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Cutting Edge: IL-17D, a Novel Member of the IL-17 Family, Stimulates Cytokine Production and Inhibits Hemopoiesis[1]

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CUTTING EDGE

Cutting Edge: IL-17D, a Novel Member of the IL-17 Family, Stimulates Cytokine Production and Inhibits Hemopoiesis[1]

Trevor Starnes,* Hal E. Broxmeyer,† Michael J. Robertson,* and Robert Hromas[2]*

A novel cytokine termed IL-17D was cloned using nested RACE PCR. It is a secreted cytokine with homology to the IL-17 family of proteins. IL-17D is preferentially expressed in skeletal muscle, brain, adipose tissue, heart, lung, and pancreas. Treatment of endothelial cells with purified rIL-17D protein stimulated the production of IL-6, IL-8, and GM-CSF. The increased expression of IL-8 was found to be NF-κB-dependent. rIL-17D also demonstrated an inhibitory effect on hemopoiesis of myeloid progenitor cells in colony formation assays. ***The Journal of Immunology*****, 2002, 169: 642–646.**

Cytokines are secreted proteins that regulate many biological activities, including hemopoiesis and the immune response (1). One recently discovered related group of cytokines is the IL-17 family (2, 3). The IL-17 family has no sequence similarity to any other known cytokines. However, a viral homologue of IL-17 was found in open reading frame 13 of herpesvirus saimiri (HVS13) (4). IL-17 binds to a type I transmembrane receptor termed IL-17R (5). IL-17R is a large ubiquitously expressed protein that also shows no sequence similarity to any other known cytokine receptors, suggesting a new ligand-receptor family (6).

Other IL-17 family members, including IL-17B (7, 8), IL-17C (7), IL-17E (9), and IL-17F (10–12), were cloned by several laboratories including ours. Each of these IL-17 family members shares four highly conserved cysteine residues that are involved in the formation of intrachain disulfide linkages. All of the IL-17 family members also have two or more cysteine residues that may be involved in interchain disulfide linkages as suggested by the homodimeric cysteine knot fold crystal structure of IL-17F (10).

Multiple functions have been reported for the IL-17 family of cytokines that mainly involve regulating the immune response. IL-17 has been shown to induce the production of IL-6, IL-8, G-CSF, GM-CSF, growth-related oncogene-α, IL-1β, TGF-β, TNF-α, PGE_2, and monocyte chemoattractant protein-1 from multiple different cell types including fibroblasts, endothelial cells, epithelial cells, keratinocytes, and macrophages (13–15). IL-17 can induce fibroblasts to secrete IL-6 and G-CSF, which can induce proliferation and differentiation of $CD34^+$ hemopoietic progenitors (16, 17). IL-17 can stimulate granulopoiesis in vivo (18) and induce murine stem cells to rescue lethally irradiated mice (19), suggesting its importance in hemopoiesis.

The IL-17 family has been linked to many disease processes including rheumatoid arthritis (20, 21), asthma (22, 23), lupus (24), allograft rejection (25, 26), tumorigenicity (27), and antitumor immunity (11, 28). The IL-17 family of proteins and their corresponding receptors represent a unique family of cytokines that is not yet fully understood.

In this study, RACE PCR was used to clone a new member of the IL-17 family termed IL-17D, which shares the highly conserved cysteine motif. Genome database searches indicate that this is likely the last undescribed IL-17 family member. IL-17D was found to regulate cytokine production in endothelial cells and showed an inhibitory effect on hemopoiesis in vitro.

Materials and Methods

Cloning

An exon structure fitting the IL-17 family motif was noted at chromosome 13p11. This sequence was used to identify primers for bidirectional RACE PCR. Nested RACE PCR was performed with minor modifications as previously described (11) using the following primers: sense 1, 5′-ccaacct gcgcagcgtgtcgccc; sense 2, 5′-tgggcctacagaatctcctacgacc; and antisense 1, 5′-ggacgcaggtgcagcccacggggatgg; antisense 2, 5′-tgacgtaggcctcggtgtag acgg. The PCR took place at 95°C for 30 s followed by 68°C for 2 min for 35 cycles in the presence of DMSO.

Recombinant protein production

The coding sequence of the mature IL-17D (minus the signal peptide) was subcloned into the pCR T7/NT TOPO TA vector (Invitrogen, Carlsbad, CA). Recombinant protein was produced in BL21 *Escherichia coli* following isopropyl β-D-thiogalactoside induction. Polyhistidine-tagged IL-17D protein was purified to homogeneity as indicated by silver stain using immobilized metal affinity chromatography (His-Bind resin; Novagen, Madison, WI). SDS-PAGE followed by Western blotting with the Anti-Express Ab (Invitrogen) at a 1/10,000 dilution followed by an anti-mouse Ig-HRP Ab (Amersham Pharmacia Biotech, Little Chalfont, U.K.) at a 1/10,000 dilution and detection with ECL^+ (Amersham Pharmacia Biotech) provided confirmation of protein identity. Endotoxin levels were determined using the E-Toxate *Limulus polyphemus* amebocyte lysate assay (Sigma-Aldrich, St. Louis, MO). There was no detectable endotoxin in the IL-17D

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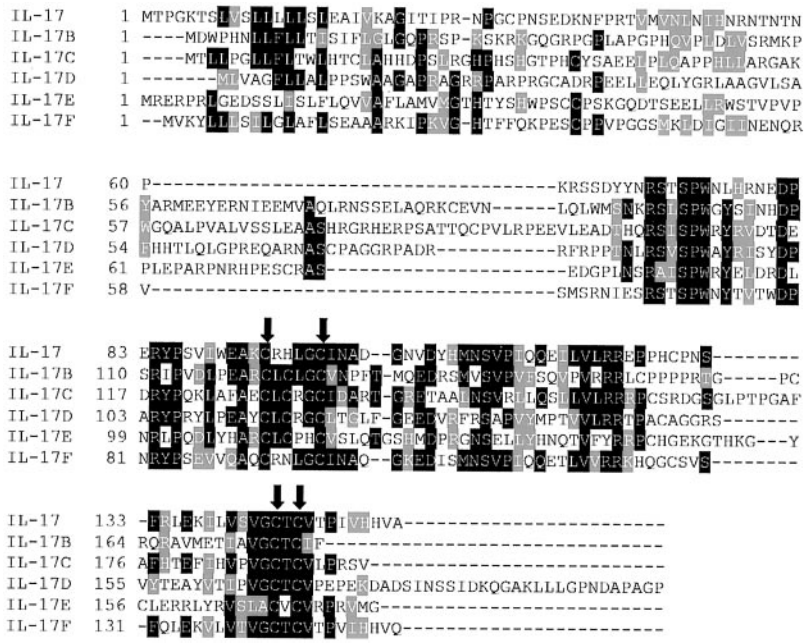

FIGURE 1. IL-17 family amino acid sequence alignment showing all six members. Arrows mark the four highly conserved cysteine residues.

protein preparation down to the levels of detection of the assay (0.06 EU/ml). IL-17 was obtained from R&D Systems (Minneapolis, MN) and IL-17E was a generous gift from A. Gurney (Genentech, San Francisco, CA).

Expression analysis

To analyze the expression pattern of IL-17D, RT-PCR was performed on multiple tissue cDNA panels (BD Biosciences, Palo Alto, CA) using the following primers: sense 3, 5′-caggtacctgcctgaagcctactgc and antisense 3, 5′-cagcaggagcttggcgccctgtttg. PCR was performed for 35 cycles at 95°C for 30 s and 68°C for 1 min. One-tenth volume of the PCR product was size fractionated by agarose gel electrophoresis, and then Southern blotted onto a nylon membrane using 0.4 N NaOH. The Southern blot was probed with ^{32}P end-labeled antisense 1 and antisense 2 primers to increase sensitivity and specificity of the expression analysis (11).

Cytokine production analysis

HUVEC (3×10^6) were plated in 100-mm plates and allowed to adhere overnight. The cells were then treated with varying concentrations of IL-17D or buffer control for 21 h at 37°C, 5% CO_2. Supernatants were harvested and tested in the LINCO*plex* human multiple cytokine ELISA (Linco Research, St. Charles, MO) (29).

Hemopoiesis

Ficoll-purified, normal human bone marrow cells obtained under Institutional Review Board (Indiana University, Indianapolis, IN) approval were assessed for granulocyte-macrophage (CFU granulocyte-macrophage (CFU-GM)),[3] erythroid (burst-forming unit-erythroid (BFU-E)), and multipotential (CFU granulocyte/erythroid/monocyte/megakaryocyte (CFU-GEMM)) progenitor cells following treatment with either IL-17D at 200 ng/ml, heat-inactivated (65°C for 30 min) IL-17D at 200 ng/ml, or control buffer as we described previously (30). Methylcellulose cultures were treated with erythropoietin (1 U/ml), steel factor (50 ng/ml), IL-3 (100 U/ml), and GM-CSF (100 U/ml).

Proliferation assay

PBMC were isolated on Ficoll gradients from cytapheresis buffy coats obtained from normal volunteer donors. Purified T cells were obtained by flow cytometric sorting of CD5$^+$ cells from these Ficoll-separated mononuclear cells. These cells were plated at 100,000 cells per well in 96-well microtiter plates in the presence of medium alone or medium containing 5 μg/ml PHA and varying concentrations of IL-17D or control buffer. The plates were incubated at 37°C, 5% CO_2 for five days followed by addition of tritiated thymidine for the last 18 h of culture. Thymidine incorporation was measured by liquid scintillation counting as described previously (31).

NF-κB activation assay

293 kidney cells (3×10^5) were plated in 6-well plates and allowed to adhere overnight. Transfection with both an IL-8 luciferase reporter construct (2 μg/well) and a *Renilla* luciferase cDNA (20 ng/well) was performed as described previously (32). Cultures were treated 24 h after transfection with TNF-α (20 ng/ml) or an IL-17 family member (100 ng/ml). Cells were harvested after 5 h of treatment and analyzed for luciferase activity using the Dual-Luciferase reporter assay system (Promega, Madison, WI) as described previously (32). Data is normalized to *Renilla* luciferase activity and presented as the fold induction in luciferase activity relative to a nontreated control.

Statistics

Values of p were calculated using the Student t test with two-tailed distribution and two-sample equal variance.

Results

The IL-17D cDNA (GenBank accession no. AF479775) was cloned using nested RACE PCR. The compiled cDNA sequence was mapped to chromosome 13p11 (contig NT_009917.7). This region of the chromosome has been linked to translocations found in Hodgkin's lymphoma (33, 34). The translated protein consists of an open reading frame 202 aa in length, making it the largest IL-17 family member. The signal peptide is composed of amino acids 1–17 with predicted cleavage between amino acids 17 and 18 (www.cbs.dtu.dk). IL-17D is most homologous to IL-17B with 27% identity (7). The highest homology among the IL-17 family members is seen in the C terminus, suggesting that the N terminus may be involved in receptor specificity. All IL-17 family members share four highly conserved cysteine residues that participate in formation of intrachain disulfide bonds (Fig. 1). IL-17D also has four other cysteine residues that may participate in interchain disulfide linkages allowing for the formation of the homodimer, as is common for other IL-17 family members (10), and seen in this study in IL-17D recombinant protein production. Unlike other members of the IL-17 family, IL-17D shows an extended C-terminal domain, which may mediate a unique receptor interaction.

[3] Abbreviations used in this paper: CFU-GM, CFU granulocyte-macrophage; BFU-E, burst-forming unit-erythroid; CFU-GEMM, CFU granulocyte/erythroid/monocyte/megakaryocyte.

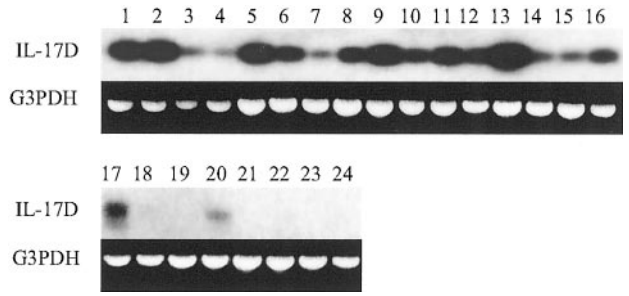

FIGURE 2. Expression analysis of IL-17D by RT-PCR followed by Southern blotting and probing with an internal primer shows high expression in skeletal muscle, brain, adipose tissue, heart, lung, and pancreas. G3PDH control PCR show relatively equal starting cDNA levels. *Lane 1*, adipocytes; *lane 2*, brain; *lane 3*, bone marrow; *lane 4*, fetal liver; *lane 5*, heart; *lane 6*, kidney; *lane 7*, total leukocytes; *lane 8*, liver; *lane 9*, lung; *lane 10*, lymph node; *lane 11*, pancreas; *lane 12*, placenta; *lane 13*, skeletal muscle; *lane 14*, spleen; *lane 15*, thymus; *lane 16*, tonsil; *lane 17*, resting $CD4^+$; *lane 18*, resting $CD8^+$; *lane 19*, resting $CD14^+$; *lane 20*, resting $CD19^+$; *lane 21*, activated $CD4^+$; *lane 22*, activated $CD8^+$; *lane 23*, activated $CD14^+$; *lane 24*, activated $CD19^+$.

Expression of IL-17D was examined using RT-PCR. IL-17D was highly expressed in skeletal muscle, brain, adipose, heart, lung, and pancreas. Lower levels of expression were also present in bone marrow, fetal liver, kidney, leukocytes, liver, lymph node, placenta, spleen, thymus, tonsil, resting $CD4^+$ T cells, and resting $CD19^+$ B cells (Fig. 2). IL-17D was poorly expressed in activated $CD4^+$ T cells, resting and activated $CD8^+$ T cells, resting and activated $CD14^+$ monocytes, and activated $CD19^+$ B cells.

rIL-17D protein was purified to homogeneity using immobilized metal affinity chromatography (Fig. 3*A*). SDS-PAGE followed by Western blot (*lane 4*) confirmed the identity of the protein and showed both the monomeric (26.3 kDa) and dimeric (52.6 kDa) forms of IL-17D.

To determine the effect of IL-17D on hemopoiesis, colony formation assays were performed with Ficoll-isolated mononuclear bone marrow cells using purified rIL-17D protein. IL-17D showed a suppressive effect on myeloid progenitor cell proliferation. At a dose of 200 ng/ml, IL-17D inhibited CFU-GM, BFU-E, and CFU-GEMM colony formation by an average of 39, 32, and 38%, respectively, when compared with buffer-treated cells cultured in methylcellulose (Fig. 3*B*). An average of 41% inhibition of CFU-GM colony formation was also seen when bone marrow cells were cultured in agar, which confirmed the methylcellulose data. Colony formation assays comparing IL-17D to heat-inactivated IL-17D showed a complete loss of the suppressive effect on hemopoiesis, suggesting that the effect seen was a function of IL-17D protein and not contaminating endotoxin.

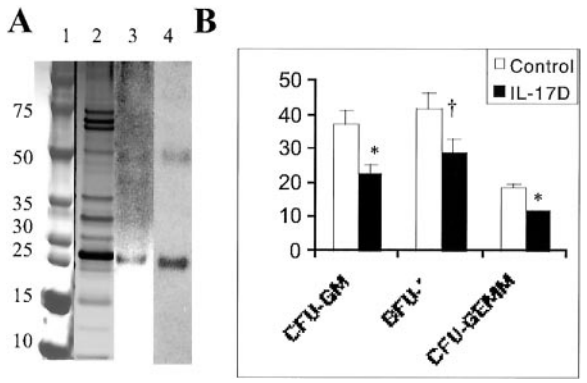

FIGURE 3. *A*, Synthesis and purification of rIL-17D. SDS-PAGE gels, *lane 1*, molecular weight marker; *lane 2*, partially purified sample showing high levels of IL-17D at ~26.3 kDa; *lane 3*, purified IL-17D showing pure monomer (26.3 kDa) and dimer (52.6 kDa) bands after silver stain; *lane 4*, Western analysis confirming the identity of the monomer and dimer protein using an Ab to the expression vector. *B*, Colony formation assay showing inhibitory effect of IL-17D (200 ng/ml) on the proliferation of myeloid progenitor CFU in methylcellulose. Control represents treatment with a similar quantity of IL-17D buffer. *, $p < 0.005$; †, $p < 0.05$.

To further investigate the function of IL-17D, endothelial cells were treated with the purified recombinant protein for 21 h and cytokine production was determined. Supernatants were harvested and cytokine production was measured using a multiple cytokine ELISA. When compared with buffer controls, IL-17D significantly increased the production of IL-6 and IL-8, by 15- and 30-fold, respectively (Fig. 4). HUVEC cells, which normally do not secrete GM-CSF, were significantly induced by IL-17D to secrete GM-CSF (Fig. 4). IL-17D did not cause a significant change in the production of IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, and TNF-α as determined in the multicytokine ELISA.

To determine a possible signaling mechanism for the induction of IL-8 production, transfection assays were completed using an NF-κB-dependent IL-8 promoter luciferase reporter construct

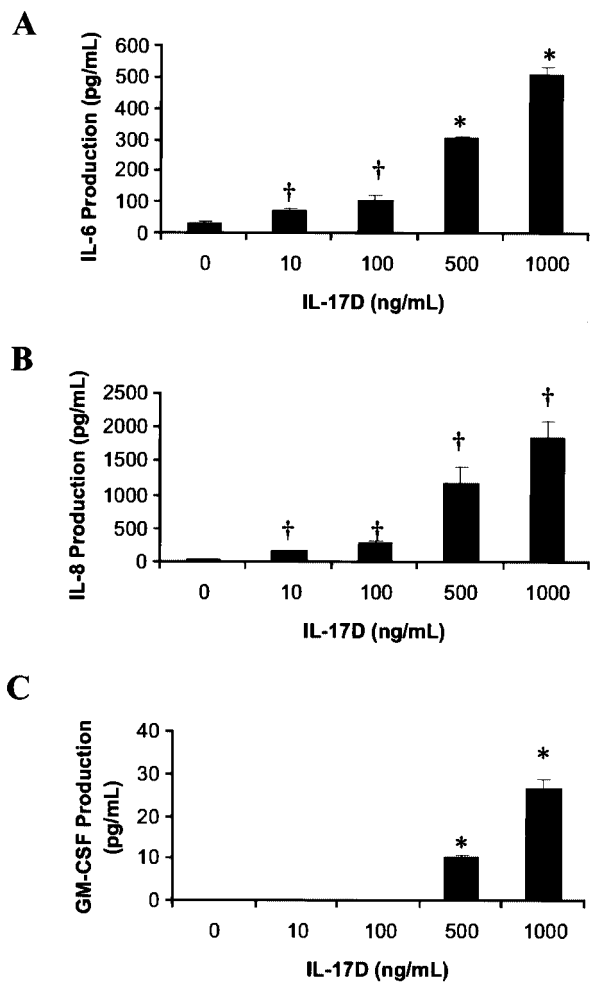

FIGURE 4. Cytokine production following IL-17D treatment of endothelial cells. *A*, IL-6; *B*, IL-8; and *C*, GM-CSF levels are increased significantly (*, $p < 0.005$; †, $p < 0.05$) in supernatant samples following treatment with varying concentrations of rIL-17D protein.

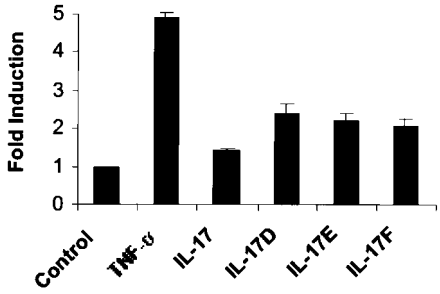

FIGURE 5. Normalized relative luciferase activity following treatment (5 h) with IL-17 proteins (100 ng/ml), TNF-α (20 ng/ml), or buffer control of 293 kidney cells transfected with the NF-κB-dependent IL-8 luciferase reporter construct.

(35). IL-17D, IL-17E, and IL-17F all showed an ~2-fold increase in the activity of the IL-8 gene promoter, whereas IL-17 and TNF-α showed a 1.4- and 4.9-fold increase, respectively (Fig. 5). This suggests that both IL-17D and IL-17F increase IL-8 production in an NF-κB-dependent fashion as was previously shown for IL-17E (9).

In assays of mitogenic activity, IL-17D did not stimulate the proliferation of resting normal PBMCs or flow cytometrically sorted $CD5^+$ T cells. Moreover, no consistent effect of IL-17D was detected on proliferation of PBMCs or $CD5^+$ T cells in response to PHA (data not shown).

Discussion

IL-17D does not appear to have the ability to stimulate the proliferation of immune cells on its own, but it does have the ability to stimulate the production of other cytokines from target tissues such as endothelial cells. This is similar to other members of the IL-17 family, which are thought to indirectly modulate the immune response by regulating cytokine production. The cytokines induced by IL-17D, such as IL-6, IL-8 and GM-CSF, are similar to those induced by other IL-17 family members (13). Certainly one role for IL-17D could be the amplification of a local primary immune response, in that it induces both myeloid growth factors and chemokines. This could rapidly produce localized infiltration of peripheral leukocytes into a lesion, and stimulate the activation and proliferation of those leukocytes once present at a specific location.

IL-17D is preferentially expressed in skeletal muscle, brain, adipose tissue, heart, lung, and pancreas, which is unusual for IL-17 family members. For example, IL-17F is only expressed in activated monocytes and activated $CD4^+$ T cells (11). It is possible that IL-17D plays a role in local immune responses that might occur in those tissues, perhaps after local structural damage such as in trauma, myocardial infarction, or stroke. It is also possible that IL-17D could be important in the growth or repair of those tissues after such structural damage.

IL-17D also suppresses the proliferation of myeloid progenitors in colony formation assays. This is in contrast to its ability to induce the production of GM-CSF, which stimulates progenitor proliferation. This inhibition may, at least in part, be due to IL-17D-induced production of inhibitory cytokines such as IL-8 (30). Levels of IL-8 produced by IL-17D stimulation of HUVEC are in the physiological range to inhibit hemopoiesis (30). However, the levels of GM-CSF produced by IL-17D treatment of HUVEC are 10-fold lower than that required for stimulation of myeloid proliferation (36). Perhaps IL-17D plays a role in the anemia of chronic disease, where prolonged immune stimulation and increased IL-17D production results in decreased hemopoiesis. Acting directly or indirectly through induced release of other cytokines, negative regulators of hemopoiesis may play critical roles in regulating the hemopoietic response to inflammation (17). IL-8 induction by IL-17D is probably mediated through NF-κB activation, as shown previously for other IL-17 family members (4, 9, 15).

Several common elements become clear from the characterization of the IL-17 family. First, they all appear to induce local cytokine production, especially chemokines, TGF family members, and hemopoietic growth factors. Second, most members of this family do not directly stimulate leukocyte proliferation, as is common among other cytokines. Finally, while distinct members of the family are expressed in different tissues, expression is often correlated with an immune response. Thus, this cytokine family plays an important role in the indirect regulation and rapid amplification of the immune response. IL-17D is likely the last IL-17 family member present within the human genome, completing the isolation and characterization of this cytokine family. The complex interrelated roles each member of the IL-17 family plays in the normal or aberrant immune response is as yet incompletely defined.

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