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IL-17 Biological Effects and Signaling Mechanisms in Human Leukemia U937 Cells

*Samuel Evans Adunyah, Richard Akomeah,
Fareed K.N. Arthur, Roland S. Cooper
and Joshua C.M. Williams*

Abstract

Human Interlekin-17 is produced by memory activated CD4+ T cells and other cells. It was initially considered unique in that its specific receptor is distinct from other cytokine receptors. IL-17 receptor is ubiquitously expressed by different cells including T cells. IL-17 plays a role in regulating growth, immune response and pro-inflammatory responses. It regulates differentiation of a subset of Th0 cells into Th-17 cells, which produce IL-17-induced cytokines. The IL-17R belongs to type 1 cytokine receptors. IL-17 belongs to a superfamily of its own, which includes IL-17A, IL-17B, IL-17C, IL-17E and IL-17F. These members of IL-17 superfamily have some sequence homology but bind to different receptors. Prior to this investigation, limited information existed on the effects of IL-17A in human leukemia cell lines. Our results show that IL-17A promotes growth, anti-apoptotic effects, chemotaxis, cytokine expression and transcriptional factor activation in leukemia cells. IL-17A activates multiple signaling pathways including PI-3 K, Jak-STAT, Raf-ERK1/2 and SRC kinase pathways, which mediate different biological effects of IL-17A in leukemia cells. Our findings implicate IL-17A in leukemia cell growth and survival, supporting potential leukemia therapy via development of anti-IL-17A drugs. This chapter focuses on IL-17A, herein referred to as IL-17.

Keywords: IL-17A, leukemia, cytokines, Jak/STAT, PI-3 K/Akt, ERK1/2 transcriptional factors

1. Introduction

IL-17 is a unique cytokine which was initially discovered through differential and subtractive screening of clones from DNA library from murine lymphoid cells and initially called T-lymphocyte associated antigen 8 (CTLA-8) [1]. IL-17 was found to have 50% sequence homology to the open reading frame 13 (ORF-13) in herpes virus Saimiri [1]. Subsequently, the human homolog of CTLA-8 was identified [1] and its incubation with human fibroblast resulted in induction of both IL-6 and IL-8. This led to renaming of the CTLA-8 human equivalent as IL-17 [2]. Human IL-17 production was also found to be limited to particular cellular elements of the immune system and that activated CD4 + T cells of the Th1/Th0 subset and

stimulated memory T cells, synthesize IL-17 [3, 4]. IL-17 is a glycosylated homodimeric protein of 30 to 35 kDa also produced by nickel-specific T lymphocytes and it regulates I-CAM-1 expression and chemokine production [5]. Unlike other cytokines, IL-17 was noted to bind to a unique receptor distinct from other cytokine receptors [1–3]. IL-17 is ubiquitously expressed in thymocytes activated by CD3 mAb, CD45R0+ population of T cells, CD8+ splenic cells in mouse cells and synovial fluid of patients with rheumatoid arthritis [6–8]. IL-17 is abundantly produced in CD4+ T cells, now known as Th-17 cells [9–11]. It is also expressed in human peripheral blood mononuclear cells (PBMC) in response to ocular lysate in patients with birdshot chorioretinopathy [12]. IL-17 has biological effects in many cells and tissues [13–15]. IL-17 induces expression and secretion of IL-1-beta, IL-6, IL-8, TNF, GM-CSF, G-CSF, ICAM-1, and PGE2 [16–20]. The molecular characterization of IL-17 receptor (IL-17R) was reported in 1997 [21]. IL-17R is a type 1 transmembrane receptor and it is a single chain, which shares some properties with IL-2R-beta chain, and GM-CSFR, all of which are type 1 membrane receptors [21, 22]. IL-17R is also expressed in synovial endothelial cells and Chondrocytes from arthritis patients [23, 24].

Five different IL-17 ligands are now characterized as members of IL-17 superfamily of cytokines and differ from other cytokines but share some sequence homology with each other [25]. Among the IL-17 super family, IL-17A is most commonly expressed in many tissues as well as in cells of hematopoietic origin including monocytes and macrophages. In addition to IL-17A, there are IL-17B, IL-17C, IL-17E and IL-17F [25, 26]. IL-17B, IL-17C and IL-17E expression are widespread in many tissues including testis, brain and kidney [25, 26]. Each IL-17 family members have their individual specific receptor as these IL-17 family members do not bind to the same receptor type [26–28]. The different members of IL-17 superfamily have different expression patterns but with similar abilities to stimulate cytokine effectors illustrating the potential for the members of the IL-17 superfamily to differentially regulate cellular responses in a wide variety of cells [28–31].

IL-17 regulates hematopoietic cell proliferation, immune response, pro-inflammatory responses [32–35] and activate specific types of T cells now known as Th-17 cells [9, 10]. These Th-17 cells play a role in host defense against extracellular pathogens by mediating recruitment of neutrophils and macrophages to infected tissues [9–11]. Th-17 cells secrete IL-17 cytokines, which in turn induce expression of IL-17-dependent cytokines. Hence, aberrant regulation of Th-17 cells may play a role in the pathogenesis of multiple inflammatory and autoimmune disorders [9–11]. IL-17 promotes chemotaxis in human monocytes and regulates angiogenesis and cytokine production in endothelial cells [12, 36–38]. Although the target cells of IL-17-mediated signaling include immune cells such as neutrophils and macrophages [32–38], majority of IL-17's biological effects were seen in cells of either epithelial or mesenchymal origin [39–43]. The role of IL-17 in immunological function was initially examined in vivo in mice by overexpressing IL-17 in the liver of mice where an enhanced granulopoiesis and leukopoiesis led to an 80% increase in splenic mass [38, 39]. IL-17-induced accumulation of neutrophils in the airways requires involvement of GM-CSF [44]. Also, regulation of endogenous stem cell by IL-17 requires both GM-CSF and Stem Cell Factor (SCF) [45]. IL-17 and G-CSF are synergistically involved in the maintenance of normal granulopoiesis [45]. The IL-17R is ubiquitously expressed [12–15] and may explain the ability of IL-17 to stimulate peripheral blood stem cells in mice [44, 45]. Also, There IL-17 plays active in vivo role in chemoattraction of cells of immune system [46, 47]. In addition, IL-17 exhibits paracrine effects in different cell types [48] whereby secreted IL-17 from T cells binds to its putative receptor on neighboring cells such as fibroblasts and trigger signaling that leads to NF-kB- mediated induction of

expression and secretion of ICAM-I, IL-2, IL-6, IL-8 [16–20] and other cytokines, which produce different biological effects [45–50]. Also some of the T-cell secreted IL-17 become sequestered and neutralized by a soluble IL-17R (sIL-17R) [51].

Clinically, IL-17 is implicated in numerous diseases including arthritis [52–54], classical Hodgkin lymphomas [55–57], multiple myeloma [58–60], airway diseases including asthma [61, 62], musculoskeletal diseases [63, 64], inflammatory bowel diseases (IBDs) [65] autoimmune diseases [66], and different types of cancer [67–69]. Significantly elevated level of IL-17 and IL-17R are found in these diseases and IL-17 and IL-17R are known to promote anti-apoptotic effects and survival mechanisms in some types of cancer [55–66]. In most cases, IL-17 itself and/or IL-17-dependent cytokines produced downstream of the IL-17R, contribute to various pathological conditions associated with these diseases [55–68]. Furthermore, Hox3/IL-17R expression ratio has been implicated in poor prognosis in some breast cancer patients undergoing tamoxifen chemotherapy as IL-17 promotes resistance to chemotherapy in breast cancer [67–71]. IL-17 is also implicated in cervical and ovarian cancer [72–74]. Similarly, expression of IL-17 R-like protein has been detected in androgen independent prostate cancer cell lines and it has been implicated in conferring resistance to apoptosis and promoting prostate cancer via MM7-induced epithelial-to-mesenchymal transition [75–77]. IL-17 is implicated in CNS and other neurological diseases [78–80], and psoriasis [81–83]. Recent reports suggest potential role for IL-17 in the “cytokine storm event” seen in advance Coronavirus Disease 2019 (COVID-19) infection with inflammation, and pro-thrombic events in severe COVID-19 patients [84–87]. Hence, there is strong interests in understanding IL-17’s biology and it roles in COVID-19 patients [85–87]. It is not surprising that IL-17’s role in these diseases [60–87], have prompted experts in pharmaceutical industries to develop anti-IL-17 type therapies for diseases in which IL-17 is implicated [88–90].

Earliest report on IL-17 induced activation of MAP kinases and NF- κ B pathways was made in chondrocytes [91]. Subsequently, IL-17 was shown to activate Raf-MAPK and Jak/STAT signaling pathways in leukemia cells [92, 93]. These reports show that IL-17 stimulates rapid phosphorylation of RAF, Erk-1/2, Jak1, Jak2, Jak3 and Stat1, Stat2 and Stat3 in human leukemia cells [92, 93]. Currently, IL-17 is known to activate and utilize multiple signaling pathways including the aforementioned as well as JNK, p38 and PI-3 K/Akt pathways to produce diverse biological effects [91–96]. Many reports have confirmed IL-17-induced activation of PI-3 K/Akt signaling mechanisms in both normal and transformed cells [96–99]. IL-17 signaling pathways are implicated in human diseases including inflammation and cancer [100]. Furthermore, TRAF and TGF-beta-1/smad2/3 signaling pathways are activated by IL-17 [99–102]. Most of the biological effects of IL-17 were initially observed in different variety of cells but to lesser extent in leukemia cells. Therefore, our rationale for initiating this study was to determine the biological effects of IL-17 in leukemia cells and elucidate the various signaling pathways utilized by IL-17 in leukemia cells. Furthermore, we wanted to determine which transcriptional factors are activated by IL-17. Finally, we wanted to determine whether IL-17 protects leukemia cells from undergoing apoptosis since previous report [75] indicated IL-17—mediates cancer cell resistance to apoptosis.

2. Experiment reagents and protocols

We purchased human U937 and THP-1 leukemia cell lines from American Tissue Culture Collection (ATCC) in Manassas, VA, USA. The cells were cultured in Roswell Park Memorial Institute-1640 media, which contained L-Glut (2 mM). Charcoal-filtered and frozen fetal bovine serum (FBS) was purchased from Atlanta Biological, Georgia, USA. Following careful thawing under sterile conditions we

heated the serum at 55 °C for 45 minutes for inactivation. After cooling, we vacuum filtered the FBS under the hood and stored 50 ml aliquots at minus 20 °C. Both streptomycin and penicillin were purchased from Invitrogen, Carlsbad, CA. Prior to using the culture media, we added FBS to a final concentration of 10% (v/v). To prevent bacterial growth in the culture media, we added penicillin (50 U/ml) and streptomycin (50 U/ml). We cultured the cells in either 25 ml or 50 ml of complete media in tissue culture flasks in a CO₂ incubator set to 5% CO₂, 37 °C and 100% humidity. Typically, we passaged the cells 8x before starting a fresh culture. U937 leukemia cells were used in most of the experiments described here.

2.1 Monocyte isolation

We purchased de-identified human blood samples from New York Blood Center, Long Island, NY and Percoll gradients from GR Health Care, Piscataway, NJ [103]. To isolate monocytes for chemotaxis assays, we isolated peripheral blood mononuclear cells (PBMC) as previously described [103]. After centrifugation to remove all red blood cells, the white blood cells were carefully retrieved and suspended in 10 ml of complete media and spread across the surface of plastic dishes. The plastic dishes were incubated in the incubator for 1.5 hours to allow monocytes to attach to the surface of the dishes. Subsequently, all non-adherent cells were carefully aspirated off and discarded. The attached monocytes were carefully scraped from the dishes and suspended in culture media. The monocytes were about 95% pure based on positive staining for CD14 marker.

2.2 Detection of cytokine expression by cytokine antibody array

To determine the effect of IL-17 on cytokine expression in leukemia cells, we performed cytokine antibody array using tissue media from untreated and IL-17 treated leukemia cells [103]. Specifically, 20million cells were either untreated or treated with IL-17 (100 ng/ml) alone or with IL-17 (100 ng/ml) plus the PI-3 K inhibitor LY20094 (20 uM) or with LY20094 (20 uM) alone for 24 hours. The tissue culture media were filtered to remove debris and their protein concentration determined by Coomassie Blue Protein Assay Kit (Pierce, IL). The culture media containing 50 ug protein from untreated and treated cells were spotted onto each of the cytokine/antibody array membranes containing antibodies for over 42 cytokines (RayBiotech, Corners, GA, USA), and incubated with gentle shaking for 2 hours at 30 °C. This allowed hybridization of each cytokine in the media to its respective cytokine antibody on the array. Next, the media was carefully removed and each membrane was washed 5x with wash buffer (provided by kit) to remove all non-specific binding. Each membrane was incubated for a specified time in the color development solution provided with kit and air dried. The dark spots representing various cytokines were visualized and quantitated by digital image scanning. The spot intensities were converted to fold change relative to the corresponding spots on the membrane of the untreated cells, which was set as 1-fold.

2.3 Western blotting detection of proteins and phosphoproteins

One million leukemia cells per ml media were either untreated (control) or treated with [92, 93] IL-17 (1 ng/ml) for 2, 5, 15, 30, 60 min or with IL-17 (100 ng/ml) at 30 °C treatments up to 48 hours. Next, the cells were rapidly pelleted by micro-centrifugation at 1,800 x g for 3 minutes. The pelleted cells were washed 3x with PBS. The final pellets were collected by centrifugation at 1800 rpm for 3 min and each pellet was lysed in 500 ul of cell lysis buffer A (containing protease

inhibitors, 0.5% Triton X-100, 50 mM NaF and 2 mM Vanadate) [7]. Total cell lysate protein concentration was determined by Coomassie Blue Protein Assay Kit (Pierce, IL) and 240 ug per sample was solubilized in 50 ul SDS gel sample buffer and resolved by 12% polyacrylamide gel electrophoresis. The protein bands were transferred to membrane and the membrane background blocked in a blocking buffer containing 5% milk. The membranes were incubated with specific antibodies to either total PI-3 K, p-PI-3 K, p-Akt^{Ser473}, p-Akt^{Thre308}, total Akt, p-STAT3, total STAT3, p-BAD, p-caspase3 or pGSK3-beta or total actin (for loading control) and protein bands detected [103]. The band intensities were scanned by digital image analyzer for quantitation and the band intensity from IL-17 treated samples compared to the intensity in the untreated sample.

2.4 Co-immunoprecipitation (co-IP) assay/Western blot

Total and phosphorylated Akt can associate with effector proteins [104, 105] via protein-protein interaction [106] and contribute to their regulation. We rationalized that if IL-17 promotes association between p-Akt and any of its downstream effectors, those proteins will be contained in the pulled down p-Akt-antibody complex and can be detected by co-IP/Western blot. To determine whether Akt/p-Akt binds to p-BAD or p-Caspase3 or p-GSK-3 (p-Akt's downstream effectors), we carried out co-IP. Specifically, 240 mg/ml protein from untreated or IL-17 treated cells were suspended in PBS (500 ul) in Eppendorf tubes and incubated with specific antibody that recognizes both total Akt and p-Akt^{Ser473} overnight with gentle shaking at 4 °C. Next, protein A agarose slurry (500 ul) was added to the complex in each tube and incubated for 2 hours at 4 °C with gentle shaking to allow protein A agarose to capture all the phosphoproteins bound to Akt/p-Akt-antibody complex. The tubes were centrifuged at 10,000 x g for 10 minutes at 4 °C, the supernatants carefully removed and the pellets were washed 5x with lysis buffer (see above). After the final wash, the phosphoprotein complexes in each tube was solubilized in SDS-gel sample buffer (50 ul) and boiled for 3 minutes to dissociate the phosphoproteins in each complex. The dissociated phosphoproteins were separated on 12% SDS-polyacrylamide gel as described above. The phosphoprotein bands were transferred to membrane and Western blotted for either p-BAD or p-Caspase3 p-GSK-3 or total Akt using specific antibodies. The band intensity for p-BAD, p-Caspase3 and p-GSK-3 were scanned with digital image analyzer. The old levels in the IL-17 treated cells calculated relative to the bands in the untreated cells. Representative results are presented.

2.5 Detection of IL-17RA in U937 leukemia cells and THP-1 leukemia cells

Specific antibody to IL-17AR was purchased from Santa Cruz, CA and goat anti-rabbit IgG-HRP antibody was from Amersham, CA. In order to detect IL-17AR, 240 ug of total cell lysate protein from 40 million cells was separated on 15% polyacrylamide gel [106]. The protein bands were transferred to nitrocellulose membrane followed by incubation in a blocking buffer containing 5% filtered non-fat milk to block non-specific sites on the membrane. Both the IL-17AR antibody and the goat-anti-rabbit antibody were used at dilutions of 1:1000. The rest of the Western blot protocols were performed as described [103, 106]. The band intensity was quantitated using digital image analyzer.

2.6 Transcription factor array

To detect transcriptional factors regulated by IL-17 stimulation in leukemia cells, we examined the profile of 54 transcriptional factors (TFs) using Panomics

Transcriptional Factor Array (1) Kit. Panomics TranSignal™ Protein/DNA Arrays simplifies the functional analysis of eukaryotic TFs and can be used to study TF activation in a variety of biological processes, including cell proliferation, differentiation, transformation, apoptosis and drug treatment [107]. The array membranes were spotted with 54 different consensus-binding sequences (oligos) and enable one to detect over 54 TFs at once in one treatment. Twenty million leukemia cells/ml were pretreated with vanadate (5 mM) for 30 minutes to inhibit endogenous phosphatases. Next, 5 million of the cells were either untreated or stimulated with IL-17 (100 ng/ml) for 4 hours in the incubator. The cells were packed by centrifugation at 1,800 x g for 3 minutes, washed 3x with PBS and gently lysed in a lysing buffer (see Western blot protocols above) without detergents by repeated aspiration through a 22-gauge needle to prevent rupturing of the nuclei. Intact nuclei were isolated by layering the cell lysate over 50% glycerol solution in Eppendorf tubes followed by centrifugation at 1,000 x g for 5 minutes. The supernatants were carefully aspirated, the nuclei pellet harvested and washed 2x with PBS. Next, the nuclei were disrupted in a nuclei lysing buffer (provided by the kit) and protein concentration determined as described above. In a slightly modified version, 12 µg of nuclei proteins in 200 µl of incubation buffer were incubated with each array membrane containing the oligos for hybridization of each oligo to its specific transcriptional factor in the nuclei extract. The membranes were washed several times and the oligo/transcriptional factor complexes (DNA/protein complexes) were detected by detection per the kit. The spots representing the various transcription factors were identified based on the charts provided by the kit. The intensities of the spots were scanned by digital image analyzer and the fold stimulation by IL-17 compared to the intensities in the untreated cells.

2.7 NF-κB/DNA and STAT3/DNA binding assays for detection of NF-κB and STAT3 activation by IL-17

To study the effect of IL-17 on NF-κB and STAT3 DNA binding functions [108, 109], 4 million leukemia cells/ml were untreated or IL-17 in time course experiments. The cells were used for specific NF-κB/DNA binding or STAT3/DNA binding assays using the NF-κB and STAT transcription factor assay kits (Active Motif, Chemicon). The kit enabled us to monitor the activation or repression of NF-κB or STAT3 proteins. The experiments were performed in triplicate.

2.8 Cell proliferation assays

To determine whether IL-17 stimulates cell proliferation in leukemia cells, 6×10^5 cells were either untreated or stimulated with IL-17 (100 ng/ml) for 48 hours [110]. The cells were harvested and aliquots were diluted into 0.4% trypan blue/PBS solution at a ratio of 1:10. The cells were counted in triplicate and the average viable cell count was recorded from each sample. We also performed MTT proliferation assay using 4×10^5 cells untreated or cells treated with IL-17 (100 µg/ml) for 48 hours in 96 well plates in triplicate. The rest of the details of the MTT assay protocols were as previously described [110].

2.9 Caspase3 activity assays as evidence for apoptosis

We purchased caspase3 assay kit from MBL International, Woburn, MA, US [111]. Sodium butyrate (NaB) is a strong inducer of apoptosis in cancer cells [112]. To determine the effects of IL-17 on NaB-induced apoptosis in leukemia cells we performed caspases3 enzymatic (colorimetric) assays in lysates from 15×10^6

untreated or NaB treated cells. Specifically, the cells were either untreated or treated with NaB (5 mM) alone, or treated with NaB (5 mM) plus IL-17 (100 ng/ml) or with IL-17 (100 ng/ml) alone in tissue culture media for up to 48 hours. At the end of the incubation, aliquot of cells were counted prior to lysing in RIPA buffer (provided in the caspase3 assay kit) and total lysate protein concentration was determined as indicated above. To measure caspase3 activity in the cell lysates, 30 ug total lysate protein from each sample was added to each experimental assay well. The enzymatic activity was measured in triplicate in microtiter plate reader according to instructions provided by the kit.

2.10 Assessment of chemotactic effects of media from IL-17 treated cells

In order to ascertain if media from IL-17 treated cells has chemotactic effects towards monocytes [103, 113], we employed the Boden Chamber chemotaxis assay method as previously described [103]. Specifically, 2000 monocytes/ml in fresh complete media were placed in the upper portion of the Boden Chamber. Equal volume of media from either untreated or IL-17 stimulated cells was put in the lower chamber to serve as a source of chemotaxis. The setup was incubated for 2-hours. Next, estimation of number of monocytes which crossed the membrane barrier to the lower chamber was performed according to the kit. Experiments were conducted in triplicate.

2.11 Assessing IL-17-induced cytokine expression by cytokine ELISA

In order to validate IL-17-induced cytokine expression observed in the cytokine antibody array, we performed cytokine ELISA assay as specified in the cytokine ELISA Kits (Ray Biotech, Norcross, GA) using equal amount of tissue culture proteins from untreated or IL-17 treated cells [103]. The ELISA monitored expression of IL-2, IL-3 and IL-8. In addition, to determine whether IL-17-induced IL-2 expression was mediated by either PI-3 K/Akt or by Jak2, in some experiments, we pre-incubated some of the cells with PI-3 K inhibitor LY20094 (20 nM) or Jak2 inhibitor AG490 (15 nM) for 30 minutes prior to stimulating the cells with IL-17 (100 ng/ml) for 24 hours.

3. Results

3.1 Effects of IL-17 on cytokine expression in human leukemia cells

To determine whether IL-17 stimulates cytokine expression in leukemia cells, we performed cytokine antibody array using tissue culture media from untreated and treated cell. As seen in **Figure 1**, within 24 hours IL-17 stimulated several fold differential expression of various cytokines in the ranking order of IL-2 > IL-3 > GRO > IL-10 > RANTES > IL-15 > IL-1. However, IL-17 failed to simulate IL-8 expression. Stimulation of cytokine expression by IL-17 was significantly inhibited by the PI-3 K inhibitor LY20094 (**Figure 1**), suggesting a role for PI-3 K in the mechanism by which IL-17 stimulates cytokine expression. Similar results were observed in THP-1 human leukemia cell line (data not shown). Also, a neutralizing antibody against of IL-17 blocked IL-17 from stimulating cytokine expression (data not shown), confirming that the observed stimulation of cytokine expression is attributed to L-17. Using ELISA assay, we confirmed stimulation of IL-2 and IL-3 expression by IL-17 without effect on IL-8 expression (**Figure 2**). The lack of effect of IL-17 on IL-8 expression seen in leukemia cells are in contrast to IL-17-induced

Figure 1.
IL-17 Stimulation of differential expression of cytokines: Inhibition by PI-3K Inhibitor (LY20094). Tissue culture media from untreated and treated cells were assayed for cytokine expression by cytokine-antibody array. Data is an average of two experiments.

Figure 2.
Effects of IL-17 on IL-2, IL-3 and IL-8 expression. Asterisk () indicates significant differences between IL-17 treated and untreated cells.*

IL-8 expression reported in different cell types [16–20]. As a follow up to our results in **Figure 1**, and our previous report that IL-17 activates Jak/STAT pathway [93], we determined whether both the PI-3 K and Jak2 mediate stimulation of specific cytokine expression by IL-17. To do so, we examined the effects of PI-3 K inhibitors LY20094 (LY) and wortmannin (WM) and Jak2 inhibitor AG490 (AG)) on IL-17-induced IL-2 expression. The ELISA array results in **Figure 3** indicate that individually LY20094 and wortmannin partially inhibited IL-17 stimulated IL-2 expression. The Jak2 inhibitor AG490 also exhibited similar inhibitory effect on ability of IL-17 to stimulate IL-2 expression. A combination of both LY20094 and AG490 completely blocked stimulation of IL-2 expression by IL-17 (not shown). These results confirmed roles for both PI-3 K and Jak2 in the mechanisms by which IL-17 stimulates IL-2 expression.

3.2 Media from IL-17 treated leukemia cells produce chemotaxis

Given that IL-17 stimulated significant expression of two chemokines (GRO and RANTES), we examined whether the culture media from IL-17 treated leukemia cells could serve as chemoattractant to human monocytes from PBMC. As seen in

Figure 3.
Inhibition of IL-17-induced IL-2 expression by PI-3K inhibitors (LY and WM) and Jak2 inhibitor (AG). Asterisk () indicates significant differences between IL-17 alone and IL-17 plus PI-3K Inhibitors (LY and WM) or Jak2 inhibitor (AG490).*

Figure 4, as compared to culture media from untreated cells, culture media from IL-17 treated cells exhibited significant time-dependent chemotaxis towards monocytes, confirming that culture media from IL-17 treated cells contains secreted chemotactic chemokines that induced chemotaxis [46].

3.3 IL-17 stimulates leukemia cell growth and protection from apoptosis

Next, we investigated the effect of IL-17 on leukemia cell proliferation. Untreated or IL-17 stimulated cells were assessed for cell growth using trypan blue exclusion and MTT assays. As shown in **Table 1**, IL-17 exhibited a time-dependent stimulation of leukemia cell growth by 3.3-fold within 48 hours. Similar results were seen in MMT assays (data not shown). Next, we examined whether IL-17 promotes leukemia cell survival and anti-apoptotic effects in leukemia cells by protecting the leukemia cells from apoptosis. The results in **Table 2** indicates that NaB alone causes significant reduction in leukemia cell survival from 100% to 52% in 24 hours. However, in the presence of IL-17, Na-induced decline in cell survival is

Figure 4.
IL-17 induced Chemotaxis. Tissue culture media from untreated IL-17 cells were assayed for chemotaxis in a Boyden Chamber with monocytes on the upper chamber.

Treatment	24 hours Cell Growth (Fold)	48 hours Cell Growth (Fold)
Cells	1.0	1.0
Cells + IL-17	2.3.0	3.3 ± 0.2

Table 1.
Effects of IL-17 on cell growth, cell survival and caspase3 activity—IL-17 promotes cell growth.

Treatment	24 hours Cell Survival (%)
Cells alone	100
Cells + NaB	52 ± 2.2
Cells + IL-17 + NaB	83 ± 1.3

Table 2.
Effects of IL-17 on cell growth, cell survival and caspase3 activity—IL-17 protects cells from butyrate-induced apoptosis.

Treatment	24 hour; Relative Caspase 3 Activity (Fold)	48 hours Relative Caspase 3 Activity (Fold)
Cells alone	1.0	1.0
Cells + NaB	3.2 ± 0.4	4.3 ± 0.1
Cells + IL-17 + NaB	1.7 ± 0.2	2.0 ± 0.3

Table 3.
Effects of IL-17 on cell growth, cell survival and caspase3 activity—IL-17 inhibits butyrate-induced caspase3 activation. Data represent mean plus/minus SD.

markedly inhibited cell survival improved from 52% to 83%. As shown in **Table 3**, NaB alone stimulated activation of caspase3 activity from 1-fold in untreated cells to 3.2 fold in 24 hours and 4.3-fold in 48 hours, indicating NaB-induced apoptosis in cells in the absence of IL-17. However, in the presence of IL-17, NaB-induced caspase3 activation is markedly reduced from 3.2-fold to 1.7-fold in 24 hours and from 4.3-fold to 2.0-fold in 48 hours. IL-17 also upgrades Bcl2 in the presence of NaB (not shown). Thus, IL-17 protects leukemia cells from undergoing apoptosis and enhances their survival. The results suggest that IL-17 may be inducing inactivation of pro-apoptotic signals while partially restoring the anti-apoptotic protein Bcl-2 expression.

3.4 IL-17 stimulates differential activation of transcription factors in leukemia cells

Within 4 hours IL-17 stimulated significant and differential activation of several transcription factors in the order of c-Myb (5.5-fold) > EGR-1 (5.0-fold) > STAT3 (4.0-fold)> > Smad3/4 (3.4-fold) > SRE (3.0 fold>CDP (2.5-.fold). IL-17 failed to activate NF-kB. Using individual transcription factor/DNA binding assays, we confirmed that STAT3/DNA binding activity is significantly enhanced by IL-17 (**Figure 5**). In contrast, IL-17 did not stimulate NF-kB/DNA binding activity in these leukemia cells (**Figure 6**). Together these results show that IL-17 differentially activates several transcriptional factors associated with regulation of cell growth, cell differentiation and apoptosis but failed to stimulate NF-kB in these cells even though IL-17 is known to stimulate NF-kB in many cell types [50]. Of note, NF-kB

Figure 5.
Activation of STAT3 Transcriptional Activity by IL-17.

Figure 6.
Lack of IL-17 stimulation of NF-kB DNA Binding Activity.

is already highly constitutively expressed in active form in these leukemia cells. This could explain why IL-17 failed to stimulate NF-kB activation further in these cells.

3.5 Direct evidence that IL-17 activates PI-3 K/Akt signaling pathway in leukemia cells

Because the PI-3 K inhibitor Ly20094 inhibited IL-17-induced cytokine expression, we examined the direct effects of IL-17 on PI-3 K and Akt phosphorylation and activation. As shown in **Figure 7a** and **7b**, in as early as 0.5 minutes, IL-17 stimulated PI-3 K tyrosine phosphorylation by 4.5-fold. PI-3 K phosphorylation and activation usually lead to downstream Akt (PKB) activation [113]. Therefore, we next examined the effects of IL-17 on Akt (PKB) phosphorylation and activation. Akt can be phosphorylated on Serine 473 (Ser⁴⁷³) and/or Threonine 308 (Thr³⁰⁸), which is in the activation domain. The western blot results in **Figure 8a** show that IL-17 stimulated Akt phosphorylation on Serine⁴⁷³ to 5-fold within 10 min in these cells. The results in **Figure 8b** show that IL-17 stimulates rapid phosphorylation of Akt on Thr³⁰⁸ with maximum effect noted at 5 minutes. Stimulation of Akt phosphorylation on Serine⁴⁷³ by IL-17 was inhibited by the PI-3 K inhibitor wortmannin (WM) (no shown). These results imply that stimulation of Akt phosphorylation by IL-17 is mediated by PI-3 K. Once Akt is activated, it phosphorylates a host of downstream effectors including BAD, Caspase3, forkhead transcription factor (FKHR), glycogen synthase kinase-3 (GSK3-beta), AFX, eNOS, TSC2, MDM2, P21/CIP1 and other downstream effectors as shown in **Figure 9a**. Dephosphorylated BAD, caspase3 and GSK3-beta play vital roles in induction of apoptosis [114].

Figure 7. Time course of IL-17-induced PI-3K phosphorylation detected by Western blot using either specific antibody for tyrosine phosphorylated PI-3K or total PI-3K as loading control. Scanned values represent ptyr-PI-3K/PI-3K ratios from 3 experiments (b). Asterisk (*) indicates significant differences between IL-17 treated and untreated cells. Results are representation form several experiments.

Figure 8. Time course of IL-17 stimulation of Akt^{Ser473} phosphorylation (a) and (b) Akt^{Thr308} Specific antibody to either Akt^{Ser473} or Akt^{Thr308} was used to monitor Akt phosphorylation by Western blot. Blots were stripped and reprobed for total Akt for loading control. The blots from 3 experiments were scanned and results are presented. Asterisk (*) indicates significant differences between IL-17 treated and untreated cells.

However, upon their phosphorylation, these pro-apoptotic proteins lose their pro-apoptotic activities [114] as phosphorylation of both BAD, caspase3 and GSK-3 leads to their inactivation. The results in **Figure 9b** and **c** show that IL-17 stimulates Akt-mediated BAD, Caspase3 and GSK-3-beta phosphorylation as p-BAD,

Figure 9. Model showing activated Akt phosphorylation of its downstream targets (a). Effect of IL-17 on Caspase3 and BAD phosphorylation (b), GSK-3-beta phosphorylation (c). In (b) and (c) cells were untreated or stimulated with IL-17 and total cell lysates were monitored for Caspase3, BAD and GSK-3-beta phosphorylation by Western blot using specific phosphoprotein antibody to each protein. Total Akt (b) or total GSK-3-beta (c) was probed for loading control. Results are representation of several experiments.

p-Caspase3 and p-GSK3-beta were contained in Akt pulled down complex from IL-17 treated cells. Phosphorylation of caspase3, BAD, GSK3-beta and STAT3 are associated with enhanced cell survival [115, 116] and could explain in part how IL-17 promotes cell survival. Also, IL-17 stimulated Akt-dependent phosphorylation of mammalian target of rapamycin (mTor) on serine 2448 (motor^{Ser 2448}) [117], which was inhibited by the Akt inhibitor SH5 (not shown).

4. Discussions

We have provided strong evidence that IL-17 stimulates significance and differential expression of IL-2, IL-3, IL-10, IL-15, GRO, and RANTES in human leukemia cells. The stimulatory effect of IL-17 on cytokine expression in these cells is similar to previous reports in non-hematopoietic cells by IL-17 [16–20]. However, IL-17 does not stimulate IL-8 expression in these cells, which contradicts early reports that IL-17 induces IL-8 expression in different cell types [17–20]. Induction of cytokine expression by IL-17 in these leukemia cells could have strong biological relevance in vivo because increases in IL-17 level in a tumor microenvironment can trigger induction of other cytokines including chemokines that could generate combination of proinflammatory, anti-inflammatory and chemotactic responses [16–20, 54]; [79–81]. IL-2 is a proinflammatory cytokine [118], which also regulates helper T cell differentiation [119]. IL-3 stimulates regulation of multipotent hematopoietic stem cell function and differentiation of all lineages as well as promote proliferation of myeloid progenitor cells [120, 121]. IL-10 is a master regulator of immunity to infection and an anti-inflammatory cytokine that can counteract the pro-inflammatory effects of IL-2 [122]. Secondly, IL-10 is known to synergize with IL-2 to promote CD8⁺ T cell cytotoxicity [123]. IL-15 is known to suppress apoptosis in T-lymphocytes by inducing Bcl2 and/or Bcl-xl in humans [124]. Perhaps, IL-15 contributes to the anti-apoptotic effect of IL-17 in these cells. Both IL-2 and IL-15 have structural and functional similarities, share the common gamma chain of their receptors and promote immune response [125]. Both GRO and RANTES are chemokines and are associated with induction of chemotaxis and recruitment of neutrophils and macrophages to sites of infection [37]. Thus, IL-17-induced GRO and RANTES expression and secretion from leukemia cells into the culture media, could account for the chemotactic effect IL-17 seen in our studies.

These leukemia cells express receptors for some of the cytokines secreted to the culture media in response to IL-17. Therefore, some of the cytokines secreted into culture media can promote both autocrine and paracrine effects on the leukemia cells. We have provided evidence that IL-17 stimulates phosphorylation of the proapoptotic proteins BAD, caspase3 and GSK3-beta, thus negating their functions. In addition, IL-17 promotes inhibition of Caspase3 activity in these leukemia cells. Furthermore, IL-17 enhances Akt phosphorylation and activation, which are associated with cell survival [126]. The ability of IL-17 to enhance protection of the leukemia cells from apoptosis implies that elevated IL-17 levels in a tumor microenvironment could lead to promotion of leukemia cell proliferation and survival, both of which could potentially produce poor prognosis in leukemia patients. Another interesting outcome of this study is that IL-17 stimulates activation of several transcriptional factors including cMyb, EGR-1, STAT3, Smad3/4, SRE, CDP, which are known to regulate proliferation, differentiation and survival [115, 127, 128]. This effect of IL-17 could in part contribute to the mechanism growth promotion and survival in these leukemia cells. Stimulation of smad3/4 transcriptional factors of the TGF-beta signaling pathway [102, 115] by IL-17 may point to potential cross talk between IL-17 and TGF-beta-induced signaling pathways to synergize

their biological effects [127, 128]. The lack of activation of NF- κ B by IL-17 in these leukemia cells is not surprising since typically these leukemia cells constitutively express high levels of active NF- κ B, which could explain the apparent lack of NF- κ B response to IL-17. Lack of NF- κ B activation by IL-17 in these cells is in contrast to IL-17-induced NF- κ B activation reported in many cells [50].

We have provided ample evidence that IL-17 activates and utilizes the Jak/STAT signaling pathway in these leukemia cells. In this pathway, IL-17 stimulates phosphorylation of Jak1, Jak2 and Jak3, STAT1, STAT2, and STAT3 [55, 93, 97]. We have also shown that Jak2 partially mediates IL-17-induced IL-2 expression. Furthermore, IL-17 strongly stimulates phosphorylation and activation of PI-3 K/ Akt pathway and promoting Akt-mediated phosphorylation of its downstream effectors. Another interesting observation is that Akt-partially mediates stimulation of IL-2 expression and secretion by IL-17. Also, IL-17 promotes phospho-Akt's association with BAD, caspase3 and GSK3-beta, supporting Akt-mediated phosphorylation of these proteins in IL-17 treated cells. These observations could in part explain how IL-17 promotes anti-apoptosis and survival in these leukemia cells [75–77, 126].

IL-17 stimulates activation of Raf–MEK–ERK1/2 pathway [92–95, 101], which could partially account for the growth promoting effects of IL-17 in leukemia cells. Previous thesis research in our laboratory revealed that IL-17 stimulates activation of LCK [129] and PKC [130]. Also, IL-17 promotes association between LCK and the p85 subunit of the PI-3 K, thus providing another mechanism for PI-3 K activation by IL-17 via LCK, a member of the Src kinases family [129]. Activation of PKC by IL-17 is associated with enhanced PKC ability to regulate cell cycle progression in leukemia cells [130]. As indicated earlier, IL-17 is profoundly implicated in many human diseases [55–74], thus supporting the suggestion that design and production of anti-IL-17 drugs could lead to better strategies for development of new therapies for those diseases [88–90]. Although the recent reports implicating IL-17 in the mechanism of the “cytokine storm” event in COVID-19 infection is far from

Figure 10. Model showing activated memory T cell secreted IL-17: Paracrine mechanism of how secreted IL-17 activates cytokine expression and secretion in leukemia cells.

Figure 11. Model showing multiple signaling mechanisms used by IL-17 in Leukemia Cells.

conclusion, there are calls for development of anti-IL-17 drugs as adjunct therapy for diseases in which IL-17 plays an active role [87, 131]. IL-17-enhanced leukemia cell growth, survival and anti-apoptosis strengthens the argument in favor of inclusion of leukemia in the list of human diseases for which anti-IL-17 adjunct therapy should be considered. Our model in **Figure 10** explains the paracrine role of T-cell secreted IL-17 in leukemia cells. Elucidation of the multiple signaling mechanisms of IL-17 in leukemia cells in our study and illustrated in **Figure 11** further enrich our knowledge on the biological effects and mechanisms of IL-17.

5. Conclusion

Our studies on effects and mechanisms of IL-17 in human U937 leukemia cells revealed that these cells express IL-17A receptor and IL-17 stimulates cell growth, survival, chemotaxis and differential expression of cytokines. These results suggest that IL-17 could trigger expression and secretion of various cytokines including chemotactic chemokines in leukemia patients. Also, IL-17 promotes anti-apoptotic effects in these cells. If these biological effects of IL-17 described here, were to occur in leukemia patients, IL-17 could promote poor prognosis in the patients. Furthermore, IL-17 stimulates differential activation of several transcriptional factors including c-Myb, EGR-1, STAT3, smad3/4 CDP and SRE but not NF-kB in these cells. Lastly, multiple signaling pathways including PI-3 K/Akt, Jak/STAT, Raf-MEK-ERK-1/2 and Lck signaling pathways differentially mediate the biological effects of IL-17 in the U937 leukemia cells. Any of these pathways could serve as a target for anti-IL-17 drugs.

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Author details

Samuel Evans Adunyah*, Richard Akomeah, Fareed K.N. Arthur, Roland S. Cooper and Joshua C.M. Williams
Department of Biochemistry, Cancer Biology, Neuroscience and Pharmacology,
Meharry Medical College, Nashville, TN, USA

*Address all correspondence to: sadunyah@mmc.edu

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