Chapter

IL-21 Signaling and Induction of Cytokine Expression in Human Leukemia Cells and Monocytes

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Abstract

Interleukin-21 (IL-21) is produced by activated T cells and it plays many diverse roles by regulating the functions of normal and abnormal cells. Its roles include regulation of proliferation, promotion of immune system and activation of apoptosis in B cells. IL-21R is a type-1 cytokine receptor and belongs to the IL-2R and IL-15R family. The signaling mechanisms of IL-21 in different cell types have been identified. However, we know less about the biological effects of IL-21 and its signaling mechanisms in leukemia cells and monocytes. In this chapter, we will focus on IL-21's biological effects and signaling pathways as well as discuss the potential implications and applications of IL-21 in leukemia cells. In these cells, IL-21 does not promote proliferation but enhances apoptosis and chemotaxis. Furthermore, IL-21 promotes differential expression of many cytokines including interleukins and chemokines. IL-21 activates both the Raf-ERK-MAPK and the Jak/STAT signaling pathways. These pathways mediate some of the effects of IL-21. Lastly, IL-21 also promotes activation of the STAT3 promoter and other transcriptional factors. These findings may be relevant to IL-21's potential clinical implications and applications.

Keywords: IL-21, Leukemia, monocyte, ERK, Jak/STAT

1. Introduction

Interleukin-21 (IL-21) was first identified in 2000 through screening of a cDNA library from CD3+ human T cells [1]. Subsequently, the investigators identified a specific clone as containing pro-proliferative effects that was sensitive to neutralization by the soluble IL-21R. Later investigators characterized this clone as a factor, which was subsequently renamed IL-21 [2]. The sequence of this new cytokine gene has an open reading frame that encodes for a 163 amino acid peptide, later confirmed as IL-21. Matured IL-21 is 15 kDa and it has four helix bundle cytokine domains and two pairs of cysteine residues showing significant homology to IL-2, IL-4, IL-15 and GM-CSF [2–4]. Based on these structural characteristics, [1] IL-21 was placed in the IL-2 family. The IL-21 gene is located at chromosome 4q26–27 [1] and found to be approximately 180 kb from the IL-2 gene. In contrast, the human IL-15 gene is located on chromosome 4q31. Clearly, there is some commonality regarding the organization, gene structure, and location of these cytokines [1].

Human IL-21 has 62% homology with murine IL-21 in addition to their two pairs of cysteine residues and well conserved WSEWS motif [5] even though the murine IL-21 gene is located on chromosome 3 [1].

Initial report indicated that IL-21 is produced exclusively by CD4+ T cells during their response to stimulation by anti-CD3 and anti-CD8 antibodies [6]. However, other cell types are known to produce IL-21 [2–5]. IL-21 has effects on many cell types including human leukemia cells and human monocytes [2–7]. The IL-21 receptor (IL-21R) is expressed on both CD4+ and CD8+ T cells in which IL-21 stimulates proliferation [1]. In addition, IL-21 promotes allogeneic-specific proliferation of lymph node T cells, enhances cytolytic activity, induces IFN-γ production in T cells [2, 3] and enhances clonal expression of antigen-activated T cells [5, 6]. IL-21 also enhances naïve OT-1 cell response, enhances the affinity of the antigen-specific CD8+ T cells, and regulates the functions of both T cells and B-lymphocytes [1, 6, 7]. It also plays important roles in B cell proliferation, differentiation and class switching [8]. IL-21 causes induction of apoptosis of resting primary B cells via downregulation of anti-apoptotic factors, Bcl-2 and Bcl-xL [9] an effect which differentiates IL-21 from other members of the IL-21 family known to promote proliferative and survival [2, 3, 7, 8].

In B-chronic lymphocytic leukemia cells (B-CLL) IL-21 enhances production of granzyme B, which mediates B-CCL apoptosis when these cells were co-treated with IL-21 and CpG oligodeoxynucleotide [9]. Thus, IL-21 has anti-proliferative effects on B lymphoma cell line [3, 10, 11] suggesting that IL-21 has potential anti-tumor activity against B-cell malignancies. IL-21 enhances transcription of genes that encode IL-21R, and IL-18R, all of which are involved in innate immunity [10–13]. IL-21 expression is Ca^{2+}-dependent and that the IL-21 gene has three NFAT binding sites, which may be involved in regulation of the IL-21 gene promoter function by calcium ionophore [14]. In contrast to its known anti-proliferative effects [9, 10, 12, 13], IL-21 promotes growth, survival and induces DNA synthesis in human myeloma cell lines via Jak1/STAT2/3 mechanism [12]. In B cells, co-stimulation by IL-21 leads to three effects: growth arrest, apoptosis or growth [12–16]. Furthermore, IL-21 promotes maturation of NK cells [14], enhances differentiation of NK cells via co-stimulation with either IL-2, or IL-15 or IL-18 and regulates the functions of many different cell types [17–20].

IL-21 receptor (IL-21R) was first discovered in 2000 [1, 2] by investigators who identified a clone structure from the human chromosome 16p11 that led to their discovery of a protein of 538 amino acids [9]. This protein of 60 kDa was later identified as the type 1 receptor with about 62% homology to the IL-4 receptor (39 kDa) whose gene is located on chromosome 16 [1, 16]. The IL-21R has two subunits, alpha and gamma and it is expressed in B cells, T cells, dendritic cells, NK cells, myeloma cell lines, lymphoma cell lines (IM-9, NK-92 and Jarkat cells), and lymphoid tissues including spleen and thymus in both CD4+ and CD8+ T cells [17, 18]. IL-21R is upregulated upon T cell receptor activation [17, 18]. IL-21R is also expressed and upregulated in epidermis of systemic sclerosis patients [18]. IL-21R has two pairs of conserved cysteine residues in its extracellular domain and a WSXWS motif proximal to its transmembrane domain. In addition, it has two Box 1 and 2 motifs, which serve as docking sites for cytoplasmic Jak kinases involved in transducing cellular signaling from the IL-21R [21–25]. The γ-chain of IL-21R is an essential component of the IL-2 family of receptors that is critical for signaling as anti-γc antibodies blocked B cell proliferation induced by IL-21 and $CD4^0$, indicating that the gamma chain is involved in IL-21R signaling mechanisms [25, 26]. Furthermore, the Jak inhibitor WH-P131 blocked IL-21-induced proliferation of BaF3 cells via IL-21R confirming a role for Jak3 in IL-21R signaling. In addition, inhibition of the IL-21γc subunit by monoclonal antibody blocked IL-21-induced activation of Jak1, Jak3, and STAT1,

STAT2, STAT3 and STAT5 thus further supporting a critical role for IL-21 γc subunit in IL-21 signaling [26].

IL-21R associates with BCL-6 translocation in B cell lymphomas and expression of IL-21R is upregulated by $CD4^0$ leading to transduction of pro-apoptotic signals [27, 28]. In addition, IL-21R plays important roles in immunoglobulin production and decreased in IL-21R expression is associated with decline in normal antibody production [28]. Furthermore, there is a decrease in IL-21R expression on B-lymphocytes in patients with systemic lupus erythromatosis (SLE) [29]. IL-21 synergizes with IL-15 to induce expansion of NK cells [1] and synergizes with IL-15 and IL-18 to upregulate IFNγ mRNA synthesis and production in NK and T cells. IL-21 also synergizes with IL-15 to promote proliferation of memory and naïve CD8+ [18], Furthermore, IL-21 has positive effect on tumor regression in B16 melanoma cells [24] as well as synergizes with IL-15 to delay CD11b bone marrow cell apoptosis [30]. In addition, IL-21 augments proliferation of IL-6-dependent human myeloma cells expressing IL-21R and synergizes with TNF to enhance myeloma cell mitogenesis [3, 13]. These synergistic effects of IL-21 led to consideration of IL-21 as a potential therapeutic agent for many cell malignancies [30]. In contrast to its synergetic effects, IL-21 antagonizes IL-4's ability to induce isotype switching in B cells from IgM to IgG and inhibits IFN-γ production by Th1 cells an [31, 32].

In BAB/C mice undergoing immunization, IL-21 caused reduction in antigen-induced eosinophil recruitment into the airway of the mice without affecting antigen-induced lymphocyte and macrophage recruitment [30]. However, another study [33] showed that IL-21 causes a decrease in antiovabumin IgE and IgG production, implying that IL-21 has anti-inflammatory effects in asthmatic patients and could serve as a potential therapy against asthma. The role of IL-21 in Crohn's disease (CD) has been reported [34]. Since Steven Rosenberg and his team [35] reported that IL-2 has anti-tumor in cancer patients, there has been extensive interest on whether members of IL-2 family of cytokines could generate similar effects. Subsequently, there are many reported that IL-21 alone or in combination with other cytokines produces anti-growth effect in various animal cancer models and on a variety of solid tumors including tumors without IL-21R [11, 36]. IL-21 is essential for IFN-γ induced expression of CXC chemokines, known to inhibit tumor angiogenesis. In addition, IL-21 induces tumor rejection by specific CTL and IFN-γ-dependent CXC chemokines in synergistic manner [37]. This anti-tumor effect of IL-21 is independent of the systemic cytokine release by known inflammatory mediators like IFN-y, IL-4, IL-10 and IL-12 [38]. Either alone or in combination with IL-23, IL-21 exhibits strong anti-tumor and anti-metastasis effects in kidney renal cell carcinoma in BALB/C mice and in human esophageal carcinoma tumors [39, 40]. Similarly, combination treatment with IL-15 and IL-21 promoted nearly 100% rejection of head and neck squamous cell carcinoma (HNSCC) in 30% of the experimental animals [41, 42], indicating potential role of IL-21 and IL-15 in minimizing transfection of animals with HNSCC [41, 42]. Similar reports in metastatic lymphoma animal models have indicate that IL-21 can act with other cytokines in combination therapy in the treatment of metastatic lymphoma tumors [43]. IL-21 enhances the synthesis of MIP-3α, a T cell chemoattractant and induce chemotaxis in intestinal epithelial cells (HT-29), indicating that IL-21 enhances immune cell response and could serve as an effective addition to cancer immunotherapy [43–45].

Our rationale for examining IL-21 effects in human myeloid monocytic leukemia cells and monocytes was based on the observations that IL-21 plays relevant roles in many types of cancer including solid tumors and lymphoma [36–43, 45]. However, little is known about the biologic effects of IL-21 in leukemia cells. In

addition, while the signaling pathways of IL-21 in many cell types are known, not much is known about the signaling pathway utilized by IL-21 in leukemia cells and monocytes. Furthermore, knowledge on potential cytokine induction ability of IL-21 in leukemia cells and monocytes was lacking. Our earlier preliminary report indicates that STAT3, Smad1, Smad2, and Smad3 are regulated by IL-21 in U937 leukemia cells [46]. Additionally, IL-21 activates Jak/STAT and MAPK pathways in different cell types [47]. Therefore, the main purpose of our study was to determine the biological effects of IL-21 in human U937 leukemia and monocytes and to gain more insight into whether the Jak/STAT and MAPK-signaling pathways mediate IL-21's specific biologic effects in these cells.

2. Materials and methods

U937 myeloid monocytic leukemia cells were obtained from ATCC and cultured in RPMI-1640 media containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biological. GA) in the presence of 5 U/ml penicillin +50 U/ml of streptomycin (Invitrogen, CA). The cultures were maintained in 5% CO_2 at 37°C and 100% humidity. HeLa/STAT3-luc cells were created by co0transfection of pSTAT3-luc and pHygromycin into human cervical epithelial HeLa cells. These cells were maintained in the same culture in the presence of 100 μg/ml of hygromycin B (Roche, NJ) [7].

2.1 Monocyte isolation

Human PBMC were isolated from anonymous health individuals (NY Blood Center, Long Island, NY) by Ficoll gradient centrifugation at 400 × *g* for40 minutes at 20°C followed by Percoll gradient (GR Health Care, Piscataway, NJ) [7]. The mononuclear cells were recovered in RPMI media supplemented with 10% FBS, 100 μg of streptomycin per ml and 100 U of penicillin per ml. The white blood cells were layered onto plastic dishes and allowed to adhere at 37°C for 90 minutes to allow the suspended cells to adhere. At the end of the incubation period, the non-adherent cells were carefully discarded and the adherent monocytes were carefully removed and suspended in culture media. The monocytes were about 95% pure based on positive staining for CD14 marker.

2.2 Western blotting detection of proteins and phosphoproteins

Sixty million (6×10^6) U937 leukemia cells or monocytes were untreated (control) or treated with IL-21 (50 or 100 ng/ml) in a time course (2, 5, 15, 30, 60 minutes) experiments [48, 49]. At the end of the time course, the cells washed 2× with PBS and collected as pellet by centrifugation at 1800 rpm for 3 minutes. The cell pellets were washed two times with cold PBS and lysed in 500 μl of lysis buffer A (containing protease inhibitors, 0.5% Triton X-100, 50 mM NaF and 2 mM Vanadate) as described in [7]. Total cell lysate protein concentration was determined by Coomassie Blue Protein Assay Kit (Pierce, IL). Equal amount of lysate proteins (120 μg per sample) was resolved by 12% polyacrylamide gel electrophoresis followed by transfer of the proteins to membrane and blotted against specific antibodies to total Jak2 or Jak3 or Tyk2 or p-Jak2 or p-Jak3 or p-Tyk2 as previously described [7]. The immunoblot band intensities were scanned via spot densitometry analysis for quantitation and scanned intensity values from the IL-21 treated samples were compared to the intensity in the untreated samples.

2.3 Detection of IL-21R on U937 Leukemia cells and monocytes

Twenty million cells (20×10^6 cells) untreated or treated with 50 ng/ml of IL-21 for 24 hours were lysed in lysing buffer and protein the concentration was determined by Coomassie Blue protein determination assay kit [50]. Exactly, 200 μg/lane of total cell lysate was separated by 15% polyacrylamide gel electrophoresis and the proteins were subsequently transferred to nitrocellulose membrane. Following blocking of the background on the membrane, for detection of IL-21R, the nitrocellulose membranes containing transferred protein bands were incubated with 1:1000 dilution of IL-21 receptor primary antibody (Novus Biologicals) at 4°C in blocking buffer (TBS, 0.1% Tween 20, 5% nonfat dry milk) for 1 hour. Next, the membrane was washed 3x in water and incubated in milk blocking buffer containing 1:3000 dilution of goat anti-rabbit IgG HRP antibody (Amersham, CA) for 1 hour at room temperature. This was followed by washing with TBS buffer containing 0.5% Tween 20. Finally, the membrane was incubated for 1 minute with enhanced chemiluminescence (ECL) solution and exposed to Kodak film and band intensities were scanned via spot densitometry analysis.

2.4 Detection of Jak1 activation by Jak1 ELISA

To detect Jak1 by ELISA, 96 well plates were pre-coated with 10 μg/ml poly lysine for 30 minutes [51]. U937 cells were seeded at 20×10^3/well in 96 well plates and incubated in serum free medium overnight. Subsequently, the cells were either untreated or treated with 50 or 100 ng/ml of IL-21 for 2, 15, 30 or 60 minutes followed by rapid removal of the culture medium by aspiration. The cells in the wells were fixed with 100 μl of 8% formaldehyde in PBS for 20 minutes at room temperature. After removal of the formaldehyde all the wells were washed three times with 200 μl of wash buffer (0.1% Triton X-100 in PBS). After washing, 100 μl of quenching buffer (wash buffer with 1% hydrogen peroxide and 0.1% azide) was added to each well and incubated for 20 minutes at room temperature. Then, the wells were washed two times with wash buffer followed by incubation of the wells with 100 μl of antibody blocking buffer for 1 hour at room temperature. After removal of the antibody blocking buffer the wells were washed with 200 μl wash buffer followed by addition in 40 μl of diluted specific p-Jak1 or total Jak1 antibody. The wells were covered with parafilm, and incubated overnight at 4°C. The primary antibodies were removed by aspiration, and wells were washed three times with wash buffer. Next, 100 μl of diluted secondary antibody (goat-anti-rabbit HRP) was added to each well and incubated for 1 hour at room temperature with head-to-tail shaking. After the incubation, the secondary antibody was removed and wells were washed three times with wash buffer and once with PBS. The wells were aspirated to remove all traces of liquid, and 100 μl of developing solution was added to each well. The wells were incubated for 15 minutes at room temperature, while monitoring the color development. Thereafter, 100 μl of stop solution was added to each well. Lastly, absorbance was read at 450 nm using a microtiter plate reader. Experiments were conducted in triplicate.

2.5 STAT/DNA binding assays for detection of STAT activation by IL-21

To determine whetherIL-21 induces activation of STAT proteins, untreated and IL-21 treated U937 leukemia cells from time course experiments for specific STAT/DNA binding assays using the STAT transcription factor assay kits (Active Motif, Chemicon) [52, 53]. Following stimulation of cells with IL-21, we used the kit to

monitor the activation or repression of several STAT proteins. The experiments were performed in triplicate.

2.6 Cell cycle analysis for cell growth

U937 cells (1×10^6) were either untreated or treated with 50 ng/ml of IL-21 for 24 or 48 hours [54, 55]. The cells were packed by centrifugation at 325 × *g* for 5 minutes, washed with cold PBS and suspended in 200 μl PBS. The cell suspension was mixed with 500 μl of ice cold ethanol and incubated on ice for 30 minutes. Next, the cells were harvested by centrifugation at 325 × *g* for 5 minutes and the pellets were suspended RNAse solution (containing 1 mg RNAse/ml in 0.2 M sodium phosphate buffer, pH 7.0) and 50 μl of propidium iodide (PI) solution (0.5 mg PI/ml in water). The samples were incubated at room temperature for 30 minutes. The tubes containing the cell suspensions were wrapped in aluminum foil and stored overnight at 4°C for equilibration. The cells were subjected to FACScan analysis using Cell Quest Pro (Becton Dickinson Immunocytometry system) and ModFitLT (v3.1, Versity Software House, Inc) to obtain G0, G1, S, and G2M population. For each analysis only single cells were analyzed. The data acquisition was set to collect a minimum of 104 cells within the single region established for each specimen using FL2-A vs. FL2-W plots. Data was analyzed using FL2-A. After establishing optimum conditions for data analysis, instrument settings were maintained constant throughout for all analysis.

2.7 Cell proliferation assays

To determine whether IL-21 stimulates cell proliferation in U937 cells, 6×10^6 cells were either untreated or stimulated with 100 ng/ml of IL-21 for up to 48 hours [56]. The cells were harvested at 24 or 48-hour time point. Aliquots of the harvested cells from each time point were diluted into 0.4% trypan blue/PBS solution at a ratio of 1:10. The cells were counted in triplicate and the average was recorded as the cell number for each sample. To validate the results of the trypan blue cell proliferation assay we performed MTT assay. Briefly, 6×10^6 cells were untreated or treated with 100 ng/ml of IL-21 for 24, 48 or 72 hours in 96 well plates. Next, we added 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to each of the wells. The plates were incubated at 37°C to permit reduction of the MTT by the electrons from the viable cells. Four hours later, the MTT formazan produced in wells containing live cells appeared as black, fuzzy crystals at the bottom of the wells. Next, we added isopropanol and HCl (100 μl) to each of the wells and mixed thoroughly. After 1 hour, the absorbance of stable blue color developed in the upper phase of the cell pellets (indicative of cell viability) was measured in a microplate reader at 750 nm.

2.8 Caspase9 and Caspase3 activity assays as evidence for apoptosis

To determine whether IL-21 induces apoptosis in human U937 leukemia cells and human monocytes, we performed caspase9 and caspases3 enzymatic (colorimetric) assays in cell lysates from untreated and IL-21 treated cells. Leukemia cells (10×10^6) and human monocytes (10×10^6) were pretreated with orthovanade (5 mM) for 30 minutes [57–59]. The cells were either untreated or treated with 100 ng/ml of IL-21 for 24 or 48 hours. The cells were lysed in RIPA buffer, and total lysate protein concentration was determined as indicated above. To assess the lysate for caspases activities, 20 μg total lysate protein from each sample was added to each experimental assay well. For positive caspase activity, we used 20 μg of total

cell lysates from U937 or monocytes treated with sodium butyrate, which is a strong inducer of apoptosis. Caspase9 and caspases3 activities were measured using specific assay kits (MBL International, Woburn, MA) using specific substrate (p-LEHD conjugate) for caspases9 and caspases3 (DEVD-pNA) respectively. The absorbance was read at 405 nm with microplate reader.

2.9 Chemotaxis assay

To determine whether culture medium from IL-21 causes chemotaxis, we placed culture media from untreated U937 cells or from U937 leukemia cells treated with IL-21 for 24 hours in the lower chamber of the Boden Chamber to serve as chemoattractant [60]. Human monocytes ((2×10^5) suspended in RPMI medium) were placed in the upper chamber. After 2-hour incubation in the Boyden Chamber, monocytes in both the lower and upper chambers counted as instructed by the kit supplier.

2.10 Luciferase assay

To determine whether IL-21 modulates Stat3 promoter function, we performed STAT3-Reporter gene analysis [61, 62]. STAT3-Reporter HeLa stable cells (Panomics, CA.) placed in 96 well plates for overnight incubation. The cells were either untreated or stimulated with different concentrations of IL-21 for different time points up to 24 hours. In some cases the cells were stimulated with different concentration of IL-21 (0.2, 5, 10, 12.5, 50, 75 or 100 μg/ml) for to 8–24 hours. As a positive control for the STAT3-Reporter gene activation, we treated some of the cells with Oncostatin M (Sigma, St. Louis) separately. At the end of the treatments, the medium was aspirated and the cells washed two times with PBS. The cells from each well were lysed in 100 μl lysing buffer and equal amount of protein in 20 μl of the lysate were added to 100 μl of Luciferase Assay Reagent (Promega) and the luciferin expression (as indicated by light production) was measured with a TD-20/20 luminometer.

3. Results

3.1 Effects of IL-21 on cell proliferation and apoptosis in both human leukemia cells and human monocytes

To determine whether IL-21 stimulates cell proliferation in myeloid monocytic leukemia cells, we performed proliferation assays on untreated and IL-21 treated leukemia cells. As shown in **Figure 1a**, IL-21 does not stimulate cell proliferation in human U937 myeloid monocytic leukemia. These cells express IL-21R. Therefore, it was surprising that IL-21 did not promote proliferation as indicated by the outcomes of both the trypan blue and MTT assays in cells treated with IL-21 for up to 48-hours. Furthermore, our results from cell cycle analysis (**Figure 1b**) show that IL-21 does not promote cell cycle progression during a 48-hour treatment. In contrast, IL-21 enhances accumulation of the leukemia cells at G0/G1 phase (**Figure 1c**), suggesting IL-21-induces apoptosis in the leukemia cells. This observation was validated by the results from specific caspase9 (**Figure 2a** and **b**) and caspases3 enzymatic assays (**Table 1**). As can be seen, IL-21stimulates several fold activation of both caspases9 and caspases3 activities in the IL-21 treated U937 cells. IL-21 produced far less apoptotic effect in monocytes as compared to the control (untreated) cells. The ability of IL-21 to stimulate apoptosis shown here is similar to an earlier reportin B cells [17].

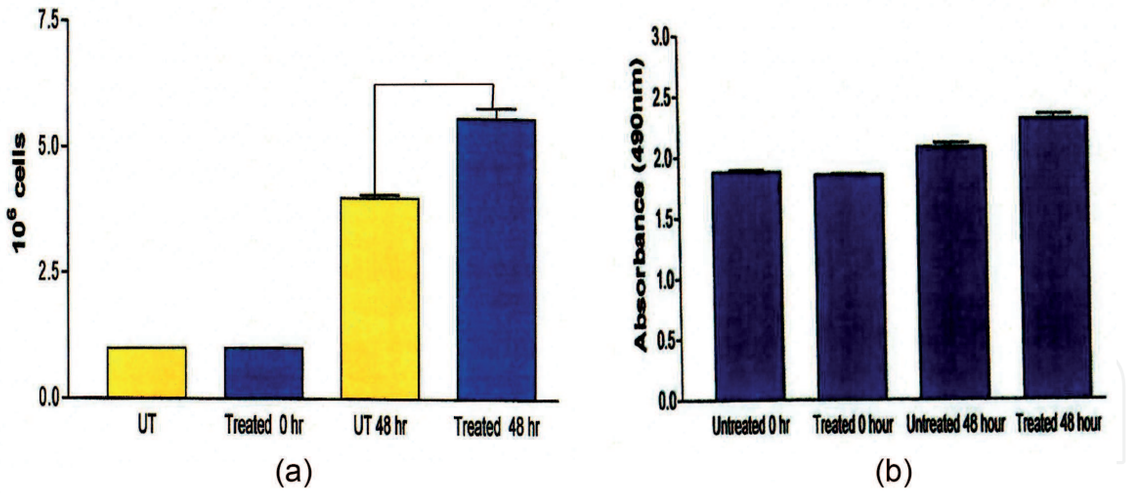

Figure 1.
(a and b) Effects of Il-21 on U937 leukemia cell growth.

3.2 Effects of IL-21 on cytokine and chemokine expression and ERK1/2 activity in human leukemia cells and monocytes

In our earlier reports [7, 46] we should that in both U937 leukemia cells and monocytes, IL-21 promoted time-dependent differential expression of several cytokines in the order of induction: IL7 > IL-15 > IL-2, IL-1, IFN-γ, TGFβ > GM-CSF and chemokines in the order: IL-8 > RANTES, IP-10 > MIP-1a > Eotaxin. Similar results were found in monocytes although to different extent. Furthermore, IL-21 triggers rapid phosphorylation of ERK-1/2 between 2 and 60 minutes in both leukemia cells and monocytes. IL-21-induced ERK1/2 phosphorylation is associated with ERK-1/2 enzymatic activation. The stimulatory effect of IL-21 on ERK-1/2 activation is significantly blocked by a neutralizing antibody against the IL-21R or impartially inhibited by the MEK inhibitor U0126 [7]. These results show that IL-21-induced interleukin and chemokine expression is partially mediated by ERK-1/2-dependent pathway.

3.3 IL-21 utilizes Jak-STAT signaling pathway

Next, we investigated whether IL-21 activates specific members of the Jak/Stat signaling pathway. Cell lysates from untreated cells or IL-21 stimulated cells were analyzed by Western Blot analysis using monoclonal antibody against specific members of the Jak/Stat signaling pathway. In some cases, we performed ELISA assay using specific ELISA assay kit (Ray Biotech Life, Peachtree Corner, Georgia, U.S.A) for specific members of the Jak/Stat signaling pathway [48, 49, 51, 63]. Our results (**Figure 3**) indicate IL-21 causes differential activation of the Jak kinases. Notably, Jak1 and Jak2 are rapidly activated in response to IL-21 stimulation; maximum stimulation occurring within 2–5 minutes. Even though Jak3 and Tyk2 are also activated in response to IL-21, the effect is delayed with maximum stimulation by IL-21 occurring at between 15 and 60 minutes. Because IL-21 stimulates significant activation of the Jak kinases, we examined whether specific members of the Stat family are also activated downstream of the Jak kinases. As shown (**Figure 4**), IL-21 differentially activates specific members of the STAT family. The order of activation was STAT2 > STAT4 > STAT6 > STAT3>. Clearly, IL-2 does not activate STAT1 and STAT5. In order to determine whether Jak2 and or Jak3 is responsible for activating Stat3 and Stat4, we examined the effect of Jak2 inhibitor and Jak3 inhibitor on STAT3 and STAT4 activation. Our results (**Figure 5**) show that in the presence of the

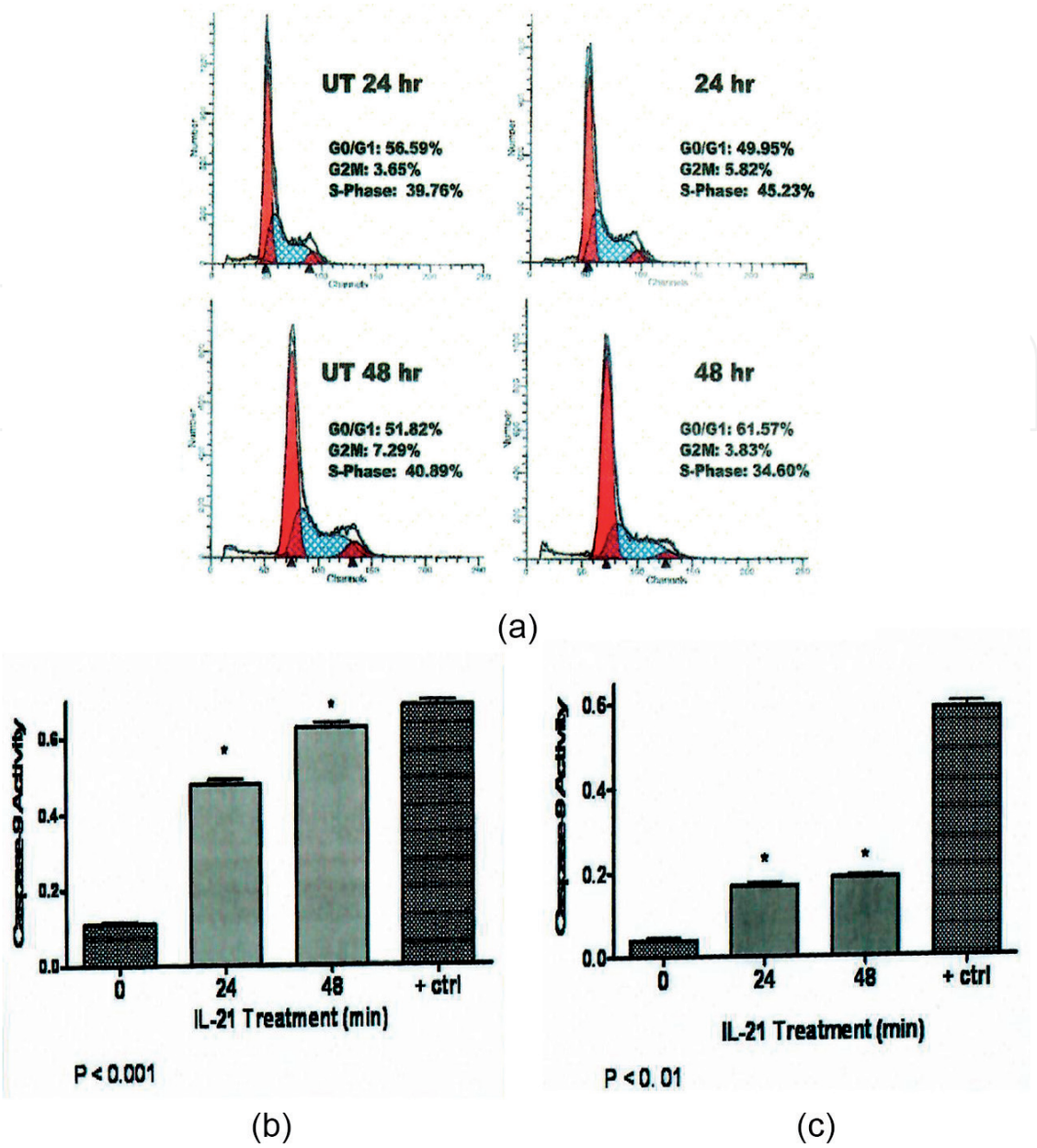

Figure 2.
(a) Effect of IL-21 on cell cycle progression. (b) IL-21-induced apoptosis in U937 leukemia cells. Asterisk (*) indicates significant difference between IL-21 treatment cells and untreated cells. (c) IL-21-induced apoptosis in monocytes. Asterisk (*) indicate significant difference between IL-21 treatment cells and untreated cells.

Experimental condition	U937 cells Caspase3 (fold activation)	Monocytes Caspase3 (fold activation)
Untreated	1	1
Cells + IL-21 (24 hours)	5.3	2.0
Cells + IL-21 (48 hours)	8.2	2.5

IL-21-Induced apoptosis in U937 cells and human monocytes. Leukemia cells (1×10^6) Or monocytes (1×10^6) were untreated (UT) or treated with 100 ng/ml of IL-21 for 24 or 48 hours and cell lysate from each experiment was assayed for caspase3 activity. Data represents an average of two experiments.

Table 1.
IL-21-induced apoptosis in U937 leukemia cells and monocytes.

Jak2inhibitor, there is significant decline in both STAT3 activation. Clearly, our results show that both Jak2 and Jak3 are responsible for STAT3 activation [63]. In contrast, the Jak2 inhibitor and Jak3 inhibitor were only slightly effective in inhibiting STAT4

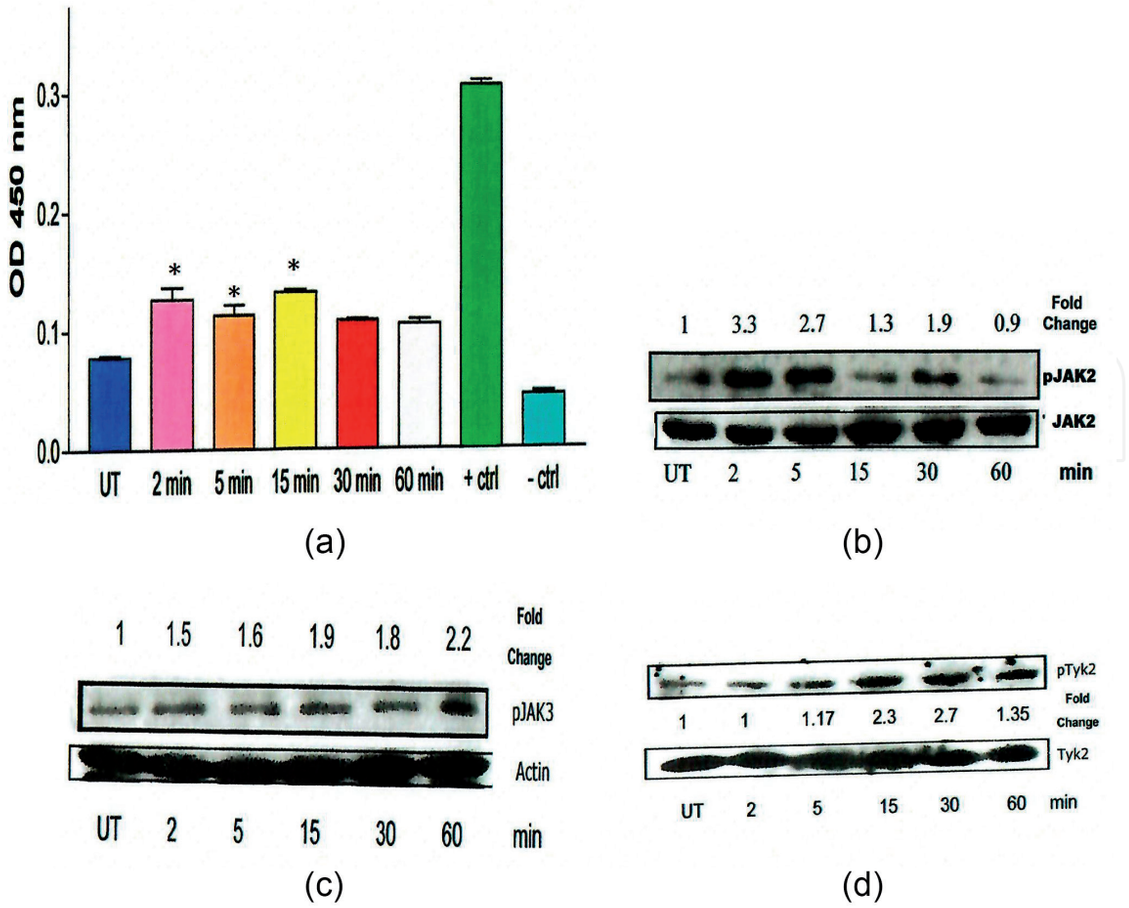

Figure 3.
(a) Effect of IL-21 on Jak1 activation. (b-d) Time course of IL-21-induced differential activation of Jak2, Jak3 and Tyk2.

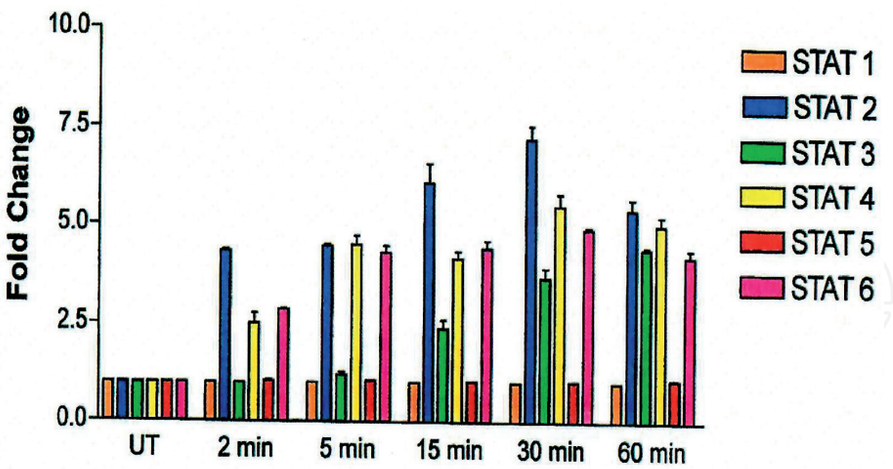

Figure 4.
IL-21 differentially activates STAT1, STAT2, STAT3, STAT4, STAT5 and STAt6.

activation. Hence, our results suggest Jak2 and Jak3may be involved in activating STAT3 [63]. These observations led us to conclude that, Jak2, Jak3 and STAT3 may be relevant for IL-21 induced signaling in leukemia cells and monocytes [63, 64].

3.3.1 Effect of IL-21 on chemotaxis: Role of ERK-1 and Jak/STAT proteins

In our previous report, we showed that IL-21 stimulates differential induction of cytokine and chemokine expression [7, 40] and that the effect is partially dependent

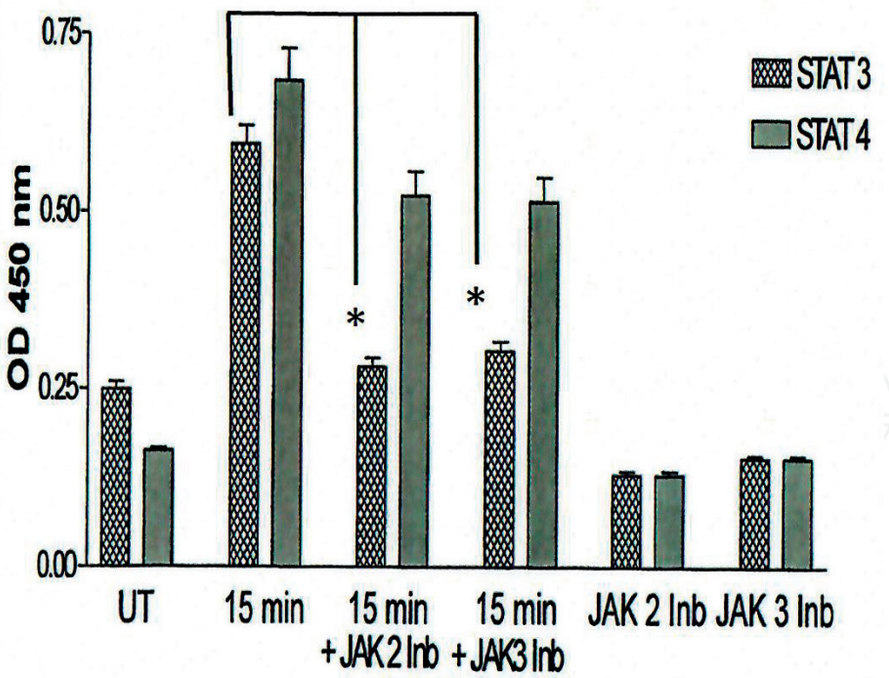

Figure 5.
Effect of Jak2 and Jak3 inhibitors on IL-21-induced STAT activation. Asterisk (*) indicates significant difference between IL-21 treatment alone and either IL-21 treatment in the presence of Jak2 inhibitor or Jak3 inhibitor.

on ERK1/2. Given that some of the chemokines including RANTES, MIP-1α could induce chemotaxis, we determined if the ERK-1/2 and Jak/Stat signaling pathways contributes to induction of chemotaxis. Therefore, we examined the effect of pre-incubation of cells with either ERK1/2 inhibitor or Jak2 inhibitor or with both prior to stimulation of the cells with IL-21 and assayed the cells for chemotaxis. The results (**Table 2**) confirmed that both the ERK1/2 and Jak2 contribute significantly towards IL-21-induced chemotaxis, suggesting that both pathways also contribute to IL-21-induced cytokine and chemokines expression. Thus, IL-21 utilizes both the ERK-1/2 MAPK and Jak/Stat signaling mechanisms for transducing its signal downstream the IL-21R. Perhaps, applications of anti ERK-1/2 and anti-Jak kinases agents could be relevant in blocking unwanted IL-21 effects in leukemia patients.

Experimental condition	Chemotaxis (fold change)
Untreated	1
Cells + IL-21	6.3 ± 0.2
Cells + U0126 + IL-21	3.5 ± 0.3
Cells + AG490 + IL-21	2.5 ± 0.1
Cells + U0126 + AG490 + IL-21	1.3 ± 0.1
Cells + U0126	0.9
Cells + Ag490	0.8

Evidence of involvement of ERK-1/2 and Jak2 in IL-21-induced chemotaxis. U937 leukemia cells were either untreated (UT) or treated with 100 ng/ml of IL-21 in the presence of either MEK inhibitor (U0126) or Jak2 inhibitor (AG490) or with both inhibitors. The effect of the each inhibitor alone was determined. Cells were incubated for 8 hours and culture medium from each experiment was placed in the bottom insert to serve as chemoattractant to monocytes (2×10^5) placed in the upper cup for Boyden Chamber chemotaxis assay. Data represents the mean plus SD from three experiments.

Table 2.
Evidence of involvement of ERK-1/2 and Jak2 in IL-21-induced chemotaxis.

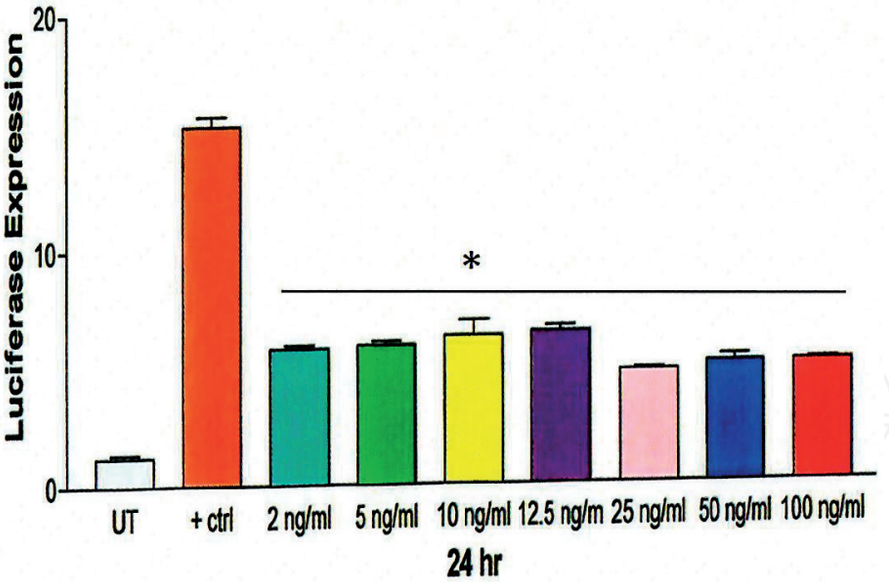

Figure 6.
Effect of IL-21 on STAT3 promoter cloned upstream of luciferase gene in STAT3-reporter-stable Hela cells. Asterisk (*) indicates significant difference between IL-21 treatment cells and untreated cells.

3.3.2 Effect of IL-21 on STAT3 promoter function

After showing that IL-21 regulates STAT3 phosphorylation and its DNA binding activity, we were curious whether IL-21 is capable of regulating the STAT3 promoter function. To address this question, we used a construct containing the STAT3 promoter cloned upstream the Luciferase gene (pSTAT3-Luc) in HeLa cells from human cervical cancer epithelial cells. We allowed cells to recover overnight and divided them into equal number of cells. The cells were either untreated or treated with different concentrations of IL-21 in time course experiments up to 24 hours. Luciferase assay was performed to determine the reporter gene function [61, 62]. The results in **Figure 6** indicate IL-21 induces a 2.5-fold increase in Luciferase activity in IL-21 treated cells as compared to untreated cells. From these results, we conclude that in addition to posttranslational regulation of STAT3, IL-21 is able to activate STAT3 promoter function. In addition, to STAT3 promoter regulation, we also noted that IL-21 regulates activation of several transcriptional factors including AP-2, Smad3 and 4, Pax-5, C-Myc, MEF-1 and CRE [46] that the effects of IL-21 were partially dependent on STAT3 activation since siRNA knockdown of STAT3 led to inhibition of IL-21's effects. By activating these transcriptional factors, IL-21 has broader regulatory roles that will likely impact on expression of genes downstream of these transcriptional factors with potential implicating consequences on the biology of different cell types.

4. Discussions

We have provided strong evidence that IL-21 does not promote proliferation in U937 myeloid monocytic leukemia cells. We have made nearly similar observations in THP-1 and HL-60 leukemia cell lines. However, IL-21 strongly promotes apoptosis in leukemia cells as has been reported for B cells [58]. In contrast, monocytes are somehow resistant to the apoptosis inducing effects of IL-21. These results are novel and could have interesting clinical implications and applications. Activation of caspases9 and caspases3 by IL-21 reported here, could point to

involvement of either extrinsic or intrinsic apoptotic pathway or both and warrant further investigation. Currently, studies are underway to elucidate the potential involvement of mitochondrial in the mechanism of IL-21-induced apoptosis in these cells. Based on its ability to induce apoptosis in leukemia cells while sparing monocytes, IL-21 could be considered as potential anti-leukemia agent to kill myeloid monocytic leukemic cells [58, 59] or in combination with pro-apoptotic agents such as butyrate [65]. Likewise, a combination therapy of IL-21 with other cytokines such as IL-34, which promotes monocyte-like differentiation in leukemia cells, could be beneficial as anti-leukemia agent [66]. The stimulatory role of IL-21 in chemotaxis and invasion of cancer cells could be explored for development of anti-IL-21 drugs to combat IL-21-mediated invasive cancers as previously suggested [67–69].

In both leukemia cells and monocytes, IL-21 induces differential expression of IL-2, IL-7, IL-15, GM-CSF, TGF-β and IFNγ, which are known to regulate diverse cell functions. IL-15 is an effective immunotherapy in conjunction with IL-2. TGF-β induction by IL-21 is also very important since TGF-β is a regulator of cell growth and differentiation in many different cell types. GM-CSF is a promoter of colony formation during bone marrow hematopoietic differentiation as well as a stimulator of specific cell-mediated cytotoxicity against tumor targets. IFNγ and IL-7 are both relevant for regulation of cellular functions [7]. Thus, some of the effects can be beneficial in promoting immune defense in organisms in response to infection by pathogens.

The chemokines including IL-8, RANTES and MIP-1α, secreted to the microenvironment by leukemia cells and monocytes in response to IL-21 in leukemia could trigger of chemotaxis [7] as well as mediate cell–cell communication [70]. The inflammatory and pro-inflammatory cytokines and chemokines produced in response to IL-21 could enhance anti-body secretion and inflammatory responses [71, 72], as well as promote inflammatory diseases including arthritis and SLE [71–74]. Many of the cytokines induced by IL-21 in these cells could be relevant for immunotherapy. In effect, IL-21 could emerge as an effective adjunct immunotherapy for many types of cancer via T-cell activation, thus leading to increasing cytotoxic effects of NK cells [29]. However, we should not overlook the possibility that some of the cytokines and chemokines induced by IL-21 could alter the tumor microenvironment, enhance NK cell antitumor activity against MHC Class-I-deficient tumors, enhance protection against enteric microbial infections, mediate radiation-improved IL-21 cancer therapy, promote major oncological applications and triggering detrimental side effects including inflammation in cancer patients and Crohn's disease [74–80].

Our study was first to provide evidence that activation of the Raf-MEK-MAPK pathway by IL-21 is relevant for some of the biological effects of IL-21 in leukemia cells and monocytes [7]. As shown, IL-21 induces rapid activation of ERK1/2, an effect, which can either be blocked by a neutralizing antibody against the IL-21R or by the MEK inhibitor U0126. Furthermore, our study outcomes suggest that inhibition of ERK-1/2 leads to significant reduction of IL-21-induced cytokine and chemokine expression in leukemia cells and monocytes. However, inhibition of ERK-1/2 by the MEK inhibitor U0126 only partially abrogated IL-21-induced cytokine and chemokine expression, suggesting involvement of signaling pathway (s) independent of Raf-MEK-ERK-1/2.

The Jak/STAT signaling pathway plays major roles in the mechanisms of regulation of cellular functions. Therefore, we examined whether specific members of this pathway are activated by IL-21 in leukemia cells. Our results show that IL-21 activates Jak2, Jak 3 and Tyk2, which are involved in activating several STAT proteins including STAT2, STAT3, STAT4 and STAT6 in both U937 leukemia

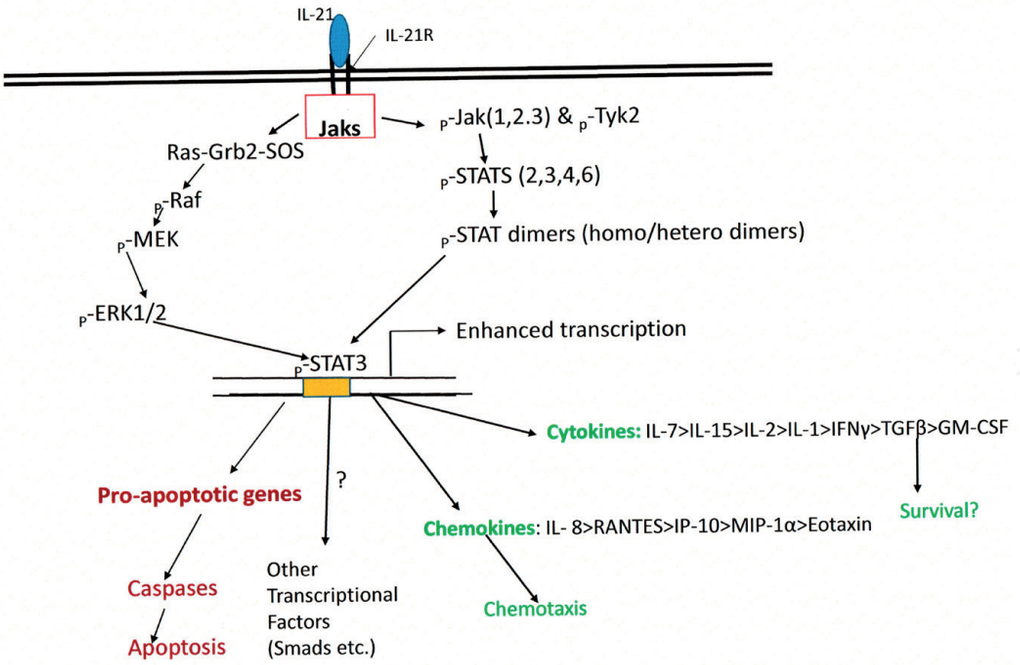

Figure 7.
Proposed signaling model for IL-21 in leukemia cells and monocytes.

cells and human monocytes. Using pharmacological inhibitors specific to either Jak2 or Jak3, we found that Jak2 and Jak3 play critical roles in mediating phosphorylation and activation of STAT3. Furthermore, the induction of chemotaxis by IL-21 is dependent on activation of both ERK-1/2 and Jak2. Our study also revealed that IL-21 activates Jak1 and Tyk2 as well as STAT1, STAT2 and STAT4 similar to earlier reports [71–75]. Taken together, both the Raf-MEM-ERK1/2 and Jak/STAT signaling pathways mediate IL-21-induced cytokine and chemokine expression in leukemia cells and monocytes. However, we are yet to elucidate the roles of STAT1, STAT2 and STAT4 in the biological effects of IL-21 in leukemia cells and monocytes. It remains possible that some of these STAT proteins activated by IL-21 in these cells will have opposing roles as reported in CD+ T cells [75].

The involvement of both the Raf-MEK-ERK and Jak/Stat signaling pathways in mediating signaling from the IL-21R and receptors of IL-2 and IL-15 is well documented [76, 77]. However, our results were among the first to clearly implicate both of these two signaling pathways in the mechanism by which IL-21 induces expression of cytokine and chemokines in leukemia cells and normal monocytes. These observations are interesting in view of the fact that both of these two signaling pathways support cell survival and proliferation and yet IL-21 fails to promote proliferation in the leukemia cells. Additionally, we have shown that IL-21 activates the STAT3 promoter suggesting that genes including anti-apoptotic and pro-apoptotic genes with STAT3 promoter can be regulated by IL-21 in leukemia cells. In addition, IL-21 regulates other transcriptional factors including Smad3/4, AP-2, Pax-5, C-myc, MEF-1 and CRE via partial STAT3-dependent mechanism. As illustrated by our model (**Figure 7**) upon binding to its receptor, IL-21 activates multiple signaling pathways, which are critical to the observed IL-21's biological effects in leukemia cells and monocytes.

The potential for IL-21 to play a role in immunotherapy including its role in cancer therapy is extensively documented [77–82]. Our work points to the need to expand research on IL-21 in different leukemia cells with the anticipation that the outcomes will provide new ideas for exploring IL-21 in combination therapy for

leukemia. Lastly, we propose that targeting the major pathways modulated by IL-21 could lead to new opportunities for treating leukemia.

5. Conclusion

Our findings indicate that both U937 leukemia cells and human monocytes express IL-21R. In these cells, IL-21 induces differential expression of various cytokines and chemokines and promotes chemotaxis. These biological effects of IL-21 could be associated with either negative clinical implications or positive clinical applications in leukemia patients. The Raf-MEM-ERK1/2 pathway and the Jak-STAT pathway play critical roles in induction of cytokine and chemokine expression by IL-21. Activation of the Jak2 and STAT3 is germane to these effects. Furthermore, IL-21 activates apoptosis in U937 leukemia cells with little or no apoptotic effect on human monocytes. The inability of IL-21 to induce apoptosis in human monocytes while inducing significant apoptosis in leukemia cells could form the basis for future application of IL-21 as a potential therapeutic factor for various types of leukemia. Lastly, the ability of IL-21 to regulate the STAT3 promoter function suggests that genes including pro-apoptotic or anti-apoptotic genes with STAT3 promoter sequences are likely to be modulated by IL-21.

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