Fig. 1D) suggested that some TLR7-signaling properties were present in tolerized cells. As described in the Introduction, a number of signaling pathways are activated when TLR7 encounters an agonist, such as S28690. The state of activation of these pathways can be determined by immunoblotting with antibodies against phosphorylated forms of important components of the pathway. Activation of the p38, SAPK, and MAPK pathways were determined using antibodies against phosphorylated p38, JNK, and ERK. NF-κB activation was indicated with phospho-IκB antibodies.

As shown in the examples in Fig. 2B, initial stimulation with S28690 resulted in strong phosphorylation of IκB, along with the 46- and 54-kDa JNK isoforms (which arise by differential mRNA splicing; ref. 16). CLL cells initially stimulated with S28690 also expressed phosphorylated p42/p44 MAPK (ERK1 and ERK2) and phosphorylated p38, although the degree of activation of the latter two pathways seemed weaker than NF-κB and SAPK.

Strikingly, JNK phosphorylation was inhibited strongly in CLL cells that were restimulated with S28690. Increased levels of phosphorylated IκB were sometimes observed in CLL cells that had
been exposed to S28690 for 24 h (Fig. 2B) but did not change after restimulation with S28690, in contrast to the strong activation seen after primary stimulation. Activation of the p38 MAPK pathway (evidenced by the appearance of phosphorylated p38) was also inhibited in reactivated CLL cells (Fig. 2B).

The situation with the classic MAPK pathway was different. As shown in the examples in Fig. 2B [and consistent with increased IL6 and IL10 production by some reactivated CLL cells (Fig. 1D)], TLR7-tolerized CLL cells expressed increased levels of phosphorylated p42/p44 ERK-1/2 proteins in response to restimulation with S28690, despite the decreased TLR7 receptor expression.

**Defective receptor kinase activity in TLR7-tolerized CLL cells.** Decreased phosphorylation of JNK isoforms in restimulated TLR7-tolerized cells could reflect defective kinase or enhanced phosphatase activity. If increased activity of phosphatases caused the decreased phosphorylation of JNK at the times indicated in Fig. 2B, then high levels of the phosphorylated forms should be seen transiently after reactivation with S28690. Accordingly, phospho-JNK levels in tolerized cells were determined 1, 10, 30, and 60 min after reactivation with S28690 (Fig. 2C). No or only weak JNK phosphorylation was seen after reactivation, in contrast to primary stimulation of these cells (Fig. 3C, lane 2). These results suggested that impaired SAPK activation in tolerized CLL cells was due mainly to defective kinase activity of the TLR7 signaling complex.

**Expression of IRAK1 and IRAK4 in TLR7-tolerized cells.** As described in the Introduction, signal propagation through TLR7 is dependent on IRAK1 and IRAK4 (17). IRAK1 expression decreases after stimulation with IL1 and some TLR ligands, which may account for some tolerant states (such as endotoxin tolerance; ref. 18). Similarly, IRAK4 protein levels are down-regulated after prolonged stimulation through TLR-2, TLR-4, or TLR-9 (potentially through cleavage of IRAK4 by a protease induced by NF-κB activation; ref. 19). Therefore, we considered that decreased levels of IRAK1 or IRAK4 could account for defective signaling in response to reactivation with S28690 in TLR7-tolerized cells. As shown in Fig. 3A, IRAK4 levels did not change in response to stimulation with S28690. However, IRAK1 levels decreased in TLR7-tolerized cells (Fig. 3A, top).

**Transcriptional regulation of TLR7 tolerization.** The TLR7-tolerized state developed over at least 6 to 8 h (data not shown), suggesting it might depend on the synthesis of proteins that inhibited subsequent signaling through TLR7. To determine if TLR7 tolerization was regulated through transcriptional mechanisms, CLL cells were stimulated for 6 h with S28690 in the presence of actinomycin D (which inhibits mRNA transcription) before restimulation with S28690 (Fig. 3B). SAPK activation was restored partially by actinomycin D, suggesting that the tolerized state was under partial transcriptional control. Similar experiments with protein synthesis inhibitors, such as cycloheximide, are not shown because these inhibitors activate SAPK pathways directly (16).

The above results suggested that TLR7 tolerization was transcriptionally regulated (Fig. 3B) and characterized by impaired SAPK activation (Fig. 2). Because primary stimulation of TLR7 caused the activation of NF-κB (Fig. 2) and transcriptional targets of NF-κB can inhibit SAPK activation (20), NF-κB signaling inhibitors were used to address the possibility that activation of NF-κB by the initial stimulation with S28690 accounted for the subsequent development of TLR7 tolerization.

Dexamethasone is an inhibitor of NF-κB–mediated gene transcription (21). CAPE prevents nuclear NF-κB translocation (22). MG-132, a 26S proteasome inhibitor, prevents degradation of IκB (23). As shown in Fig. 3C (lanes 5 and 6), treatment of CLL cells with MG-132 during primary stimulation with S28690 ameliorated the block in JNK phosphorylation upon reactivation with S28690. Dexamethasone and CAPE also restored JNK phosphorylation partially in TLR7-tolerized cells (Fig. 3C, lanes 7, 8, 9, and 10). Taken together, the results suggested that initial activation of NF-κB by S28690 contributed to the block in JNK phosphorylation that characterized TLR7-tolerized cells.

**Figure 2.** TLR7 expression and signal transduction in tolerized cells. A, PBMCs and CLL cells were cultured alone or tolerized with S28690. After 24 h, numbers of TLR7 mRNA transcripts (relative to HPRT transcripts) were determined by real-time PCR. Left and right, individual results from nine CLL patients and nine normal donors, respectively; these suggest that receptor levels were lower in tolerized cells. B, purified CLL cells were cultured alone (lanes 1 and 2) or with S28690 (1 μg/ml; lanes 3 and 4) for 24 h. The cells were then washed thrice and cultured alone (lanes 1 and 3) or activated with S28690 (lanes 2 and 4). After 1 h, changes in the phosphorylation status of JNK, p42/p44 ERK, p38, and IκB were determined by immunoblotting. C, JNK phosphorylation levels in tolerized cells were determined 1, 10, 30, and 60 min after stimulation with S28690. Dexamethasone and CAPE also restored JNK phosphorylation partially in TLR7-tolerized cells (Fig. 3C, lanes 7, 8, 9, and 10). Taken together, the results suggested that initial activation of NF-κB by S28690 contributed to the block in JNK phosphorylation that characterized TLR7-tolerized cells.

Taken together, these results suggested that TLR7 expression and the activation of the SAPK and NF-κB signaling pathways were decreased in TLR7-tolerized cells.
Enhanced sensitivity of TLR7-tolerized CLL cells to chemo-therapeutic agents. Previously, we showed that CLL cells exposed to S28690 for 2 days were killed more easily by cytotoxic T cells (CTL; ref. 14). Because, in retrospect, these cells had been tolerized, we considered that their sensitivity to CTLs might extend to cytotoxic drugs. Vincristine is a microtubule inhibitor with activity against CLL cells \textit{in vitro} (24). A dose of 0.1 \textmu g/mL of vincristine was found to be relatively nontoxic to CLL cells (shown in Fig. 4A, \textit{top two panels}, and summarized in Fig. 4B), in contrast to doses of 1 \textmu g/mL or more which killed most cells within 24 h (data not shown). S28690 alone increased the death of CLL cells somewhat (Fig. 4A and compare with Fig. 4A, \textit{first and third panels}). Remarkably, TLR7-tolerized tumor cells were highly sensitive to low doses of vincristine (Fig. 4B and compare with Fig. 4A, \textit{second and fourth panels}).

In separate experiments, the enhanced susceptibility of TLR7-tolerized cells to vincristine was found to extend to low doses of Adriamycin (data not shown), an anthracycline that kills tumor cells by a variety of mechanisms, including DNA intercalation and induction of oxidative stress (25). However, fludarabine, a purine analogue that is active against CLL cells \textit{in vivo} (26), did not exhibit enhanced activity against TLR7-tolerized CLL cells \textit{in vitro} (data not shown).

Although TLR7-tolerized CLL cells were uniformly more sensitive to low doses of vincristine, variations in the magnitude of killing by vincristine were apparent (Fig. 4B). However, no obvious correlations were noted with the underlying cytogenetic abnormalities or CD38 expression (Table 1), which are known to be important biological parameters associated with variations in the clinical behavior of CLL cells (27).

To determine if the timing of exposure to S28690 affected the resulting sensitivity to vincristine (28), CLL cells were simultaneously treated with vincristine and S28690. In marked contrast to prior treatment with S28690, acute treatment protected CLL cells from 10-fold higher doses of vincristine (Fig. 4C). This finding suggested that entry into the tolerized state was necessary for cells to become sensitized to vincristine.

Mechanism of enhanced sensitivity of TLR7-tolerized CLL cells to vincristine. As revealed in Fig. 4A, low doses of vincristine caused tolerized CLL cells to become mainly necrotic. Because energetic conditions are thought to determine the mode of cell death (29), the less nutrient-rich serum-free conditions used in these experiments may have promoted necrosis. Indeed, addition of serum increased the percentage of annexin-V\textsuperscript{+}7-AAD\textsuperscript{−} cells but did not alter the enhanced sensitivity of tolerized cells to vincristine. Moreover, the enhanced death was not mediated mainly by classic mitochondrial or death receptor pathways (30) because cleavage of caspase-8 and caspase-9 occurred at around the same time after the release of cytochrome c from the mitochondria, and death could not be blocked by Fas or TNF antibodies, or by permeability pore transition inhibitors such as cyclosporin A (data not shown).

Strong JNK signaling, especially in the setting of aberrant NF-\textkappa{B} activation, is thought to kill cells (20, 31). Because microtubule disruption is known to activate JNK signaling (32), we examined vincristine-induced signaling in TLR7-tolerized CLL cells (Fig. 5). Low doses of vincristine caused small increases in the phosphorylated p54 isoform of JNK (Fig. 5A; compare \textit{lanes 1 and 3, top}). Activation of JNK, implied by the presence of the phosphorylated form, was also indicated by the small increase in the level of phosphorylated c-Jun, a substrate of JNK. Strikingly, the same dose of vincristine produced much higher levels of phospho-JNK and phospho-c-Jun in TLR7-tolerized cells (compare \textit{lanes 3 and 7}). The elevated levels of phospho-c-Jun also reflected increased total c-Jun levels in TLR7-tolerized cells (compare \textit{lanes 5–8 with lanes 1–4}).

Discussion

The results in this paper suggest that stimulation through TLR7 produces a state of tolerization that is characterized by impaired production of TNF\alpha (Fig. 1) and aberrant activation of SAPK and
NF-κB signaling pathways (Fig. 2). Importantly, this tolerized state seems to confer enhanced sensitivity to chemotherapeutic agents (Figs. 4 and 5) and CTLs (14).

Not all immunomodulatory agents seem capable of sensitizing CLL cells to chemotherapy. For example, exposure to type 1 IFNs (28) for 48 h protected CLL cells from low doses of vincristine (data not shown), in marked contrast to S28690 (Fig. 4).

The failure to make TNFα by TLR7-tolerized cells may be due to blocked SAPK and NF-κB activation because the TNFα promoter contains AP-1 and NF-κB sites (33). However, the tolerized state does not involve a complete shutdown of signaling through TLR7. Cytokines, such as IL6 and IL10 (Fig. 1D), were made, and the MAPK pathway (as shown by phosphorylation of ERK proteins; Fig. 2B) was still activated in TLR7-tolerized cells restimulated with S28690.

The mechanism of TLR7 tolerization seems to be multifactorial. An altered balance between proinflammatory cytokines (TNFα) and anti-inflammatory cytokines (IL10; Fig. 1D) could lead to impaired inflammatory signal transduction. Decreased expression of TLR7 mRNA (and presumably TLR7 protein; Fig. 2A) and IRAK1 (Fig. 3A) would be expected to decrease the magnitude of signaling through TLR7 upon reactivation. In addition, the initial activation of NF-κB through TLR7 seemed to induce inhibitors of TLR7 by transcriptional mechanisms because TLR7 tolerization could be blocked by actinomycin D and NF-κB inhibitors (Fig. 3B and C). Despite the apparent absence of a unique mechanism for TLR7 tolerization, the complexity and diversity of the possibilities attest to the probable biological importance of the phenomenon.

The observation that a state of “tolerance” follows activation with a variety of TLR agonists (including endotoxin and imidazooquinolines) suggests that it has been important for multicellular organisms to develop methods to limit production of potentially harmful, inflammatory cytokines caused by TLR-mediated responses. Endotoxin tolerance also involves multiple mechanisms.

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**Figure 4.** Sensitization of TLR7-tolerized CLL cells to killing by vincristine. A, purified CLL cells were cultured alone or tolerized with S28690 (1 μg/mL) for 2 d. The cells were then harvested, washed, and recultured, with or without vincristine (0.1 μg/mL) for 18 h. They were then stained with annexin-V and 7-AAD and analyzed by flow cytometry. Numbers in the top right quadrants of the dot plots, the total percentages of annexin-V- and 7-AAD- cells, indicating apoptotic and necrotic cells, respectively. B, summary of results for 20 individual patients. Top, raw data for patients 1, 6, 9, 21, 25, 26, 42, 46, 62, 69, 74, 78, 88, 89, 102, 110, 119, 121, 122, and 123; bottom, averages. Numbers over the double-headed arrows, P values for differences between the sample means. Tolerized cells were significantly more sensitive to the lytic effects of vincristine. C, CLL cells from eight different patients were treated with toxic doses of vincristine (1 μg/mL) with or without S28690 at the same time. The spontaneous and specific death percentages were determined after 18 h by flow cytometry (specific death is the difference between the percentage of 7-AAD- and annexin-V- cells in the presence of vincristine and the spontaneous death percentage, defined as the percentage of 7-AAD- and annexin-V- cells without cytotoxic drug treatment). In contrast to the tolerized state, acute treatment with S28690 protected CLL cells from vincristine-induced death. The P values indicate that the differences were statistically significant.
increased expression of negative regulators of TLR signaling, such as IRAK-M, and members of the suppressor of cytokine signaling protein family (15). TLR7 tolerization resembles endotoxin tolerance in several ways, but there seem to be some mechanistic differences. The disappearance of germinal center kinase, an upstream activator of JNK, was seen in reactivated endotoxin-tolerant PBMCs, but not in TLR7-tolerized CLL cells (data not shown; ref. 34). Similarly, IRAK4 disappears in endotoxin-tolerant cells (19) but not in TLR7-tolerized cells (Fig. 34).

Cytokines (such as IFNγ and the granulocyte macrophage colony-stimulating factor) have been reported to overcome endotoxin tolerance (35), but type I IFNs could not reverse TLR7 tolerization (data not shown).

The physiologic significance of the tolerized state that follows strong TLR activation, particularly the TLR7-tolerized state, is not clear. Endotoxin-tolerant macrophages and dendritic cells that arise in patients after Gram-negative septicemia may cause immunosuppression associated with enhanced susceptibility to subsequent infections (36). Daily injections of CpG oligonucleotides would presumably tolerate TLR9-expressing cells and cause a generalized state of immunosuppression, associated with the destruction of lymph node follicles (37). We found previously that TLR7-tolerized CLL cells (i.e., CLL cells treated with S28690 for 2 days) could not stimulate T cells to proliferate, despite strong expression of costimulatory molecules, but were more susceptible to killing by CTLs (14). Taken together, these observations suggest that tolerization may mark cells to be cleared more easily by CTLs during viral infections, when pathogen-associated molecular patterns (such as single-stranded RNA and oxidized guanosine) are present.

Although TLR7 tolerization may have evolved to facilitate antiviral immunity, induction of tolerization in TLR7+ cancer cells may potentially be used to increase their susceptibility to cytotoxic chemotherapeutic drugs or CTLs that have been activated by a vaccine (38). Clearance of leukemic skin infiltrates by topical imidazoquinolines (3) might be explained partly by TLR7-tolerization of the vaccine (38). Clearance of leukemic skin infiltrates by topical imidazoquinolines (3) might be explained partly by TLR7-tolerization (38). Therefore, we were interested in testing whether tolerization may mark cells to be cleared more easily by CTLs during viral infections, when pathogen-associated molecular patterns (such as single-stranded RNA and oxidized guanosine) are present.

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discrimination of signal transduction pathways by the TLR, reduced expression of IRAK1, reduced production of TNFα relative to anti-inflammatory cytokines, such as IL10 and TGFβ, and increased expression of negative regulators of TLR signaling, such as IRAK-M, and members of the suppressor of cytokine signaling protein family (15). TLR7 tolerization resembles endotoxin tolerance in several ways, but there seem to be some mechanistic differences. The disappearance of germinal center kinase, an upstream activator of JNK, was seen in reactivated endotoxin-tolerant PBMCs, but not in TLR7-tolerized CLL cells (data not shown; ref. 34). Similarly, IRAK4 disappears in endotoxin-tolerant cells (19) but not in TLR7-tolerized cells (Fig. 34).

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Although TLR7 tolerization may have evolved to facilitate antiviral immunity, induction of tolerization in TLR7+ cancer cells may potentially be used to increase their susceptibility to cytotoxic chemotherapeutic drugs or CTLs that have been activated by a vaccine (38). Clearance of leukemic skin infiltrates by topical imidazoquinolines (3) might be explained partly by TLR7-tolerization in target tumor cells. Clinical studies are needed to provide more information about the utility of TLR7 tolerization in management strategies for CLL and, by extension, other B cell lymphomas.

Figure 5. Enhanced vincristine-induced JNK activation in TLR7-tolerized CLL cells. A, purified CLL cells were cultured alone or tolerized with S28690 (1 μg/mL) for 48 h. The cells were then washed and recultured in the presence or absence of vincristine (0.1 μg/mL), as well as the specific JNK inhibitor SP600125 (20 μM). After 1 h, lysates were collected and levels of phosphorylated JNK and c-Jun levels, along with total levels of JunD, c-Jun, and β-actin, were determined by immunoblotting. The results show that JNK activation (as measured by phosphorylated JNK levels and reflected by enhanced phosphorylation of c-Jun) in response to an otherwise sublytic dose of vincristine was enhanced in TLR7-tolerized cells (compare lanes 3 and 7). The results with CLL cells from patient 25 are shown and are representative of six other samples. B, control and tolerized CLL cells from the indicated patients were treated with vincristine (0.1 μg/mL) for 18 h in the presence or absence of SP600125. The percentage of necrotic and apoptotic cells was then determined by staining with annexin-V and 7-AAD. Specific death was determined by subtracting the percentage of dead cells without vincristine from the percentage of dead cells in the presence of vincristine. The results show that increased sensitivity of tolerized cells to vincristine could be blocked partially by SP600125.

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