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Sensitization of IL-2 Signaling through TLR-7 Enhances B Lymphoma Cell Immunogenicity[1]

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The innate ability of B lymphoma cells to escape control by tumor-reactive T cells must be overcome to develop effective immunotherapies for these diseases. Because signals from both the innate and adaptive immune systems direct the acquisition of strong immunogenicity by professional APCs, the effects of IL-2 and the TLR-7 agonist, S28690, on the immunogenic properties of chronic lymphocytic leukemia (CLL) B cells were studied. IL-2 with S28690 caused CLL cells to proliferate and increased their expression of B7-family members, production of TNF-α and IL-10, and levels of tyrosine-phosphorylated STAT-1 and STAT-3 proteins. S28690 increased CD25 expression on CLL cells and sensitized them to IL-2 signaling. However, IL-2 did not change TLR-7 expression or signaling in CLL cells. The ability to stimulate T cell proliferation required additional activation of protein kinase C, which inhibited tumor cell proliferation, "switched off" IL-10 production, and caused essentially all CLL cells (regardless of clinical stage) to acquire a $CD83^{high}CD80^{high}CD86^{high}CD54^{high}$ surface phenotype marked by the activation of STAT-1 without STAT-3. These findings suggest that TLR-7 "licenses" human B cells to respond to cytokines of the adaptive immune system (such as IL-2) and provide a strategy to increase the immunogenicity of lymphoma cells for therapeutic purposes. ***The Journal of Immunology,*** **2006, 176: 3830–3839.**

Chronic lymphocytic leukemia (CLL),[3] the most common leukemia in the western world (1), is incurable with conventional cytotoxic chemotherapy but may be sensitive to T cell-based immunotherapies (2). However, despite the expression of high levels of MHC molecules, at least one tumor Ag (the Ig Id), and sometimes even costimulatory molecules (3, 4), CLL cells are weakly immunogenic (or unable to sustain a strong type 1 immune response) and escape control by natural (5) or vaccine-activated (6) tumor-reactive T cells. Consequently, the development of clinically relevant methods to increase the immunogenicity of CLL cells is an important goal.

Immunogenicity is a complex phenotype caused by signaling pathways that regulate the expression of costimulatory molecules, proinflammatory cytokines and chemokines, and ability to stimulate T cell proliferation and differentiation. Costimulatory molecules include CD80 and CD86, which are B7-family members that bind to CD28 on T cells and enhance their expression of anti-apoptotic and cytokine genes (7), CD54, which stabilizes T and APC contacts (8), and CD83, a characteristic dendritic cell (DC) marker (9). ICOS-L and PDL-1 are additional B7 family members that regulate the behavior of activated T cells (10), and 4-1BB ligand (4-1BBL) is a CD28-independent costimulatory factor, primarily for $CD8^+$ T cells (11).

Important transcription factors that positively regulate the immunogenic phenotype include members of the NF-κB family (12) and the STAT family member, STAT-1 (13). Other members of the STAT family (particularly STAT-3) are negative regulators of immunogenicity, and cause production of immunosuppressive factors such as IL-10 (14, 15). Previously, we showed that both IL-2 (a cytokine mediator of adaptive immunity) (4) and the imidazoquinoline, S28690 (a synthetic TLR-7 agonist which mediates innate immunity) (16, 17), could enhance some aspects of CLL immunogenicity, but required additional signals (mainly from activators of protein kinase C (PKC) family members; Ref. 3) to make CLL cells able to stimulate T cell proliferation.

The high-affinity IL-2R consists of the α (CD25), β, and common γ (γ_c) chains (18) and is expressed by many CLL cells (4). Although IL-2R signaling has not been well-characterized in CLL cells, IL-2 activates the MAPK pathway that involves ERK-1/2, along with STAT-1, -3, -5a, and -5b, in most IL-2 responder cells, and the p38 phosphorelay pathway in T cells (19, 20). Like other TLRs (21), TLR-7 activates NF-κB, p38 MAPK, and the stress-activated protein kinase (SAPK) pathway that involves JNK-1 and -2 (22). Given that IL-2 and S28690 individually activate only some of the signaling pathways required for strong immunogenicity, and that highly immunogenic cells such as DCs incorporate information from both the innate and adaptive immune systems (23), the effects of combinations of IL-2 and S28690 on the immunogenicity of CLL cells were studied in this paper.

Materials and Methods

Blood samples

Heparinized blood (30–40 ml) was collected from consenting CLL patients (diagnosed by a persistent monoclonal elevation of $CD19^+CD5^+IgM^{low}$ lymphocytes; Ref. 1). Patients were untreated at the time of analysis and their

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[3] Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; DC, dendritic cell; SAPK, stress-activated protein kinase; PDB, phorbol dibutyrate; MLR, mixed lymphocyte response; 4-1BBL, 4-1BB ligand; MFI, mean fluorescence intensity; PKC, protein kinase C; PFA, paraformaldehyde.

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clinical characteristics and identification numbers are described in Table I. Protocols were approved by the local review board.

Abs and reagents

PE- or FITC-labeled CD80, CD86, CD54, CD83, CD25, 4-1BBL, CD5, and CD19 Abs were purchased from BD Pharmingen. PE-labeled anti-ICOS-L and PDL-1 and unlabeled CD80 and CD86 Abs for blocking experiments were obtained from eBioscience. Class I and II MHC Abs were from clones W6/32 and IVA12, respectively, obtained from the American Type Culture Collection and purified in our laboratory. Isotype control Abs for blocking experiments were obtained from BD Pharmingen. Phorbol dibutyrate (PDB) was obtained from Sigma-Aldrich, and stock solutions (5 mg/ml) were made in DMSO. Clinical grade IL-2 (Chiron) was purchased from the hospital pharmacy. S28690 and the inactive control imidazoquinoline, S26424 (17), were obtained from 3M Pharmaceuticals. The powder was dissolved in AIM-V medium (Invitrogen Life Technologies) (with 33% DMSO) at 1.3 mg/ml and stored in the dark at 4°C. Abs against STAT-1, STAT-3, STAT-5, JNK, p38, p42/p44 ERK, IκB, the tyrosine-phosphorylated forms of STAT-1, STAT-3, and STAT-5, and the serine/threonine-phosphorylated forms of JNK, p38, ERK, and IκB were obtained from Cell Signaling Technology. β-Actin Abs were obtained from Sigma-Aldrich.

Cell purification

CLL and T cells were isolated from fresh blood by negative selection (RosetteSep; StemCell Technologies) as described previously (5).

Activation of CLL cells

Purified CLL cells (1.5×10^6 cells/ml) were cultured in serum-free AIM-V medium plus 2-ME (Sigma-Aldrich) (5×10^{-5} M) in 6- or 24-well plates (BD Labware) at 37°C in 5% CO_2 for the times indicated in the figure legends. S26424, S28690, IL-2, or PDB were used at 0.1 μg/ml, 0.1 μg/ml, 500 U/ml, or 10 ng/ml, respectively. These concentrations were determined by the effects of the individual immunomodulators on CD80 and CD86 expression (for S28690 and IL-2) or CD83 (for PDB) (data not shown). S26424 (the control compound for S28690) did not have measurable effects on CLL cells so AIM-V medium, alone, was used as a control for most experiments.

Table I. *Clinical properties of CLL patients*

Patient No.	Sex	Rai Stage[a]	Age (years)	WBC	Duration	Treatment[b]	CD38 (%)	Cytogenetics
1	F	I	49	32	1.5	None	10	13q−
2	F	I	52	27	7	None	8	T12[c], 13q−
3	M	0	59	15	1	None	3	Nd
4	M	0	59	27	1	None	9	Nd
5	F	0	40	23	2	None	4	Nd
6	F	0	32	22	1	None	Nd	Normal
9	M	0	45	13	2	None	6	Normal
11	F	I	39	25	7	None	25	Nd
14	M	0	49	12	1	None	Nd	Nd
15	F	0	73	19	11	None	8	Nd
16	M	I	42	34	7	None	Nd	Nd
17	M	II	57	146	6	Rad	32	Nd
19	M	II	69	121	2	None	1	Nd
20	F	IV	61	70	2	Ch	11	T12
21	M	II	63	68	6	None	5	13q−
22	M	I/II	58	22	1	None	45	T12, 11q−
23	M	IV	64	125	7	Ch,S	2	T12, 11q−
25	F	IV	55	87	7	S	1	13q−
26	F	I/II	52	47	1	Ch	9	Nd
28	M	I	59	77	4	None	Nd	13q−
29	F	I/II	77	37	4	None	46	13q−
30	F	III	70	142	4	Ch, F	3	T12
31	M	IV	59	59	10	F	14	13q−
32	M	IV	54	125	3	Ch, F	Nd	Nd
38	M	IV	50	130	2	F, Ch, CHOP	7	Nd
39	F	III	56	123	7	F, R	6	Nd
40	M	IV	53	25	1	F, CHOP	Nd	Nd
41	M	IV	66	364	10	Ch, F, S	93	Nd
43	M	IV	47	512	7	Ch, F	48	Nd
44	F	IV	48	98	11	None	2	13q−
45	F	III	77	164	3	Ch, F	23	Nd
46	M	IV	55	121	6	Ch	2	13q−
47	M	IV	66	113	5	Ch, CHOP	Nd	Nd
67	F	0	56	13	2	None	Nd	Nd
73	M	0	85	15	2	None		Nd
75	M	I	64	44	3	None		Normal
79	M	0	82	12	1	None	1	13q−
98	M	I	85	13	5	None	7	Nd
99	M	II	59	31	3	None	Nd	Nd
100	M	IV	61	45	10	Ch, F, Rads, P	Nd	Nd
101	M	IV	64	89	8	F, Ch, CHOP	Nd	Nd
102	M	IV	62	150	8	F, Ch, CHOP	Nd	Nd
103	M	III	49	69	3	None	3	Normal
104	F	0	56	12	2	None	1	Nd
105	M	IV	66	9	2	Ch, P	Nd	11q−
106	F	III	64	153	5	Ch, FC	Nd	Normal

[a] Rai stage 0, lymphocytosis; I, with adenopathy; II, with hepatosplenomegaly; III, with anemia; IV, with thrombocytopenia (1).
[b] Ch, chlorambucil; P, prednisone; F, fludarabine; S, splenectomy; Rads, local radiation; R, rituxan; CHOP, cyclophosphamide/vincristine/adriamycin/prednisone.
[c] T12, Trisomy 12; Nd, not done.

Mixed lymphocyte responses (MLRs)

T cells were isolated from CLL patients and adjusted to 5×10^5 cells/ml in AIM-V medium. Activated CLL cells were washed at least four times (to remove residual immunomodulators), irradiated (2500 cGy), and suspended at 5×10^5 cells/ml (or lower concentrations) in AIM-V. Responders and stimulators were mixed in a 1:1 (v:v) ratio and cultured in 96-well round-bottom plates (BD Labware) without cytokines or serum. Proliferation was measured 4–6 days later in a colorimetric assay (5). In some experiments, the activated CLL cells were lightly fixed (5 min) in 1% paraformaldehyde (and then washed extensively before suspension in AIM-V medium) before being placed in the T cell cultures. It has been shown previously that APCs can present Ag even when fixed (24).

Flow cytometry and DNA analysis

Surface immunophenotyping was performed as described previously (5). For analysis of DNA content, CLL cells ($\sim 1 \times 10^6$) were washed and fixed in 70% ethanol at −20°C for several days at 10^6 cells/ml. The cells were then washed and resuspended in 1 ml of Ca^{+2}, Mg^{+2}-free PBS to which 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml RNase were added, and incubated for 1 h at 37°C (to allow the escape of low m.w. DNA through the permeabilized membranes). Cells were then washed, resuspended in staining buffer (0.1 mM EDTA, 0.1% Triton X-100, and 50 μg/ml propidium iodide; Sigma-Aldrich) at room temperature in the dark for 4–12 h, filtered through nylon mesh, and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Western blots

Proteins were extracted from activated CLL cells and immunoblotting was performed as described previously (3), using anti-rabbit and anti-mouse IgG1 secondary Abs, as required. Blots were stripped for 15–30 min at 37°C in Restore Western blot stripping buffer (Pierce), washed once at room temperature, and then blocked with 10% milk for 1 h. Chemiluminescence signals were detected using Supersignal West Pico Luminal Enhancer and Stable Peroxide Solution (Pierce) and a GS-700 Imaging densitometer with MultiAnalyst software (Bio-Rad).

Isolation of total RNA and synthesis of cDNA

Total RNA from activated CLL cells was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. To remove contaminating genomic DNA, 10 μg of RNA were incubated with 10 U of RNase-free DNase I (Promega) for 30 min at 37°C. The RNA concentration was determined in a spectrophotometer at 260 nm.

cDNA was synthesized with the Superscript First Strand Synthesis System (Invitrogen Life Technologies) in a 20-μl reaction containing 3 μg of total RNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 0.5 μg of oligo dT_{18}, 0.5 mM dATP, dGTP, dCTP, and dTTP, and 200 U of Superscript II Reverse Transcriptase. The priming oligonucleotide was annealed to total RNA by incubating the mixture at 70°C for 5 min and cooling to 4°C. Reverse transcription was conducted at 42°C for 2 h and the cDNA was stored at −20°C.

Real-time PCR

The following primers were used to amplify cDNA: TLR-7 forward, 5′-CTAAAGACCCAGCTGTGACCAG-3′, TLR-7 reverse, 5′-CCAGTC CCTTTCCTCGAGACAT-3′; hypoxanthine phosphoribosyltransferase (HPRT) forward, 5′-GAGGATTTGGAAAGGGTGTT-3′, HPRT reverse, 5′-ACAATAGCTCTTCAGTCTGA-3′.

PCR was performed on a DNA engine Opticon System (MJ Research) using SYBR Green I as a double-strand DNA-specific binding dye. PCRs were cycled 40 times after initial denaturation (95°C, 15 min) according to the following parameters: denaturation at 95°C for 15 s, primer annealing at 57°C for 20 s, and extension at 72°C for 20 s. Fluorescent data were acquired during each extension phase. After each reaction, a melting curve analysis was performed by cooling the samples to 4°C and then heating them to 95°C at 0.2°C/s. Fast loss of fluorescence is uniquely observed at the denaturing/melting temperature of the amplified DNA fragment. Standard curves were generated from serial 10-fold dilutions of DNA made with the above primers.

Cytokine measurement

Cytokine levels in culture supernatants (from CLL cells activated for 48 h) were determined by a multianalyte fluorescent bead assay with a Luminex-100 system (Luminex). Kits allowing measurement of CCL3, CCL4, CCL5, CXCL10, IL-6, GM-CSF, IFN-γ, IL-10, and TNF-α were used, according to the manufacturer's instructions (R&D Systems). Individual cytokine concentrations were determined from standard curves using Bio-Plex 2.0 software (Bio-Rad). Assays were linear between 3 and 15,000 pg/ml. TNF-α was also measured with ELISA kits from Pierce, according to the manufacturer's instructions.

Statistical analysis

The Student t test was used to determine p values for differences between sample means.

Results

Effect of IL-2 and S28690 on proliferation and costimulatory molecule expression by CLL cells

As reported previously, both IL-2 (at doses above 500 U/ml) (4) and S28690 (at doses above 0.1 μg/ml) (16) change the shape and surface molecule expression of CLL cells. However, neither IL-2 nor S28690, alone, caused CLL cells to proliferate, as measured by counting them at the end of the culture period (Fig. 1*A*). In contrast, the combination of IL-2 and S28690 increased cell numbers significantly after at least 3 days of culture (Fig. 1*A*). This net increase in CLL cells appeared to result from increased proliferation (rather than resistance to apoptosis) as more cells were found in the G_2-S phase of the cell cycle (Fig. 1*B*) without an increase in subdiploid DNA (representing apoptotic cells). Note that spontaneous CLL cell death is not usually observed in the time frame of these experiments under serum-free conditions (3, 25).

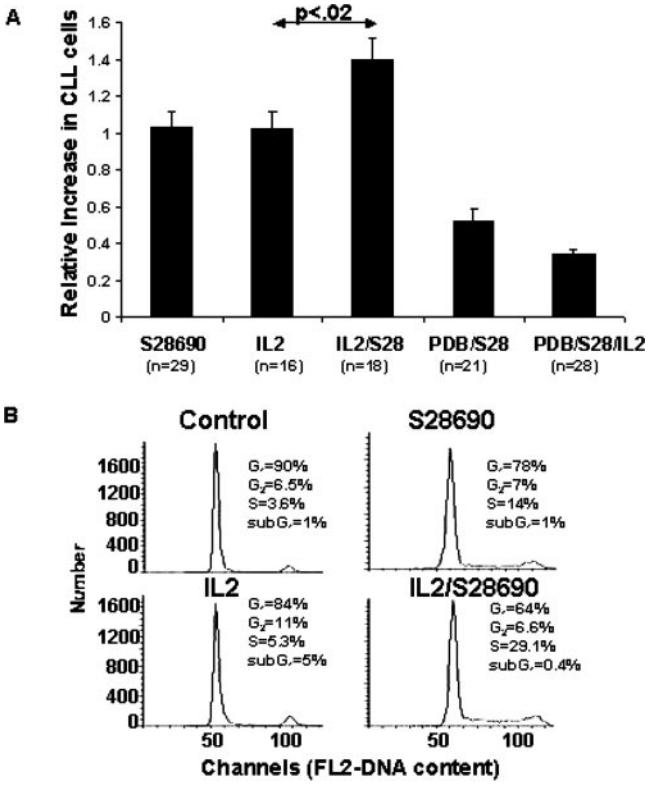

FIGURE 1. Effect of S28690 and IL-2 on proliferation of CLL cells. *A*, Purified CLL cells (1×10^6 cells/ml) from the indicated numbers of patients were cultured with combinations of S28690 (0.1 μg/ml), IL-2 (5000 U/ml), or PDB (10 ng/ml) for 3–5 days. At the end of the culture period, viable cells were counted manually in a hemocytometer. The number of activated cells divided by the number of cells cultured alone was then determined for each patient sample. The average and SEs of these measurements are shown in the graph. *B*, DNA content histograms show that CLL cells (from patient 104) cultured alone, or with IL-2 or S28690 alone, were mainly in the G_1-G_0 phase of the cell cycle while a significantly higher number were in cycle in the presence of both IL-2 and S28690. Little subdiploid DNA was apparent in these cultures. Similar results were obtained with CLL cells from four other patient samples.

IL-2 and S28690 individually increased the percentages of CLL cells that expressed CD80 and CD86, as well as the mean fluorescence intensity (MFI) of expression of these molecules (Fig. 2). S28690 appeared to be more potent in this regard and also increased CD83 expression on CLL cells more than IL-2. As with proliferation, the effects of IL-2 and S28690 on costimulatory molecule expression were additive (Fig. 2), suggesting they were mediated by different mechanisms.

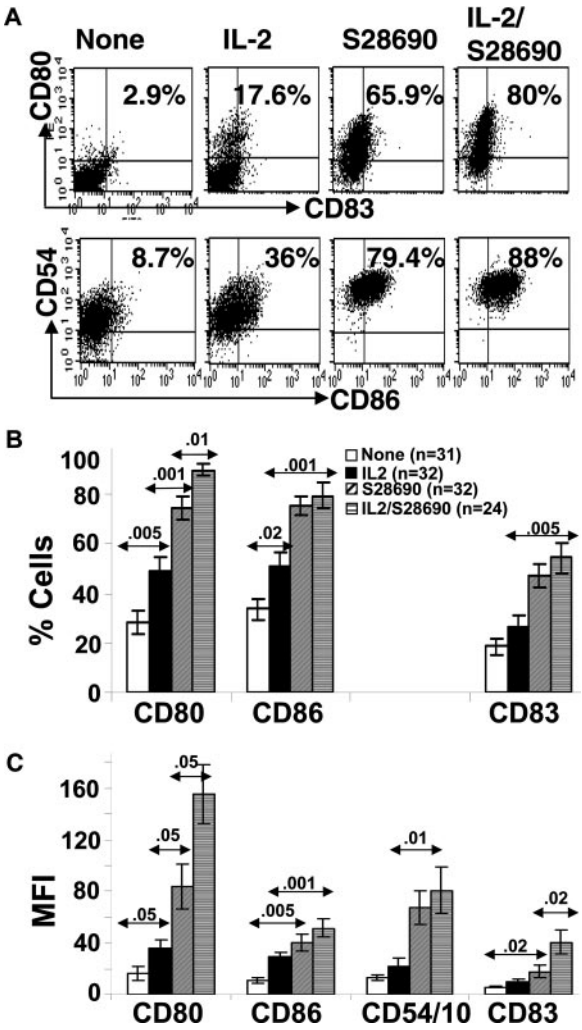

FIGURE 2. Effect of S28690 and IL-2 on costimulatory molecule expression by CLL cells. Purified CLL cells were cultured alone, or with IL-2, S28690, or both IL-2 and S28690, for 3–4 days. Expression of CD80, CD86, CD54, and CD83 were then determined by flow cytometry. *A*, An example of one patient sample is shown. The numbers in the dot plots in the *upper* and *lower rows* are the percentages of $CD80^+$ (sum of the *right* and *left upper quadrants*) and $CD86^+$ (sum of the *right upper* and *lower quadrants*) CLL cells, respectively. *B*, The percentage of cells expressing the different costimulatory molecules (indicated by staining intensity above the first decade of log fluorescence) (*left graph*) and the MFI of expression (*right graph*) were determined for CLL cells from the number of patients indicated in the graph legend. The average and SE of the results for each different costimulatory molecule are shown. For CD54, only the MFI (divided by 10) is shown because essentially all CLL cells express this molecule. The magnitude of CD80 expression was increased especially by S28690 in combination with IL-2. The numbers over the double-headed arrows are the *p* values for the differences between sample means.

Activation of signaling pathways in CLL cells by IL-2 and S28690

The cooperation between IL-2 and S28690 in causing cell proliferation and increased costimulatory molecule expression arises presumably through interactions between their respective signaling pathways. As described in the introduction, TLR-7 activates NF-κB, p38, and JNK (22), while IL-2 activates ERK and STAT family members, including STAT-1, 3, 5a, and 5b (20). Abs against phosphorylated forms of important pathway molecules can be used to indicate the state of activation of the signaling pathways.

As shown in Fig. 3*A*, S28690 treatment caused rapid phosphorylation of IκB, p38, and both JNK isoforms (suggesting activation of the NF-κB, p38, and SAPK pathways). IL-2 increased the phosphorylation of p42 and p44 ERK, but none of the other signaling pathways. The early signaling events that accompanied simultaneous treatment of CLL cells with IL-2 and S28690 were a composite of these effects. Thus, all the signaling pathways studied were activated, with NF-κB, p38, and JNK signaling at similar levels as cells treated with S28690 alone, and ERK signaling at the levels resulting from treatment with IL-2 alone (Fig. 3*A*).

Unlike T cells (20), IL-2 did not appear to activate JAK/STAT signaling directly in CLL cells (Figs. 3*B* and 4*C*). Similarly, TLR-7 does not phosphorylate STAT proteins directly (21), although autocrine production of cytokines in response to S28690

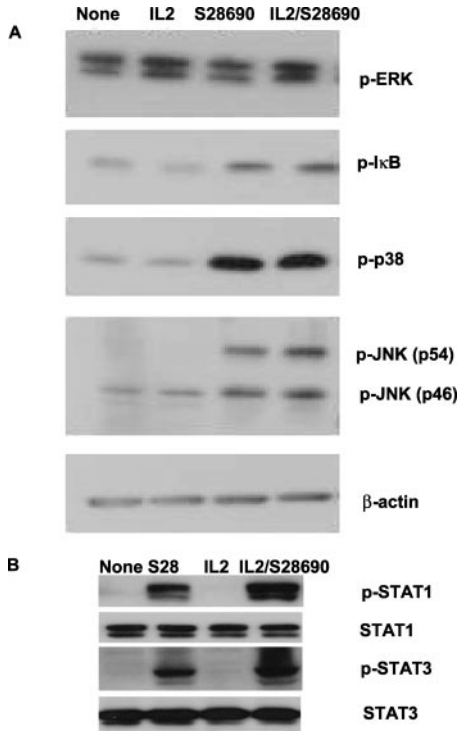

FIGURE 3. Effect of IL-2 and S28690 on signaling pathways in CLL cells. *A*, Purified CLL cells were cultured alone or treated with IL-2, S28690, or both IL-2 and S28690. After 1 h, changes in the phosphorylation status of JNK, p42/44 ERK, p38, and IκB were determined by immunoblotting with phosphospecific Abs as described in *Materials and Methods*. The blots were first probed with phosphospecific Abs and then stripped and probed with a pan-specific JNK, p42/p44, or p38 Ab (data not shown) or a β-actin Ab as a loading control. A representative example of the results obtained with the CLL cells of patient 79 is shown. The results were similar for four additional patient samples. *B*, CLL cells were cultured for 24 h in the presence or absence of IL-2 and/or S28690 and phosphorylated STAT-1 and STAT-3 levels were determined by immunoblotting. The results were similar for six additional samples.

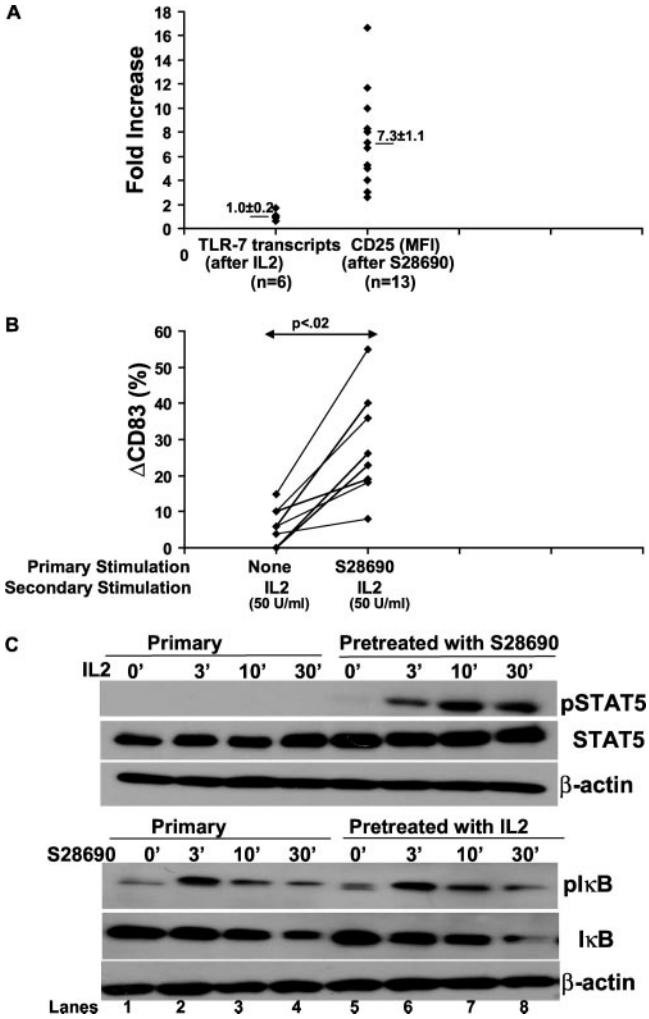

FIGURE 4. Enhancement of IL-2R expression and responses by TLR-7 activation. *A*, CLL cells from the indicated numbers of patients were cultured for 2 days alone, with S28690, or with IL-2. CD25 expression was then determined by flow cytometry and TLR-7 mRNA expression by real-time PCR, as described in *Materials and Methods*. The MFI of CD25 expression after treatment with S28690 was divided by the MFI of CD25 expression without stimulation. For TLR-7, the transcript number after treatment with IL-2 was divided by the transcript number in cells that were cultured alone. The results from the individual samples are shown, along with the average and SE, and indicate that S28690 increased markedly the expression of CD25 but IL-2 did not change the expression of TLR-7, at the mRNA level. *B*, CLL cells were cultured overnight alone or with S28690 to increase CD25 expression levels. The cells were then washed and either cultured alone or stimulated with low doses of IL-2 (50 U/ml) for 2 days. The expression of CD83 was then measured by flow cytometry. The difference in the percentage of $CD83^+$ cells, with or without IL-2 treatment, was determined for each patient sample. The results show that S28690 increased the response of CLL cells to IL-2 in this assay, concomitant with the increase in CD25 expression. The *p* value is the significance of the differences between the changes in the two groups. *C*, CLL cells from patient no. 73 were cultured for 24 h alone (*lanes 1–4*) or with S28690 (*lanes 5–8*, *top panels*), or IL-2 (*lanes 5–8*, *lower panels*). The cells were then washed and stimulated with IL-2 (*top panels*) or S28690 (*bottom panels*). Tyrosine-phosphorylated STAT-5 levels (representing IL-2R signaling) or serine-phosphorylated IκB levels (representing TLR-7 signaling) were then determined 0, 3, 10, and 30 min later by immunoblotting, as described in *Materials and Methods*. STAT-5 was phosphorylated by IL-2 only in CLL cells that had been pretreated with S28690 (compare *lanes 5–8* with *lanes 1–4*, *upper panels*). In contrast, IL-2 did not augment TLR-7 signaling (compare *lanes 1–4* and *5–8*, *bottom panels*). This experiment was repeated with three other patient samples with similar results.

results in phosphorylation of STAT-1 and STAT-3 after several hours (16). Consistent with an absence of early interactions between the signaling pathways, simultaneous treatment with IL-2 and S28690 did not directly phosphorylate STAT-1 or STAT-3 (data not shown). However, tyrosine-phosphorylated STAT-1 and STAT-3 levels were increased after 24 h (Fig. 3*B*).

Enhancement of IL-2 signaling in CLL cells by S28690

Because IL-2 and S28690 appeared to have independent effects on early signaling events (Fig. 3*A*), but increased proliferation (Fig. 1), costimulatory molecule expression (Fig. 2), and phosphorylated STAT-1 and STAT-3 levels (Fig. 3*B*) after more prolonged culture periods, we considered that IL-2 might increase TLR-7 levels (and the effects of S28690 in culture) or that S28690 might increase IL-2R levels (and the effects of IL-2), or both.

Treatment with S28690 resulted in strong up-regulation of CD25 on CLL cells (Fig. 4*A*). Increases in CD25 expression were variable but did not appear to correlate with clinical staging differences. Five patients (patients 3, 4, 6, 9, 15) had stage 0 disease, two (patients 11, 17) had stage I/II disease, and six (patients 31, 32, 38, 43, 46, 47) had stage III/IV disease (Table I). The average and SE of the S28690-induced changes in CD25 expression for these groups were 7.0 ± 0.6 ($n = 5$), 6.5 ± 2.5 ($n = 2$), and 8.0 ± 2.5 ($n = 6$), respectively. In contrast, IL-2 did not affect TLR-7 expression, at the mRNA level (Fig. 4*A*). Note that attempts to quantitate TLR-7 protein levels with existing commercial Abs by immunoblotting were unsuccessful, perhaps because of low expression of TLR-7. These results suggested that S28690 could enhance IL-2 signaling, but not the reverse.

To determine whether the increased CD25 expression caused by pretreatment with S28690 affected IL-2 signaling, we compared changes in CD83 expression on CLL cells stimulated with a low dose of IL-2 (50 U/ml, which is 10–100 times lower than in the studies shown in Figs. 1–3) before or after treatment with S28690 (Fig. 4*B*). Only a small number of CLL cells acquired CD83 expression when treated with low doses of IL-2 alone. However, when the cells were first exposed to S28690, the number that acquired CD83 expression in response to IL-2 was much higher (Fig. 4*B*), even considering that CD83 expression had been increased by the preliminary culture in S28690 (Fig. 2*B*). Similar results were seen with CD80 and CD86 expression (data not shown).

To demonstrate further that S28690 caused enhanced IL-2 signaling, early phosphorylation of STAT proteins was studied. As mentioned previously, IL-2 phosphorylates and activates STAT-5 in many IL-2 responder cells, but not CLL cells (see *lanes 1–4*, *upper panels*, Fig. 4*C*). When CLL cells were cultured with S28690 overnight, treatment with IL-2 now resulted in rapid phosphorylation of STAT-5 (*lanes 5–8*, *upper panels*, Fig. 4*C*). In contrast, using NF-κB activation to represent TLR-7 signaling, treatment overnight with IL-2 did not lead to enhanced phosphorylation of IκB in response to S28690 (compare *lanes 5–8* with *lanes 1–4*, *lower panels*, Fig. 4*C*).

Taken together with the absence of an effect on TLR-7 gene expression (Fig. 4*A*), these results suggested that IL-2 did not sensitize CLL cells to TLR-7 signaling, whereas S28690 sensitized CLL cells to IL-2 (in part through increasing IL-2R expression (Fig. 4*A*).

Effect of PKC agonists on costimulatory function and phenotype of CLL cells treated with IL-2 and S28690

In accordance with their increased expression of costimulatory molecules, CLL cells treated with both IL-2 and S28690 were better able to support T cell proliferation (measured in MLRs) than CLL cells treated with either agent alone (Fig. 5*A*). However, their

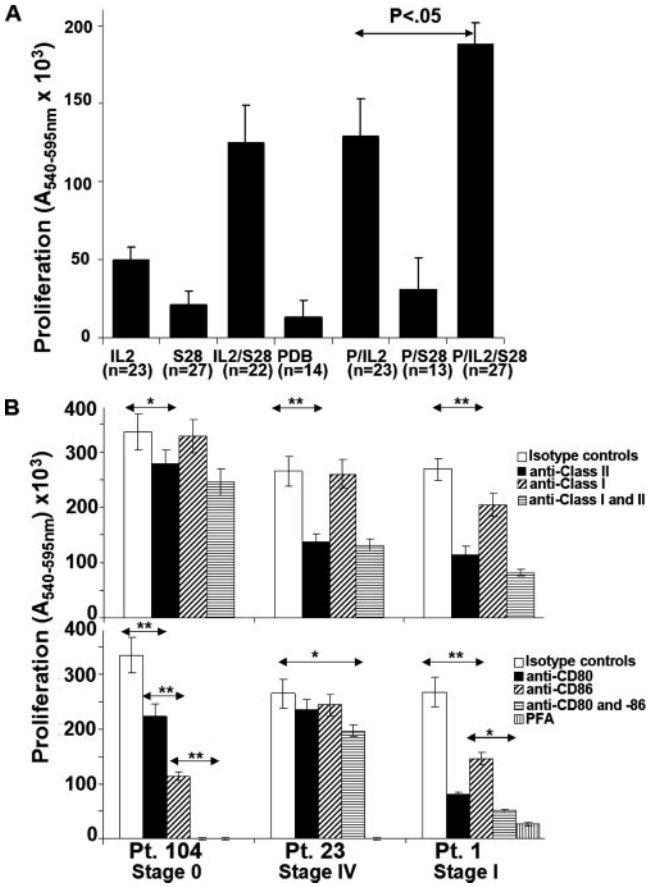

FIGURE 5. Effect of S28690 and IL-2 on the ability of CLL cells to stimulate T cell proliferation. *A*, CLL cells were purified from individual patients and cultured alone or with IL-2, S28690, IL-2 and S28690, PDB, PDB and IL-2, PDB and S28690, or PDB, IL-2, and S28690 as described in *Materials and Methods*. After 3–4 days, the cells were harvested, washed extensively, irradiated (2500 cGy), and used to stimulate T cells from CLL patients (obtained at the same time as the tumor cells and rested in culture until added to the MLRs). After 5–6 days, Alamar blue was added and proliferation was measured in an OD colorimetric microplate reader at wavelengths of 540 (reduced state) and 595 (oxidized state) nm. The difference between these readings was used as a measure of the number of viable cells in the culture. After subtracting the proliferation induced by nonactivated CLL cell stimulators, the results from each individual experiment were used to generate the average proliferation and SE from the number of patients indicated on the *x*-axis. IL-2 was required for CLL cells to acquire strong stimulatory capabilities and interacted with both PDB and S28690 to increase the stimulatory capabilities of CLL cells. *B*, CLL cells from patients 104, 23, and 1 were activated with PDB, S28690, and IL-2 and used as stimulators in MLRs. Blocking class I MHC, class II MHC, CD80, and CD86 Abs (each at 20 μg/ml), as well as isotype control Abs, were added to some wells at the start of the MLR cultures. In some cases, the CLL cells were first fixed with PFA (to inhibit their ability to secrete cytokines) before use as stimulators in the MLRs. Proliferation was then measured using Alamar blue. The average of three replicate wells (after subtracting the background proliferation of the responders and stimulators) and SE is shown for each patient. *, $p < 0.05$; **, $p < 0.01$.

stimulatory ability was still rather weak. Previously, we had found that the costimulatory ability of CLL cells treated with S28690 (16) or IL-2 alone (4) could be enhanced significantly by concomitant stimulation with PKC agonists (such as phorbol esters, Bryostatin, or a synthetic Bryostatin analog called Picolog; Ref. 26) through a number of mechanisms, including increased expression of the DC marker, CD83. Because CLL cells treated with IL-2 and S28690 did not increase CD83 expression maximally (Fig. 2*B*), the effect of additional treatment with PDB was then studied.

PDB decreased the numbers of CLL cells after treatment with IL-2 and S28690 (Fig. 1) but caused them to become better stimulators of T cell proliferation (Fig. 5*A*). Interestingly, CLL cells treated with both PDB and S28690 remained weak stimulators of T cell proliferation, despite increased expression of CD83. Hence, IL-2 appeared to be required for CLL cells to acquire strong T cell stimulatory ability.

Blocking experiments were conducted to determine whether the enhanced proliferation of T cells resulting from coculture with CLL cells treated with PDB, S28690, and IL-2 resulted from antigenic signaling, or was nonspecific and caused simply by high levels of cytokines in the presence of increased costimulatory molecule expression (Fig. 5*B*). T cell proliferation was inhibited mainly by Abs against class II MHC molecules (Fig. 5*B*, *top graph*), suggesting that $CD4^+$ T cells were stimulated mainly by the activated CLL cells. Consistent with this, $CD4^+$ T cell numbers increased more than $CD8^+$ T cells (measured by manual counting in a hemocytometer and flow cytometry) at the end of the culture period (average numbers and SEs with CLL cells from patients 1, 9, 23, and 104 as stimulators were: initial $CD4^+$ cells: $(90 \pm 3) \times 10^4$/ml. Final $CD4^+$ cells: $(213 \pm 13) \times 10^4$/ml. Initial $CD8^+$ cells: $(5 \pm 2) \times 10^4$/ml. Final $CD8^+$ cells: $(23 \pm 4) \times 10^4$/ml). Blocking Abs against CD80 and CD86 independently inhibited T cell proliferation. This inhibition was stronger in the presence of both Abs (Fig. 5*B*, *bottom graph*), suggesting that the two costimulatory molecules (along with class II MHC) contributed to the increased stimulatory ability of CLL cells treated with PDB, S28690, and IL-2. However, cytokine production may also play a role in the enhanced T cell stimulation. When the activated CLL cells were fixed with paraformaldehyde (PFA) (to prevent cytokine production while preserving cell membrane protein expression), the ability to stimulate T cell proliferation was inhibited strongly (Fig. 5*B*, *bottom graph*, vertical bars).

Taken together, these results suggested that CLL cells treated with PDB, S28690, and IL-2 acquired properties of strong APCs and stimulated T cell proliferation through expression of Ag, costimulatory molecules (particularly CD80 and CD86), and cytokines. Note that CLL cells from patients with all clinical stages of the disease (Fig. 5*B*) could stimulate T cell proliferation in this manner, upon activation with PDB, S28690, and IL-2.

Costimulatory molecule expression by CLL cells treated with S28690, IL-2, and PKC agonists

PDB, alone, caused ~90% of CLL cells to express CD83 (Fig. 6*B*, clear bars). PDB also increased the number of $CD80^+$ and $CD86^+$ CLL cells (the latter more than the former), as well as the expression of 4-1BBL and PDL-1 (Fig. 6*C*, clear bars). CD54 and ICOS-L expression were affected only marginally by PDB (Fig. 6, *B* and *C*).

Addition of IL-2 during activation of CLL cells with PDB mainly increased the MFI of CD80 and CD54 expression (Fig. 6*B*, solid bars). A slightly greater percentage of $CD80^+$ cells was obtained when CLL cells were activated with both PDB and S28690 (Fig. 6*B*, diagonal bars). The addition of IL-2 to S28690 and PDB increased strongly the MFI of CD80 expression (compared with CD86 (Fig. 6*B*)), as well as CD54. Remarkably, regardless of clinical stage or cytogenetic abnormalities (Table I), and in accordance with their strong T cell stimulatory ability (Fig. 5), CLL cells treated with IL-2, S28690, and PDB acquired uniformly a $CD83^{high}CD80^{high}CD86^{high}CD54^{high}$ cell surface phenotype (Fig. 6, *A* and *B*, horizontal bars).

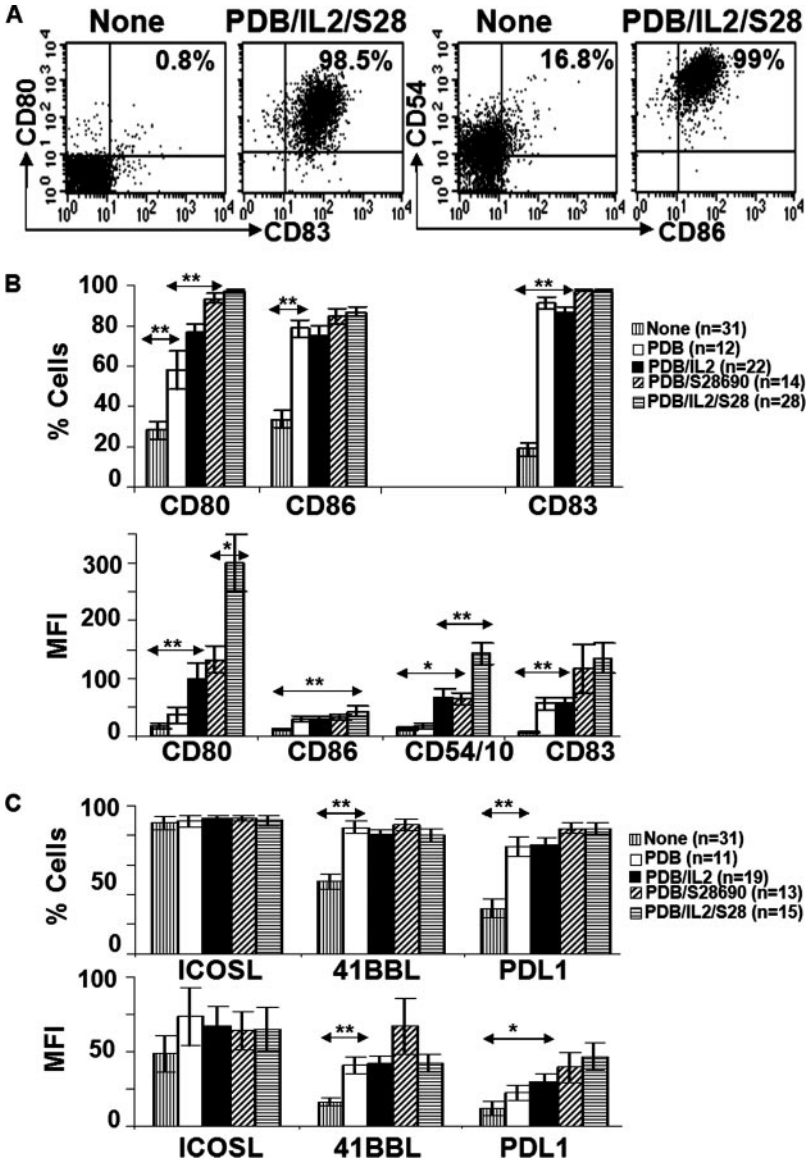

FIGURE 6. Effect of PKC agonists on costimulatory molecule expression by CLL cells treated with S28690 and IL-2. *A*, CLL cells from a representative patient were cultured alone, or with S28690, IL-2, and PDB, for 3 days. CD80, CD83, CD54, and CD86 expression was then determined by flow cytometry. The percentages refer to CD80 (sum of the *right* and *left upper quadrants*) (*left dot plots*) and to CD86 (sum of the *right upper* and *lower quadrants*) (*right dot plots*). *B*, Summary of the flow cytometric evaluation of the percentage of CLL cells expressing CD80, CD83, CD54, and CD86 (and the MFI of expression) after culture alone, with PDB, PDB and IL-2, PDB and S28690, or PDB, IL-2 and S262690. The average and SE of the results from the number of patients indicated in the graph legend are shown. For CD54, only the MFI (divided by 10) is shown because essentially all CLL cells express this molecule. The results indicated that PDB and S28690 caused nearly 100% of CLL cells to acquire CD80, CD86, and CD83 expression. Addition of IL-2 affected mainly the magnitude of CD80 and CD54 expression. *C*, Summary of similar flow cytometric evaluation of ICOS-L, 4-1BBL, and PDL-1 expression. PDB, with or without IL-2, and/or S28690 increased the expression of 4-1BBL and PDL-1 but not to the same degree as CD80, CD86, CD54, and CD83. *, $p < 0.05$; **, $p < 0.01$.

Cytokine production by CLL cells treated with S28690, IL-2, and PKC agonists

The increased phosphorylation of STAT1 and STAT3 after 24 h in CLL cells treated with IL-2 and S28690 (Fig. 3*B*) was consistent with autocrine production of cytokines. In addition, immunogenic cells promote activated T cell proliferation and differentiation by secreting cytokines and chemokines, as well as expressing costimulatory molecules (27). Accordingly, we measured the production of a number of cytokines and chemokines (relevant to induction of effective antitumor immunity; Ref. 28) by CLL cells activated with various combinations of PDB, S28690, and IL-2 (Fig. 7). CLL samples from 19 different patients (nos. 1, 9, 20, 23, 25, 26, 38, 67, 73, 75, 98, 99, 100, 101, 102, 103, 104, 105, 106), representing all clinical disease stages, were studied.

CLL cells treated with IL-2, alone, made very few cytokines or chemokines. As reported previously (16), CLL cells invariably made TNF-α (Fig. 7*A*, *top left graph*) and lesser amounts of IL-10 (Fig. 7*A*, *middle left graph*) in response to S28690. In the presence of IL-2, S28690-induced TNF-α production was somewhat decreased. Remarkably, IL-10 production was increased significantly when CLL cells were treated with both IL-2 and S28690 (Fig. 7*A*, *middle left graph*).

PKC agonists had dramatic effects on the production of these cytokines. Although a paucity of cytokines was made by CLL cells treated with PDB alone, TNF-α production was increased considerably after treatment with both PDB and S28690, and especially after PDB, S28690, and IL-2 (Fig. 7*A*, *top left graph*). At the same time, IL-10 production by CLL cells treated with S28690 and IL-2 was inhibited strongly by concomitant treatment with PDB. These changes in the balance of TNF-α and IL-10 production caused by PDB were highly statistically significant (Fig. 7*B*, *top left graph*).

The effects of these immunomodulatory agents on the production of other chemokines and cytokines were more variable. Chemokines (such as RANTES (CCL5) (Fig. 7*A*, *bottom left graph*), MIP-1-α (CCL3), MIP-1-β (CCL4), and IFN-γ-inducible protein-10 (CXCL10) (data not shown)) generally followed the pattern of TNF-α, and tended to be produced in greater amounts when CLL cells were treated with PDB, S28690, and IL2, consistent with the increased ability of these cells to stimulate T cell proliferation (Fig. 5). IL-6 was sometimes very high when CLL cells were treated with S28690 (with or without IL-2), but did not appear to be affected especially by concomitant stimulation with PDB (Fig. 7*A*, *top right graph*). CLL cells have been reported to make the type 1 immune cytokine, IFN-γ (29). IFN-γ production

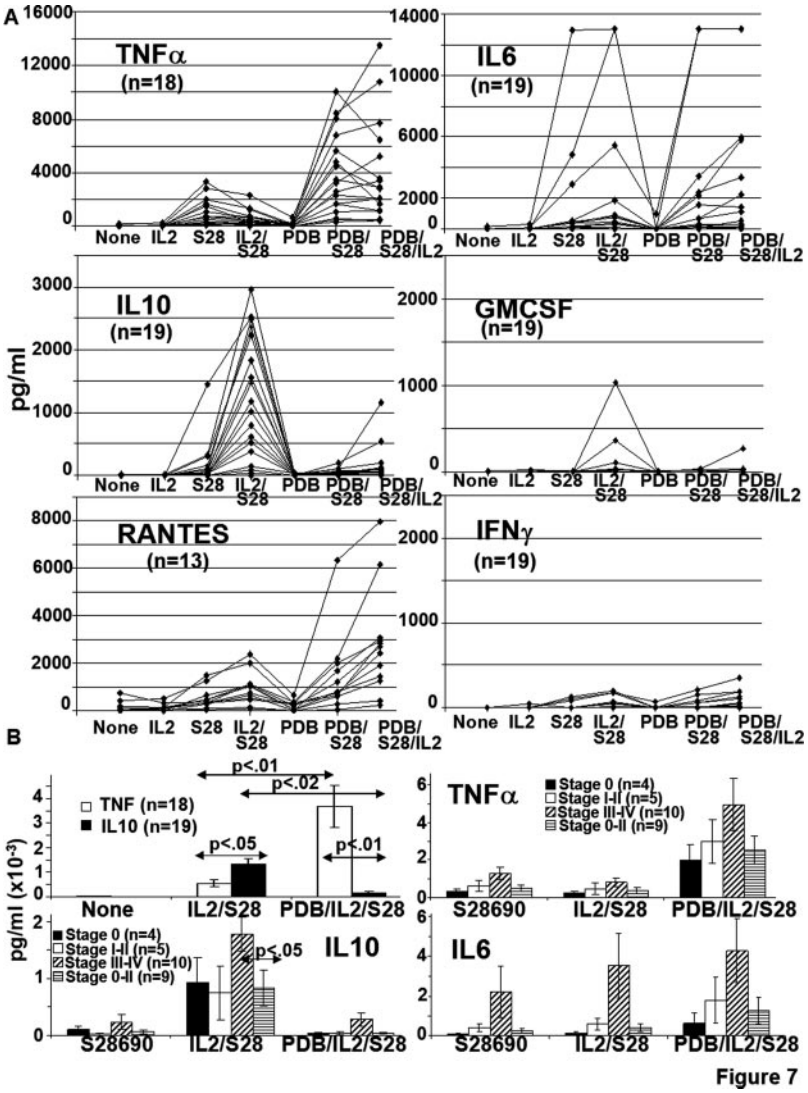

FIGURE 7. Effect of S28690, IL-2, and PDB on cytokine and chemokine production by CLL cells. *A*, CLL cells from patients 9, 67, 73, and 104 (stage 0 disease), 1, 26, 75, 98, and 99 (stage I-II disease) and 20, 23, 25, 38, 100, 101, 102, 103, 105, and 106 (stage III-IV disease) were cultured alone or with IL-2, S28690, IL-2, and S28690, PDB, PDB and S28690, or PDB, IL-2, and S28690 for 48 h. Supernatants were then harvested for cytokine measurements as described in *Materials and Methods*. The results for TNF-α, IL-10, RANTES (CCL5), IL-6, GM-CSF, and IFN-γ are shown. Each line represents results for a different patient sample. Note that TNF-α production by stimulated CLL cells from patient 75 was off-scale and is not shown. RANTES levels were measured only from 13 patient samples. *B*, The averages and SEs of TNF-α (□) and IL-10 (■) produced by CLL cells alone, or treated with IL-2 and S28690, or PDB, IL-2, and S28690 are shown (*top left*). The averages and SEs of TNF-α (*top right*), IL-10 (*bottom left*), and IL-6 (*bottom right*) production by stage 0 CLL cells (■), stage I-II CLL cells (□), stage III-IV CLL cells (▨), and the combination of stage 0 and stage I-II CLL cells (▤), after stimulation with S28690, IL-2, and S28690, and PDB, IL-2, and S26890, are shown. The numbers over the double-headed arrows are the *p* values for the differences between sample means.

Figure 7

was both uncommon and low in the CLL samples studied here, but tended to be increased by treatment with S28690, IL-2, and PDB (Fig. 7*A*, *bottom right graph*). GM-CSF production was also infrequent, but tended to be increased more by CLL cells treated with IL-2 and S28690 (Fig. 7*A*, *middle right graph*).

Although cytokine and chemokine production appeared to follow general patterns, the magnitude of production was quite variable. Such variability could be related potentially to biological differences between the tumor samples, reflected in the clinical stages of the patients. Using the Rai clinical staging system for CLL (1), samples were grouped into stage 0 (which may never require treatment), stage I-II (which has a mean survival of 7–10 years), and stage III-IV (which has a mean survival of <5 years) (see legend to Table I). For purposes of establishing statistical significance, stages 0, I, and II ("low-risk" disease) were also compared with stages III and IV ("high-risk" disease).

On this basis, CLL cells from stage III and IV patients made significantly higher levels of the immunosuppressive cytokine, IL-10, when stimulated with IL-2 and S28690 (Fig. 7*B*, *bottom left graph*). Production of most other cytokines (e.g., IL-6 (Fig. 7*B*, *bottom right graph*)) also tended to be higher when advanced stage CLL cells were treated with S28690 (with or without PDB or IL-2), and approached (but did not reach) statistical significance (i.e., $p < 0.05$).

Interestingly, TNF-α production did not seem to be affected as much by the clinical stage (Fig. 7*B*, *top right graph*). Importantly, PDB was able to shut off the production of IL-10 (Fig. 7*B*, *bottom left graph*), while increasing the production of TNF-α (Fig. 7*B*, *top right graph*), regardless of clinical stage.

STAT-1 and STAT-3 activation in CLL cells treated with S28690, IL-2, and PKC agonists

Because CLL cells treated with S28690, IL-2, and PDB appeared to become strong APCs, their expression of tyrosine-phosphorylated STAT-1 and -3 protein levels was measured (in view of the relationship of these signaling molecules with tumor immunogenicity; Refs. 14, 15) (Fig. 8). Consistent with previous results (Fig. 3*B*), S28690 (with or without IL-2) increased activated STAT-1 and especially STAT-3 levels (*lanes 3* and *7*) (which was shown previously to be due to autocrine production of IL-6 and -10; Ref. 16).

PDB alone did not activate STAT-1 or STAT-3, and promoted STAT-1 activation by IL-2 (*lanes 5* and *6*). Remarkably, PDB strongly inhibited STAT-1 and STAT-3 activation by S28690 (*lanes 3* and *4*). STAT-1 activation (presumably by IL-2) was maintained in CLL cells treated with PDB, IL-2, and S28690.

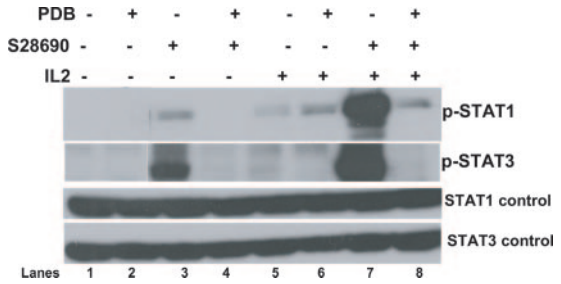

FIGURE 8. Effect of S28690, IL-2, and PDB on STAT-1 and STAT-3 activation in CLL cells. CLL cells were cultured for 24 h alone (*lane 1*), with PDB (*lane 2*), with S28690 (*lane 3*), with PDB and S28690 (*lane 4*), with IL-2 (*lane 5*), with PDB and IL-2 (*lane 6*), with IL-2 and S28690 (*lane 7*) and with PDB, IL-2, and S28690 (*lane 8*). Tyrosine-phosphorylated STAT-1 and STAT-3 levels were then determined by immunoblotting, as described in *Materials and Methods*. Similar results were obtained using three different patient samples.

Therefore (in accordance with other features of strong immunogenicity), gene transcription regulated by STAT-1 appeared to be favored in CLL cells treated with IL-2, S28690, and PDB.

Discussion

The results in this article suggest that CLL cells treated with a TLR-7 agonist, IL-2, and a PKC agonist differentiate into cells that resemble DCs, with uniformly high expression of costimulatory molecules (Fig. 6), and ability to stimulate T cell proliferation (Fig. 5). Each of the individual immunomodulators appeared to affect specific aspects of costimulation. For example, IL-2 promoted STAT-1 activation (Fig. 8). S28690 activated NF-κB, SAPK, and p38 signaling pathways (Fig. 3*A*), and PKC agonists induced CD83 expression (Fig. 6), turned off IL-10 production (Fig. 7), and inhibited STAT-3 activation (Fig. 8). However, all three agonists were required to cause CLL cells to undergo DC-like maturation.

IL-2 and S28690 initially provided only additive signaling to CLL cells (Fig. 3), perhaps because of the locations of the respective receptors. The IL-2R is found on the plasma membrane while TLR-7 is in the endosomal compartment (30). However, IL-2 and S28690 signaling became more connected over time. TLR-7 activation caused increased expression of CD25, leading to enhanced signaling through the IL-2R (Fig. 4). The results of these interactions between S28690 and IL-2 included increased proliferation (Fig. 1), expression of costimulatory (Fig. 2), and activated STAT-1 and STAT-3 molecules (Fig. 3*B*), and production of cytokines (Fig. 7) by CLL cells.

IL-2 and S28690 had especially striking effects on IL-10 production (Fig. 7*A*), particularly by more aggressive CLL cells from patients with advanced stage disease (Fig. 7*B*). Although the mechanism is unclear (but probably reflects signaling aberrations caused by the cytogenetic abnormalities associated with CLL progression; Ref. 31), this observation may have pathogenic implications, because IL-10 has immunosuppressive properties and promotes the development of regulatory T cells that can inhibit strong type 1 responses, required for effective antitumor immunity (32). It is possible that endogenous stimulation of CLL cells by IL-2 (or IL-2 family members; Ref. 4) and endogenous TLR-7 agonists (such as oxidized guanosines (30) or single-stranded RNA (33)), produced in response to episodes of infection (both clinical and subclinical), may lead to enhanced production of IL-10 and contribute to the poor prognosis of patients with stage III and IV disease.

Despite these potent interactions between IL-2 and S28690, additional activation by PKC agonists was required to cause CLL cells to become highly immunogenic. The immunogenic importance of PKC (likely the PKCβ isozyme) has been documented previously (3, 4, 16, 34), although the mechanism is not entirely clear. PKC agonists increased CD83 expression (Fig. 6), inhibited the proliferation of CLL cells treated with IL-2 and S28690 (Fig. 1), "switched off" IL-10 production (Fig. 7), and significantly altered the relative amounts of phosphorylated STAT-1 and STAT-3 (Fig. 8). These latter effects may reflect inhibitory phosphorylation of STAT-3-activating cytokine receptors by phorbol esters (35). Given the importance of STAT-3 as a negative regulator of DC and tumor cell immunogenicity (14, 15), we suggest that turning off STAT-3 activation (and IL-10 production) was critical for making the CLL cells highly immunogenic.

Although the results in this paper describe a method to increase the immunogenicity of B cell tumors, they may have broader implications for human B cell immunology. The enhancement of IL-2 signaling by S28690 (a synthetic analog of the natural TLR-7 ligand, single stranded RNA (33)) (Fig. 4), may represent a model for the priming of an adaptive immune response to a systemic viral infection. The "one-way" nature of this interaction (i.e., priming of IL-2 responses by S28690 but not of TLR-7 responses by IL-2) (Fig. 4) may represent an immunological control mechanism to localize the effects of activated T cells (represented by IL-2) to sites of active viral infections (represented by treatment with S28690) and contrasts markedly with the ability of type 1 IFNs (which are cytokines of innate immunity) to increase TLR-7 expression (36). Nevertheless, B cells required concomitant stimulation with PKC agonists to become highly immunogenic. Because phorbol esters can mimic signaling through the BCR (37), the requirement for simultaneous PKC signaling may represent another point of immunological control, which ensures that only Ag-activated B cells acquire strong immunogenicity in the presence of high levels of innate and adaptive immune stimulators to avoid nonspecific immune activity and the development of autoimmune diseases. We speculate further that IL-2 and TLR-7 signaling (in the absence of PKC activation) together cause strong production of IL-10 (Fig. 7) as an additional mechanism to suppress nonspecific immunity.

Along with providing a potential model for understanding human tolerance and immunogenicity mechanisms, the results described in this paper may also aid in devising immunotherapeutic strategies for CLL. Despite the fact that CLL cells from different patients are heterogeneous (characterized by different cytogenetic abnormalities, mutation status of the rearranged Ig locus, or expression of CD38 and Zap70; Ref. 31), and respond variably to IL-2 (4), S28690 (16), and phorbol esters as single agents (Fig. 7), CLL cells uniformly became highly immunogenic when treated with all three of these agents. The absence of significant heterogeneity in patient response, coupled with the ease, rapidity, and reproducibility of the method, suggest the use of imidazoquinolines, along with IL-2 and clinically relevant PKC agonists (such as Bryostatin-1 (3) or Picolog (26)), to make DC-like CLL cells for autologous tumor vaccines in vitro, or (depending on clinical toxicity) to turn CLL cells into endogenous vaccines in vivo.

Disclosures

R. L. Miller is employed by 3M Pharmaceuticals, whose (potential) product, compound S28690, was studied in the present work. S28690 is a member of a class of compounds being developed by 3M Pharmaceuticals for commercial purposes.

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