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IL-17 Stimulates Granulopoiesis in Mice: Use of an Alternate, Novel Gene Therapy-Derived Method for In Vivo Evaluation of Cytokines[1]

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IL-17 is a novel cytokine secreted principally by $CD4^+$ T cells. It has been shown to support the growth of hemopoietic progenitors in vitro; however, its in vivo effects are presently unknown. Adenovirus-mediated gene transfer of the murine IL-17 cDNA targeted to the liver (5×10^9 plaque-forming units (PFU) intravenous) resulted in a transiently transgenic phenotype, with dramatic effects on in vivo granulopoiesis. Initially, there was a significant increase (fivefold) in the peripheral white blood count (WBC), including a 10-fold rise in the absolute neutrophil count. This was associated with a doubling in the spleen size over 7–14 days after gene transfer, which returned to near baseline by day 21, although the white blood cell count remained elevated. There was a profound stimulation of splenic hemopoiesis as demonstrated by an increase in total cellularity by 50% 7 days after gene transfer and an increase in hemopoietic colony formation. A maximal increase in frequency of high proliferative potential colonies (HPPC) (11-fold) and CFU-granulocyte-macrophage (GM) and CFU-granulocyte-erythrocyte-megakaryocyte-monocyte (GEMM) (CFU) (6-fold) was seen on day 3 after IL-17 gene transfer. Both CFU and HPPC remained significantly elevated in the spleen throughout day 21, but at reduced levels compared with day 3. Bone marrow CFU and HPPC were elevated on day 3 only by 75% and 25%, respectively, without changes in total cellularity. Thus, murine IL-17 is a cytokine that can stimulate granulopoiesis in vivo. Since IL-17 is principally produced by $CD4^+$ T cells, this cytokine could have therapeutic implications in AIDS-related bone marrow failure and opportunistic infections. *The Journal of Immunology,* **1998, 161: 6383–6389.**

The immune system is known to be linked to hemopoiesis, which is partly mediated through T cells that exert regulatory function on hemopoiesis via secretion of both stimulating and inhibitory cytokines (IL-2, IL-3, IL-6, IL-8, and granulocyte-macrophage CSF (GM-CSF)[3] (1). However, these cytokines appear to be only a portion of a complex cytokine network that link the immune system to hemopoiesis. Specific regulatory functions of T cells and their role in hemopoiesis have not been elucidated. Recently, a novel cytokine, IL-17, was purified and cloned (2–4). Its production was found to be restricted to activated $CD4^+$ lymphocytes. Although IL-17 does not appear to have a direct effect on human CD34-selected, umbilical cord blood-derived stem cells, coculture of these precursor cells with a fibroblast feeder layer and IL-17 induced proliferation and differentiation (4). These effects were associated with the increased release of hemopoietic active cytokines from the fibroblast feeder cells (IL-1, G-CSF, IFN-γ, IL-6, and IL-8). IL-17-mediated release of cytokines appears to be cell specific. With macrophages, Jovanovic et al. showed a different cytokine profile (TNF-α, IL-1β, IL-1Ra, IL-6, IL-10, and IL-12) (5).

We hypothesized that IL-17 might have a potential regulatory role in hemopoiesis. Repeated attempts to generate transgenic mice that ubiquitously overexpress mIL-17 failed. Therefore, to investigate the in vivo effects of this novel cytokine, we overexpressed the cytokine in vivo using a recombinant adenovirus expression system. This expression system has previously been shown to result in sustained and effective cytokine production in vivo, resulting in a definitive phenotype (6, 7). This novel gene transfer approach permits the study of cytokines that may be otherwise limited by the inability to generate transgenic mice or by the lack of biologically active protein for in vivo use. Moreover, this strategy is not theoretically strain limited, as is transgenics. Furthermore, this gene transfer approach allows the study of cytokines in adult animals without the confounding effects of overexpression during early development.

Adenoviral-mediated cytokine delivery of the mIL-17 cDNA resulted in a leukocytosis predominantly consisting of neutrophils, splenomegaly, and increased cellularity of the spleen. Our data suggest that mIL-17 is a cytokine with in vivo activity and that its transient overexpression can stimulate both medullary and extramedullary hemopoiesis in mice resulting in granulopoiesis.

The data presented in this work demonstrate that mIL-17 exerts important effects on hemopoiesis and secondary granulopoiesis in vivo. These results can potentially be utilized to develop treatment

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[3] Abbreviations used in this paper: GM, granulocyte-macrophage; m, murine; BM, bone marrow; Ad, adenovirus; GEMM, granulocyte-erythrocyte-megakaryocyte-monocyte; HPPC, high proliferative potential colonies; AP, alkaline phosphatase; PFU, plaque-forming unit; Luc, luciferase; h, human; WBC, white blood count; ANC, absolute neutrophil count.

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strategies for patients with a compromised bone marrow status, such as aplastic anemia, either cancer treatment-induced or AIDS-related.

Materials and Methods

Construction of Ad-mIL-17

The mIL-17 cDNA was amplified from an mIL-17 cDNA clone provided by Immunex Corporation (Seattle, WA) by PCR using KlenTaq (Clontech, Palo Alto, CA) and specific primers. The 5′ primer was modified to contain a Kozak consensus sequence immediately before the start codon and contained a *Kpn*I site at the 5′ end. The 3′ primer contained an *Xba*I site in the 3′ end. The cDNA template contained an IL-7 leader sequence as previously described (3). A single 498-bp PCR product was obtained and cloned into PGEM-T (Promega, Madison, WI) and subsequently digested with *Kpn*I and *Xba*I, and the insert was then cloned into pACCMV.PLA (6). The sequence of this construct (pACCmIL-17) was verified by dideoxynucleotide thermal cycling sequencing. This vector was cotransfected into 911 cells with *Xba*I-restricted AdCMVLacZ DNA (6) using calcium-phosphate precipitation (8), and plaques were screened by blue-white selection as described by Schaack et al. (9). Ad-mIL-17 clones were screened by PCR, and protein production was confirmed by a mIL-17 bioassay. One of these clones was chosen for all in vivo studies. Protein expression was confirmed by measurement of mIL-17 bioactivity in virally infected 911 cell supernatants (see below). One clone was used for all subsequent experiments.

Viruses were propagated on 911 cells using endotoxin-free conditions and purified by CsCl as previously described (6, 10). mIL-17 production was measured in 911 cell supernatants as previously reported and described below (2, 3). Virus preparations were screened for replication-competent adenovirus by propagation on A549 cells. This assay has a sensitivity of 1 contaminant per 10^8 PFU. All viral preparations had a PFU:particle ratio of $<$100:1. All lots of recombinant adenovirus contained less than 1 endotoxin unit/ml as measured by the *Limulus* amebocyte lysate assay (Bio Whittaker, Walkersville, MD).

Measurement of mIL-17 by bioassay

mIL-17 plasma levels were determined using the previously described biologic assay by Fossiez et al. (4). Briefly, cell supernatants were incubated with 50,000 BALB/c 3T3 fibroblasts for 16 h. mIL-17-induced mIL-6 release was measured using a commercially available sandwich ELISA (Biosource International, Camarillo, CA).

Plasma samples obtained from individual animals were serially diluted in media and incubated in 96-well plates following the recommendations of the manufacturer. For each experiment, a serial dilution of recombinant mIL17 (R&D Systems, Minneapolis, MN) was treated in an identical manner for the purpose of establishing a standard curve. BALB/c 3T3 cells were harvested at 85–90% confluency by trypsinization with 0.5× Trypsin solution (Life Technologies, Gaithersburg, MD) and resuspended in assay medium. Cells (5×10^4) were added to each well, resulting in a total volume of 150 μl, and the plates were incubated overnight at 37.0°C with 5% CO_2 in a humidified chamber. mIL-6 secretion was measured in the supernatant of each well following the manufacturer's instructions with an mIL-6 ELISA kit purchased from Biosource International. The specificity of mIL-17-induced mIL-6 release in biologic samples was confirmed by performing assays with and without BALB/C 3T3 fibroblasts as well as by incubation with a polyclonal anti-mIL-17 Ab (R&D Systems). Data are reported in mIL-17 U/ml. One mIL-17 unit is defined as the amount that results in release of 1 pg/ml of mIL-17-dependent mIL-6 secretion in this assay.

Western blot for mIL-17

Samples were loaded and separated on a precast 4–20% gradient Tris-Glycine Gel (Novex, San Diego, CA) (1 μl of plasma, 5 μl of supernatant of Ad-mIL-17-infected producer cells, 10 ng of recombinant mIL-17 (R&D Systems). The gel was transferred onto a nitrocellulose membrane (Millipore, Bedford, MA) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). After blocking with 5% skim milk for 1 h, the membrane was incubated overnight with goat anti-mIL-17 (R&D Systems) at a concentration of 0.2 μg/ml before incubation with a secondary alkaline phosphatase (AP)-conjugated detection Ab (rabbit anti-goat IgG-AP conjugate (Sigma, St. Louis, MO). The blot was developed using an AP conjugate substrate kit following the recommendations of the manufacturer (Bio-Rad).

PCR specific for Ad-mIL17

Genomic DNA was extracted from homogenized liver tissue with the QIAamp Tissue kit (Quiagen, Chatsworth, CA). PCR reactions were performed on individual animals from Ad-mIL-17- and AdCMVLuc-treated groups using 100 ng DNA. Primers were designed to span the IL-7 leader sequence as well as the mIL-17 cDNA (17-A, 5′-TGG-TAC-CTC-CAC-CAT-GTT-CCA-TGT-TTC-3′; 17-B, 5′-GTC-TAG-ACT-TAG-GAC-GCA-TGG-CG-3′). This primer design conferred specificity for the mIL-17 gene contained in Ad-mIL-17 genome, but not the endogenous mIL-17 gene. The reaction mix was prepared from a kit following the recommendations of the manufacturer (Promega). The PCR was performed for 40 cycles in a DNA thermocycler (PTC-100, MJ Research, Watertown, MA) using the following conditions: 94.0°C for 1 min, 60.0°C for 1 min, 72.0°C for 1 min, and final 7-min extension at 72°C after completion of cycle 40. After elecrophoretic separation on a 1.5% agarose gel, the ethidium bromide-stained PCR products were examined under the UV light.

Animals

Male C57BL/6 mice ages 4–6 wk were purchased from Hilltop Laboratories (Scottdale, PA) and housed with food and water ad libitum in the vivarium of the Louisiana State University Medical Center (LSUMC). The animals were injected into the internal jugluar vein with 5×10^9 PFU of recombinant adenovirus encoding either mIL-17 (Ad-mIL-17) or luciferase (AdCMVLuc) and were sacrificed at 3, 7, 14, or 21 days. Organs were harvested under sterile conditions; blood was collected via cardiac puncture and transferred into heparinized tubes. Spleens were ground after extraction between two slides, and cells were transferred into medium. Bone marrow cells from both femurs were also flushed into medium (DMEM/15% FBS, Life Technologies). RBC were lysed in splenic specimens with Tris-buffered NH_4Cl. Nucleated cell counts were performed using a hemocytometer and trypan blue staining to account for viability of cells. Smears from peripheral blood were prepared using standard techniques and stained with a modified Wright-Giemsa stain.

Colony forming assays

Methylcellulose cultures for CFU-GM, CFU-GEMM, and CFU-HPPC were performed with MethoCult GF 3434 from Stem Cell Technologies (Vancouver, Canada) following the instructions of the manufacturer (components: 0.9% methylcellulose, 15% FBS, 1% albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml iron-saturated human transferrin, 10^{-4} M 2-ME, 2 mM L-glutamine, 10 ng/ml rmIL-3, 10 ng/ml rhIL-6, 50 ng/ml rm stem cell factor (SCF), and 3 U/ml rhEpo). Briefly, 2×10^4 bone marrow cells or 2.5×10^5 spleen cells were suspended in 1 cc of methylcellulose medium and plated in 35-mm tissue culture dishes (Fisher, Pittsburgh, PA). The dishes were placed into a dedicated incubator and maintained there at 37°C under a 5% CO_2 atmosphere and saturated humidified conditions. Analysis for colony formation was conducted at day 10 for CFU-GM and day 15 for CFU-GEMM using an inverted microscope, with definition of a colony consisting of an accumulation of at least 50 cells.

FACS analysis

Cells (10^5) were washed and incubated on ice with anti-CD45 (neutrophil) and analyzed on a Coulter Epics Elite flow cytometer (Hialeah, FL) as previously described (10).

Statistical analysis

Means of data were calculated by ANOVA with Fisher's follow-up testing. A value of $p < 0.05$ was considered statistically significant.

G-CSF ELISA

Plasma mG-CSF protein concentrations were determined using a specific enzyme-linked immunoassay developed in our laboratory using previously described procedures (11). Murine recombinant G-CSF (mG-CSF) was generously provided by Amgen (Thousand Oaks, CA).

Abs against this protein were produced in rabbits, purified over protein A-Sepharose columns (Pierce, Rockford, IL) and dialyzed against PBS. An aliquot was biotinylated with NHS-LC-biotin (Pierce), and the biotinylated Ab was separated from the unbound biotin by dialysis against PBS. The ELISA was performed using native and biotinylated forms of the Ab as the capture and detection Abs, respectively. For this purpose, the wells of a 96-well plate (Nunc-Immunoplate Maxisorb, Neptune, NJ) were coated with 50 μl of 10 μg/ml capture Ab and incubated overnight at 4°C. All subsequent steps were performed at 25°C. The plates were then washed with wash buffer (0.05% Tween 20 in PBS), and blocked with 2% BSA in wash buffer for 2 h. mG-CSF standards and samples were diluted in a dilution buffer containing an appropriate concentration of FCS in wash buffer to yield similar protein concentrations. Both standards (50–1200

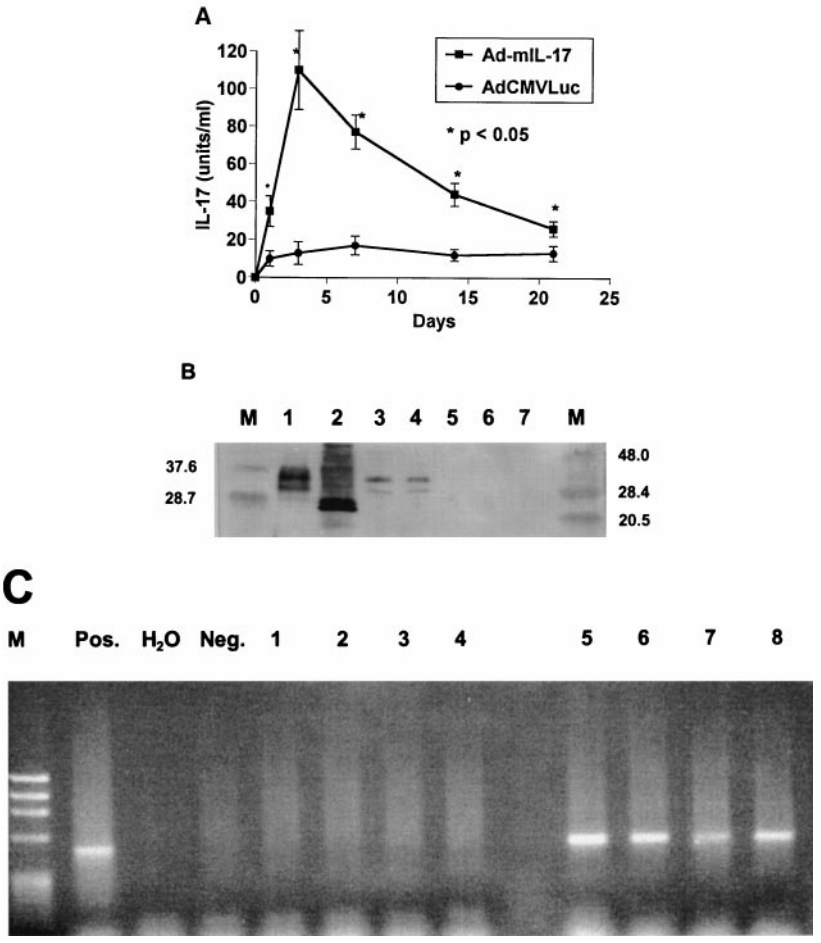

FIGURE 1. *A*, IL-17 pharmacokinetics. Serum obtained from individual mice ($n = 4$ per group) was analyzed at serial time points for mIL-17 bioactivity as described in *Materials and Methods*. Specificity of the reaction was confirmed with anti-mIL-17 serum, which in this assay completely inhibited mIL-17 bioactivity. No mIL-17 expression was detected in control animals; therefore, the depicted AdCMVLuc curve is identical with background mIL-17 levels (AdCMVLuc- or PBS-treated animals). Data are expressed as mIL-17 units and represent the mean value of individual animals ± SEM. *, Value at least $p < 0.05$. *B*, mIL-17 detection in plasma by Western blot. Plasma from individual Ad-mIL-17-treated animals was analyzed for mIL-17 protein at different time points as outlined in *Materials and Methods*: day 3 (*lane 3*), day 7 (*lane 4*), day 14 (*lane 5*), and day 21 (*lane 6*). *Lane 7* is plasma from an untreated control animal. Supernatant from Ad-mIL-17-infected 911 producer cells and recombinant *E. coli*-produced mIL-17 are depicted in *lanes 1* and *2*, respectively. *C*, Demonstration of mIL-17 gene transfer to the liver. Presence of the transferred mIL-17 gene in the liver of treated mice was confirmed at serial time points. A specific 477-bp product (pos. = positive control) is visualized in Ad-mIL-17-treated animals over a period of 21 days (days 3, 7, 14, and 21: *lanes 5-8*), but not in control animals (H_2O, PBS treated, negative mouse, and AdCMVLuc-treated mice (days 3, 7, 14, and 21: *lanes 1-4*)).

pg/ml) and samples (50 μl) were added to wells, and the plates were incubated for 1 h. After washing, 50 μl of biotinylated anti-mG-CSF IgG (10 μg/ml in wash buffer containing 2% FCS) was added, and the plates were incubated for 1 h. Wells were then washed and incubated for 1 h, after adding 100 μl of 0.1 μg/ml peroxidase-conjugated streptavidin (Jackson Laboratories, West Grove, PA) in wash buffer plus 2% FCS. After washing the plate, 200 μl of 1-step turbo TMB-ELISA (Pierce) was added as substrate and color was allowed to develop for 30 min in the dark. After stopping the reaction with 50 μl of 3 M H_2SO_4, plates were read at 450 nm. mG-CSF concentrations were calculated from the standard curve using log-log linear regression. The assay had an interassay coefficient of variation of 4.4%, and the assay failed to detect 1200 pg/ml homologous mouse proteins (growth hormone, prolactin) or murine GM-CSF, IL-3, or IL-6, or *Escherichia coli* LPS.

Results

Expression of mIL-17 in vivo

In vivo production of mIL-17 was confirmed after gene transfer using the mIL-17 bioassay and was specific for Ad-mIL-17-treated animals. mIL-17 levels peaked on day three at 110 units (±10.5), and, although from thereon they steadily declined, they were significantly elevated until day 21 (Fig. 1*A*). Serum from Ad-mIL-17-treated mice stimulated mIL-6 release in the bioassay, and this was completely blocked with serum from rabbits immunized against mIL-17, but not with preimmune or inactivated (boiled) immune rabbit serum (data not shown). mIL-6 serum levels in Ad-mIL-17-treated animals were minimally elevated over AdCMVLuc-treated mice throughout the experiment, indicating minimal in vivo stimulation of mIL-6 through mIL-17 (data not shown). No mIL-17 expression was detected in control animals; therefore, the depicted AdCMVLuc curve is identical with background mIL-17 levels (AdCMVLuc- or PBS-treated animals) (data not shown). mIL-17 bioactivity in serum was highly correlated with the presence of mIL-17 protein in plasma by Western blot (Fig. 1*B*). A signal specific for mIL-17 at molecular mass of 34 kDa was detected by Western blot analysis in plasma of animals treated with Ad-mIL-17 on days 3 and 7 (Fig. 1*B*, *lanes 3* and *4*, respectively). No mIL-17 could be detected by Western blot on days 14 or 21 after gene transfer. No signal was seen in AdCMVLuc-treated control animals at any time point of the study (data not shown).

Persistence of the transferred mIL-17 gene

Presence of the transferred mIL-17 gene in the liver was determined with a specific PCR reaction, which did not detect genomic mIL-17. A 477-bp product was detected only in Ad-mIL-17-treated animals, but not in AdCMVLuc- or PBS-treated animals. Persistence of the gene was seen throughout the duration of the experiment (21 days) (Fig. 1*C*).

Animals

No abnormalities, such as behavioral changes or alterations in activity or food intake, or evidence of morbidity was observed in mIL-17-treated mice over an observation period of 4 weeks. Spleens were enlarged by up to twofold between days 7 and 21 in mIL-17-treated animals (Fig. 2).

Peripheral blood

The peripheral white blood count (WBC) increased by threefold at 3 days and by sixfold on day 7. It remained elevated at fourfold through day 21 (Fig. 3*A*). This was mainly due to the expansion of mature granulocytes comprising 65% and 58% of the WBC at 48 h

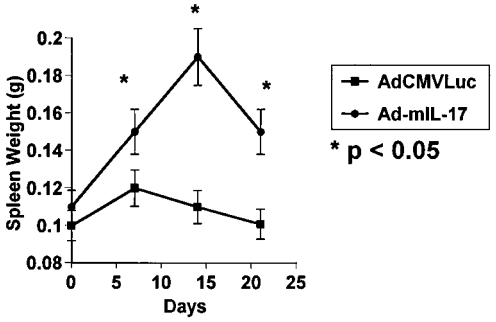

FIGURE 2. Overexpression of mIL-17 induces splenomegaly. Spleens of four individual animals per time point were harvested after sacrifice and weighed. Data represent the mean value of individual animals ± SEM. *, Indicates a value of at least $p < 0.05$.

and 72 h, respectively. The absolute neutrophil count (ANC) comprised 61% at the peak of the WBC on day 7 (Fig. 3*B*). Morphologic analysis was confirmed with FACS using the lineage-specific mAb anti-CD45 (data not shown). This biologic response of granulopoiesis to administration of Ad-mIL-17 followed a statistically significant dose response, with peak values on day 7 (WBC increased twofold with 1×10^9 PFU Ad-mIL-17, threefold with 2×10^9, and sixfold with 5×10^9). This was not observed in AdCMVLuc-treated animals (data not shown). Total lymphocyte numbers increased by twofold on days 7 and 14 in Ad-mIL17-treated animals.

CFUs and precursor frequency

Bone marrow. Frequency of CFUs (CFU-GM and -GEMM) in bone marrow of mIL-17-treated animals increased by approxi-

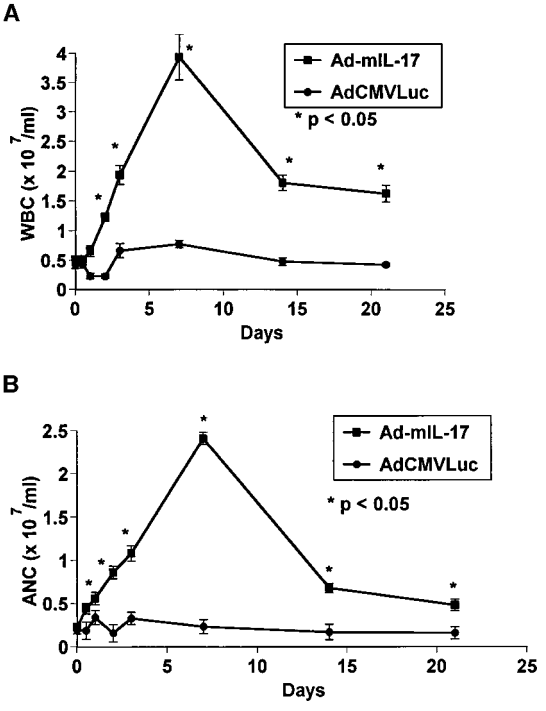

FIGURE 3. mIL-17 causes leukocytosis. Peripheral blood from four individual animals per group was evaluated at serial time points for WBC (*A*) and ANC (*B*). The data represent the mean value of results obtained from individual animals ± SEM. Four separate experiments using the same number of animals showed similar results. *, Indicates a value of at least $p < 0.05$.

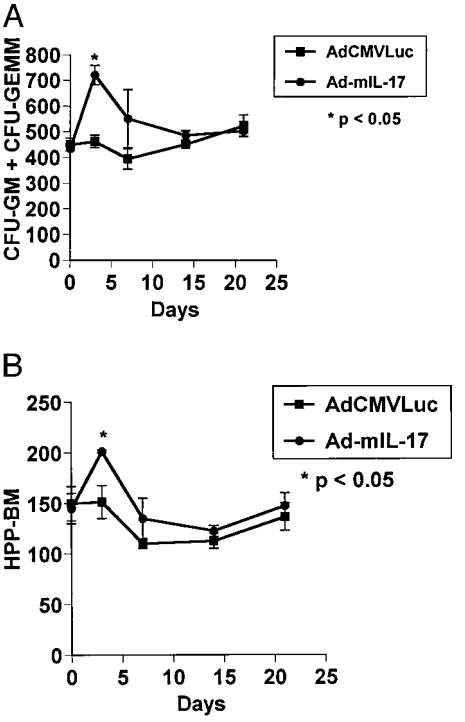

FIGURE 4. IL-17 increases bone marrow colony forming units. Bone marrow colonies were plated from two individual animals per data point as described in *Materials and Methods*, and CFU-GM and CFU-GEEM (*A*) and HPPC (*B*) were enumerated. Data represent the mean value (CFU/10^5 cells) of individual animals ± SEM. *, Indicates a value of at least $p < 0.05$. The experiment was repeated twice with similar results.

mately 75% on day 3 only (Fig. 4*A*). Frequency of HPPC increased by 25% on day 3 only (Fig. 4*B*). Total bone marrow cellularity did not change significantly for the duration of 21 days (Table I).

Spleen. The absolute number of CFUs (CFU-GM and GEMM) increased by eightfold with an increase in frequency of CFU by sixfold on day 3 (Fig. 5*A*). The absolute number of HPPC increased by 16-fold, and the frequency of HPPC increased by 11-fold on day 3 (Fig. 5*B*). On day 7 a 50% increase in total cellularity was seen with a 2.8-fold increase in absolute number of CFUs and a fivefold increase in absolute number of HPPC (Table I). This correlated with a 1.9-fold increase in CFU frequency and a 3.5-fold increase in HPPC frequency. On day 14, total cellularity was still increased by 50%, which returned to baseline on day 21 (Table 1). Absolute number of CFUs remained elevated at two- to threefold on days 14 and 21, whereas absolute HPPC frequency was increased by sixfold. Frequency of CFU was double on days 14 and 21, frequency of HPPC was increased by four- to fivefold on days 14 and 21 (Fig. 5*B*). Total cellularity of the spleen increased by approximately 50% in mIL-17-treated animals on days 7 and 14 (Table I).

G-CSF serum levels

Ad-mIL-17 resulted in a significant increase in G-CSF with a peak of 3706 pg/ml (±398), at 24 h after Ad-mIL-17 gene transfer (Fig. 6). No G-CSF was detected in PBS-treated mice (data not shown). Following this peak, G-CSF levels returned to undetectable levels by 72 h in Ad-mIL-17-treated animals. AdCMVLuc-treated control animals showed a modest elevation in G-CSF at 24 h, which returned to undetectable levels at 72 h. No G-CSF was detected in either group at days 3, 7, 14, and 21 after gene transfer.

Table I. *mIL-17 increases total spleen cellularity*[a]

Cell	Day 0	3	7	14	21
Spleen Ad-IL-17	10.4 ± 2.1	12.44 ± 1.9	18.92* ± 2.2	18.46 ± 2.4*	13.24 ± 2.6
Spleen Ad-luc	9.8 ± 1.9	8.13 ± 2.3	12.54 ± 2.3	13.2 ± 1.8	12.84 ± 3.2
BM Ad-IL-17	1.1 ± 0.3	1.05 ± 0.23	1.65 ± 0.19	2.42 ± 0.46	2.26 ± 0.33
BM Ad-luc	1.3 ± 0.22	1.46 ± 0.31	1.96 ± 0.42	1.96 ± 0.39	2.35 ± 0.4

[a] Spleen and bone marrow cells from three individual animals per group were obtained and processed at different time points as outlined in *Materials and Methods.* Numbers are expressed as cells $\times 10^7$. The data represent the mean value of results from individual animals ± SEM. Two subsequent experiments using the same number of animals showed similar results.

*, Indicates a value of at least $p < 0.05$.

Discussion

The purpose of this study was to investigate the in vivo effects of mIL-17. Since IL-17 can release cytokines that both stimulate and inhibit hemopoiesis in vitro, we hypothesized that this cytokine would have a regulatory role on hemopoiesis in vivo. One well-established method to study in vivo activity of cytokines is the administration as recombinant protein. This reagent was not available at the time of initiation of this work, and *E. coli*-derived mIL-17 has just recently been commercially released. Thus far, no mIL-17 molecule with in vivo activity has been reported.

Another powerful methodology to study the in vivo effects of cytokines is the generation of transgenic animals. In these animals the cytokine is constitutively overexpressed, resulting in continuously elevated plasma levels. Several attempts by our group to generate transgenic mice ubiquitously overexpressing mIL-17 mice failed, and it appeared that it resulted in no or dysfunctional pregnancies (data not shown). Due to the lack of pharmacologically active compound and the inability to generate a transgenic animal, we overexpressed the protein using an in vivo adenoviral expression system as previously described (6).

Recombinant adenovirus is a gene transfer vector with high expression characteristics in mammalian cells. Previously, our group reported sustained high levels of a TNF-inhibitor gene for 4 wk after injection into mice of a recombinant adenovirus vector encoding a novel TNF-R:Fc fusion protein. Overexpression of this gene resulted in a phenotype similar to the 55-kDa TNF receptor knockout mouse (6). In an effort to further prolong transgene expression, others and we have investigated the use of less immunogenic vectors and/or immunomodulation strategies of the animal, which result in gene expression for up to 1 yr (12).

To investigate the effects of mIL-17 in vivo, a first-generation, E1-deleted, recombinant adenovirus was engineered to express the mIL-17 gene. Intravenous, liver-directed delivery resulted in sustained and effective production of biologically active mIL-17 in mice, which followed a dose-response pattern. The treatment with Ad-mIL-17 was well tolerated and without apparent morbidity. We found this cytokine to exert profound stimulatory effects on hemopoiesis. Morphologically, the most significant effects were observed in the peripheral blood and the spleens of treated animals. The stimulation of peripheral granulopoiesis was associated with both increases in total cellularity of the spleen and in early precursors as measured by colony forming assays. The increase in total number of spleen-derived HPPC over the entire duration of the experiment was not associated with a decrease in marrow precursors. Although both the total cellularity and absolute precursor number remained constant in the bone marrow, the precursor frequency increased on day three. The question of the origin of the splenic precursor cells was not addressed in this study. Although we did not investigate the possibility of progenitor redistribution from the marrow to the spleen, it is likely that both marrow and

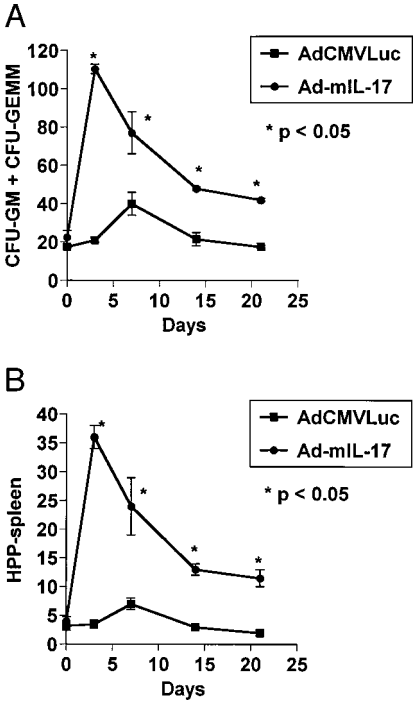

FIGURE 5. IL-17 increases splenic colony forming units. Splenocyte colonies were plated from two individual animals per data point as described in *Materials and Methods*, and CFU-GM and CFU-GEMM (*A*) and HPPC (*B*) were enumerated. Data represent the mean value (CFU/10^5 cells) of individual animals ± SEM. *, Indicates a value of at least $p < 0.05$. The experiment was repeated twice with similar results.

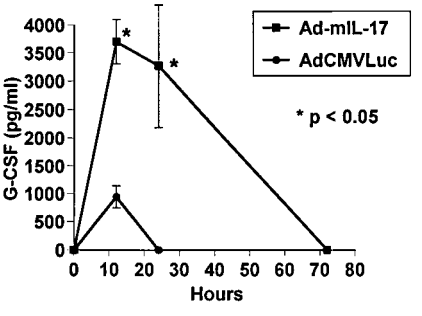

FIGURE 6. IL-17 induces transiently elevated G-CSF serum levels. Serum content of G-CSF was determined in four individual animals per group as outlined in *Materials and Methods*. No G-CSF was detected in either group on days 7, 14, or 21. Data represent the mean value of individual animals ± SEM. *, Indicates a value of at least $p < 0.05$. The experiment was repeated once with similar results.

splenic hemopoiesis were stimulated independently. However, the transient increase in marrow precursor frequency on day 3 may indicate a spill of precursor cells that have migrated to the spleen. No significant increase in bone marrow cellularity or precursor frequency was found thereafter. The increase of mature neutrophils in peripheral blood of treated animals was most pronounced on day 7. This response lagged behind the maximum increase of marrow and spleen CFUs, which was seen on day 3. This suggests that the increased mature elements observed in the periphery originated from marrow and splenic progenitors. Complete clearance of adenovirus-infected cells eventually occurs via both cellular and humoral immune responses in previously not sensitized, immunocompetent animals (13, 14). However, persistence of the transgene by PCR, over at least a 3-wk period as demonstrated, is comparable to systemically administered adenoviral vectors encoding reporter genes (13, 14), indicating that mIL-17 most likely does not enhance the immune-mediated elimination of adenovirus-infected hepatocytes. The pharmacokinetic profile of Ad-mIL-17, with a peak at day 3 followed by a steady decline in serum levels through day 21, is similar to the profile of other secreted proteins previously published using adenovirus-mediated gene transfer (6, 13, 14). Although mIL-17 was detected by Western blot on days 3 and 7, it could not be detected thereafter with this technique, indicating less sensitivity compared with the bioassay. Human IL-17 is reported as 20- to 30-kDa homodimeric variably glycosylated polypeptide (4). The high sequence homology of human and mIL-17 suggests conservation of the proteins. Both in vitro and in vivo adenoviral-expressed mIL-17 proteins were identical in size at 34 kDa. In comparison, the prokaryotic-expressed recombinant mIL-17 was smaller in size (26 kDa), which is most likely due to glycosylation differences in the eukaryotic-expressed protein. The eukaryotic mIL-17 is visualized as a double band, which, we believe, is due to variable glycosylation of the protein. The systemic administration of recombinant adenovirus results in virus distribution to selected organs with 90% viral uptake and protein expression in the liver; the rest is equally distributed in lung and in spleen (Refs. 12 and 15; our unpublished observations). No virus was detected in bone marrow cells. Therefore, we believe the observed results are due to a systemic effect of secreted mIL-17 rather than a local, organ-restricted effect.

Among the cytokines that mIL-17 can release in vitro, G-CSF is the most active compound in stimulating granulopoiesis (4). To investigate its potential role in mIL-17-mediated granulopoiesis, serum levels were measured. We observed a rapid rise in G-CSF levels after Ad-mIL-17 administration, which peaked at day 2. No further G-CSF was detected after day 3. We also observed a modest, but detectable, increase in G-CSF in AdCMVLuc-treated animals, indicating possibly in part a virus-mediated mechanism of G-CSF release. However, measuring the area under the curve, these levels were significantly less than those seen in Ad-mIL-17-treated animals. Thus, we feel that the increase in G-CSF is specific to mIL-17, although we cannot rule out a synergistic effect of mIL-17 and adenovirus transduction in mG-CSF responses. A significant increase in neutrophils is observed in mice within 6–24 h of G-CSF administration, which returns to baseline within 24 h of treatment cessation (16, 17). Although the pharmacokinetic profile seen in our experiments could be associated with neutrophilia on days 2 and 3, it does not explain the granulopoiesis with maximum response at day 7, occurring 4 days after disappearance of G-CSF. Layton et al. reported that, despite the continuous administration of G-CSF, levels of the cytokine decreased with increasing neutrophil counts. This inverse relation was explained by the increased availability of G-CSF receptors on developing granulocytes, thus binding free G-CSF (18). Although in our assay the mG-CSF levels became undetectable, we cannot entirely exclude elevated tissue-bound mG-CSF in vivo, which would not be detected by the ELISA in plasma.

Moulinex et al. demonstrated that both marrow cellularity and marrow CFUs decline significantly between days 2 and 5 after repeated G-CSF injections (16). In contrast, marrow cellularity of Ad-mIL-17-treated mice did not change significantly throughout the experiments, and a significant increase in marrow-derived CFUs was seen on day 3. Although these findings do not exclude a potential role of G-CSF in mIL-17-mediated effects on hemopoiesis, they suggest additional or other mechanisms, possibly other cytokines (4, 19, 20).

Although acute G-CSF elevation followed by neutrophilia is seen with endotoxin treatment, this possibility is considered unlikely since all vector lots tested negative for endotoxin, in both control virus and Ad-mIL-17 virus preparations, using a highly sensitive assay. Vectors were propagated in endotoxin-free conditions using endotoxin-free media and a certified lot of serum. Lastly, luciferase controls did not display the hematologic response observed in Ad-mIL-17-treated animals. However, to entirely rule out the possibility of endotoxin contamination, experiments could be performed in endotoxin-resistant mouse strains.

mIL-6 release from fibroblasts has been developed as a bioassay for mIL-17 measurement; however, surprisingly, serum levels of Ad-mIL-17-treated animals were not, or were only minimally, elevated over control animals throughout the experiments. Although mIL-6 was reported to cause mild peripheral neutrophilia in vivo at pathophysiologically relevant doses (5), Katayama et al. demonstrated antagonistic effects of the combination of IL-6 and G-CSF in vitro (21). Therefore, it appears that the Ad-mIL-17-mediated granulopoiesis is largely mIL-6 independent.

IL-17 is known to stimulate the production of a variety of cytokines in vitro, some with stimulatory, and others with inhibitory, effects on hemopoiesis. The cytokine profile varies with the cell type that is exposed to IL-17, thus adding further complexity to its possible in vivo actions (4, 20). Although at the present time we have looked only at mIL-17-mediated induction of G-CSF and mIL-6, to further define potential in vivo mechanism and interaction with other cytokines, we are currently investigating mIL-17-mediated effects in animals pretreated with a variety of specific anti-cytokine Abs. Specifically, candidate cytokines we are currently investigating in our laboratory are soluble c-*kit* ligand or stem cell factor and Flt2/Flk3 ligand. Although our work focused on correlation and evaluation of the most obvious in vivo response to mIL-17 administration, which is induction of granulopoiesis, other potential effects on the hemopoietic system by mIL-17 are quite likely, considering the stimulation of precursor cells that need to be addressed separately. Interestingly, mIL-17-mediated in vivo stimulation of myelopoiesis was associated with a doubling of peripheral blood lymphocytes. This was not associated with changes of lymphocyte frequency scored in histologic spleen sections, although absolute lymphocyte numbers increased due to increased total spleen cellularity (data not shown). The effects of mIL-17 on lymphopoiesis and lymphocyte subset populations is presently being investigated in our laboratory. Moreover, similar in vivo studies are in progress to evaluate hematologic consequences of mIL-17 long-term expression. This is being investigated in SCID mice that lack immune-mediated elimination of adenovirus vectors.

In summary, we demonstrated in this study that overexpression of mIL-17 has a profound stimulatory effect on hemopoiesis, resulting in secondary leukocytosis. Recombinant adenovirus technology provides a novel tool that can overcome difficulties in

studying the biology of cytokines where a transgenic animal cannot be established and pharmacologically active preparations for in vivo use are not available. Moreover, mIL17 can be expressed in adult animals, whereas expression during embryogenesis appears to be lethal. These results support further research and development of this cytokine for potential clinical applications that are caused by leukopenia, such as serious bacterial and fungal infections, for conditions that are associated with bone marrow failure, such as AIDS and aplastic anemia, and for transplantation- and cancer treatment-induced toxicities.

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