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

Paul Schwarzenberger, Weitao Huang, Peng Ye, Peter Oliver, Misty Manuel, Zili Zhang, Gregory Bagby, Steve Nelson and Jay K. Kolls

_J Immunol_ 2000; 164:4783-4789; doi: 10.4049/jimmunol.164.9.4783
http://www.jimmunol.org/content/164/9/4783

References  This article cites 32 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/164/9/4783.full#ref-list-1

Subscription  Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Requirement of Endogenous Stem Cell Factor and Granulocyte-Colony-Stimulating Factor for IL-17-Mediated Granulopoiesis

Paul Schwarzenberger,†† 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 S/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.

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). RBC were lysed in splenic specific media with Tris-buffered NH₄Cl. Nucleated cell counts were performed in a FACSscan (Becton-Dickinson, San Jose, CA). Cell line purity was determined by antibody staining.

Animals

WCB6F1/Mg6/Mg6-d (S/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 × 10⁴ 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 NH₄Cl. Nucleated cell counts were performed...
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 (Diff-Quick; Baxter, Deerfield, IL). For G-CSF neutralization studies in vivo, mice were injected with anti-G-CSF Ab or preimmune IgG (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 Immunex (Seattle, WA) following the instructions of the manufacturer. Briefly, 8– to 10-lb rabbits (Charles River Laboratories, Wilmington, MA) were injected with the immunization preparation constituted 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 capability were measured using a bioassay previously described by Dale et al. (16) and our group (17). Using the standard titrated 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/108 PFU. All preparations were screened for replication competent adenovirus by propagation on A549 cells using a standard tritiated thymidine incorporation assay, the proliferative response of G-CSF in NSF-60 cells was completely blocked with purified anti-G-CSF.

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. After washing the 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).

Methylcellulose cultures for CFU-GM, CFU-granulocyte-erythrocyte-megakaryocyte-monocye (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 t-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 × 104 BM cells or 2.5 × 104 spleen cells were suspended in 1 ml 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% CO2 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. After washing the 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. 1A). Peak secretion of G-CSF occurred at an IL-17 concentration of 20 ng/ml. Heat-inactivated rmIL-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 < 0.0001) as well as a significant narrowing of the distribution of cells expressing high levels of SCF (Fig. 1B). 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 rmIL-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 rmIL-17 treatment (data not shown).
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 ± 6 ± 370 pg/ml vs 670 ± 6 ± 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).

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. 3A). 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. 3B). 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 ± 1.05 vs 3.5 ± 0.5 × 10^7/ml, p < 0.01) (Fig. 3A). Similar to littermate control
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. 3B).

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. 5A). 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. 5B, 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. 5A). 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. 5B).

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-G-CSF 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. 6B). Anti-G-CSF also significantly attenuated AdmIL-17-mediated increases in ANC (Fig. 6B). 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.

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.
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 ± 63 vs 528 ± 63) (Fig. 7A). 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 induces 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
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).

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.

Acknowledgments

We thank Dr. Kenneth Dorshkind for generously providing the S17 cell line and Dr. Paul Kincade for generously providing the BMS2 cell line. We also thank Amgen for supporting our research with a gift of...
rmG-CSF. We also express gratitude to Dr. Melanie Spriggs (Immunex, Seattle, WA) for her generously sharing data and reagents.

References


Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017