Differential Effects of Interleukin-2 and Interleukin-15 versus Interleukin-21 on CD4+ Cutaneous T-Cell Lymphoma Cells

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Abstract

In this study, we compared the effects of interleukin-2 (IL-2), IL-15, and IL-21 on gene expression, activation of cell signaling pathways, and functional properties of cells derived from CD4+ cutaneous T-cell lymphoma (CTCL). Whereas both IL-2 and IL-15 modulated, in a CTCL cell line, the expression of >1,000 gene transcripts by at least 2-fold, IL-21 up-regulated <40 genes. All three cytokines induced tyrosine phosphorylation of Jak1 and Jak3 in CTCL cell lines and native leukemic (Sezary) cells. However, only IL-2 and IL-15 strongly activated signal transducers and activators of transcription 5, phosphoinositide 3-kinase/Akt, and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK signaling pathways in the cell lines and mitogen-primed native cells. In contrast, IL-21 selectively activated signal transducers and activators of transcription 3. Whereas all three cytokines protected CTCL cells from apoptosis, only IL-2 and IL-15 promoted their proliferation. The effects of the cytokine stimulation were Jak3 kinase– and Jak1 kinase– dependent. These findings document the vastly different effect of IL-2 and IL-15 versus IL-21 on CTCL cells. They also suggest two novel therapeutic approaches to CTCL and, possibly, other CD4+ T-cell lymphomas: inhibition of the Jak1/Jak3 kinase complex and, given the known strong immunostimulatory properties of IL-21 on CD8+ T, natural killer, and B cells, application of this cytokine to boost an immune response against malignant CD4+ T cells. [Cancer Res 2008;68(4):1083–91]

Introduction

Among the several cytokines that signal through the receptors that share the common γ chain (γc), interleukin-2 (IL-2) is by far the best characterized. It signals through the receptor that, in addition to the γc chain, contains β chain, the second signal-transducing component and, in the case of high-affinity receptors, also the IL-2–specific nonsignaling α chain. In normal immune cells, IL-2 has been shown to activate Jak/signal transducers and activators of transcription (STAT) signaling, phosphoinositide 3-kinase (PI3K)/Akt, and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling pathways (1). IL-15 shares with IL-2 signaling via receptor with both γc and β chains and, similar to IL-2, also uses the third, IL-15–specific, nontransducing α chain. Consequently, IL-2 and IL-15 share a number of properties, including stimulation of T-cell, natural killer (NK)–cell, and B-cell proliferation and functional maturation, but certain features unique to each of these cytokines have also been described (1, 2). IL-21 is also quite pleiotropic and displays a spectrum of effects on immune cells (3), with its ability to increase cytotoxicity of both NK (4) and CD8+ T cells (5) being the best defined. The IL-21 receptor has, in addition to the γc, its own distinct signal-transducing α chain. All three cytokines activate Jak1 and Jak3 kinases that phosphorylate and, hence, activate the respective cytokine receptors, as well as the signaling proteins that dock to the activated receptors.

T-cell lymphomas represent a heterogeneous group of biologically and clinically distinct lymphoproliferative disorders with the majority derived from the CD4+ helper/inducer T-cell subset. Primary cutaneous T-cell lymphomas (CTCL) represent the most frequently occurring type (6, 7); they display a tendency to progress over time to the more advanced forms including development of peripheral blood involvement, a leukemic phase also called Sezary syndrome. The current study was aimed at comparing the effects of IL-2, IL-15, and IL-21 on the CTCL cells with regard to modulation of the gene expression, activation of signaling pathways, and cell function. We have used in the study two CTCL-derived, IL-2–dependent cell lines, Sez-4 (8) and SeAx (9), as well as the primary leukemic (Sezary) CTCL cells.

Materials and Methods

CTCL cell lines and patients and healthy donor CD4+ T cells. IL-2–dependent T-cell lines Sez-4 and SeAx were derived from CTCL patients (8, 9). The cell lines were cultured at 37°C and 5% CO_2 in the presence of 200 units of IL-2 in standard RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sez-4) or 10% heat-inactivated human serum (SeAx), 1% penicillin/streptomycin/fungizone mixture, and 2 mmol/L L-glutamine. Peripheral blood mononuclear cells from patients and healthy donors were obtained after Ficoll density-gradient centrifugation from heparinized blood samples. The CTCL patients were diagnosed as having peripheral blood involvement (leukemic phase/Sezary syndrome) on the basis of clinical, histopathologic, and immunophenotypic criteria. The leukemic CTCL cell populations of the four patients analyzed were >90% pure as determined by flow cytometry analysis using the CD4/CD8 ratio and CD7 and/or CD26 loss by the CD4+ T-cell population as the criteria. The control, normal CD4+ T cells were purified from the mononuclear cell population by negative purification using CD4+ T-cell isolation kit (Miltenyi Biotec). The kit permitted removal of the non-CD4+ T lymphocytes by applying a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD20, CD36, CD43, and CD235a (glycophorin A) and avidin-coated magnetic microbeads. To obtain primed cells, leukemic CTCL cells were cultured for 7 days in the presence of a mitogen phytohemagglutinin

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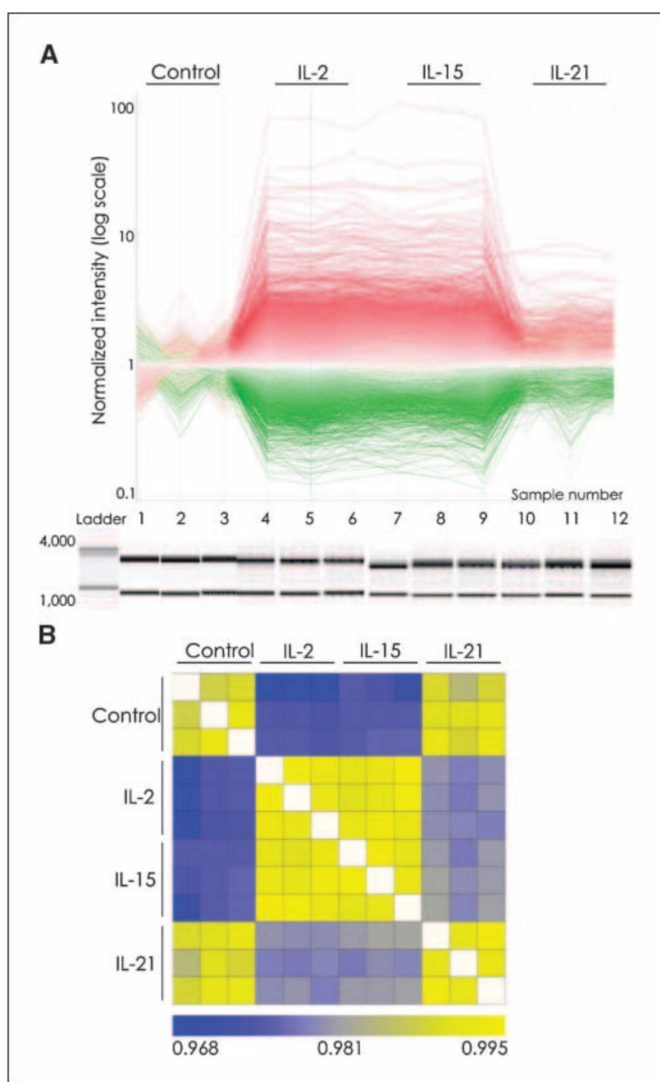

Figure 1. Expression profile of the genes induced by the γc-signaling cytokines in CTCL cell line. CTCL-derived, IL-2–dependent Sez-4 cell line was deprived of IL-2 for 16 h and cultured for 4 h in triplicate with medium or pretested saturating doses of IL-2 (200 units), IL-15 (20 ng/mL), or IL-21 (100 ng/mL). After cell harvest and total RNA isolation, the samples were checked for RNA quality, reverse-transcribed to cDNA with simultaneous labeling, and hybridized to Affymetrix HG-U133 Plus 2 arrays that contain DNA probes corresponding to >47,000 gene coding sequences. The results were processed in GeneSpring and further analyzed using Partek Genomics Suite, Spotfire, and GeneSpring programs. *A,* gene expression pattern in the triplicate samples stimulated with the depicted cytokines or medium alone (*top*). *Bottom,* RNA quality control of the samples. *B,* intensity plot of the gene expression data correlations. Values were calculated using Pearson correlation for a prefiltered set of transcripts. The color scale represents the range of the pair-wise correlations among the examined samples.

(PHA-L; Sigma) used at 5μg/mL. Donation of blood by patients and healthy donors was conformed to the University of Pennsylvania Institutional Review Board–approved protocols, and informed consent was obtained.

Cytokines. Recombinant human rhIL-2 (Bender MedSystems) was used at the dose of 200 units, rhIL-15 (R&D Systems) at the dose of 20 ng/mL, and rhIL-21 (Biosource) at 100 ng/mL.

Kinase inhibitors. Pan-Jak (Jak I; Calbiochem) is a quinolin derivative with the structure 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[*h*]-imidaz[4,5-*f*] isoquinolin-7-one. It inhibits enzymatic function of all four members of the Jak family with the IC_{50} of 15 nmol/L for Jak1, 1 nmol/L for Jak2, 5 nmol/L for Jak3, and 1 nmol/L for Tyk2 in *in vitro* kinase activity inhibition assay (10). Jak3-specific inhibitor was synthesized according to the published structure (11) and displays the *in vitro* IC_{50} kinase activity inhibition of Jak3 at 2 nmol/L for Jak3, 20 nmol/L for Jak2, and 100 nmol/L for Jak1.

Gene expression analysis. Sez-4 cell line was starved of IL-2 for 16 h, washed twice, and placed into six-well plates in 10 mL RPMI (10% FBS) for 2 h followed by addition of IL-2 (200 units), IL-15 (20 ng/mL), or IL-21 (100 ng/mL) or medium alone for 4 h. In the Jak-signaling inhibition experiments, IL-2–starved Sez-4 cells were pretreated for 30 min with pan-Jak (300 nmol/L), Jak3 (300 nmol/L), or drug solvent and cultured with IL-2 (200 units) or medium alone for 4 h. All experimental groups were examined in triplicates. Cells were harvested, and total RNA was isolated using a TRIZOL and Qiagen RNeasy protocol from the Penn Microarray Facility. RNA integrity and quality were assessed on an Agilent 2100 Bioanalyzer (Agilent). The mRNA was reverse-transcribed using a poly(t)-T7 promoter primer, amplified in a linear fashion by *in vitro* transcription with biotin labeling and hybridized to the U133 Plus 2.0 array chips that contain >54,000 probe sets covering >47,000 transcripts. The chips were washed, stained, and scanned according to the manufacturer's instructions. Normalized expression values were calculated using GCRMA (GeneSpring GX 7.3.1, Agilent). Present/absent/marginal flag calls were calculated using the MAS5 algorithm (GeneChip Operating Software, Affymetrix) and all MAS5 quality control variables were within reference ranges. Genes that appeared with a present flag in all three samples of at least one condition in the experiment were taken for further analysis. Principal component analysis was used to assess variability of data. Differentially expressed genes were identified using ANOVA. False discovery rate test correction was performed using Benjamini-Hochberg step-up method (Partek Genomics Suite 6.2, Partek, Inc.). For further analysis, we used gene lists that are estimated to have 1% false positive genes (false discovery rate, 0.01).

Western blot. The cells were washed briefly in PBS, centrifuged, and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA] supplemented with 0.5 mmol/L phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktails I and II from Sigma and protease inhibitor cocktail from Roche, according to the manufacturer's specifications. For normalization of the gel loading, the protein extracts were assayed with Lowry method (Bio-Rad Dc protein assay). Typically, 5 to 50 mg of the protein per lane was loaded. To examine protein phosphorylation, the membranes were incubated with the antibodies specific for Jak1 Y1022/1023, STAT3 Y705, STAT5 Y694, Akt Thr308, ERK1/2 T202/Y204 (all from Cell Signaling). 4G10 (Santa Cruz Biotechnology) mouse monoclonal antibody was used to detect total tyrosine phosphorylation. To detect total protein, we used anti-Jak1 (Cell Signaling), anti-Jak3 (Santa Cruz), and anti-actin (Santa Cruz) antibodies. The membranes were incubated with the appropriate secondary peroxidase-conjugated antibodies. The blots were developed using the enhanced chemiluminescence (ECL) plus Western blotting detection system from Amersham.

Immunoprecipitation. Cells were lysed with lysis buffer that contained protease and phosphatase inhibitors. Next, immunoprecipitation with the Jak3 rabbit polyclonal antibody (Santa Cruz) and 20 μL protein G agarose (Life Technologies-Bethesda Research Laboratories) was performed for 1 h at 4°C. Immunoprecipitates were washed, boiled, suspended in reducing SDS loading buffer, separated on an 11% polyacrylamide/SDS gel, and transferred electrophoretically to hybridization transfer membranes. The membranes were blocked with 5% milk in TBST buffer for 30 min and incubated with relevant primary antibody overnight. Then they were washed, incubated with peroxidase-conjugated secondary antibody, and washed again. Blots were developed using the ECL chemiluminescence reagent (Pierce).

Small interfering RNA assay. Mixture of four Jak1-specific or Jak3-specific small interfering RNA (siRNA) or nonsense siRNA (all purchased from Dharmacon) was introduced into cells at 100 nmol/L by lipofection with the new generation Lipofectamine (DMRIE-C; Invitrogen). The procedure was repeated after 24 h, and the cells were cultured for additional 24 h. The cells were harvested at one time point 48 h after first transfection. Cells were cultured in the presence of IL-2. Cells were extensively washed after 48 h and placed in six-well plates for 2 h; IL-2 and

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IL-21 were added for 20 min for Western blot analysis and for 24 h for the cell proliferation and apoptosis assays.

Cell proliferation assay. After the cell culture for 24 h in presence of IL-2, IL-15, or IL-21, cell proliferation was evaluated by detection of bromodeoxyuridine (BrdUrd) incorporation using the commercially available kit cell proliferation ELISA (Roche) according to manufacturer's protocol. In brief, cells were seeded in 96-well plates (Corning) at concentration of 1×10^4 cells per well in RPMI medium supplemented with 10% FBS and labeled with BrdUrd (Roche) for 4 h. After the plate centrifugation (10 min at $300 \times g$), supernatant removal, and plate drying, the cells were fixed and the DNA was denaturated by addition of 200 mL FixDenat reagent. The amount of incorporated BrdUrd was determined by incubation with a specific antibody conjugated with peroxidase followed by colorimetric conversion of the substrate and absorbance evaluation in the ELISA plate reader.

Cell apoptosis (TUNEL) assay. We used the ApoAlert DNA fragmentation assay kit from BD Bioscience according to the manufacturer's protocol. In brief, cells were cultured at 0.5×10^6 cells/mL for 24 h with IL-2 or IL-21. The cells were collected, washed twice in PBS, and fixed with 1% formaldehyde/PBS. After the wash, cells were permeabilized with 70% ice-cold ethanol for at least 2 h, washed, and incubated in TdT incubation buffer for 1 h at 37°C. The reaction was stopped by adding 20 mmol/L EDTA, and the cells were washed twice in 0.1% Triton X-100/bovine serum albumin/PBS. Finally, samples were resuspended in 0.5 mL of PI/RNase/PBS, collected and analyzed by flow cytometry (FACSort BD) using the CellQuest PRO software.

Results

Gene expression profiling of cultured CTCL in response to stimulation by IL-2, IL-15, and IL-21. To determine expression of which gene is modulated in CTCL cells by the key γc-signaling cytokines, we performed whole-genome gene expression analysis in the CTCL-derived, IL-2–dependent Sez-4 cell line after IL-2 withdrawal and subsequent stimulation with IL-2, IL-15, IL-21, or medium alone. As shown in Fig. 1*A*, IL-2 and IL-15, which act via receptors that share the entire γc/β chain signal transducing complex, were potent modulators of gene expression in these cultured CTCL cells. In contrast, IL-21 that uses a heterodimeric receptor composed of the γc and IL-21–specific α chain showed a very limited stimulatory capacity. Similar results were obtained in separate experiments, as well as with another CTCL-derived cell line SeAx (data not presented). The correlative intensity plot analysis of the results (Fig. 1*B*) showed that the overall gene expression patterns induced by IL-2 and IL-15 were indistinguishable from each other but markedly differed from the expression patterns seen in the IL-21–stimulated and medium-stimulated cells. In turn, direct comparison of the IL-21–treated and medium-treated cells revealed great similarity in the gene expression patterns between these two groups. IL-21–induced cells were, however, less different from the IL-2–induced and IL-15–induced cells than the medium-treated cells, indicating that IL-21 affects expression of a subset of genes modulated by the other two γc-signaling cytokines. Applying filter thresholds of at least 2-fold change in gene expression and a false discovery rate of 0.01, >1,000 gene transcripts were identified as modulated by both IL-2 and IL-15 with >700 being up-regulated and >300 being down-regulated. In striking contrast, IL-21 modulated expression of <40 gene transcripts, all of which were up-regulated. The entire microarray data set is available in GEO database under the accession number GSE8685.

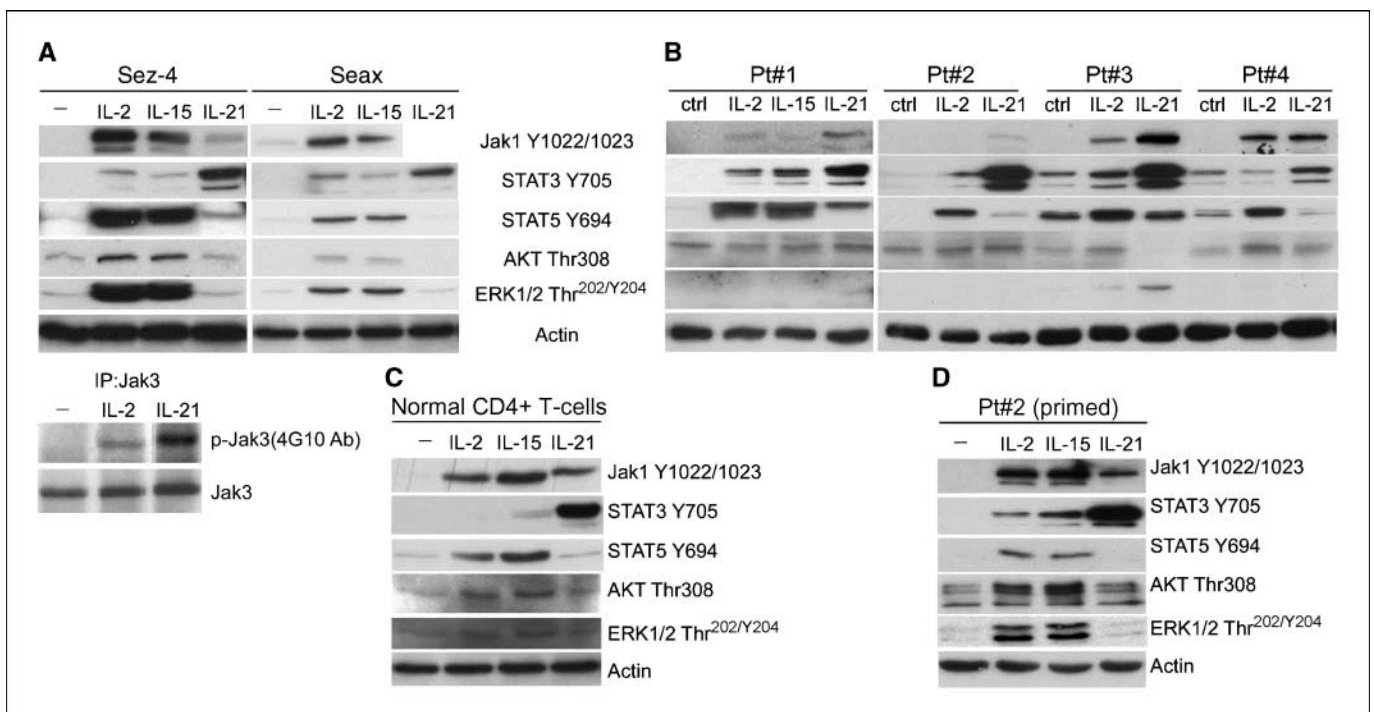

Figure 2. Cell signal transduction pathways activated by the γc-signaling cytokines in CTCL and normal CD4+ T cells. IL-2–dependent CTCL-derived cell lines Sez-4 and SeAx that were starved of IL-12 for 16 h (*A*), circulating malignant CD4+ T cells derived directly from four patients with the advanced leukemic phase of CTCL (*B*), circulating normal CD4+ T cells (*C*), and leukemic CTCL cells primed by preculture with a mitogen (*D*) were stimulated with medium alone, IL-2 (200 units), IL-15 (20 ng/mL), or IL-21 (100 ng/mL) for 20 min. The cell lysates were either directly analyzed by Western blotting with phosphorylated specific antibodies against the depicted tyrosine or threonine moieties of the listed proteins and control total actin-specific antibody or (*A, bottom*) immunoprecipitated with an anti-Jak3 antibody before Western blotting with the broad specificity anti–phosphorylated tyrosine 4G10 antibody.

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Table 1. Cellular pathways and functional programs modulated by IL-2 in the CTCL cells

	Genes
Signaling pathways	
Cytokine-cytokine interactions	Up-regulated: *TNFS10*, *IL2RB*, *VEGF*, *ACVR1B*, *IL10RA*, *IL10*, *CCL3*, *LOC643930*, *CXCL12*, *TNFRSF1B*, *CD40LG*, *CSF2*, *IL17RB*, *IL13*, *TNFRS21*, *TNFRS9*, *LIF*, *CSF2RB*, *TNFRSF18*, *BMP2*, *IL1A*, *TNFRSF10A*, *TNFRSF12A*, *TNFRSF11*, *IL5*, *IL2RA*, *IL18RAP*, *CCR4*, *TNFRSF4*, *OSM*, *TNFRSF8*, *IL8*, *IL24*, *TGFB1*, *TNFSF14*
	Down-regulated: *TNFRSF11A*, *FAS*, *PDGFC*
Jak/STAT	Up-regulated: *CISH*, *BCL2L1*, *SOCS2*, *IL2RB*, *CCND1*, *IL10RA*, *IL10*, *STAT3*, *PIM1*, *SPRED2*, *CSF2*, *IL13*, *LIF*, *CSF2R*, *CCDN3*, *IL5*, *CD25*, *CCDN2*, *MYC*, *OSM*, *SOCS1*, *SPRED1*, *IL24*
	Down-regulated: *PIK3R3*, *SOS1*, *JAK2*, *STAM*
MAPK	Up-regulated: *ZAK*, *PTPN7*, *FGF7*, *FGFR1*, *MAPKAPK2*, *ACVR1B*, *RRAS2*, *GADD45B*, *DUSP4*, *PRKX*, *IL1A*, *DUSP7*, *DUSP2*, *HSPA1B*, *MYC*, *DUSP6*, *TGFB1*, *JUND*
	Down-regulated: *SOS1*, *CACNG3*, *CASP1*, *FAS*, *CACNA1E*, *MAPK2K6*
Metabolic pathways	
Purine	Up-regulated: *UMPS*, *DHODH*, *UCK2*, *POLR3K*, *POLR1B*, *POLR1C*, *POLR2D*, *POLE2*, *CTPS*, *NP*, *PUS1*
	Down-regulated: *NT53*
Pyrimidine	Up-regulated: *LOC645619*, *GART*, *POLR3K*, *ADCY9*, *POLR1C*, *POLR1B*, *PDE4A*, *POLR2D*, *ADCY3*, *POLE2*, *NP*, *PPAT*
	Down-regulated: *ENPP2*, *PD34D*, *NT5E*, *PDE3B*
Biological functions	
Cell cycle regulation/ cell proliferation	Up-regulated: *BCCIP*, *BCL2*, *CCND1*, *CCND2*, *CCND3*, *CCNE1*, *CCNE2*, *CDC25A*, *CDC6*, *CDK5R1*, *DKC1*, *DLST*, *DUSP4*, *DUSP6*, *EEF1E1*, *FGF2*, *GNL3*, *HK2*, *IL1A*, *IL2RA*, *IL8*, *MYC*, *NOL1*, *PINX1*, *PTCH*, *RGC32*, *RHOB*, *TGFB1*, *VEGF*, *WT1*, *ZAK*
	Down-regulated: *ATM*, *CCNG1*, *CCNG2*, *CDKN1B*, *CDKN1C*, *CDKN2C*, *ING4*, *JAK2*, *MAP2K6*, *PDGFC*, *PLCB1*, *PTPRC*, *RGPD4*, *SESN1*, *SESN3*, *TFDP2*
Apoptotic cell death	Up-regulated: *AHR*, *AXUD1*, *BAG1*, *BCL2*, *BCL2L1*, *BIRC3*, *CARD10*, *CARD9*, *CD40LG*, *CDK5R1*, *EEF1E1*, *ELMO3*, *GADD45B*, *HSPA1B*, *HSPD1*, IER3, IL10, IL1A, IL24, IL2RA, IL2RB, PDCD11, PDCD2L, PEA15, PHLDA1, PHLDA2, PIM1, PTRH2, RHOB, SERPINB9, *SOCS2*, *SPP1*, *TGFB1*, *TNFAIP8*, *TNFRSF10A*, *TNFRSF12A*, *TNFRSF18*, *TNFRSF1B*, *TNFRSF21*, *TNFRSF9*, *TNFSF10*, *TNFSF14*, *TRIB3*, *VEGF*, *ZAK*
	Down-regulated: *ATM*, *CARD6*, *CASP1*, *DAPK1*, *FAS*, *FOXO3A*, *IHPK2*, *ING4*, *LGALS12*, *PDCD4*, *PROK2*, *PTPRC*, *RFFL*, *TP53INP1*

By mining KEGG and Gene Ontology databases using the Database for Annotation, Visualization, and Integrated Discovery tool (12), we assigned some of the IL-2/IL-15–modulated genes into signaling and metabolic pathways, as well as functional groups (Table 1). The most prominent group represented the cytokine/cytokine receptor genes, suggesting that IL-2 and IL-15 strongly affect the ability of the cells to respond to the other cytokines. Many of the currently classifiable genes represented the known targets of the Jak/STAT and mitogen-activated protein kinase (MAPK) signaling pathways, confirming the role of IL-2 and IL-15 as potent inducers of these pathways (1). The cytokines also exerted an effect on genes involved in purine and pyrimidine metabolism, suggesting the presence of a proproliferative signal induced by IL-2 and IL-15. Accordingly, many of the modulated genes were assigned to cell proliferation and cell cycle progression programs. In addition, a set of the modulated genes grouped within the apoptotic cell death program suggested the capacity of IL-2 and IL-15 to protect cell viability. Due to their very low number, categorization of the IL-21–modulated genes was much more difficult. However, several (*CD25*, *Pim-1*, *Pim-2*, *BATF*, *STAT3*, *IL-5*) could be identified as targets of STAT3 (8, 13–15) and, with exception of IL-5, assigned to the cell apoptosis program (16–20).

Impact of IL-2, IL-15, and IL-21 on activation of Jak/STAT, PI3K/Akt, and MEK/ERK signaling pathways and cellular growth and survival. To examine directly the effects of the γc-signaling cytokines on cell signal transduction pathways in the malignant T cells, we determined, in the two CTCL-derived, IL-2–dependent cell lines Sez-4 and SeAx, the activation status of key pathways upon cell stimulation with IL-2, IL-15, and IL-21 (Fig. 2*A*). Similar to the gene expression data, there were profound differences between IL-2 and IL-15 compared with IL-21 in activation of the Jak/STAT, PI3K/Akt, and MEK/ERK pathways. Whereas IL-2 and IL-15 induced strong phosphorylation of STAT5, Akt, and ERK1/2 and very weak phosphorylation of STAT3, IL-21 strongly phosphorylated STAT3 but not STAT5, Akt, or ERK1/2. As expected, the cytokines phosphorylated Jak1 and Jak3, although to a different degree. To show that a similar pattern of differential activation of signaling pathways is induced by the γc-signaling cytokines also in the uncultured, freshly isolated CTCL cells, we examined circulating malignant cells from four patients with the leukemic phase of the lymphoma (Fig. 2*B*). As with the CTCL-derived cell lines, the primary malignant cells displayed strong phosphorylation of STAT3 in response to IL-21 and strong phosphorylation of STAT5 upon stimulation with IL-2 or IL-15. Interestingly, essentially no phosphorylation of either Akt or ERK1/2 in response to any of the cytokines could be detected.

These findings raised at least two interesting questions. First, whether the differential pathway activation by IL-2 and IL-21 is the distinct feature of CTCL cells or can be seen in CD4+ T cells in general. Second, what is the possible cause of the difference in the PI3K/Akt and MEK/ERK pathway activation between the CTCL-derived cell lines and primary CTCL cells. To answer the former

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question, we examined CD4+ T cells isolated from a healthy individual. As shown in Fig. 2*C*, the pathway activation pattern was the same as that of the native CTCL cells with IL-21 inducing strong phosphorylation of STAT3 and IL-2 and IL-15 stimulating strongly STAT5. This observation indicates that the differential response to the cytokines is an intrinsic feature of CD4+ T cells rather than a unique property of the malignant CTCL cells. In regard to the differential activation of the PI3K/Akt and MEK/ERK pathways by the primary and cultured CTCL cells, we reasoned that this might reflect a difference in the cell activation status with the circulating leukemic cells being in the more quiescent state. To address this possibility, we preactivated the leukemic CTCL cells with a mitogen (PHA) for 7days. As shown in Fig. 2*D*, the preactivation with PHA changed the cytokine response pattern of the leukemic CTCL cells. Similar to the CTCL-derived cell lines and in contrast to the freshly isolated cells, the primed cells phosphorylated both Akt and ERK1/2 in response to IL-2 and IL-15 but not to IL-21.

To determine the biological effects of IL-2 and IL-21 on the CTCL cells, we examined their influence on cell growth and survival. Whereas IL-2 strongly stimulated proliferation of both cultured (Fig. 3*A*) and primary (Fig. 3*B*) CTCL cells, IL-21 had very limited to absent effect on their proliferation. In contrast, both cytokines displayed similar degree of antiapoptotic activity in these cells (Fig. 3*C* and *D*).

Role of Jak1 and Jak3 kinases in IL-2–mediated and IL-21–mediated cell activation. We next examined the role of Jak1 and Jak3 in response to the γc-signaling cytokines of the CTCL cells by taking advantage of the availability of highly potent and specific Jak inhibitors. Whereas the kinases, in particular Jak3, play a critical role in the function of normal T lymphocytes, their relative importance in malignant T cells remains poorly characterized (8, 21, 22). Initially, we performed gene expression analysis in the Sez-4 cells stimulated with IL-2 in the presence of pan-Jak and Jak3 inhibitors. Whereas the former inhibits *in vitro* kinase activity of all four members of the Jak family at low nanomolar doses (IC_{50} inhibition at 15 nmol/L for Jak1, 1 nmol/L for Jak2, 5 nmol/L for Jak3, and 1 nmol/L for Tyk2; ref. 10), the latter is as potent but much more specific for Jak3 (IC_{50} for Jak3 at 2 nmol/L, 20 nmol/L for Jak2, and 100 nmol/L for Jak1; ref. 11). As schematically depicted in the similarity matrix plot analysis (Fig. 4*A*), both pan-Jak and Jak3 inhibitors very profoundly suppressed the IL-2–mediated modulation of gene expression (see GEO database, accession number GSE8687, for the entire data set). The overall gene expression pattern of cells treated with IL-2 along with the pan-Jak or Jak3 inhibitor was not only indistinguishable from each

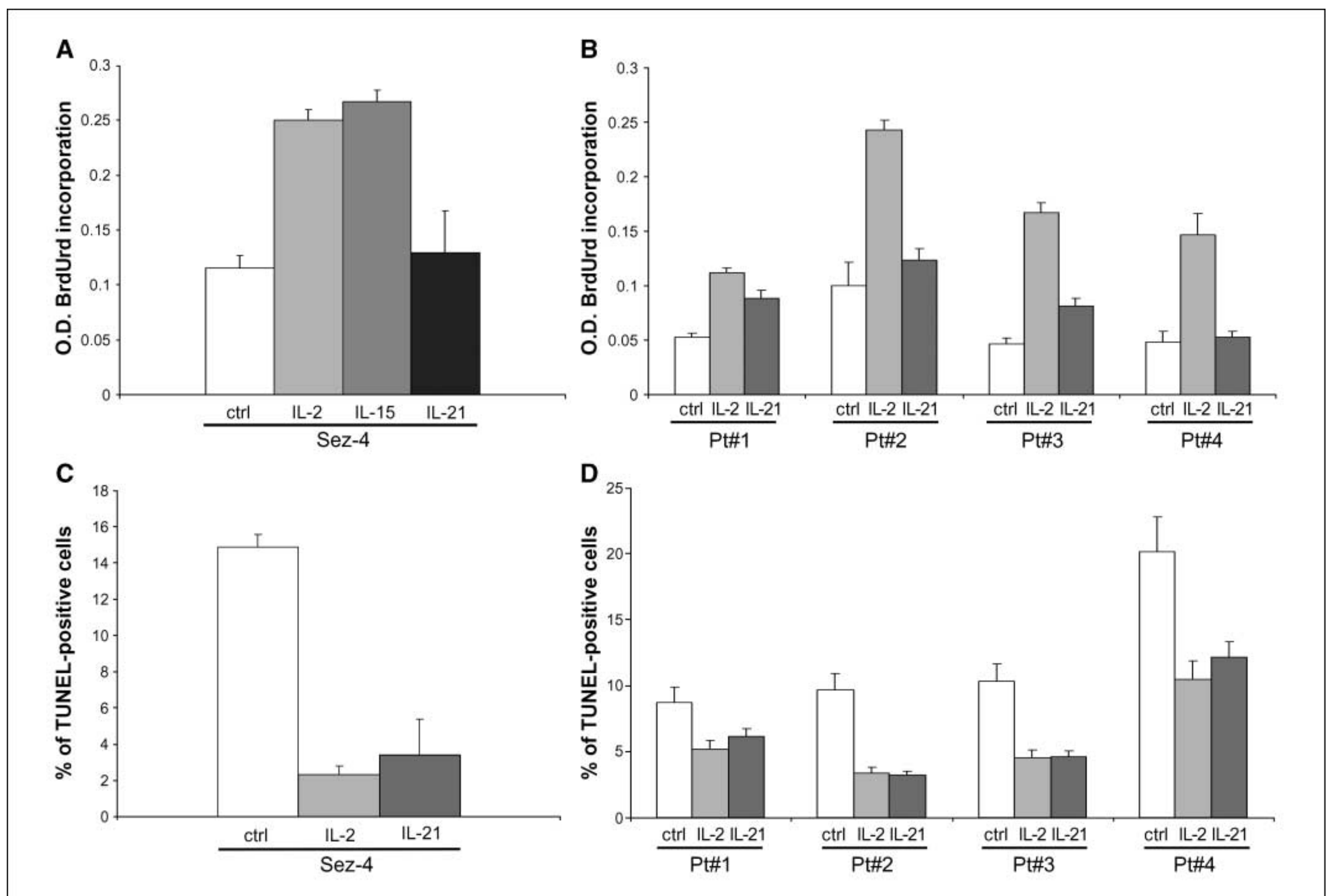

Figure 3. Cytokine-induced proliferation and inhibition of apoptosis in CTCL cells. The IL-2–dependent CTCL Sez-4 cell line starved of IL-2 and the isolated primary leukemic (Sezary) CTCL cells were cultured with medium alone or stimulated with the depicted γc-signaling cytokines and examined for proliferative rate in a BrdUrd uptake assay (*A* and *B*) and apoptotic cell death rate in a TUNEL staining assay (*C* and *D*).

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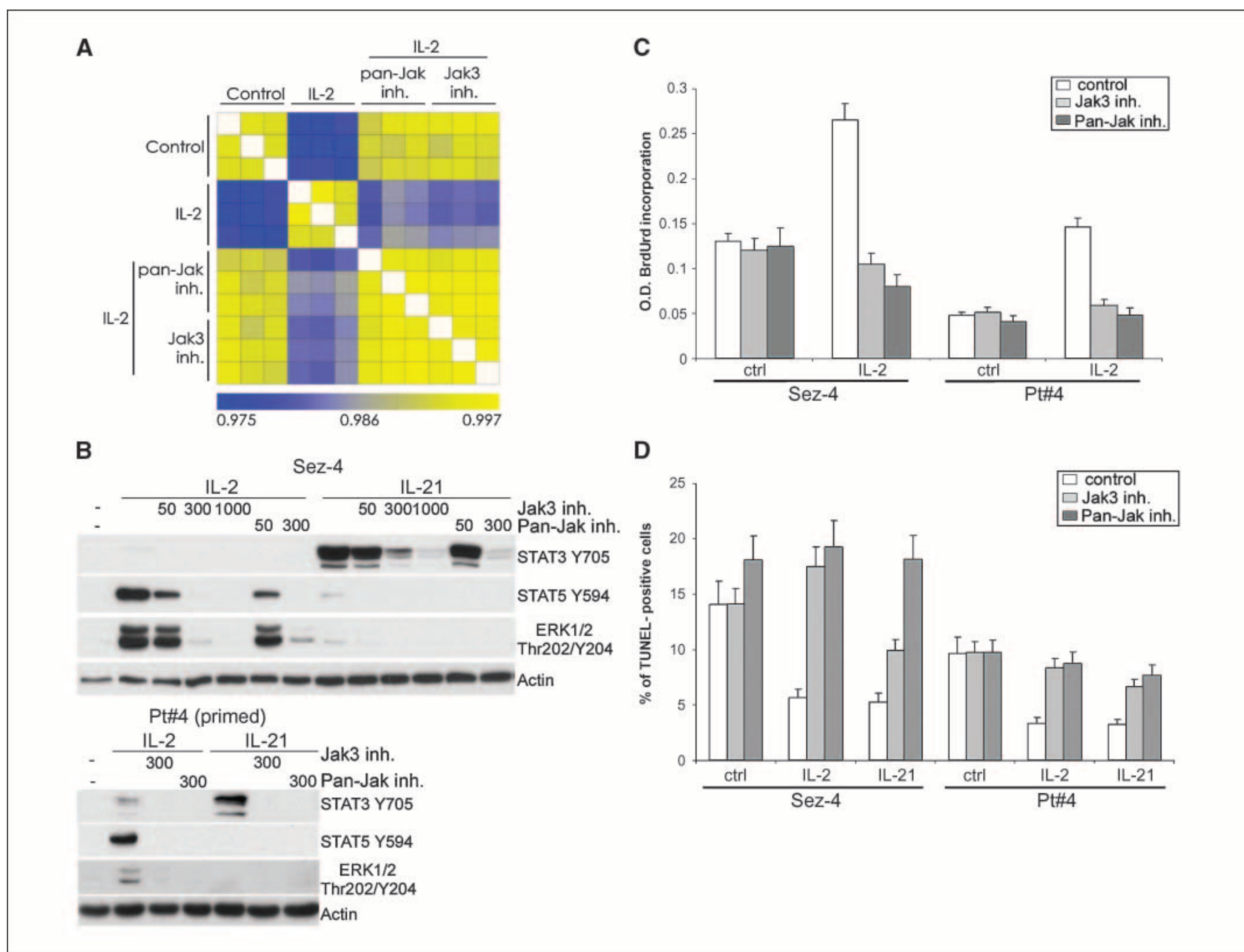

Figure 4. Effects of Jak inhibitors on gene expression, signal transduction, and biological functions of cytokine-stimulated CTCL cells. *A,* IL-2–depleted Sez-4 cells were cultured in triplicates, pretreated for 30 min with pan-Jak (300 nmol/L) inhibitor, Jak3 inhibitor (300 nmol/L), or drug vehicle alone and stimulated by IL-2 (200 units) added for 4 h to all but the control cultures. After cell harvest and total RNA isolation, all samples were checked for the RNA quality, converted to cDNA amplified and labeled and hybridized to Affymetrix HG-U133 Plus 2 arrays. The results are depicted as intensity plot of the gene expression data correlation. The color scale represents the range of the pair-wise correlations among the samples. *B-D,* IL-2–starved Sez-4 and mitogen-primed leukemic CTCL cells were cultured with 200 units of IL-2 or 100 ng/mL IL-21 in the presence of the pan-Jak and Jak3 inhibitor at the listed concentrations. Western blot analysis (*B*) of the Sez-4 and primed leukemic CTCL cell lysates was performed with the depicted phosphorylated specific and control antibodies. Determination of the proliferative (*C*) and apoptotic cell death rate (*D*) in the same CTCL cell populations.

other but also from the control, IL-2 untreated cells. From >1,000 gene transcripts modulated by IL-2, there were only 35 and 14 different transcripts for pan-Jak and Jak3 inhibitor, respectively, expression of which differed by >2.0-fold from the control, medium-treated cells. However, for most of these relatively few genes, there was a profound decrease in expression compared with the IL-2 only–treated cells, e.g., expression of *IL-2Rα* gene dropped from >17-fold above the background to <4-fold and expression of *CISH* from >26-fold to around 3-fold upon cell treatment with either of the Jak inhibitors. This substantial decrease suggests an incompleteness of Jak inhibition rather than the presence of Jak-independent signaling involved in modulation of these genes. Regardless, the observed profound suppression of the IL-2–modulated genes indicates that Jak kinases or at least Jak3 is critical for the IL-2–induced changes in gene expression in the CTCL cells.

To characterize in greater detail the role of Jaks in the γc-transduced signaling in CTCL cells, we examined the effect of the Jak inhibitors used at various concentrations on activation of STATs and ERK and modulation of cell functions in response to IL-2 and IL-21. When applied at 300 nmol/L, both pan-Jak and Jak3 inhibitors profoundly suppressed in the Sez-4 cells the IL-21–induced phosphorylation of STAT3 (Fig. 4*B, top*). The same inhibitor concentrations abrogated the IL-2–induced phosphorylation of STAT5 and ERK1/2. Similar results were obtained with the primary mitogen-preactivated CTCL cells (Fig. 4*B, bottom*). On the cell function level, the inhibitors displayed similarly profound suppression of the IL-2–induced cell proliferation (Fig. 4*C*) and of the IL-2–mediated or IL-21–mediated protection from apoptosis (Fig. 4*D*).

To determine the role of Jak1 in the γc-transduced signaling and to confirm the key role of the Jaks by a different experimental approach, we treated Sez-4 cells with Jak1 and Jak3 siRNA. As

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shown in Fig. 5*A*, depletion of either of the kinases markedly inhibited IL-2–induced phosphorylation of STAT5 and ERK1/2 and the IL-21–induced STAT3 phosphorylation. Loss of Jak1 and Jak3 expression also affected to a similar degree the IL-2–induced cell proliferation (Fig. 5*B*), as well as the IL-2–promoted and IL-21–promoted cell survival (Fig. 5*C*). These observations show that both Jak1 and Jak3 play a critical role in IL-2–induced and IL-21–induced cell signaling in CTCL cells.

Discussion

In this study, we have characterized the effect on the CTCL cell lines and primary leukemic (Sezary) cells of three key cytokines that signal through receptors containing the γc chain and are critical for development and function of various normal immune cells. We have found that IL-2 and IL-15 markedly changed expression by of >1,000 gene transcripts (with over 700 being up-regulated and over 300 being down-regulated) in a CTCL-derived cell line. In contrast, IL-21 increased expression of <40 genes. Proteins encoded by some of the IL-2/IL-15–regulated genes belonged to the Jak/STAT and MAPK signaling pathways, and many were classifiable as the pathway-induced members of the cell proliferation, cell cycle, and apoptosis functional programs. At least five of the IL-21–modulated genes represented known targets of STAT3, and their protein products are known to protect cells from apoptosis. Whereas all three cytokines induced Jak1 and Jak3 phosphorylation, IL-2 and IL-15 induced strong STAT5 but not STAT3 phosphorylation. In contrast, IL-21 had the opposite effect on STAT3 and STAT5. Furthermore, IL-2 and IL-15, but not IL-21, activated PI3K/Akt and MEK/ERK pathways as determined by Akt and ERK1/2 phosphorylation. Similar observations were made in the primary CTCL cells derived directly from patients with the leukemic form of the lymphoma (Sezary syndrome) with the caveat that the leukemic primary cells required mitogen-induced priming to activate the PI3K/Akt and MEK/ERK pathways. As expected, IL-2 and IL-15 induced proliferation of both cultured and native CTCL cells and protected them from the apoptotic cell death (23), whereas IL-21 had essentially no proproliferative effect but protected the cells from apoptosis. The CTCL cell signaling in response to the cytokines was entirely Jak1 and Jak3 kinase dependent because high-specificity pan-Jak and Jak3 inhibitors abrogated the IL-2–induced gene expression modulation, as well as the IL-2–triggered and IL-21–triggered signal transduction by not only the Jak/STAT but also, in the case of the IL-2, MEK/ERK pathway. Jak inhibitors suppressed also the IL-2–promoted and IL-21–promoted cell proliferation and protection from cell apoptosis. Jak1 and Jak3 siRNA-mediated depletion inhibited all the examined signaling pathways, as well as cell growth and survival.

Although there are some biological differences in the function of IL-2 and IL-15 (1, 2), it is not particularly surprising that IL-2 and IL-15 modulated in the CTCL cells essentially overlapping sets of genes and activated the same signaling pathways, given that their receptors share the entire signal transduction complex of the β and γc chains. A similar overlap in the gene expression pattern (24) and signaling pathway activation (2, 25, 26) was found also in normal murine CD8+ T cells stimulated with these cytokines. Both cytokines are also highly mitogenic for these cells (2, 26) and CTCL cells (23). The detailed study of the biochemical mechanisms underlying the noted differences of IL-2 and IL-15 effects on murine CD8+ T cells has found that the differences affect protein synthesis, are quantitative rather than qualitative in nature, and tend to occur upon prolonged cell stimulation likely due to the

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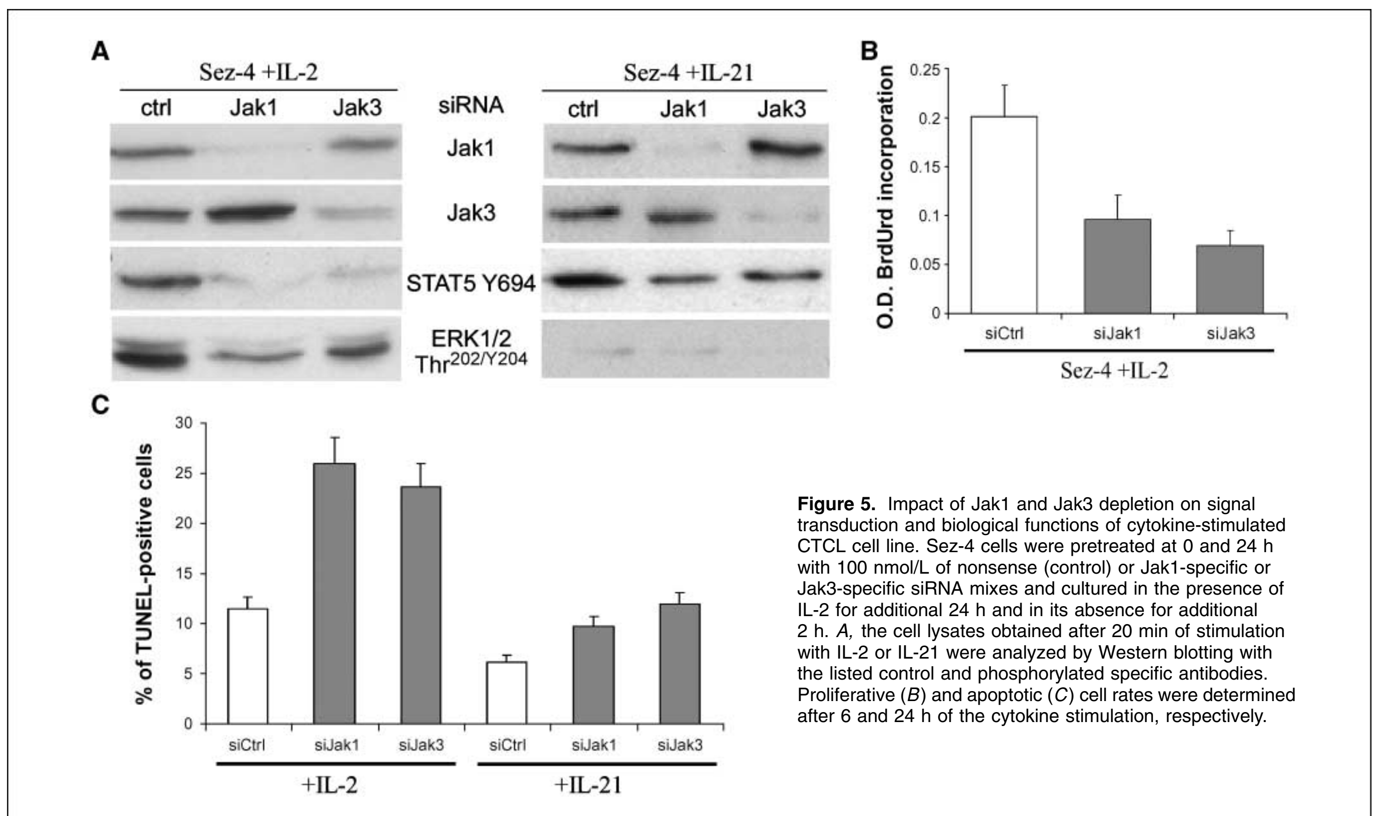

Figure 5. Impact of Jak1 and Jak3 depletion on signal transduction and biological functions of cytokine-stimulated CTCL cell line. Sez-4 cells were pretreated at 0 and 24 h with 100 nmol/L of nonsense (control) or Jak1-specific or Jak3-specific siRNA mixes and cultured in the presence of IL-2 for additional 24 h and in its absence for additional 2 h. *A,* the cell lysates obtained after 20 min of stimulation with IL-2 or IL-21 were analyzed by Western blotting with the listed control and phosphorylated specific antibodies. Proliferative (*B*) and apoptotic (*C*) cell rates were determined after 6 and 24 h of the cytokine stimulation, respectively.

differential ability of these cytokines to regulate sustained expression of their respective IL-2–specific and IL-15–specific α-chain receptor subunits (2).

It is remarkable that, in contrast to STAT5 activation, the primary leukemic CTCL cells required transient preactivation to efficiently activate the PI3K/Akt and MEK/ERK pathways in response to IL-2. This observation is very reminiscent of the finding that normal murine CD4+CD25+ T cells also required priming to activate the PI3K/Akt pathway upon IL-2 stimulation (27). These combined observations indicate that the final effect of IL-2 and likely other cytokines and cellular stimuli are dependent on the discrete functional status of the targeted cells.

The profound difference in the effect on the CTCL cells of IL-21 compared with IL-2 and IL-15 was quite surprising considering that not only their respective receptors share the γc chain but also that IL-21 receptor's α chain displays a high degree of homology to the IL-2 and IL-15 receptor's β chain. Consequently, both types of receptors have very similar signaling units and activate the same members of the Jak kinase family Jak1 and Jak3. The weak response to IL-21 seems, however, strictly cell type–specific because in naive murine CD8+ T cells, IL-21 activated at least as many genes as IL-15 (5). Although, similar to our findings, IL-21 induced strong phosphorylation of STAT3 in the murine CD8+ T cells, it also induced somewhat weaker phosphorylation of STAT5, STAT1, Src homology and collagen, and Akt (26). Finally, IL-21 promoted functional maturation and proliferation of the cells (5, 26).

Despite the overall very limited effect of IL-21 on the CTCL cells, our data show that the cytokine is active in such cells given its effect on gene expression, cell signaling, and protection from apoptotic cell death. The results also indicate that STAT3 is the key effector protein of the IL-21–induced signaling in the CTCL cells. In addition to STAT3 being strongly and selectively phosphorylated, the "gene expression signature" of STAT3 was the only one we could discern within the set of genes regulated by IL-21 as presented in the Results. Furthermore, many of the STAT3 signature genes have well-documented antiapoptotic effect (16–20). Our recent observation that siRNA-mediated STAT3 depletion performed in the CTCL cells that display persistent activation of the factor triggered massive apoptotic cell death (28) strongly supports this notion.

Our findings that IL-2 and IL-15 are highly stimulatory for CTCL cells and that IL-21 is not may have important translational implications for CTCL and, possibly, other CD4+ T cell–derived lymphomas. Namely, they provide rationale for targeting the Jak1/Jak3 complex associated with the γc-containing cytokine receptors, on one hand, and for applying IL-21 as a booster of the immune response, on the other hand. As we show, the small molecule inhibitors that target either the entire Jak family (10) or highly selectively Jak3 (11) profoundly inhibit gene expression, cell signaling, and function of CTCL cells. The fact that the Jak3 inhibitor was at least as effective as the pan-Jak inhibitor indicates that targeted inactivation of Jak3 is sufficient to suppress in CTCL cells signals generated by the IL-2 type, γc-signaling cytokines. Given the high specificity of the Jak3 inhibitor, originally developed as an immunosuppressive agent to prevent transplant rejection (11, 29), and the highly restricted expression of the γc to immune cells, targeting Jak3 therapeutically in CTCL possibly, other CD4+ T-cell lymphomas might prove particularly effective and relatively devoid of side effects. In regard to IL-21, its very limited effect on the CTCL cell gene expression, cell signaling, and proliferation coupled with the highly stimulatory effect of the cytokine on normal immune CD8+ T cells (5, 26, 30) and NK cells (31) strongly suggest that IL-21 may prove highly efficacious in therapy of this type of T-cell lymphoma despite the noted antiapoptotic effect on the CTCL cells (Fig. 3). The IL-21–promoted long-term survival of mice bearing various tumors including a wide spread, systemic lymphoma (30–36) strongly supports this notion. Finally, it is also conceivable that the appropriately timed application of the Jak3 inhibitor to suppress growth and survival of the CTCL cells alternated with IL-21 administration to boost the antilymphoma immune response may display a particularly beneficial therapeutic effect in CTCL and similar T-cell lymphomas.

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