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Evidence for the Involvement of JAK/STAT Pathway in the Signaling Mechanism of Interleukin-17

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Interleukin-17 is a T-cell-derived pro-inflammatory cytokine, exhibiting multiple biological activities in a variety of cells and believed to fine tune all general phases of hematopoietic response. However, the signaling mechanism of this novel cytokine remains unknown. Here, we report for the first time that the early signaling events triggered by interleukin-17 involve tyrosine phosphorylation of several members of the JAK and STAT proteins in human U937 monocytic leukemia cells. Immunoprecipitation with specific antibodies followed by Western blot analysis with antiphosphotyrosine antibody has shown that in U937 cells, interleukin-17 induces time-dependent stimulation of tyrosine phosphorylation of JAK 1, 2 and 3, Tyk 2 and STAT 1, 2, 3 and 4 within 0.5 to 30 min. Interleukin-17-mediated tyrosine phosphorylation of these proteins strongly suggests that the JAK/STAT signaling pathway may play a major role in transducing signals from interleukin-17 receptors to the nucleus.

Interleukin-17 (IL-17), which was earlier known as CTLA8, is a T-cell-expressed pleiotropic cytokine that appears to be part of a unique ligand-receptor system that regulates the production of various cytokines (1–3). IL-17 was originally cloned from a T cell hybridoma produced by fusion of a mouse cytotoxic T cell clone and a rat T cell lymphoma (4, 5). The human IL-17 was cloned based on its homology to the rodent sequence and to an Open reading frame (ORF) of Herpes virus saimiri (HVS) (1, 5, 6). Besides the rat and human factors, mouse IL-17 has also been cloned (7, 8).

Human IL-17 is secreted by $CD4^+$ activated memory T cells and is a variably glycosylated, 20–30 kDa homodimeric polypeptide (1, 6). The 136 amino acid mature segment of human IL-17 containing one potential N-linked glycosylation site shows a sequence identity of 62.5% and 58% to the mouse and rat sequences respectively (8). The HVS-ORF gene which consists of 151 amino acid residues, has a 57% amino acid residue identity with the rat IL-17 (4, 5, 8) and 72% identity with the human IL-17 (1, 5, 8). The receptor for IL-17 also has been recently isolated from mouse IL4 thymoma cells and has been characterized as a type-I transmembrane glycoprotein of 120 kDa (7, 9).

Though the biological functions of IL-17 have not been fully elucidated, the limited literature available on IL-17 so far suggest that it could function as a major vehicle by which T cells communicate with the hematopoietic system (2). Fibroblasts, when cultured in the presence of IL-17, are able to sustain $CD34^+$ hematopoietic progenitor cells and direct their maturation towards neutrophils (5, 6). IL-17 has also been found to induce the release of IL-6, IL-8, and G-CSF, cytokines which regulate hematopoiesis (10) and also to have synergistic or antagonistic effects with other cytokines (11). However, the signaling pathway(s) utilized by IL-17 in various cells still remain unknown.

It is well documented that cytokines and growth factors transduce signals across the cell membrane that result in the expression of early response genes. One of the key signaling pathways that is instrumental in achieving this is the JAK/STAT signal transduction pathway that involves Janus kinases (JAKs) and Signal transducers and activators of transcription (STATs) (12, 13). First, one or more members of the JAK family (JAK 1, 2, 3 and Tyk 2) of tyrosine kinases associated with a transmembrane receptor is phosphorylated and activated after ligand-receptor interaction. Activated JAKs recruit and phosphorylate one or more of the STAT proteins on tyrosine residues. Tyrosine phosphorylated STATs dimerize, translocate to the nucleus and bind specific promoter elements to regulate gene expression. Thus, the STAT proteins function as a direct link between growth factor receptors and the nucleus by performing a dual role, first as signal transducers by acting as substrates of the JAKs, and after phosphorylation, homo or hetero dimerization, and nuclear translocation, by acting as transcriptional activators. The STAT family includes at least six

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members that can be activated by one or more JAK family kinase in response to specific cytokines (13).

Because of its significance, we wanted to know whether the JAK/STAT pathway plays a role in IL-17R mediated signaling in U937 cells. As revealed by immunoprecipitation and Western blot analysis, here, we report for the first time that in U937 cells, IL-17 mediates rapid tyrosine phosphorylation of Tyk 2, JAK 1, 2, and 3, and STAT 1, 2, 3 and 4, suggesting that the JAK/STAT pathway may be involved in mediating the biological effects of IL-17.

MATERIALS AND METHODS

U937 cells. Human leukemia U937 cells obtained from ATCC were maintained at 37°C under 5% CO_2 tension in RPMI 1640 medium containing 10% FBS and 50 units/ml each of penicillin and streptomycin. Viability of cells was estimated to be greater than 95% by Trypan blue dye exclusion. Cells (3×10^6 per treatment) were pretreated with 5 mM orthovanadate for 30 minutes at 37°C to inhibit endogenous phosphotases. Thereafter, aliquots of vanadate pretreated cells were exposed to various doses of IL-17 for varying periods of time at 37°C. Following treatment, cells were rapidly packed by micro-centrifugation at 13,000 rpm for 20 sec, washed twice in PBS and were lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM Na Vanadate, 1% Brij-35, 0.5% Triton X-100, 10 μg/ml each of aprotinin and Leupeptin, 1 mM PMSF and 1 μM of Microcystin) by extensive vortexing. Samples were spun down to remove cell debris and the total protein content was determined using Coomassie protein assay reagent. For all further experiments, known quantity of the protein samples were used (19).

Human recombinant IL-17 was purchased from R & D Systems, Minneapolis, MN and diluted into tissue culture medium in the absence of fetal bovine serum (FBS). FBS was from Atlanta Biologicals, Atlanta, GA, and Pepstatin A, leupeptin, aprotinin, phenyl methyl sulfonyl fluoride (PMSF), microcystin and EDTA were products of Sigma Chemicals, St. Louis, MO. The anti-phosphotyrosine antibody coupled to horseradish peroxidase (RC20) and antibodies to STAT 1, 2, 3 and 4, JAK 1 and Tyk 2 were from Transduction Labs, Lexington, KY, and antibodies to JAK 2 and 3 from Upstate Biotechnology, Lake Placid, NY. Coomassie protein assay reagent and Supersignal CL-HRP detection reagent were purchased from Pierce, Rockford, IL; protein A-agarose from Boehringer-Mannhein and anti-mouse Ig peroxidase linked antibody from Amersham, Arlington Heights, IL.

Immunoprecipitation and Western blotting. To determine whether IL-17 stimulation of U937 cells causes changes in the tyrosine levels of members of the JAK/STAT family of proteins, we immunoprecipitated the proteins with specific antibodies to Tyk2, JAK and STAT proteins. Briefly, 500 μg of lysate proteins were incubated with 3–5 μg of antibody (as recommended by the manufacturer) with gentle shaking at 4°C for either 3 hours or overnight. Thirty microliters of Protein A agarose were used to capture the immunocomplex. The resulting Protein A agarose-immunocomplex pellet was resuspended in SDS-gel sample buffer and extensively vortexed and boiled for 3 minutes to solubilize the bound proteins. The samples were divided into two equal portions and were analyzed on either 10 or 12% polyacrylamide SDS gels in duplicate along with known molecular weight markers. Protein bands were transferred to nitrocellulose filters by electrotransfer at 4°C and the transfer efficiency was verified by Ponceau staining (0.4% in 5% TCA). The filters with the protein bands were subjected to RC20 Western blot for detection of phosphotyrosine bands as follows. The filters were destained and incubated in TBST blocking buffer (TBS containing 0.1% Tween 20 and 1 to 4% BSA) for 30 minutes at 37°C. Subsequently, the filters were immunoblotted in a blocking solution containing anti-phosphotyrosine antibody (RC20 in a dilution of 1/2500) for 30 minutes at 37°C. Filters were then washed in TBST for 30 minutes at 37°C with changes in buffer every 10 minutes. Finally, they were incubated in supersignal CL-HRP detection reagent for one minute followed by exposure to X-ray film.

To verify that the samples resolved on gels contained equal amounts of proteins, the membrane filters containing the phosphotyrosine blots were stripped by incubation with gentle shaking at 40°C in Tris buffer (0.5 M, pH 6.8) containing 2% SDS and 100 mM β-mercaptoethanol for 15 min. This was followed by 6 washings in a wash buffer (TBST). For detection of the protein levels, after blocking, each filter was immunoblotted in blocking solution containing the specific primary antibody for 30 minutes at 37°C. After 2 to 3 washes with TBST, the filters were exposed to the secondary antibody (anti-mouse IgG-HRP) for 30 minutes at 37°C. The filters were then incubated in supersignal CL-HRP detection reagent for one minute followed by exposure to X-ray film (19).

RESULTS

The role of tyrosine phosphorylation in cytokine-receptor signaling cascades in regulation of cellular functions is very well established as evidenced by the fact that the initial signaling events triggered by several cytokines including IL-2, IL-3, IL-4, IL-6, IL-13 and IL-15 involve rapid induction of phosphorylation of proteins on tyrosine residues (14–18). Also, it is well established that cytokine-mediated phosphorylation of key signaling molecules including the JAK and STAT proteins occurs on tyrosine residues (12, 13, 33). In our earlier studies, it was established that in U937 cells, the optimal dose of IL-17 required to induce tyrosine phosphorylation of various cellular proteins is either 0.5 or 1.0 ng/ml (19).

IL-17 has been considered to play a role in fine tuning all the phases of hematopoiesis including differentiation (2). Because the JAK/STAT pathway has been demonstrated to play relevant roles in transducing signaling from molecules which regulate differentiation (20, 30), our next goal was to determine whether this pathway is utilized by IL-17. To address this issue, U937 cells were exposed to IL-17 (0.5 ng/ml) for various time points. Cellular proteins were immunoprecipitated with specific antibodies to JAK 1, 2 and 3, Tyk 2 and STAT 1 to 4 proteins respectively and Western blotted to RC20, an anti-phosphotyrosine antibody coupled to horse-radish peroxidase (Figs. 1 to 8). Figure 1 shows that in comparison to the untreated control, there was an immediate increase to 1.7 fold in JAK 1 phosphotyrosine level in IL-17 stimulated cells within 0.5 min. Following a slight drop to 1.4 fold at 2 min, increases (2.2 and 2.1 fold) were noticeable at the later time points. Next, we examined the effects of IL-17 on JAK 2 and JAK 3 tyrosine phosphorylation. The results (Figs. 2 and 3) indicate that IL-17 induces maximum increase in tyrosine phosphorylation of JAK 2 (1.7 fold) and JAK 3 (2.2 fold) at 5 and 10 min respectively. We also looked into the changes in the tyrosine

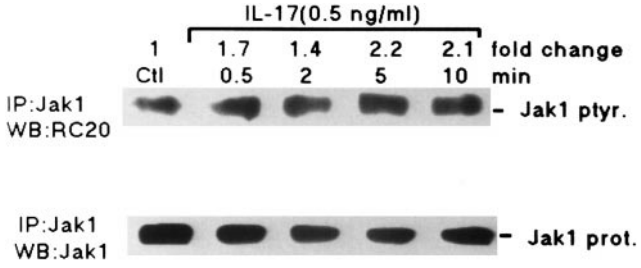

FIG. 1. Effects of IL-17 on JAK 1 tyrosine phosphorylation. Human U937 cells (3×10^6) were either untreated or treated with 0.5 ng/ml of IL-17 for various periods of time. Total cell extract (500 μg protein) was immunoprecipitated with anti-JAK 1 antibody. Immunocomplexes were captured on Protein-A agarose and samples were analyzed for tyrosine phosphorylation using RC20. JAK 1 protein was detected by Western blot hybridization of the stripped membrane with anti-JAK 1 as the primary antibody followed by anti-mouse IgG-HRP as the secondary antibody.

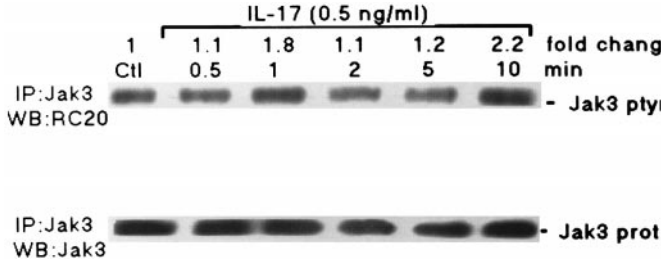

FIG. 3. Effects of IL-17 on JAK 3 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-JAK 3 and probed with RC20. Probes were stripped and reprobed for JAK 3 protein by Western blot hybridization.

phosphorylation levels of Tyk 2 in IL-17 treated U937 cells and observed that following an immediate maximum increase to 1.7 fold by 0.5 min, there was a gradual decline until the last time point of 10 min. Except in the case of JAK 2, with all other members of the JAK family, an increase in tyrosine phosphorylation was seen within 0.5 min.

It was noted that changes in JAK 1, 2 and 3 phosphotyrosine levels were not due to alterations in protein levels. These results suggest that JAK 1, 2 and 3 proteins undergo rapid alteration of tyrosine phosphorylation in response to IL-17 and provide an important evidence that perhaps these proteins may play a key role in transducing signals from IL-17 downstream.

Cytokine-mediated activation of the JAK tyrosine kinases enables these kinases to mediate the tyrosine phosphorylation of specific STAT proteins (30). Therefore, in further analysis, we examined the ability of IL-17 to mediate tyrosine phosphorylation of specific STAT molecules. IL-17-induced stimulation (2.6 fold) of STAT 1 tyrosine phosphorylation was noticeable within 0.5 min. The stimulatory effects of IL-17 on STAT 1 showed an undulating pattern as could be observed by the decline in levels at 1 and 10 min and an increase at the 5 and 30 min time points (Fig. 5). The results in Figs. 6 and 7 demonstrate that a gradual but steady increase in the levels of tyrosine phosphorylation was noticeable with STAT 2 and STAT 3 proteins. The maximum levels were reached at 30 and 10 min time points for STAT 2 (4.5 fold) and STAT 3 (3.8 fold) respectively. In STAT 4 (Fig. 8), tyrosine phosphorylation was found to be rapidly activated by IL-17 as evidenced by the 2.6 fold elevation in the level within 0.5 min. This was followed by a steady decline till 10 min. In all of these cases, the protein levels of the STAT molecules did not contribute to the changes in phosphotyrosine levels (Figs. 5 to 8). The above results indicating the increase in tyrosine phosphorylation levels of several members of the JAK/STAT family strongly suggests their role in the signaling pathway of IL-17 in U937 cells.

DISCUSSION

The limited literature available on the biological activity of IL-17 indicate that it can be considered the newest T cell derived hematopoietic cytokine, that might ultimately have the ability to coordinate or impact all general phases of a hematopoietic response (2). To understand how this cytokine may have an overall control on hematopoiesis, it is essential to elucidate its signal transduction pathway(s). As a major step in this

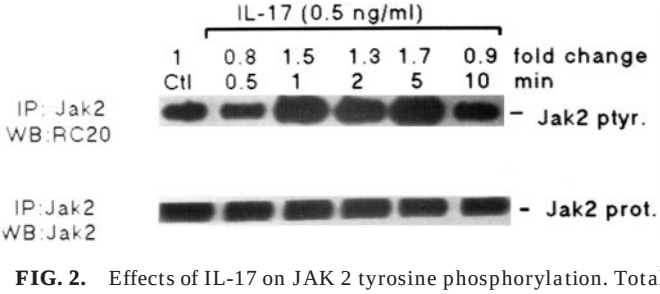

FIG. 2. Effects of IL-17 on JAK 2 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-JAK 2 antibody and probed with RC20. Probes were stripped and reprobed for JAK 2 protein by Western blot hybridization.

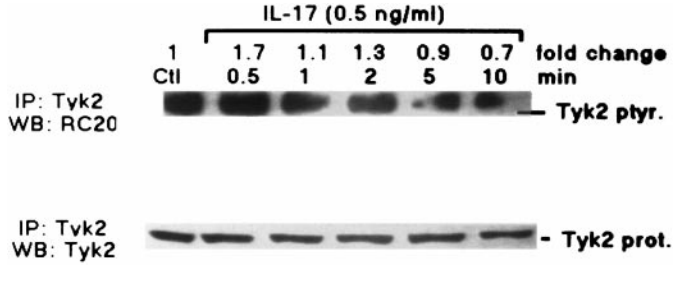

FIG. 4. Effects of IL-17 on Tyk 2 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-Tyk 2 and probed with RC20. Probes were stripped and reprobed for Tyk 2 protein by Western blot hybridization.

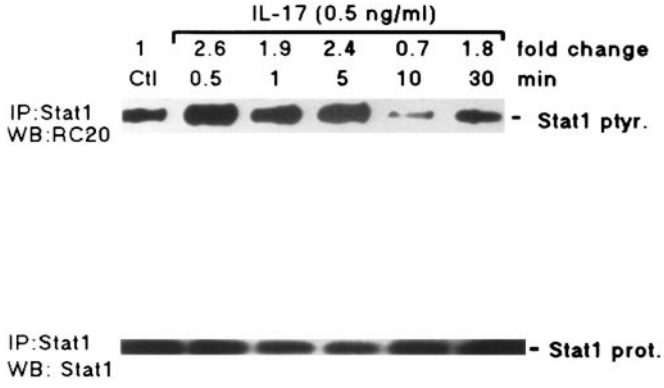

FIG. 5. Effects of IL-17 on STAT 1 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-STAT 1 antibody and probed with RC20. Probes were stripped and reprobed for STAT 1 protein by Western blot hybridization.

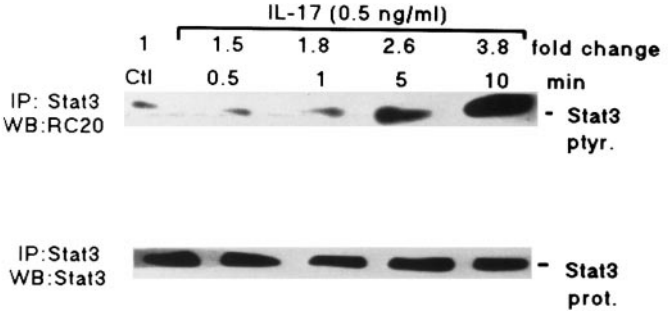

FIG. 7. Effects of IL-17 on STAT 3 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-STAT 3 antibody and probed with RC20. Probes were stripped and reprobed for STAT 3 protein by Western blot hybridization.

direction, we are providing the first evidence that the mechanism of action of IL-17 involves rapid activation of tyrosine phosphorylation of several key signaling molecules belonging to the JAK/STAT family.

Activation of the JAK family kinases followed by STAT protein phosphorylation, represents a defined signal transduction pathway that directly links cytokine receptors to gene expression (12). The binding of many cytokines and growth factors like IL-2 & IL-12 (21), IL-3 (22), IL-4 & IL-13 (23), IL-6 (24), IL-15 (25), EGF (26), LIF (27), G-CSF (28) and GM-CSF (29) to their receptors activates JAKs which are protein tyrosine kinases that are physically associated with the receptors. This is usually followed by rapid tyrosine phosphorylation of STATs which subsequently dimerize (resulting in enhancement of their DNA binding abilities) and translocate to the nucleus, where they activate transcription (31). Whereas the STAT proteins possess SH2 & SH3 domains, the Tyk/JAK kinases have two putative catalytic domains (of which only the carboxyl one is active) but lack SH2 & SH3 domains (32). Of the several conserved structural and functional domains shared by all the STATs, the most critical one is the SH2 domain, which is essential for the recruitment of STATs to activate receptor complexes and for interaction with the JAKs, which phosphorylate the STATs (13, 33).

Early tyrosine phosphorylation events are necessary for the functioning of either the JAK/STAT or the Ras/MAPK pathways. The results from our study clearly indicate that the major pathway(s) utilized by IL-17 include the JAK/STAT pathway as evidenced by the rapid stimulation of phosphorylation of the JAKs (JAK 1, 2 and 3, Tyk 2 and the STAT proteins (STAT 1, 2, 3, and 4). Increases in tyrosine phosphorylation of these molecules was noticed as early as 0.5 min in most of these proteins. Thus, treatment of cells with IL-17 appears to lead to immediate activation of JAK 1, 2 and 3 and Tyk 2.

It has been shown that one or more STAT proteins are activated in response to all cytokines that utilize cytokine receptor superfamily members (30, 31). The STAT family is also characterized by association with one or more members of the JAK family, whereby ligand binding results in aggregation of receptor chains and the associated JAKs, allowing transphosphorylation and activation of JAK catalytic activity. JAK activation is required for initiation of multiple signaling

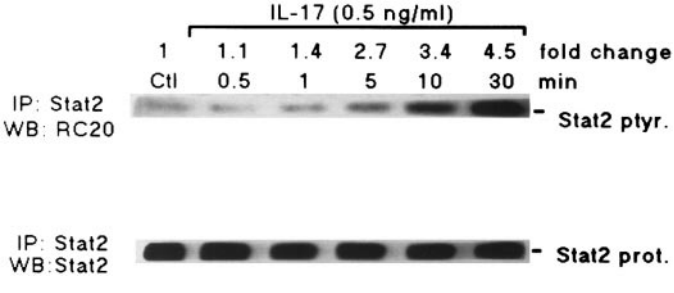

FIG. 6. Effects of IL-17 on STAT 2 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-STAT 2 antibody and probed with RC20. Probes were stripped and reprobed for STAT 2 protein by Western blot hybridization.

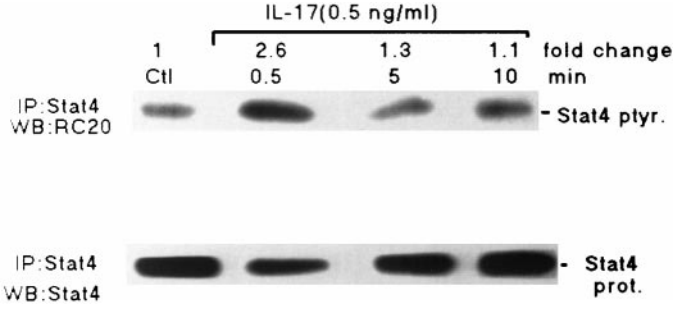

FIG. 8. Effects of IL-17 on STAT 4 tyrosine phosphorylation. Total cell extract (500 μg protein) was immunoprecipitated with anti-STAT 4 antibody and probed with RC20. Probes were stripped and reprobed for STAT 4 protein by Western blot hybridization.

pathways including the Ras/MAPK pathway in response to cytokines (30) and JAK 2 has been specifically shown to play a key role in this process (34, 35). Consistent with this, studies in our lab have shown that cRaf, a major player in the Ras/MAPK pathway to be involved in the signaling of U937 cells by IL-17 (19). The fact that we were able to observe activation of JAK 1, 2 and 3 in our present study strongly suggests that the JAKs, especially Jak 2 could be a major factor in initiating the multiple signaling pathways induced by IL-17 in U937 cells.

The function of STAT proteins and the mechanism through which specificity is reached in each particular cytokine signaling system have not yet been clarified. However, there is growing evidence that STAT proteins are likely to play an important role in mitogenesis, survival or differentiation (12). The STAT proteins are differentially phosphorylated in response to different cytokines in different cells. Activation of different STAT proteins leads to induction or stimulation of expression of a variety of genes which participate in tissue injury, inflammation and immune response processes (30).

The STAT family includes at least six members that can be activated by one or more JAK family kinase in response to specific cytokines. Each cytokine receptor tends to signal through a subset of STATs, typically leading to the phosphorylation of one to three different STATs. For example, IL-3 and GM-CSF induce tyrosine phosphorylation of STAT 1, 5 and 6 predominantly (12). However, it is interesting to note that in our study there was stimulation of four of the six known STAT proteins (STAT 1 to 4). In the above context, the stimulation of phosphorylation of STAT 1, 2, 3 and 4 by IL-17 in U937 cells gives rise to many questions. Since each STAT member is associated with a specific biological function, it would be of interest to know if indeed, IL-17 controls a wide variety of tissue and cellular functions by activating a specific or several members of the STAT family. Certainly, it will be of interest to characterize the nature of the role played by individual members of the JAK/STAT pathway in the mechanism of action of IL-17.

As suggested by previous workers, it is likely that IL-17 functions as a true pleiotropic cytokine which controls several functions like inflammation, immunity and hematopoiesis. IL-17 is known to induce secretion of IL-6, IL-8, G-CSF and PGE2, molecules which have different functions at various stages of the hematopoietic process (10, 19). Furthermore, murine IL-17 is known to stimulate activation of the transcription factor NFkB, which is known to regulate a number of gene products involved in cell activation and growth control (7). Thus, by stimulating the release of a number of cytokines, IL-17 can have broad effects that can be seen as a summation of all events, some of which have the potential to counterbalance each other.

Clearly, this report provides the first evidence indicating the possible role of JAK/STAT pathway in the signaling mechanism of IL-17 in U937 cells.

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