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Requirement of Endogenous Stem Cell Factor and Granulocyte-Colony-Stimulating Factor for IL-17-Mediated Granulopoiesis[1]

Paul Schwarzenberger,[2]*† Weitao Huang,*† Peng Ye,*† Peter Oliver,*† Misty Manuel,*† Zili Zhang,*†‡ Gregory Bagby,§ Steve Nelson,† and Jay K. Kolls*†‡

IL-17 is a novel, CD4$^+$ T cell-restricted cytokine. In vivo, it stimulates hematopoiesis and causes neutrophilia consisting of mature granulocytes. In this study, we show that IL-17-mediated granulopoiesis requires G-CSF release and the presence or induction of the transmembrane form of stem cell factor (SCF) for optimal granulopoiesis. However, IL-17 also protects mice from G-CSF neutralization-induced neutropenia. G-CSF neutralization completely reversed IL-17-induced BM progenitor expansion, whereas splenic CFU-GM/CFU-granulocyte-erythrocyte-megakaryocyte-monocyte was only reduced by 50% in both *Sl/Sld* and littermate control mice. Thus, there remained a significant SCF/G-CSF-independent effect of IL-17 on splenic granulopoiesis, resulting in a preservation of mature circulating granulocytes. IL-17 is a cytokine that potentially interconnects lymphocytic and myeloid host defense and may have potential for therapeutic development. ***The Journal of Immunology*****, 2000, 164: 4783–4789.**

Recently, a novel T-cell derived cytokine with homology to Herpesvirus saimiri was cloned by Rouvier et al. (1) and initially named CTLA-8. Its production was found to be restricted to activated CD4$^+$ lymphocytes and was subsequently renamed IL-17 (2–4). Specific regulatory functions of T cells and their role in hematopoiesis have not been elucidated, although it is known that T cells exert regulatory function on hematopoiesis via secretion of both stimulating and inhibitory cytokines (IL-2, IL-3, IL6, IL-8, and GM-CSF) (5). However, these cytokines appear to be only a portion of an intricate cytokine network that links the immune system to hematopoiesis.

In a previous publication, we demonstrated that in vivo overexpression of murine IL-17 (mIL-17)[3] stimulates hematopoiesis, particularly granulopoiesis (6). Furthermore, we demonstrated that mIL-17 stimulates the release of G-CSF. The release of G-CSF preceded the induction of granulopoiesis; however, the peak expression occurred several days before the peak increase in absolute neutrophil count (ANC). Based on these data, we hypothesized that other growth factors may be involved in IL-17-induced granulopoiesis. Specifically, we investigated G-CSF and stem cell factor (SCF) because SCF is known to strongly synergize with G-CSF in stimulating granulopoiesis (7–11). In this study, we show that IL-17 induces enhanced expression of the transmembrane form of SCF in the bone marrow (BM)-derived stroma cell lines BMS2, S17, and NIH3T3. Moreover, these cell lines secrete G-CSF in response to IL-17 in a dose-dependent fashion. To test the hypothesis that IL-17 induced increases in membrane-bound SCF are required for granulopoiesis in vivo, we investigated IL-17-mediated granulopoiesis in Steel-Dickie mice (*Sl/Sld*), which have a mutation where the transmembrane and cytoplasmic domains of SCF are deleted. A truncated soluble form of SCF is produced, but functional transmembrane SCF is absent in these animals (12, 13). To study a synergistic role of G-CSF with the transmembrane form of SCF, G-CSF was neutralized in *Sl/Sld* mice and littermate control mice overexpressing IL-17.

Materials and Methods

Cell lines

NIH3T3 cells were obtained from American Type Culture Collection (Manassas, VA). S17 cells were a generous gift from Dr. Kenneth Dorshkind (Los Angeles, CA) (14). BMS2 cells were a generous gift from Dr. Paul Kincade (Oklahoma City, OK) (15). All cells were grown in IMDM (Life Technologies, Gaithersburg, MD) with 10% FBS (Life Technologies) with penicillin/streptomycin (50 U/50 μg/ml; Life Technologies). Recombinant mIL-17 was purchased from R&D Systems (Minneapolis, MN).

Animals

WCB6F$_1$/J Mgf^{Sl}/Mgf$^{Sl\text{-}d}$ (*Sl/Sld*) mice and their littermate controls were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in a dedicated specific pathogen-free room in the vivarium of Louisiana State University Health Science Center with filter top cages. Autoclaved food and water was provided ad libitum. Sterility was monitored using sentinel animals that were periodically bled and tested for common mouse pathogens. To achieve overexpression of IL-17, animals were injected in the internal jugular vein with 5×10^9 PFU of recombinant adenovirus encoding either mIL-17 (Ad-mIL17), the control gene luciferase (AdCMVLuc), or PBS and were sacrificed at 7 days, the time of maximal induction of granulopoiesis (6). Organs were harvested under sterile conditions. Blood was collected via cardiac puncture or retro-orbitally and transferred into heparinized tubes. Spleens were excised and ground between two slides and cells were transferred into medium. BM cells from both femurs were flushed into medium (DMEM/15% FBS; Life Technologies). RBC were lysed in splenic specimens with Tris-buffered NH_4Cl. Nucleated cell counts were performed

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[3] Abbreviations used in this paper: mIL-17, murine IL-17; ANC, absolute neutrophil count; SCF, stem cell factor; Ad-mIL-17, adenovirus-encoding mIL-17; AdCMVLuc, adenovirus-encoding luciferase; rm, recombinant murine; BM, bone marrow; WBC, white blood cell count; GEMM, granulocyte-erythrocyte-megakaryocyte-monocyte; HPP, high proliferative potential.

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using a hematocytometer and trypan blue staining to account for the viability of cells. Smears from peripheral blood were prepared using standard techniques and stained with a modified Wright-Giemsa stain (DiffQuick; Baxter, Deerfield, IL). For G-CSF neutralization studies in vivo, mice were injected with anti-G-CSF Ab or preimmune (IgG) serum at 3 mg 1 h before and 3 days after adenovirus injection. With this dose of anti-G-CSF, no G-CSF could be detected at any time point in animals of any treatment group.

Anti-CSF Ab purification

Polyclonal rabbit anti-G-CSF serum was generated using an immunization kit purchased from Ribi (Hamilton, MT), following the instructions of the manufacturer. Briefly, 8- to 10-lb rabbits (Charles River Laboratories, Wilmington, MA) were injected with the immunization preparation reconstituted with recombinant murine G-CSF (Amgen, Thousand Oaks, CA). Administration was performed intradermally (0.3 ml), i.m. (0.4 ml), s.c. (0.1 ml), and i.p. (0.2 ml). Rabbits were boosted 4 and 8 wk after the initial immunization and bled 10–14 days after each boost. Before use, rabbit plasma was purified over a Sepharose A column (Bio-Rad, Hercules, CA). Activity of the Ab was tested each time by using a bioassay: G-CSF activity and anti-G-CSF neutralization capacity were measured using a bioassay previously described by Dale et al. (16) and our group (17). Using the standard tritiated thymidine incorporation assay, the proliferative response of G-CSF in NSF-60 cells was completely blocked with purified anti-G-CSF.

Construction of Ad-mIL-17

The construction of mIL-17 adenovirus has been described in detail by our group (6). Briefly, viruses were propagated on 911 cells using endotoxin-free conditions and CsCl density purification as described previously (18, 19). mIL-17 production was measured in 911 cell supernatants using a bioassay 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/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 (BioWhittaker, Walkersville, MD).

G-CSF ELISA

Plasma G-CSF protein concentrations were determined using a specific enzyme-linked immunoassay developed in our laboratory using described procedures previously reported in detail (6). Briefly, the ELISA was performed using native and biotinylated forms of the above described anti-G-CSF rabbit polyclonal Ab as the capture and detection Abs, respectively. G-CSF concentrations were calculated from a standard curve of recombinant G-CSF (Amgen) 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.

Measurement of mIL-17 by bioassay

The biological assay previously described by Fossiez et al. (4) and our group (6) was used to validate mIL-17 expression in vivo. mIL-17 and mIL-6 concentrations were calculated from standard curves using log-log linear regression. One mIL-17 unit is defined as the amount that results in release of 1 pg/ml mIL-17-dependent mIL-6 secretion in this assay.

SCF ELISA

The ELISA was performed following instructions provided and outlined by R&D Systems. ImmunoModule plates (Nalge Nunc International, Naperville, IL) were coated overnight with a rat anti-mouse SCF mAb (Genzyme, Cambridge, MA) at 2 μg/ml in 100 μl of PBS. The plates were rinsed in PBS and blocked with 1% BSA for 2–4 h. The plates were rinsed, dried, and stored at 4°C. Culture supernatants (50 μl) were diluted in 50 μl of 0.1% BSA, PBS, and 0.05% Tween 20 in PBS (pH 7.4). After an overnight incubation (4°C), the wells were rinsed three times with PBS and 0.05% Tween 20 (wash buffer). A biotinylated goat anti-murine SCF (100 μl of 200 ng/ml; R&D Systems) was applied to the wells for 1–2 h at room temperature. The wells were rinsed three times with wash buffer, and a 1/5000 dilution of neutravidin/HRP (Pierce, Rockford, IL) was applied for 30 min at room temperature. The wells were rinsed four times with wash buffer and incubated with 100 μl of a 1:1 solution of HRP substrate (R&D Systems). After 5–20 min, the reaction was stopped with 25 μl of 0.18 N H_2SO4, and the results were read on a Dynatech Platereader (Biotech Instruments, Winooski, VT) at 450 nm. A known standard of rmSCF (R&D Systems) was diluted in culture medium for establishing a standard curve. SCF concentrations were determined using log-log linear regression. Sensitivity of the assay was determined up to 20 pg/ml.

Colony-forming assays

Methylcellulose cultures for CFU-GM, CFU-granulocyte-erythrocyte-megakaryocyte-monocyte (GEMM), and CFU-high proliferative potential (HPP) were performed with MethoCult GF 3434 from Stem Cell Technologies (Vancouver, Canada) as described previously (6) (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 recombinant human IL-6, 50 ng/ml rmSCF, and 3 U/ml recombinant human erythropoietin). Briefly, 2×10^4 BM cells or 2.5×10^5 spleen cells were suspended in 1ml 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 defining a colony consisting as an accumulation of at least 50 cells. CFU-HPP is defined as an accumulation of at least 200 cells.

FACS analysis

Murine BM stroma cell lines (BMS2, S17, NIH3T3) were cultured in 6-well plates (Corning, Corning, NY). The cells were collected by removing the medium and incubating the cells for 15–30 min with 5 mM EDTA in PBS. The cells were spun down and ~10^6 cells were resuspended in PBS with 1% BSA (wash buffer) containing normal goat IgG (10 μg/100 μl; Sigma, St. Louis, MO). After 15 min, biotinylated goat anti-mouse SCF (0.5 μg/100 μl; R&D Systems) was added to the solution and incubated at 4°C for 1 h. The cells were washed and incubated for 30 min in wash buffer containing 10 μg/ml solution of streptavidin conjugated with PE (PharMingen, San Diego, CA). The cells were washed with wash buffer and analyzed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Histograms and mean channel fluorescence were generated and analyzed using CellQuest Software (Becton Dickinson).

Data analysis

Comparisons between the means were analyzed by ANOVA using the statistical software package StatView (Calabasas, CA). A $p < 0.05$ was considered to be statistically significant. Kolmogorov-Smirnov statistics were performed for flow cytometric analysis, using CellQuest software (Becton Dickinson). A $p < 0.05$ was considered to be statistically significant.

Results

IL-17 induces G-CSF release and enhances cell surface SCF expression in murine BM stroma cells in vitro

rmIL-17 induced G-CSF secretion over 24 h in a dose-dependent manner in several BM-derived stroma cell lines (BMS2, NIH3T3, and S17, Fig. 1*A*). Peak secretion of G-CSF occurred at an IL-17 concentration of 20 ng/ml. Heat-inactivated mIL-17 failed to result in any detectable G-CSF secretion (data not shown). To investigate whether IL-17 increased SCF expression in these cells, S17, BMS2, or NIH 3T3 cells were incubated for 24 h with 40 ng/ml recombinant murine IL-17 or heat-inactivated IL-17.

FACS analysis of S17 cells exposed to IL-17 demonstrated a significant increase in surface SCF as measured by mean channel fluorescence (isotype control: 6.4, rmIL-17: 250.6, rmIL-17-heat inactivated: 215.8; $p < 0.001$) as well as a significant narrowing of the distribution of cells expressing high levels of SCF (Fig. 1*B*). Similar results were seen in BMS2 and NIH3T3 cells (data not shown). To confirm these findings with an independent assay, cell extracts were analyzed for cell-associated SCF by ELISA 24 h after mIL-17 incubation (40 ng/ml). Cell-associated SCF increased 35 ± 5% in S17 cells (750 ± 79 vs 1180 ± 140 pg/ml; $p < 0.0001$, $n = 4$). Similar increases in cell-associated SCF by ELISA were observed in BMS2 cells (32 ± 7%) and NIH3T3 cells (70 ± 12%). No soluble SCF was detectable in cell supernatants 24 h after mIL-17 treatment (data not shown).

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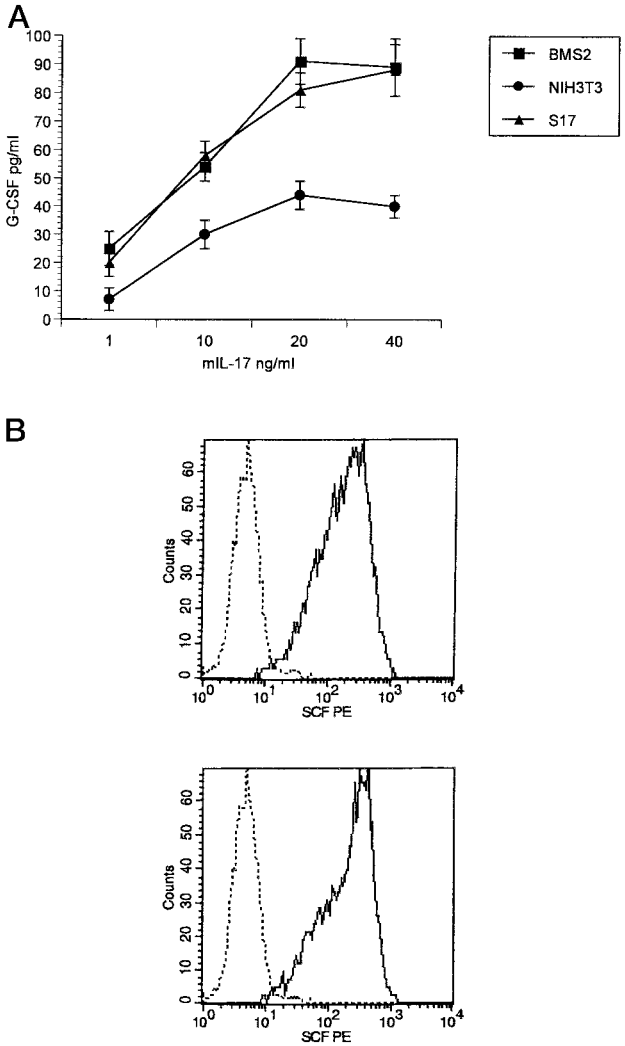

FIGURE 1. IL-17 induces G-CSF release and enhances cell surface SCF expression on murine BM stroma cells in vitro. The murine BM stroma cell lines BMS2, NIH3T3, and S17 were incubated with increasing doses of recombinant mIL-17. At 24 h, supernatants were collected and analyzed for G-CSF content by ELISA as outlined in *Materials and Methods*. No G-CSF could be measured with heat-inactivated mIL-17 (*A*). The BM stroma cell line S17 was incubated with recombinant mIL-17 at 40 ng/ml for 24 h and analyzed by flow cytometry with an Ab against SCF. Heat-inactivated mIL-17 was used for controls. Mean channel fluorescence is plotted on the abscissa (*B*). Data points represent the results of triplicate experiments.

In vivo induction of G-CSF by IL-17

G-CSF was measured in the plasma of mice at 1 and 7 days. Administration of Ad-mIL17 resulted in a significant increase in plasma G-CSF within 24 h in both littermate controls and *Sl/Sld* animals to similar levels (1025 ± 370 pg/ml vs 670 ± 290 pg/ml, respectively, $p > 0.05$, $n = 6$). The administration of anti G-CSF effectively neutralized circulating G-CSF since no G-CSF could be measured in these animals (data not shown). Similar to our previous report (6), AdCMVLuc-treated control animals had a small but detectable G-CSF response at 24 h, constituting <5% than in the AdIL-17-treated animals. No G-CSF could be measured in the littermate control groups at day 7 (AdmIL-17 and AdCMVLuc treated). However, in the AdmIL-17-treated *Sl/Sld* animals G-CSF levels were significantly elevated at 220 ± 31 pg/ml 7 days after vector administration. No G-CSF was detected in AdCMVLuc-treated *Sl/Sld* mice (Fig. 2).

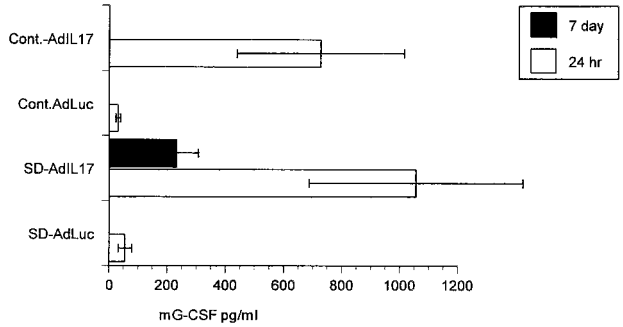

FIGURE 2. In vivo G-CSF measurements: G-CSF was measured by ELISA in plasma of control and *Sl/Sld* mice at 1 and 7 days after administration of AdmIL-17 or AdCMVLuc. Data points constitute the means ± SE obtained from six individual animals per group.

IL-17 increases granulocytes in control and in Sl/Sld *mice*

Overexpression of mIL-17 for 7 days by adenovirus-mediated gene transfer in control animals resulted in a 6.5-fold increase in white blood cell count (WBC) (AdCMVLuc vs AdmIL-17, 4.6 ± 0.6 vs 30.7 ± 2.4 × 10^7/ml) (Fig. 3*A*). The increase in WBC was largely due to an increase in granulocytes as measured by the ANC. The mean ANC value in AdCMVLuc-treated animals was 1.6 ± 0.32 × 10^7/ml. In comparison, in AdmIL-17-treated animals, the ANC rose to 23.5 ± 2.9 × 10^7/ml or a 14.5-fold increase over the AdCMVLuc group ($p < 0.0001$; Fig. 3*B*). There was no statistical difference in ANC between AdCMVLuc and PBS controls (data not shown). Overexpression of mIL-17 in *Sl/Sld* mice resulted in a lesser but significant 2-fold increase of the total WBC compared with the AdCMVLuc-treated animals (7.8 + 0.45 vs 3.5 + 0.5 × 10^7/ml, $p < 0.01$) (Fig. 3*A*). Similar to littermate control

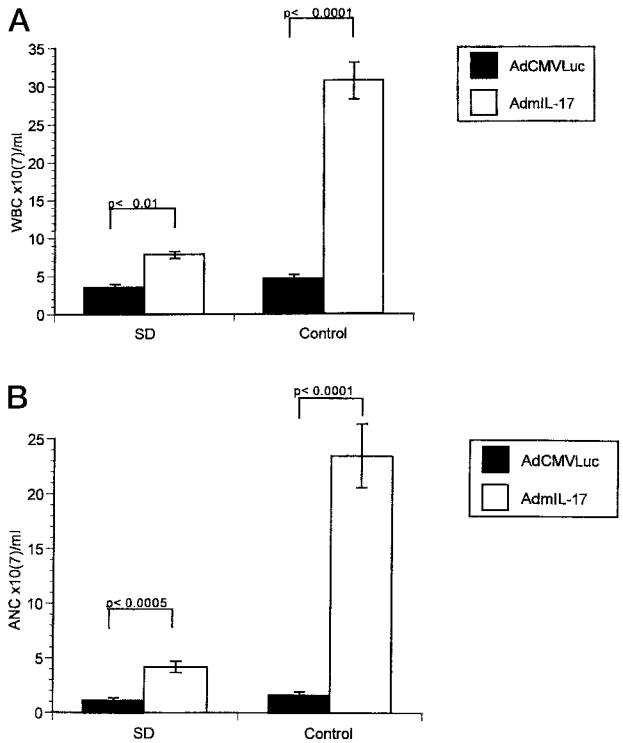

FIGURE 3. mIL-17 increases granulocytes in control and in *Sl/Sld* mice**.** Normal control and *Sl/Sld* mice were treated with AdmIL-17 or AdCMVLuc. Seven days later, peripheral blood was analyzed for total WBC (*A*) and the ANC (*B*). The *p* values are indicated above the bars. Data points constitute the means ± SE obtained from 8 to 12 individual animals.

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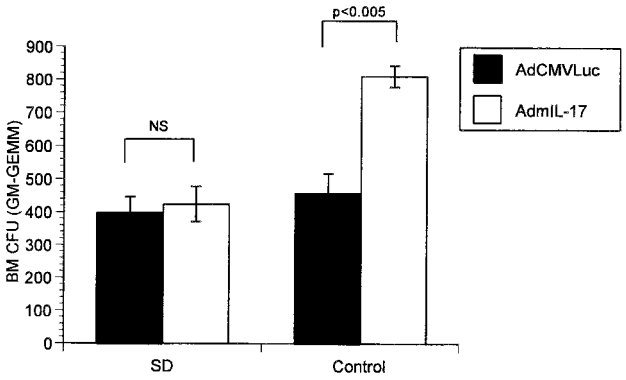

FIGURE 4. AdmIL-17 increases BM CFU (CFU-GM/CFU-GEMM) in control animals but not in *Sl/Sld* mice: Normal control and *Sl/Sld* mice were treated with AdmIL-17 or AdCMVLuc. BM precursors were measured for each individual animal by colony-forming assay (CFU-GM/CFU-GEMM) and calculated for the nuclear cellular fraction of total BM flushed from both femurs. The *p* values are indicated above the bars. Data points constitute the means ± SE ($\times 10^3$) obtained from 16 individual animals.

animals, this was mainly due to expansion of mature granulocytes. The ANC in AdmIL-17-treated *Sl/Sld* animals rose to 4.2 ± 0.52 × 10^7/ml (or a 4-fold increase) compared with AdCMVLuc treatment (1.1 ± 0.24 × 10^7/ml; Fig. 3*B*).

AdmIL-17 increases BM CFU in control animals but not in Sl/Sld *mice*

As previously reported by our laboratory in C57BL/6 mice, no changes were observed in BM cellularity 7 days after AdmIL-17 or AdCMVLuc administration in littermate controls or *Sl/Sld* animals (6). However, as previously observed with C57BL/6 mice, AdmIL-17-treated littermate controls demonstrated a significant increase in absolute CFU (-GM and -GEMM, 458 ± 60 vs 811 ± 33, $p < 0.005$) (Fig. 4). No statistically significant effect was observed on CFU-HPP (data not shown). In contrast to littermate controls, *Sl/Sld* mice failed to show an increase in CFU (-GM and -GEMM) after AdmIL-17 administration compared AdCMVLuc controls (399 ± 48 vs 425 ± 53). Furthermore, no IL-17-induced changes were seen with BM CFU-HPP (data not shown).

AdmIL-17 increases CFU (-GM, -GEMM, and -HPP) spleen in control mice and to a lesser extent in Sl/Sld *animals*

AdmIL-17 administration in littermate controls resulted in a significant increase in splenic cellularity by 1.4-fold compared with AdCMVLuc-treated animals ($p < 0.05$). Moreover, AdmIL-17 administration resulted in a total splenic CFU (-GM and -GEMM) increase from 1336 ± 60 to 4086 ± 188, $p < 0.0001$)compared with the AdCMVLuc group (3-fold increase) (Fig. 5*A*). Absolute splenic CFU-HPP demonstrated a more dramatic 5-fold increase from 211 ± 17 to 1022 ± 110 in AdmIL-17-treated animals over AdCMVLuc controls (Fig. 5*B*, $p < 0.0001$). AdmIL-17 administration in *Sl/Sld* mice resulted in a significant 3.4-fold increase in splenic CFU (-GM and -GEMM) from 840 ± 136 to 2846 ± 99 ($p < 0.001$) compared with AdCMVLuc controls (Fig. 5*A*). There was also a significant 3.6-fold increase in CFU-HPP in *Sl/Sld* mice after AdmIL-17 treatment from 176 ± 17 to 638 ± 144 ($p < 0.001$, Fig. 5*B*).

Requirement of G-CSF and SCF for IL-17-induced granulopoiesis

The studies performed in *Sl/Sld* mice suggested a critical role of the membrane-bound form of SCF in IL-17-mediated granulopoiesis. However, in *Sl/Sld* mice, there remained a significant increase in mature neutrophils as well as neutrophil precursors. As G-CSF has been shown to synergize with SCF in inducing granulopoiesis, we investigated the role of endogenous G-CSF in IL-17-mediated granulopoiesis. Control or *Sl/Sld* mice were injected with anti-GCSF or nonimmune IgG before administration of PBS, AdCMVLuc, or AdmIL-17. Statistically significant differences were not found between the AdCMVLuc group compared with the PBS controls in any of the experimental outcomes; therefore, only AdCMVLuc control data are shown as controls. Dale and colleagues. (20) previously reported neutropenia in dogs with the development of neutralizing anti-G-CSF Abs . Similar to their report, over the 7-day experiment, anti-G-CSF administration resulted in mild but statistically significant neutropenia in both AdCMVLuc-treated *Sl/Sld* and littermate control animals (Fig. 6*B*). Anti-G-CSF also significantly attenuated AdmIL-17-mediated increases in ANC (Fig. 6*B*). The greatest attenuation by anti-G-CSF, in IL-17-mediated increases in WBC and ANC, was seen in the AdmIL-17-treated control mice (Fig. 6), suggesting that there is synergism between G-CSF and SCF in IL-17-induced granulopoiesis. This is further supported by the fact that AdmIL-17 induced only modest increases in WBC and ANC in *Sl/Sld* mice (Fig. 6) despite the fact that these mice release G-CSF in response to IL-17.

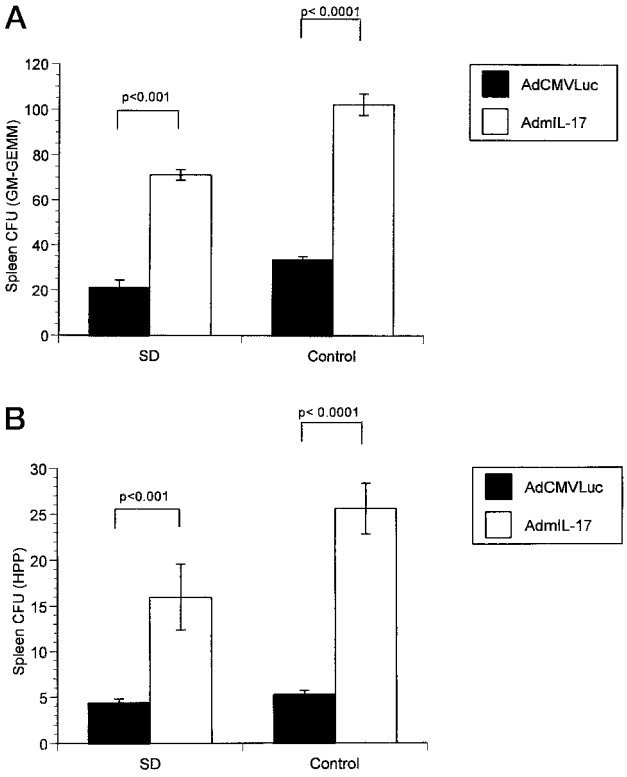

FIGURE 5. AdmIL-17 increases CFU (-GM, -GEMM) spleen in both control and *Sl/Sld* animals: Normal control and *Sl/Sld* mice were treated with AdmIL-17 or AdCMVLuc. Spleen precursors were measured for each animal by colony-forming assay and calculated for the nuclear cellular fraction of the entire organ (*A*, CFU-GM/CFU-GEMM; *B*, CFU-HPP). The *p* values are indicated above the bars. Data points constitute the means ± SE ($\times 10^3$) obtained from 16 individual animals per group.

Despite the evidence to support a synergistic role of SCF and G-CSF in IL-17-induced granulopoiesis, we also observed a significant SCF- and G-CSF-independent effect of IL-17. AdmIL-17, in the presence of anti-G-CSF, resulted in a significant increase in both WBC and ANC in both control and *Sl/Sld* mice compared with AdCMVLuc-treated animals treated with anti-G-CSF (Fig.

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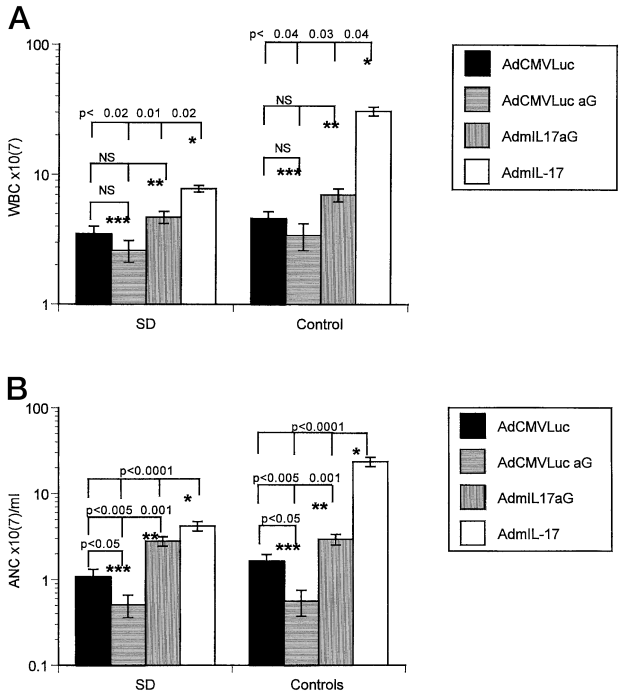

FIGURE 6. IL-17 protects both control and *Sl/Sld* animals from anti-G-CSF-induced neutropenia. Normal control and *Sl/Sld* mice were treated with AdmIL-17 or AdCMVLuc. Endogenous G-CSF was neutralized with anti-G-CSF (aG). Seven days later, peripheral blood was analyzed for total WBC (*A*) and the ANC (*B*). *, *p* values between the AdmIL-17- treated groups; **, *p* values between the AdmIL-17aG groups; and ***, *p* values between the AdCMVLuc aG-treated groups. Data points constitute the means ± SE obtained from 8 to 12 individual animals.

6). Thus, IL-17 protected both control and *Sl/Sld* mice from anti-G-CSF-induced neutropenia (Fig. 6).

One week of G-CSF neutralization did not have a significant effect on BM cellularity or BM precursor frequency (CFU-GM, -GEMM, and -HPP) in AdCMVLuc-treated control animals (AdCMVLuc vs AdCMVLuc anti-G-CSF: 440 ± 50 vs 458 ± 60) or *Sl/Sld* animals (356 ± 43 vs 349 ± 48). However, anti-G-CSF treatment completely reversed the AdmIL-17-induced BM precursor expansion seen in control animals (CFU-GM, CFU-GEMM, AdmIL-17 vs AdmIL-17 anti-G-CSF, 811 ± 33 vs 528 ± 63) (Fig. 7*A*). Similarly, 1 week of G-CSF neutralization did not affect splenic cellularity or precursor frequency in either control or *Sl/Sld* mice (AdCMVLuc vs AdCMVLuc anti-G-CSF, littermate control: 33.4 ± 1.5 vs 32.2 ± 1.9 for CFU-GM/CFU-GEMM, and 5.3 + 0.4 vs 6 + 0.9 for CFU- HPP; *Sl/Sld* mice: 21 ± 3 vs 26.9 ± 3.4 for CFU-GM/CFU- GEMM, and 4.4 ± 0.5 vs 5.4 ± 0.8 for CFU-HPP). Furthermore, anti-GCSF treatment also attenuated the AdmIL-17-induced precursor expansion in the spleen in both littermate control and *Sl/Sld* mice (littermate control: AdmIL-17 vs AdmIL-17 anti-G-CSF 102 ± 4.7 vs 69.4 ± 4.3 for CFU-GM/CFU-GEMM, and 25.6 ± 2.8 vs 10.5 ± 0.9 for CFU-HPP; *Sl/Sld*: AdmIL-17 vs AdmIL-17anti G-CSF 71.2 ± 4.7 vs 44.5 ± 4.3 for CFU-GM/CFU-GEMM, and 16 ± 2.8 vs 11.5 ± 0.9 for CFU-HPP). In this study, in contrast to BM, splenic precursors remained statistically significantly elevated in both strains over AdCMVLuc-treated controls (Fig. 7, *B* and *C*).

Discussion

We have previously demonstrated that overexpression of IL-17 utilizing adenoviral-mediated gene transfer in C57BL/6 mice in-

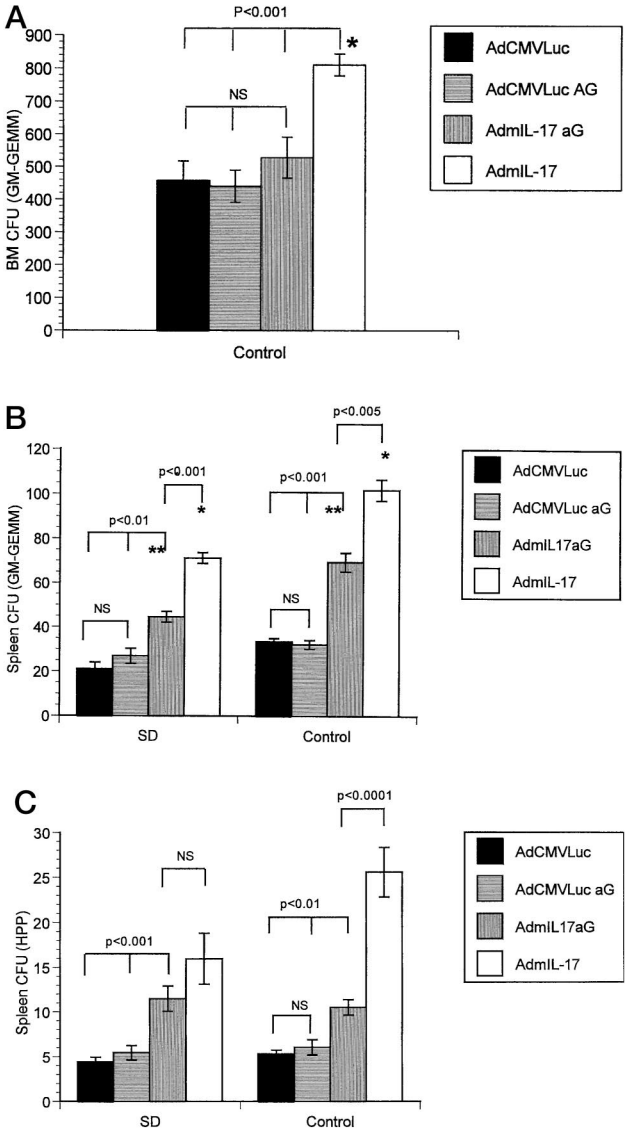

FIGURE 7. G-CSF neutralization completely reverses AdmIL-17-induced BM precursor expansion (CFU-GM/CFU-GEMM) in control mice and partially reverses splenic precursor expansion (CFU-GM, -GEMM, and -HPP) in both control and in *Sl/Sld* mice: Normal control and *Sl/Sld* mice were treated with AdmIL-17 or AdCMVLuc. Anti-G-CSF was administered 1 h before and 3 days after adenovirus injection. BM or spleen precursors were measured for each individual animal by colony-forming assay and calculated for the nuclear cellular fraction of the entire organ (*A*, BM CFU-GM/CFU-GEMM; *B*, spleen CFU-GM/CFU-GEMM; and *C*, spleen CFU-HPP). *, *p* values between the AdmIL-17-treated groups; **, *p* values between the AdmIL-17 aG-treated group. Data points constitute the means ± SE ($\times 10^3$) of 14 individual animals.

duces dose-dependent granulopoiesis in vivo (6). The peripheral neutrophilia in IL-17-treated mice consisted of multisegmented and morphologically mature-appearing granulocytes (6).

We hypothesized that IL-17 acts on BM stroma cells by inducing the release of G-CSF and SCF. Furthermore, we hypothesized that these cytokines mediate proliferation and differentiation of myelopoietic progenitors.

Our in vitro results show that mIL-17 induces the release of G-CSF from several BM stroma cell lines and the induction of the membrane-associated form of SCF on stroma cells. We have previously demonstrated that in C57BL/6 mice, the in vivo IL-17-induced increase in plasma G-CSF levels was transient and that

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G-CSF became undetectable at 48 h in the blood, which coincided with the rising ANC. This was in spite of the continuous systemic overexpression of bioactive mIL-17, which was detected for over 2 wk (6). Results in this series of experiments were consistent with our previous report, and at day 7 we could not detect G-CSF in normal *Sl/Sld* littermate controls (Fig. 2). Interestingly, we observed reduced but significant levels of G-CSF in the plasma of *Sl/Sld* mice 7 days after AdmIL-17 administration. This persistence of G-CSF in the plasma was also associated with a significantly attenuated IL-17-induced increase in ANC (Fig. 3*B*). Layton et al. (21) observed that despite the continuous infusion of G-CSF into animals, G-CSF protein could no longer be detected in plasma as neutrophilia developed. The authors demonstrated that the expanded population of neutrophils could more effectively absorb and bind G-CSF (21). These data suggest that IL-17-induced increase in granulocytes contribute to the clearance of G-CSF in this model.

Our data demonstrate three key points of IL-17-induced granulopoiesis. First, membrane SCF is required for optimal IL-17-induced granulopoiesis as *Sl/Sld* mice have a reduced ANC, an absent increase in BM CFU, and reduced absolute splenic precursors after IL-17 overexpression compared with littermate controls. Second, endogenous G-CSF is also required and synergizes with SCF to effect optimal IL-17-induced granulopoiesis. Evidence to support this is the fact that anti-G-CSF blocks IL-17 increases in ANC by 82% in littermate control mice and by 28% in *Sl/Sld* mice. Moreover, anti-G-CSF blocked IL-17 increases in BM CFU completely as well as it attenuated splenic CFU-GEMM and -HPP responses. These data indicate that G-CSF secretion is predominantly responsible for IL-17-mediated stimulation of BM hematopoiesis and largely responsible for splenic hematopoiesis. However, the presence of the transmembrane form of SCF amplifies this response synergistically and *Sl/Sld* mice are able to secrete G-CSF in response to IL17, yet they lack efficient IL-17-induced granulopoiesis. These studies support the conclusion that IL-17-mediated effects on the progenitor compartments spleen and BM are to a large extent the result of G-CSF secretion which is augmented by the induction or presence of transmembrane SCF. In fact, Ulich et al. (11) combined exogenous administration of SCF and G-CSF and observed a dramatic synergistic effect in stimulation of both medullary and extramedullary hematopoiesis and neutrophilia. Third, there is a SCF-/G-CSF-independent effect of IL-17 on increasing the peripheral blood ANC since IL-17 can increase and stabilize the ANC in the presence of anti-G-CSF. The IL-17-mediated neutrophil increase was similar by 6-fold in both anti-G-CSF-treated *Sl/Sld* and littermate control mice.

There exist several possibilities to explain this finding. IL-17 could induce myeloid progenitor differentiation, irrespective of the presence of G-CSF or SCF, although these growth factors appear to amplify the mIL-17-mediated response in synergism. This effect of mIL-17 could be a direct effect or an effect that is caused by release of other cytokines such as IL-3 induction (22, 23), GM-CSF (24, 25), or IL-6 (26). Another possibility is that IL-17 makes the mature neutrophil more resistant to apoptosis. G-CSF has been reported to protect against neutrophil apoptosis (27–30). Thus, IL-17 may protect against neutrophil apoptosis in the absence of G-CSF.

The significantly decreased IL-17-mediated response on granulopoiesis in *Sl/Sld* mice indicates the requirement of transmembrane SCF for its optimal effect. For instance, expansion of BM HPP progenitor cells was observed in control mice but not in the BM of *Sl/Sld* mice. Although IL-17 caused substantially enhanced absolute splenic precursor expansion in controls compared with *Sl/Sld* mice, the differences for the relative or fold increases were less pronounced between strains. Splenic precursors increased by 3.6-fold vs 4.9-fold for CFU-HPP and 3.4-fold vs 3.1-fold for CFU-GM/-GEMM in *Sl/Sld* vs control mice, respectively.

The data presented suggest a differential effect of IL-17 on different compartments of granulopoiesis. G-CSF neutralization completely reversed the IL-17-mediated effect on BM precursor expansion. Although spleen precursor frequency was also reversed completely with anti-G-CSF treatment, the absolute splenic precursor number remained significantly elevated in both control and *Sl/Sld mice.* Thus, IL-17 is capable of expanding the myeloid progenitor pool both via G-CSF and SCF induction as well as by a G-CSF- and SCF-independent effect.

The IL-17-mediated, G-CSF- and SCF-independent effects were most pronounced on peripheral neutrophils: Although 1 wk of G-CSF neutralization substantially reduced ANC in both control and *Sl/Sld* animals, IL-17 expression increased granulocytes equally by 6-fold in both animal strains. Thus, IL-17 can independently increase neutrophils in the absence of known key stimulators of granulopoiesis, G-CSF, and SCF.

G-CSF was recently found to be a response cytokine for emergency situations securing uninterrupted supply of neutrophils and macrophages by “BM priming” (31, 32). Little is known about cross-talk between immune mediating cells and as to how an organism can optimize its defense mechanisms against microbial invaders. IL-17 is a cytokine found exclusively in activated T cells. It is possible that IL-17 functions as a cross-talk cytokine between the cellular and innate immune system by inducing proliferation and differentiation of myeloid progenitors in the hematopoietic organs and the periphery. Furthermore, at a site of inflammation, activated T cells could potentially accelerate neutrophil differentiation via IL-17 secretion.

Although the role of T cells in regulation of hematopoiesis is unclear, it is believed their regulatory function occurs locally within the hematopoietic microenvironment (5). The data presented could identify a potential mechanism through which T cells might regulate hematopoiesis (4). For instance, T cells were found to enhance engraftment in BM transplantation while decreased numbers of T cells in donor marrow result in failure to engraft (33). This implies that interactions occur between T cells and other hematopoietic precursor cells. These interactions occur either directly via cell to cell contact, or via cytokines as messengers, or through combined effects.

Ongoing experiments in our laboratory examine the functionality of IL-17-differentiated granulocytes. We are also examining the role of IL-17 in BM transplantation. The data presented in this work demonstrate that mIL-17 induces G-CSF and SCF in BM stroma cells. In vivo effects of mIL-17 on hematopoiesis and secondary granulopoiesis are predominantly related to the release of G-CSF and the presence of functional SCF for optimal action. Moreover, mIL-17 independently induces and accelerates neutrophil maturation from myeloid precursors in control and *Sl/Sld* mice treated with neutralizing anti G-CSF Ab. These results can potentially be utilized to develop treatment strategies for patients with a functional deficit in myelopoiesis or in patients that need a rapid increase in mature granulocytes. Such conditions include serious bacterial or fungal infections, patients with compromised hematopoiesis as seen in aplastic anemia, cancer treatment induced or AIDS related.

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