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IL-24 Induces Apoptosis of Chronic Lymphocytic Leukemia B Cells Engaged into the Cell Cycle through Dephosphorylation of STAT3 and Stabilization of p53 Expression

Alexander Sainz-Perez,1,2,* Hélène Gary-Gouy,1* Françoise Gaudin,* Ghyath Maarof,* Anne Marfaing-Koka,† Thierry de Revel,‡ and Ali Dalloul3*

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of long-lived monoclonal B cells mostly arrested at the G0/G1 phase of the cell cycle. CLL cells strongly express intracellular melanoma differentiation-associated gene-7 (MDA7)/IL-24. However, adenovirus-delivered MDA7 was reported to be cytotoxic in several tumor cell lines. We report herein that rIL-24 alone had no effect; however, sequential incubation with rIL-2 and rIL-24 reduced thymidine incorporation by 50% and induced apoptosis of CLL cells in S and G2/M phases of the cell cycle, but not of normal adult blood or tonsil B cells. IL-24 stimulated STAT3 phosphorylation in IL-24R1-transfected cells but not in normal or CLL B cells. In contrast, IL-24 reversed the IL-2-induced phosphorylation of STAT3 in CLL, and this effect was neutralized by anti-IL-24 Ab. Phospho-STAT3 inhibition of STAT3 and Stabilization of p53 Expression

Cells Engaged into the Cell Cycle through Dephosphorylation of PSTAT3 and Stabilization of p53 Expression

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(P)STAT3 and enhancement/stabilization of p53 expression, thereby mediating cell cycle arrest and apoptosis of cells in the G1/M phase of the cell cycle. This mechanism was unexpected as it is opposite to that generally attributed to members of the class II family of cytokines from studies with epithelial cell lines. Additionally, and at variance with the previously reported JAK/STAT-independent action of adenovirus MDA7 (17), we demonstrate a specific induction of apoptosis by rIL-24.

Materials and Methods

Patients

The patients enrolled in the study were diagnosed according to cytologic and immunologic analyses and followed up at the Percy Military Hospital (Clamart, France) and Antoine Béclère Hospital (Clamart, France) between 2004 and 2007. Treatment-free period (if any) was at least 3 mo. Approval for these studies was obtained from the institutional review board of the University Hospital Antoine Béclère (Paris XI). All patients gave informed consent. B lymphocytes from leukemic donors were purified and maintained in culture as previously described (14). For B cell purification, a negative B cell selection was applied using a B cell enrichment RosetteSep kit (StemCell Technologies). The mean percentage of B cells was 92.5% (89.5–98%) in patients.

Reagents

The p53 transactivation activity inhibitor pifithrin-α and the caspase inhibitors z-VAD-fmk (Calbiochem) and Tartrate resistant acid phosphatase Sigma-Aldrich) were used. Na-pervanadate was prepared by mixing equal concentrations of Na2VO3 with H2O2 (100 mM) (both from Sigma-Aldrich) for 15 min at room temperature, and serial dilutions were added to cells.

Cell culture, proliferation, cell cycle, and apoptosis

BW5147 cell lines transfected with empty vector or with IL-2Rα plus IL-20Rβ (IL-2R1 heterodimer, given by Dr. J.-C. Renaud) were expanded in IMDM with 10% FCS, stimulated, and processed in the same way as CLL and control B cells. Tonsil and donor blood B cells were negatively selected using magnetic beads (Dynal Biotech) as previously described (12). B cells from patients were cultured in RPMI 1640 medium supplemented with 10% FCS or 10% autologous serum, penicillin (100 U/ml), streptomycin (100 mg/ml), 2 mM l-glutamate, and 1 mM sodium pyruvate (In VitroGen). Cells were incubated with rIL-2 and rIL-24 (both from R&D Systems) at respective final concentrations of 50 and 100 ng/ml (or as indicated).

Proliferation assays were performed in flat-bottom 96-well plates. Cells (107/200 μl/well) were incubated at 37°C under various conditions. Each condition was done in triplicate. After 72 h, 0.5 μCi of [methyl-3H]thymidine was added to each well and incubated 18 h at 37°C. Plates were then harvested in a Wallac Printed Filtermat A with a Harvester 96 (Tomtec). Incorporation and were dried for at least 1 h. Dried Printed Filtermat was put into a harvest in a Wallac Printed Filtermat A with a Harvester 96 (Tomtec). For apoptosis studies, cells were stained either with the Apo2.7-PE mAb (Becton Dickinson) or annexin V-FITC and 5 μg plus PI (BD Biosciences). Fluorescence was measured using a FACScan flow cytometer (BD Biosciences). For cell cycle analysis, cells were synchronized with cold thymidine (100 mM) on ice for 15 min, washed, and resuspended in 300 μl of annexin V-FITC apoptosis detection kit (BD Biosciences). Cells (1 × 106) in 100 μl 1× binding buffer was stained with 5 μl of annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. Next, 400 μl of 1× binding buffer was added before FACS analysis. For cell cycle analysis, cells were synchronized with cold thymidine (100 μM) and 1 × 106 cells were washed with cold PBS 1× and resuspended in 1 ml ETOH 70% and kept on ice for at least 10 min. Cells were then centrifuged and the pellet was suspended in 1 ml PBS 1× plus RNase (40 μg) plus PI (18 μl) (all from Sigma-Aldrich) at room temperature. The percentage of doublets was estimated by forward scatter (FSC) linear vs FSC area analysis. For CFSE staining, 5 × 106 cells were washed twice in cold PBS and resuspended in PBX plus CFSE (5 μM) for 10 min at room temperature and reaction blocked with addition of FSC (2%), washed twice, and re-suspended in culture medium at different conditions.

Fluorescence was measured using a FACScan flow cytometer (BD Biosciences), and data analysis was performed using CellQuest software (BD Biosciences).

Immunoprecipitation and Western blot

CLL cells were processed from fresh blood and washed at room temperature in a serum-free RPMI with 10 mM HEPES. Lysates from fresh blood CLL cells (10 × 106), stimulated with IL-2 at different times, were precipitated with 1 μg anti-phosphotyrosine clone 4G10 mAb (Upstate Biotechnology) plus protein G-Sepharose beads (Sigma-Aldrich). Proteins were separated on SDS-PAGE gels, electrotransferred onto polyvinylidene difluoride membranes (Amersham), and blotted with anti-STAT3, STAT5, and JAK1 MAb (BD Transduction Laboratories). Blots were revealed using a goat-anti-mouse HRP-conjugate (Bio-Rad Laboratories) and ECL detection (Amersham). For p53 expression were preincubated or not with IL-2 overnight, then incubated or not with 25 μM p53 inhibitor durant 5 min and stimulated or not with IL-24 for 10 min and lysed as previously described. The primers were transcribed with anti phosphoTy705-STAT3 (B-7) mAb (Tebu-bio), dehybridized, and rebotted with anti-STAT3 mAb (BD Transduction Laboratories).

CCL cells (10 × 106) were stimulated or not with rIL-2, rIL-24, or rIL-2 plus rIL-24 and lysed at 4°C for 30 min in lysis buffer (20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 50 U/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) containing 1% Nonidet P-40 detergent. Proteins were separated on SDS-PAGE gels, electrotransferred onto polyvinylidene difluoride membranes (Amersham), and blotted with either anti-p53 mAb (DakoCytomation), anti-phospho-p53 (S15) mAb (R&D Systems), anti-p38 (A-12) mAb (Tebu-bio), or anti-Bcl-2 (C-2) mAb (Tebu-bio). Blots were revealed using a goat-anti-mouse HRP conjugate (Bio-Rad Laboratories) and ECL detection (Amersham). IL-24 was neutralized with a goat IgG-anti-human IL-24 (R&D Systems) according to the manufacturer’s instructions for 1 h at 37°C before addition to cells.

Real-time PCR

Cells (2 × 106/ml) were incubated in complete medium for 24 h under various conditions, and RNA was purified using the RNeasy Mini kit (Qiagen). Reverse transcription was done with random hexamers using SuperScript III reverse transcriptase (Invitrogen). Reverse transcription expression was analyzed by PCR using the TaqMan technology. Results were analyzed using the ABI Prism 7700 sequence detection system software (PE Applied Biosystem). Each reaction was normalized by the cycle threshold (Ct) of GAPDH cDNA expression. Relative expression was calculated by measuring the difference of normalized Ct between condition A vs condition B and applying the following formula: difference of expression between A and B = 2 CtA − CtB. TaqMan-specific primers and probes were selected using Primer Express software and specificity verified using the ABI Prism 7700 sequence detection system software. The names of amplicon size primer sequences are: p53 82 L1 ATCTACT GGCGGACAGAAGACCT, R1 GCCGGATGCTTCTCTCTTG, P1 CGGTCTCT CCCAGGACAGCAGCA; p21 73 L1 CGACTGGATGCTTCTCTTG, R1 TCTCT GGTGACAAAGTCTGAGAAG, P1 CATCCAGACGGCTTCTG; p19 70 L1 TCCGACATTGACAGAAG, R1 GCCATGGAGAGCATAGCTGAG, P1 TGGGACAGAAGCTGACACAGCA; GADDS5a 114 L1 GATAACAGGTCTGTTGCT, R1 GGAGTGCTGTGCGTCTTC, P1 CTGTCGTCTGCTC; MDM2 143 L1 TGCCAAGCTTCTCTGTGAAA, R1 TTTGATCAACCCACTTCTTCTAGC, P1 CCGGAGCTTGCCACAGCTAC, P1 TGGCGGACAGAAGCTGACACAGCA; GADDS5a 225 L1 GAAGTTGGAATGCTGGATTGCT, R1 GAAGATGTTGATGCGTCTTGGTC, PI CCGTCTGCTGCTC; TCCD 143 L1 TGGCAAGCTTCTCTGTGAAA, R1 TTGGTACACCCACTTCTTCTAGC, P1 CCGGAGCTTGCCACAGCTAC, P1 TGGCGGACAGAAGCTGACACAGCA.

Immunofluorescence staining

Cytospin samples were analyzed for PSTAT3 expression. Slides were fixed in PBS- 4% parafinoldehyde for 20 min and permeabilized with acetone/ methanol for 10 min. Slides were then washed in PBS and incubated with 1 μg/ml anti-phospho-Tyr705-STAT3 (B-7) mAb (Tebu-bio) in PBS-1% BSA for 2 h. Slides were washed in PBS then incubated with a donkey-anti-mouse R-PE conjugate (AbCys, Interchim) for 30 min and washed with PBS. Slides were then mounted with Vectashield anti-fading medium (AbCys). Fluorescent images were acquired using a charge-coupled device D701 camera (Olympus) connected to a Zeiss microscope and managed by a computer equipped with the Cell F software (Olympus).

Statistical analysis

Statistics were carried out using the two-tailed, nonparametric Wilcoxon tests using paired values.

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Table I. rIL-24 inhibits IL-2-induced thymidine incorporation in CLL B cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Medium</th>
<th>IL-2</th>
<th>IL-2 Indexb</th>
<th>IL-2 + IL-24c</th>
<th>% Inhibitiond</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98 ± 12.2</td>
<td>1,403 ± 20</td>
<td>17.3</td>
<td>859 ± 40</td>
<td>38.7</td>
</tr>
<tr>
<td>2</td>
<td>67 ± 14.8</td>
<td>1,288 ± 14.8</td>
<td>19.2</td>
<td>1,088 ± 87</td>
<td>15.5</td>
</tr>
<tr>
<td>3</td>
<td>38 ± 22</td>
<td>591 ± 93.6</td>
<td>15.5</td>
<td>456 ± 196.8</td>
<td>22.8</td>
</tr>
<tr>
<td>4</td>
<td>79 ± 22.6</td>
<td>1,042 ± 55.6</td>
<td>13.1</td>
<td>1,112 ± 28.5</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>91 ± 35</td>
<td>812 ± 12</td>
<td>8.9</td>
<td>553 ± 137</td>
<td>31.8</td>
</tr>
<tr>
<td>11</td>
<td>357 ± 58</td>
<td>11,050 ± 207</td>
<td>30.9</td>
<td>9,755 ± 415</td>
<td>11.7</td>
</tr>
<tr>
<td>12</td>
<td>98 ± 31</td>
<td>2,917 ± 274</td>
<td>30.3</td>
<td>1,915 ± 232</td>
<td>34.3</td>
</tr>
<tr>
<td>13</td>
<td>111 ± 18</td>
<td>4,160 ± 405</td>
<td>37.4</td>
<td>2,130 ± 112</td>
<td>48.8</td>
</tr>
<tr>
<td>14</td>
<td>113 ± 30</td>
<td>3,298 ± 102</td>
<td>29.2</td>
<td>2,165 ± 210</td>
<td>34.2</td>
</tr>
<tr>
<td>16</td>
<td>91 ± 11</td>
<td>17,154 ± 313</td>
<td>151</td>
<td>13,916 ± 2,474</td>
<td>18.8</td>
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<tr>
<td>17</td>
<td>209 ± 82</td>
<td>5,588 ± 709</td>
<td>26.7</td>
<td>4,246 ± 402</td>
<td>24.0</td>
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<tr>
<td>19</td>
<td>140 ± 12.6</td>
<td>2,225 ± 154</td>
<td>15.9</td>
<td>2,239 ± 136</td>
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</tr>
<tr>
<td>20</td>
<td>282 ± 57</td>
<td>27,020 ± 3,179</td>
<td>95.8</td>
<td>15,051 ± 4,169</td>
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</tr>
<tr>
<td>21</td>
<td>959 ± 220</td>
<td>78,283 ± 1,617</td>
<td>81.6</td>
<td>60,650 ± 143</td>
<td>22.5</td>
</tr>
<tr>
<td>29</td>
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<td>3,506 ± 1,124</td>
<td>48.0</td>
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<td>77.3</td>
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<tr>
<td>30</td>
<td>172 ± 32</td>
<td>35,308 ± 1,596</td>
<td>205</td>
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<tr>
<td>33e</td>
<td>153 ± 31</td>
<td>1,639 ± 266</td>
<td>10.7</td>
<td>1,576 ± 111</td>
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</tr>
<tr>
<td>33f</td>
<td>876 ± 265</td>
<td>32,487 ± 1,297</td>
<td>34.0</td>
<td>13,338 ± 996</td>
<td>59.0</td>
</tr>
<tr>
<td>Tonsil B</td>
<td>28,715 ± 4,978</td>
<td>36,221 ± 1,289</td>
<td>12.2</td>
<td>1,403 ± 20</td>
<td>38.7</td>
</tr>
<tr>
<td>Blood B</td>
<td>110 ± 30</td>
<td>294 ± 47</td>
<td>306 ± 194</td>
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<td></td>
</tr>
</tbody>
</table>

a Thymidine incorporation in cpm/10^6 cells after 3 days of culture of B cells from CLL patients, blood donors (n = 3) or pediatric tonsils (n = 3).
b Ratio of IL-2/medium counts.
c Cells were cultured overnight with IL-2, then with IL-24 for 3 more days.
d Percentage inhibition was calculated as cpm from IL-2 plus IL-24 cultures divided by cpm for IL-2 cultures.

Results

rIL-24 inhibits thymidine incorporation by activated CLL B lymphocytes

The effect of rIL-24 on CLL cell proliferation was investigated. Peripheral blood samples were collected from 33 patients with CLL with >20,000 lymphocytes/mm² and B cells were cultured for 3 days in medium or medium plus IL-2, with or without IL-24; then, [3H]-thymidine incorporation was tested. Spontaneous apoptosis was negligible in all samples cultured for 3 days (<2%) and therefore did not affect our results. As expected, most cell samples (22/33) proliferated in response to 50 ng/ml of IL-2 alone (18, 19), albeit to various degrees. The range of the IL-2 thymidine incorporation index was from 3 to 200 (Table I shows the results for B cells from 17 patients: proliferation as assessed from thymidine incorporation was 8.9–205 times that of cells cultured in medium alone). In contrast to IL-2, rIL-24 alone had no significant effect on cell proliferation, even when used at a dose of 300 ng/ml (data not shown). However, 20–100 ng/ml of IL-24 partially reversed the IL-2-induced proliferation in 14 of 17 samples: the inhibition was between 11.7% (patient 11) and 77.3% (patient 29) ([3H]-thymidine incorporation (cpm): IL-2 = 12,770; IL-2 + IL-24 = 8,868; p = 0.0004). Interestingly, cells from patient 33 underwent a Richter transformation, evolving from a CLL (33e) toward a large cell lymphoma (33f). After transformation, the cells retained strong CD5 expression and their protein phosphorylation profile on tyrosine was much more extensive than that in cells from the CLL stage (data not shown). Cells from both CLL and lymphoma stages were thawed and cultured in parallel in the same experiment. Lymphoma cells proliferated much faster than CLL cells both in medium alone and in the presence of IL-2. IL-24 inhibited both the spontaneous (data not shown) and IL-2-induced cell growth of lymphoma cells by 59% but had no effect on CLL cells (Table I). We tested combinations of IL-2 and IL-24 on six samples of B cells from tonsils or normal adult peripheral blood in parallel, and IL-24 did not exert any inhibitory activity on normal B cells.

We also tested the effect of IL-24, added to the cultures at various times before or after addition of IL-2 (Fig. 1a). The inhibitory

FIGURE 1. rIL-24 inhibits thymidine incorporation in activated CLL cells. a. IL-2 was added to cells cultured with IL-2 1 day before (D-1), at the same time (D0), or one day after (D1) IL-2. Counts of cells cultured with IL-2 were compared with those with IL-2 plus IL-24. b. IL-2 was added to CLL cells cultured for 24 h with medium alone, IL-2, IL-2 plus CD40 ligand, or anti-IgM plus CD40 ligand. For each condition, counts of cells cultured with IL-2 were compared with those without IL-24. Statistics: two-tailed Wilcoxon’s test. Thymidine (0.5 µCi/well) was added at day 3 for 15 h (n = 5–8 experiments).
and IL-24 inhibits thymidine incorporation, see above) and as-
stimulated them or not with IL-24 (sequential incubation with IL-2

tioned in normal lymphocytes and lymphoid cell lines. 

tion of JAK/STAT proteins in CLL cells in the same way as de-
ted in Fig. 2).

Thus, IL-2 signaling stimulated the phosphoryla-
tion following incubation with IL-2 and IL-24 plus IL-24 as above; proteins were extracted and submitted to Western blotting as in b.

effect of IL-24 was greater when IL-24 was added 1 day after IL-2

(\(p = 0.032\)) than when added at the same time (\(p = 0.041\)). IL-24 added to the culture 1 day before IL-2 had no inhibitory effect

(\(p = 0.687\)).

IL-24 also has an inhibitory effect on cells stimulated with either IL-2 plus CD40 ligand or anti-IgM plus CD40 ligand (Fig. 1b). This effect was similar to that on cells stimulated with IL-2 alone

(\(p = 0.01\) for all conditions). These various observations sug-
gested that IL-24 acts mainly on stimulated/proliferating cells but

not on resting cells.

**IL-24 suppresses IL-2-induced STAT3 phosphorylation on tyrosine through a phosphatase-dependent mechanism**

We studied the effects of IL-24 on IL-2 signaling in CLL cells. The IL-2 signaling pathway starts with its interaction with IL-2R, which is then phosphorylated; this triggers the phosphorylation of JAK1, which associates with the IL-2R\(\beta\) chain, and JAK3, which associates with the common \(\gamma\) chain; this results in activation of STAT5 and STAT3 (20–22). Protein extracts from CLL cells incubated with IL-2 were immunoprecipitated with anti-phosphotyrosine 4G10 Ab, and Western blots were performed with mAbs specific for proteins of the JAK/STAT pathway (Fig. 2a).

IL-2 induced a rapid phosphorylation (within 2 min, peaking at 10 min) of tyrosine residues in JAK1, STAT3, and STAT5 proteins (Fig. 2a). Thus, IL-2 signaling stimulated the phosphoryla-
tion of JAK/STAT proteins in CLL cells in the same way as de-
scribed in normal lymphocytes and lymphoid cell lines.

We cultured CLL B cells overnight with or without IL-2 and stimulated them or not with IL-24 (sequential incubation with IL-2 and IL-24 inhibits thymidine incorporation, see above) and as-

sessed STAT3 phosphorylation on tyrosine with anti-PY705-STAT3. STAT3 was extensively phosphorylated in cells incubated overnight with IL-2 (evidence of sustained activity) but not in cells stimulated with IL-24 alone (Fig. 2b). In contrast, the presence of IL-24 inhibited IL-2-induced phosphorylation of STAT3. The total amount of STAT3 was similar under all con-

ditions (Fig. 2b). The above results are shown on cells from five different patients. However, IL-24 has been reported to induce the phosphorylation of STAT3 in non-B cells (27), and this is inconsistent with our observations. We therefore investigated its action on the BW5147 thymoma cell line transfected with genes encoding an IL-24R1 heterodimer consisting of both IL-

20R\(\alpha\) and IL-20R\(\beta\) chains. We confirmed IL-24R expression in this cell line by immunofluorescence (data not shown). IL-24R-

transfected cells displayed a strong STAT3 phosphorylation in the presence of IL-24 but did not respond to IL-2, whereas the untransfected control cell line did not respond to either IL-2 or IL-24 (Fig. 2c, left). Control experiments with purified tonsil B cells were performed in parallel and failed to show any effect of IL-24 on STAT3 (Fig. 2c, right). These experiments demonstrated the specific effect of IL-24 on CLL cells and validated the activities of recombinant cytokines used.

We studied the kinetics and dose response of STAT3 phosphory-
ylation following incubation with IL-2 and IL-24. STAT3 phos-
phorylation was inhibited by IL-24 doses as low as 10 ng/ml of

IL-24 (Fig. 3b), and inhibition was rapid (2 min) (Fig. 3a). In

![FIGURE 2.](http://www.jimmunol.org/) IL-24 inhibits the phosphorylation of STAT3 on tyrosine in IL-2-stimulated CLL cells. a, Kinetics of IL-2 signaling. Cells (\(10^6\)) were cultured with rIL-2 for the indicated time, lysed, and immunoprecipitated with anti-phosphotyrosine 4G10 Ab (Ipp pTyr, left) or not (total lysate, right) and submitted to Western blotting with the Abs specific for STAT3, STAT5, and JAK1. b, IL-24 inhibits IL-2-induced PSTAT3: CLL cells from five patients were cultured with or without IL-2 overnight and stimulated or not with 100 ng/ml IL-24 for 10 min. Cells were then submitted to Western blotting with anti-PY705-STAT3, dehybridized, and blotted with anti-STAT3. c, Cells from the BW cell line transfected with empty vector or with vector carrying genes encoding the IL-24R1 heterodimer (IL-20R1 + IL-20R2 chains) (left) or B cells from tonsils (right) were incubated as in b with combinations of IL-2, IL-24, and IL-2 plus IL-24 as above; proteins were extracted and submitted to Western blotting as in b.

![FIGURE 3.](http://www.jimmunol.org/) Kinetics and specific activity of IL-24 on CLL cells. a, Right, CLL cells were cultured overnight with IL-2, and IL-24 (100 ng/ml) was added or not thereafter for 10 min; proteins were extracted, immunoprecipitated with 4G10 Ab or control mouse IgG, and submitted to Western blotting with anti-STAT3. Top left, cells were cultured overnight with IL-2 and then stimulated with IL-2 (100 ng/ml) for the indicated time, and proteins were submitted to Western blotting and probed with anti-PSTAT3, anti-STAT3, and anti-

STAT5. Bottom left, Proteins were immunoprecipitated with 4G10 Ab and probed with anti-STAT5. b, Left, CLL cells were cultured overnight with IL-2 and indicated concentrations of IL-24 were added thereafter. Right, Cells cultured overnight with IL-2 were then stimulated with IL-24 (10 ng/ml), IL-24 plus anti-IL-2, or control goat IgG, or not stimulated. Protein extracts were probed with anti-PSTAT3 and with anti-STAT3 Ab.

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were incubated or not for 5 min with and without 25 PSTAT3 to another cell compartment. To test this possibility cells phorylation of tyrosine in STAT3 rather than the translocation of (22). In parallel, we confirmed the phosphorylation of PSTAT3 became undetectable presumably due to dimerization and/or engagement of a tyrosine-phosphatase activity. reverses the inhibition by IL-24 of STAT3 phosphorylation. Left. Cells were cultured in medium alone or with IL-2 overnight, and IL-24 was added after 10 min and the cells were lysed and blotted with anti PY705-STAT3 and with anti-STAT3. Right. Cells cultured in the same conditions were supplemented with 25 μM Na-pervanadate for 5 min. The data are representative of four experiments.

FIGURE 4. IL-24 stimulates PSTAT3 dephosphorylation in IL-2-stimulated CLL cells. a. CLL cells were cultured overnight with medium alone or with IL-2. IL-24 was then added and cells were cytopsin 30 min later. Cells were fixed and stained with anti-PSTAT3 Ab plus donkey anti-mouse-PE Ab (top) or with secondary Ab alone (bottom), b. Pervanadate, an inhibitor of tyrosine-phosphatase activity, reverses the inhibition by IL-24 of STAT3 phosphorylation. Left. Cells were cultured in medium alone or with IL-2 overnight, and IL-24 was added after 10 min and the cells were lysed and blotted with anti PY705-STAT3 and with anti-STAT3. Right. Cells cultured in the same conditions were supplemented with 25 μM Na-pervanadate for 5 min. The data are representative of four experiments.

contrast to STAT3, the amounts of STAT5 in the cytosol of cells incubated overnight with IL-2 declined (Fig. 3a, lower panel) and PSTAT5 became undetectable presumably due to dimerization and translocation into the nucleus (22). In parallel, we confirmed the specificity of rIL-24 activity on IL-2-stimulated CLL cells by neutralization experiments using a polyclonal anti-human IL-24 Ab (Fig. 3b, right).

Thus, IL-24 stimulated the dephosphorylation of PSTAT3 in CLL cells that had been prestimulated with IL-2. We used immunostaining experiments to confirm this finding. Cells were incubated overnight with or without IL-2, and IL-24 was then added for 30 min. The cells were isolated from the medium by centrifugation and stained with anti-PSTAT3 Ab. PSTAT3 was much more abundant in IL-2-cultured cells than in nonstimulated cells, and its abundance declined rapidly in the presence of IL-24; it did not relocate to the nucleus (Fig. 4a). This suggested active dephosphorylation of tyrosine in STAT3 rather than the translocation of PSTAT3 to another cell compartment. To test this possibility cells were incubated or not for 5 min with and without 25 μM pervanadate, an inhibitor of protein tyrosine phosphatase activity (23), and were then stimulated for another 10 min with IL-24. Proteins were then extracted and analyzed by Western blotting. A small amount of STAT3 in untreated CLL is naturally phosphorylated on tyrosine (Fig. 4b). As expected STAT3 was phosphorylated in the presence of IL-2 and dephosphorylated by the addition of IL-24. A short incubation with pervanadate (Fig. 4b, right panel) antagonized the inhibition by IL-24 of STAT3 phosphorylation in IL-2-stimulated cells (Fig. 4b).

Therefore, the effect of IL-24 on CLL is mediated by the induction and/or engagement of a tyrosine-phosphatase activity.

IL-24 induces the apoptosis of B cells engaged into the cell cycle

We next determined whether the inhibition of thymidine incorporation into B cells reflected cell cycle block and/or apoptosis. CLL B cells were synchronized with cold thymidine for 24 h and cultured with or without IL-2, then washed and stimulated or not with IL-24 for an additional 24 h; they were then fixed and stained thereafter with PI and subjected to FACS analysis. The proportion of hypodiploid cells as assessed by the analysis of the cell cycle was very similar to that estimated by staining with annexin V (data not shown). Hypodiploid (apoptotic) cells, cells in G2/M phases of the cell cycle, and hyperdiploid cells were counted (M1 to M4 populations, respectively, in Fig. 5a and b). The number of cycling (S + G2/M + hyperdiploid) cells increased 4-fold under IL-2, whereas IL-24 alone had no significant effect (see the one representative sample in Fig. 5a). In contrast, the number of (S + G2/M + hyperdiploid) cells was reduced by more than half under IL-2 plus IL-24. A parallel increase in hypodiploid cells was observed under this condition (Fig. 5a). In this figure, we show all ungated cells. The percentage of cell doublets was estimated in eight experiments to be 4.4 ± 1.5, and these doublets localized mostly in the hyperdiploid population.

The mean ± SD percentage of cells in each phase of the cell cycle was determined (Fig. 5b). Analysis of 24 samples indicated that the most significant inhibitory effect of IL-24 in IL-2-cultured cells vs IL-2 alone was on the G2/M population (7.34 ± 2.30% vs 4.01 ± 1.4%), whereas its effect was lower in hyperdiploid cells (5.42 ± 2.85% vs 2.71 ± 1.58%). IL-24 significantly increased the proportion of the cell population that was hypodiploid (4.0 ± 1.7% vs 6.79 ± 2.8%) but increased only slightly that in G0/G1 (83.4 ± 5.3 vs 87.2 ± 5.3%) (Fig. 5b).

These results suggested that IL-24 induced apoptosis at the expense of proliferating cells. To verify this, we incubated cells with CFSE, with IL-2 and with or without IL-24; the cells were then stained with Apo2.7 mAb after 2 days. Apoptosis was evidenced in 27.7% cells that had been stimulated with IL-2 plus IL-24 and only in those that had undergone one division (CFSElow cells) but not in those that did not divide (CFSEhigh cells) (Fig. 5c). In contrast, the proportion of cells cultured with IL-2 alone and that had divided and underwent apoptosis was much lower (10.5%) (Fig. 5c). The percentage of annexin V-positive, PI-negative apoptotic cells was also higher under IL-2 plus IL-24 (20%) than under IL-2 (8%) (see the representative sample in Fig. 5c).

IL-24 induces apoptosis through stabilization/enhancement of p53 expression

Oncogenic pathways that signal through STAT3 inhibit p53 expression (24, 25). STAT3 is a latent transcription factor activated by several growth factors and cytokines (26), including cytokines of the γc family, which cause its phosphorylation on tyrosine and translocation into the nucleus. Work with nonlymphoid cells shows that several members of the class II family of cytokines, which includes IL-24 (2, 27, 28), activate STAT3; also, IL-24-mediated apoptosis was reported to be independent of STAT3 in several nonlymphoid cell lines (17). However, our results demonstrate that IL-24 abolishes IL-2 signaling in CLL.

To investigate the mechanisms of IL-24-mediated apoptosis, we incubated CLL cells with various combinations of IL-2, IL-24, zvd (a caspase inhibitor), and pifithrin (pi)-α, an inhibitor of p53 transcription (29). Cell cycle analysis was performed using cells stained with PI. The number of cells in the G2/M phase of the cell cycle was, under IL-2 plus IL-24, half that
FIGURE 5. rIL-24 induces apoptosis and decreases the population of CLL cells engaged into the S/G2/M phase of the cell cycle. 

a. CLL cells were synchronized with cold thymidine, cultured in medium only or medium supplemented with IL-24, IL-2, or IL-2 then IL-24, and then fixed and stained with PI. M1, hypodiploid cells (dead); M2, cells in G0/G1; M3, cells in S + G2/M; M4, hyperdiploid cells. The increase in the M3 population under IL-2, the decrease of this population, and the increase in the M1 population under IL-2 plus IL-24 are shown.

b. Analysis of the cell cycle. Mean ± SD (n = 8) of the M1 to M4 populations calculated as in a from CLL cells cultured as indicated. In each population, IL-2 vs IL-2 plus IL-24 conditions were compared using Wilcoxon’s test.

c. Top, Cells were incubated in CFSE, stimulated with IL-2 or IL-2 plus IL-24 and stained with Apo2.7 Ab. Bottom, Cells cultured for 3 days with IL-2 or IL-2 plus IL-24 were stained with annexin V-FITC and PI; the histogram shows the population gated on annexin V plus PI cells.
under IL-2 (3.36 ± 1.3% vs 7.1 ± 1.5%). Pft-α almost totally reversed the loss of cells from the S/G2/M phases induced by IL-24 (6.44 ± 2.2% in the presence of pft-α vs 3.36 ± 1.3% in its absence) (Fig. 6, a and b). The effect of IL-24 on the proportion of cells in the S/G2/M phase was also reduced albeit to a lesser extent by zvad (IL-2 + IL-24 + zvad: 4.8 ± 1.77%) (Fig. 6, a and b). These observations suggest that cells driven to proliferation by IL-2 undergo a p53-mediated block of the cell cycle followed by (at least partly) caspase-dependent apoptosis.

The phosphatase activator troglitazone induces p53 expression, STAT3 dephosphorylation, and apoptosis of CLL cells

We showed that IL-24-induced apoptosis in CLL is dependent on p53 expression (see above). As PSTAT3 is a repressor of p53 transcription, IL-24-induced apoptosis in CLL is presumably also dependent on PSTAT3 dephosphorylation. Therefore, a phosphatase activator may reproduce some of the effects of IL-24.

Troglitazone (TG) is an activator of protein tyrosine phosphatase 1B (PTP1B); it reduces PY705-STAT3 and promotes apoptosis in human primary gliomas (34). We tested whether TG stimulated p53 expression and apoptosis in CLL. TG induced dose-dependent dephosphorylation of PSTAT3 in CLL cells cultured in the presence of IL-2. It also induced p53 production, but had no effect on total STAT3 abundance (Fig. 7a). Cell cycle

FIGURE 6. The decrease in the S/G2/M population in IL-2 plus IL-24-cultured cells is dependent on IL-24-mediated induction of p53 expression. a and b, The pharmacological inhibitor of p53, pft-α, reverses the IL-24-mediated decrease of cells engaged into cell cycle. Cells were cultured overnight with medium or IL-2 and then supplemented with pft-α (40 μM), zvad-fmk (zvad) (10 μg/ml), IL-24, IL-24 plus pft-α, IL-24 plus zvad, or IL-24 plus anti-IL-24 for 24 h and stained with PI for cell cycle analysis. a, A representative experiment from one patient with CLL is shown. b, Percentage of CLL cells in the S/G2/M phases of the cell cycle (open histograms) or hypodiploid cells (filled histograms). Means ± SD for cells from eight different patients. For black-and-white histograms, comparisons were made between IL-2 plus IL-24 vs IL-2 and between IL-2 plus IL-24 and IL-2 plus IL-24 plus either pft-α, zvad, or anti-IL-24. Only significant results (p < 0.03) using Wilcoxon’s test are indicated (*). c, IL-24 augments p53 expression and phosphorylation in CLL. Cells were lysed after 3 days of culture as indicated and analyzed by Western blotting with anti-p53, anti-Pser15-p53, anti-Bcl-2, and anti-p38 MAP kinase Abs. d, Relative gene expression in CLL cells cultured for 48 h with medium only and medium containing IL-24, IL-2, and IL-2 plus IL-24 (histograms in that order). RNA was extracted on day 3, reverse-transcribed, amplified, and analyzed by real-time PCR. Means ± SD samples for samples from four to six different patients are shown. Values for medium- vs IL-2 plus IL-24-treated samples were compared using Wilcoxon’s test.
We report the first description of the conditions under which IL-24 exerts a proapoptotic effect on CLL as well as the associated molecular events. The effect is dose dependent and reversed at both cellular and molecular levels by anti-IL-24 Abs; it is therefore specific. CLL cells contain abundant IL-24 mRNA and protein (14), and this led us to study the effect of exogenous IL-24 on these cells. Our findings appeared not to be entirely coherent with the published (5–7) apoptotic effect of adenovirus-encoded IL-24. This effect was described as being independent of both IL-24R and the JAK/STAT pathway (17). However, we found that rIL-24 is proapoptotic, provided that CLL cells are stimulated to enter the cell cycle, and this reconciles the various diverse observations. IL-24 expression inversely correlates with the proliferation of malignant melanocytes (4, 5), which is consistent with IL-24 having protective and growth inhibition activities in terminally differentiated cells, which is the case in CLL cells (13–16). However, intracellular IL-24 did not apparently counteract the apoptotic effect of rIL-24 in IL-2-stimulated CLL cells. To explain this, we observed that IL-24 transcripts are rapidly down-regulated in activated B cells (unpublished data). We think therefore that once cells have received a proliferation signal (here by IL-2), intracellular IL-24 is down-regulated; this allows cells to enter the cell cycle. If cells are exposed shortly after a “stop signal” (here by rIL-24), they undergo apoptosis (this is also consistent with the role of p53, see below).

Note that the effects of IL-24 on B cells are similar to those of IL-4. IL-4 exerts an inhibitory effect after B cells receive an activation signal, from, for example, IL-2 (35, 36). However, IL-4 and IL-24 may inhibit B cell proliferation by different molecular mechanisms, as their respective expressions are inversely correlated. We indeed observed that IL-4 is a potent inhibitor of both CD5 and IL-24 expression in B cells (our unpublished results), which is also consistent with CD5 being an IL-24 inducer (14).

CLL cell death was diminished by zvad-fmk, and thus caspases are involved to some extent and need to be studied more in detail. In contrast, cell death was abolished by a pharmacological inhibitor of p53, and therefore the mechanism involves p53 induction.

p53 is a major transcription factor that potently inhibits cell cycle progression at G1/S and G2/M checkpoints (37, 38). Its expression and function are impaired in many tumors (39, 40) due to down-regulation or inactivation by mutations. Restoring endogenous p53 induced regression of murine liver carcinomas in vivo. The mechanism did not involve apoptosis but instead involved induction of a senescence program associated with the production of M-CSF, CCL2, CXCL1, and IL-15 (41). Note that IL-24 induces the production by human PBMC of inflammatory cytokines, including TNF-α, GM-CSF, IL-6, IL-12, and IFN-γ (42). Restoration of p53 function in vivo can lead to tumor regression through induction of cellular senescence in sarcomas and apoptosis in lymphomas (43). These studies pave the way for the use of p53 activators to treat human cancers. Here, however, we could not reverse death completely with zvad-fmk, so both senescence and apoptosis may be at work in CLL. The patterns of regulation of downstream genes indicate that p53 accumulates in cells following stress signals that stop its degradation and/or enhance its transcription and its activity as a transcription factor (Fig. 6). IL-24 may act as a stress signal that turns off the IL-2-signaling cascade and induces p53 expression, probably by inactivating the p53 transcriptional repressor PSTAT3. Other mechanisms may also be operating through the stabilization of p53 and/or inhibition of its degradation through the phosphorylation of Ser15 in p53 by any of at least eight

Analysis (Fig. 7b) and annexin V staining and cell counting (data not shown) showed that TG treatment led to the death of IL-2-stimulated cells. Note that IL-24 killed cells in S/G2/M phase but not those in G0/G1 phase of the cell cycle, whereas TG mainly affected cells in the G0/G1 phase (Fig. 7b, left). Thus, although there were some differences, TG had effects similar to those of IL-24, consistent with our observation that the inhibition of PSTAT3 is a general mechanism leading to enhanced p53 expression and cell death of proliferating cells.

**FIGURE 7.** Effect of TG on dephosphorylation of PSTAT3, p53 induction, and cell death in CLL. *a*, TG inhibits the phosphorylation of STAT3 and augments p53 expression in IL-2-cultured CLL cells. Cells cultured with IL-2 and the indicated concentrations of TG were lysed and probed by Western blotting with Abs specific for PY705-STAT3, total STAT3, p53 and Bcl-2. *b*, The same cells as in *a* were analyzed for progression through the cell cycle. *Left*, Analysis of the population in G1 gated on living populations. *Right*, Analysis of the cells in G2/M phase (M2) (n = 5).
different Ser/Thr kinases (30), including p38 MAPK, which is activated by IL-24 (14). However, the complexity of p53 regulation by posttranslational mechanisms is such that dedicated analysis is required to understand the effects of IL-24 more fully. Our experiments support the role of PSTAT3 dephosphorylation on p53 de-repression and downstream induction of cell death. TG, an anti-angiogenic drug and a PPAR-γ agonist (34) that activates the tyrosine phosphatase PTP1B, which then dephosphorylates PSTAT3, augmented p53 expression and apoptosis in IL-2-stimulated CLL cells, thereby mimicking the effect of IL-24. Inhibiting PSTAT3 to enhance p53 expression, which in the context of proliferating cells is sensed as a danger signal, may be a general mechanism operating in both normal and malignant cells. This mechanism requires p53 with no inactivating mutation or deletion that is active as a transcription factor. This is indeed the case in most CLLs (44, 45) and in cancers, as p53 mutations generally occur late in tumor progression (46, 47). Our results and working hypothesis are summarized in Fig. 8.

IL-24 is expressed in keratinocytes (48) and in inflamed skin (49). Its physiological function, if any, on lymphocytes remains obscure despite IL-24 being expressed by human PBMC and having pro-TH1 activity (42). Further work is required to determine which receptor(s) of IL-24, and phosphatases mediated its effects. In physiological conditions, SHP-1 and SHP-2, are recruited to the IL-2Rβ and dephosphorylate proteins of the JAK/STAT family, thereby terminating the IL-2-induced signal (50, 51). We indeed observed that SHP-1 is present in huge amounts in CLL (data not shown). Our molecular studies were limited to the action of IL-24 on IL-2-stimulated cells, although IL-24 inhibits cells stimulated with anti-IgM plus CD40 ligand (Fig. 1). In contrast, IL-24 was inactive on cells stimulated with anti-IgM only probably due to the poor response of CLL cells to anti-IgM alone. In conclusion, sequential activation of cells with a growth factor to trigger the proliferation followed by PSTAT3 inhibition and restoration of p53 expression by IL-24 may be useful to treat CLL and other cancers (52, 53).

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References


