Interleukin-1 Modulation of Cytokine Receptors on Human Neutrophils: In Vitro and In Vivo Studies


Interleukin-1 (IL-1) modulation of cytokine receptors (human IL-1 receptor [hIL-1R], human granulocyte colony-stimulating factor [hG-CSFR], human granulocyte-macrophage CSF receptor [hGM-CSFR], and human tumor necrosis factor receptor [hTNFR]) on human neutrophils was studied both in vitro and in vivo. In vitro, incubation of neutrophils with IL-1 at 37°C for 0.5 or 8 hours caused a reduction of IL-1 binding in a dose-dependent manner, but did not demonstrably affect binding of the other cytokines tested. In vivo, neutrophils from patients with gastrointestinal malignancies who were participating in a clinical trial of recombinant human IL-1β (rhIL-1β) demonstrated modulation of cytokine receptors in an IL-1β dose- and time-dependent manner. At the two highest dose levels of IL-1β (0.068 and 0.1 μg/kg), reduction (> 40%) of G-CSF binding and elevation (twofold to sixfold) of IL-1 binding to neutrophils was observed after 1 hour and 4 to 8 hours, respectively. In addition, IL-1β rapidly elevated G-CSF and glucocorticoid levels in plasma. Patients at the lowest dose level (0.002 μg/kg) had a less dramatic change in these parameters. Further in vitro studies showed that synthetic glucocorticoids and G-CSF synergistically up-modulated IL-1 binding to neutrophils in a dose- and time-dependent manner. Scatchard analysis of binding data showed that this in vitro synergistic modulation was due to an increase in receptor numbers, rather than an increase in binding affinity. In addition, both human umbilical cord blood and bone marrow neutrophils responded to G-CSF and dexamethasone (Dex) with a superadditive increase in IL-1 binding. Therefore, one of mechanisms for IL-1 up-modulation of IL-1R on human neutrophils in vivo was due to the fact that IL-1 rapidly elevates serum levels of G-CSF and glucocorticoids.

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MATERIALS AND METHODS

Growth factors. Recombinant human G-CSF (rhG-CSF) and rIL-6 were obtained from Amgen, Thousand Oaks, CA. Human rTNF was a gift from Dr M.A. Palladino, Genentech, San Francisco, CA. Human rIL-1, rIL-1β, and rGM-CSF were supplied by Immunex, Seattle, WA.

Chemicals and cell cultures. Jecove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) was supplemented with 3.02 g/L NaHCO₃, 25 mmol/L HEPES, pH 7.2. A single lot of fetal calf serum (FCS) (KC Biologicals, Lenexa, KS) was used throughout the present study. Chemically defined IMDM was supplemented with insulin (20 μg/mL) and transferrin (20 μg/mL). Disassociation buffer contained 120 mmol/L NaCl and 100 mmol/L acetic acid. pH 4.0. Cholesterol, prednisolone, dexamethasone (Dex), estradiol, progesterone, and hydrocortisone were purchased from Sigma, St Louis, MO, and dissolved in 100% alcohol. Concentration of steroid stock solution was 4 × 10⁻³ mol/L. N-succinimidyl-3-(4-hydroxyphenyl)-propionate was obtained from Pierce, Rockford, IL.

Murine NFS-60, WEHI-164 2F, and B-9 cell lines were gifts from Dr J.N. Ihle (St Jude Children's Research Hospital, Memphis, TN), Dr L.J. Old (Memorial Sloan-Kettering Cancer Center, New York, Thousand Oaks, CA 91320-1789).

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NY), and Dr L. Aarden (The Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands), respectively. Human U-373 cell line was purchased from American Tissue Culture collection (Rockville, MD), and human AML-193 cell line was obtained from Dr G. Rovera (Wistar Institute, Philadelphia, PA). All cell lines were cultured in IMDM with 10% FCS. For the growth factor-dependent cell lines, NFS-60 cells were supplemented with 1 ng/mL rhG-CSF, B-9 cells with 2 ng/mL rhIL-6, and AML-193 cells with 10 ng/mL rhGM-CSF.

Neutrophil purification. The rhIL-1β phase I/II clinical trial was approved by the Memorial Sloan-Kettering Cancer Institute Review Board. Human bone marrow cells and human peripheral blood were obtained from consenting healthy volunteers and patients participating in the phase I/II clinical trial of IL-1. Heparinized blood (5 to 7 mL) from patients or normal donors was layered onto 4 mL of Neutrophil Isolation Media (Los Alamos Laboratories, Los Alamos, NM) or mono-poly resolving medium (Flow Laboratories, Rockville, MD), and centrifuged at 2,000 rpm at room temperature in a Beckmann GPK centrifuge (Beckman Instrument Inc, Palo Alto, CA) for 45 minutes. Plasma was saved from each sample and frozen for subsequent analysis of cytokine levels. Neutrophils prepared by this method were greater than 98% pure and greater than 98% viable by trypsin blue exclusion.

Human umbilical cord blood was obtained from New York Hospital, New York, NY, and was fractionated by Ficoll-Hypaque medium. The cord blood neutrophil fraction was further purified by precipitation of red blood cells with dextran. This purification protocol obtained greater than 90% of neutrophils as judged by morphology.

Iodination of cytokines. Five hundred nanograms of rhIL-1, rhG-CSF, rhIL-6, rhTNF, or 3-(4-hydroxyphenyl)-propionyl-derived rhGM-CSF was iodinated by the chloramine-T method as previously described. The 3-(4-hydroxyphenyl)-propionyl-derived rhGM-CSF was prepared by incubation of 20 μg rhGM-CSF with 4.5 nmol N-succinimidyl-3-(4-hydroxyphenyl)-propionate in 0.1 mol/L borate buffer, pH 8.5, at 4°C overnight as described by Bolton and Hunter. The purity of labeled cytokines was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli. The specific activity of labeled cytokines, usually between 150 and 250 μCi/μg, was determined by self-displacement analysis as described by Calvo et al.1 Human U-373, AML-193, murine B-9, NFS-60, and WEHI-164-2F cell lines responded to IL-1, GM-CSF, IL-6, G-CSF, and TNF, respectively, and were used to determine the recovery of bioactivity of labeled cytokines. To increase the sensitivity of these bioassays, either chemically defined IMDM or IMDM with 0.2% FCS was used with the exception of rhGM-CSF.

Evaluation of cytokine and glucocorticoid levels. Cytokine levels were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits for hGM-CSF and hTNF (RD Systems, Minneapolis, MN), as well as hG-CSF and hIL-1β (Amgen Diagnostics, Thousand Oaks, CA). Sensitivities of GM-CSF, TNF, G-CSF, and IL-6 assays were 25, 4.8, 50, and 10 pg/mL, respectively. Plasma corticosterone levels were determined by a licensed clinical laboratory by radioimmunoassay (Memorial Sloan-Kettering Hospital). Corticosterone represents the predominant glucocorticoid produced by patients.

RESULTS

IL-1 modulation of cytokine receptors on human neutrophils in vitro. Neutrophils obtained from healthy donors were incubated with various doses of IL-1 at 37°C for either 0.5 or 8 hours before binding assays for IL-1, GM-CSF, TNF, and G-CSF at 4°C. The results (Fig 1) showed that IL-1 reduced its own binding in a dose-dependent manner after an 8-hour incubation, but did not dramatically affect the binding of other cytokines. IL-1, 2 and 20 ng/mL, reduced greater than 48% and greater than 78% of IL-1 binding, respectively. A similar result was also observed in neutrophils incubated with IL-1 for 0.5 hours (data not shown).

IL-1β modulation of cytokine receptors on human peripheral blood neutrophils in vivo. Peripheral blood from patients participating in a clinical trial of intravenously administered doses of IL-1β infused over 30 minutes was collected at the indicated time points and neutrophils were purified. Binding assays were performed at 4°C. At an IL-1β dose of 0.002 μg/kg, IL-1 affected (either increased or decreased) receptor binding for various cytokines to neutrophils from patients no. 002 and 004 by less than 25% (Fig 2A). One exception was GM-CSF binding, which decreased by greater than 60% in patient 004 after 1 hour. On the other hand, G-CSF, TNF, and IL-1 binding to neutrophils from one patient (003) decreased by greater than 45%, greater than 50%, and greater than 45%, respectively, within 3 hours. At an IL-1β dose of 0.068 μg/kg, G-CSF binding in all four patients was reduced by greater than 60% between 1 and 2 hours (Fig 2B). IL-1 binding increased twofold to sixfold in three patients after 6 to 8 hours. There were less dramatic changes in GM-CSF binding and TNF binding. Neutrophils from two patients were evaluated at the 0.1-μg/kg dose. G-CSF binding decreased by greater than 60% within 2 hours (Fig 2C). A twofold increase of IL-1 binding to neutrophils was observed in one patient (303) after 4 hours. In contrast, modulation of TNF and GM-CSF binding was less striking.

Effect of IL-1 on cytokine and glucocorticoid production in vivo. A difference between in vivo and in vitro effects of
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IL-1 on G-CSF and IL-1 binding to neutrophils implied that in vivo IL-1 might induce secondary molecule(s) to modulate expression of cytokine receptors on neutrophils. To further resolve a mechanism for IL-1 modulation of cytokine binding in vivo, plasma levels of cytokine and glucocorticoid in the IL-1-treated patients were determined. At an IL-1 dose of 0.002 μg/kg, G-CSF levels increased from less than 50 pg/mL to 400 pg/mL after 1 to 4 hours, and glucocorticoid levels increased from 120 ng/mL to 200 to 300 ng/mL after 2 to 3 hours (Fig 3A). At the two highest doses of IL-1, the glucocorticoid levels in serum increased 2.1- to 3.5-fold (from a range of 77 to 108 ng/mL to a range of 234 to 378 ng/mL) after 2 to 4 hours, and G-CSF levels increased from less than 50 pg/mL to 0.8 to 4.7 ng/mL after 2 to 4 hours (Fig 3B and C). There was no induction of IL-6 at the lowest dose level (data not shown); however, an increase in immunoreactive IL-6 over baseline values was observed at the 0.068 pg/kg (peak values, 54 to 298 pg/mL) and the 0.1 pg/mL level (peak value, 245 to 1950 pg/mL) after 2 to 4 hours. There was no change in TNF or GM-CSF in any of these patients as judged by ELISA (data not shown).

Glucocorticoids and G-CSF modulation of IL-1 binding to human neutrophils in vitro. To test whether glucocorticoids and G-CSF were indeed involved in the up-modulation of IL-1 binding, neutrophils from healthy donors were incubated in the presence or absence of various doses of G-CSF, 10^{-7} mol/L Dex, or a combination of the two at 37°C for 8 hours before binding assays. The results (Fig 4) showed that G-CSF and Dex synergistically increased IL-1 binding to neutrophils in a G-CSF dose-dependent manner. The 125I-IL-1 binding to control neutrophils was 459 cpm. Dex increased IL-1 binding more than threefold relative to control neutrophils. G-CSF at doses of 2 and 20 ng/mL increased IL-1 binding by greater than 9% and greater than 30%, respectively. In the presence of Dex, 2 and 20 ng/mL of G-CSF increased IL-1 binding 6.1- and 9.5-fold, respectively.

To determine whether this modulation was specifically induced by glucocorticoids, neutrophils were incubated in the presence or absence of various doses of steroids, 20 ng/mL G-CSF, or a combination of the two at 37°C for 8 hours before binding assays. As shown in Fig 5A and B, 125I-IL-1 binding capacity of neutrophils was markedly up-modulated by the combination of G-CSF and synthetic glucocorticoids such as Dex, prednisolone, and hydrocortisone, but not other steroids. The 125I-IL-1 binding to control neutrophils was 1,720 cpm. At a dose of 10^{-7} mol/L, hydrocortisone, prednisolone, and Dex increased IL-1 binding by greater than 0.7-, 1.8-, and 1.9-fold, respectively, relative to that of control. The IL-1 binding to G-CSF-treated neutrophils was 1.2-fold that of control. In the presence of G-CSF, 10^{-7} mol/L hydrocortisone, prednisolone, and Dex, IL-1 binding increased by more than 1.4-, 3.9-, and 4.4-fold, respectively. Cholesterol, estradiol, and progesterone, either alone or in combination with G-CSF, did not demonstrably affect IL-1 binding. This synergistic induction of IL-1 binding was also a glucocorticoid dose-dependent process. In the presence of G-CSF, 125I-IL-1 binding to 10^{-5} and 10^{-6} mol/L Dex-treated neutrophils was 3,900 cpm and 8,500 cpm, respectively. The same experiments were repeated twice on human peripheral blood neutrophils and once on human bone marrow neutrophils, and a similar pattern was obtained (data not shown).

Kinetic analysis of G-CSF and glucocorticoid modulation of IL-1 binding on neutrophil in vitro. Kinetics of G-CSF and glucocorticoid modulation of IL-1 binding was performed by incubation of neutrophils in the absence (control) or presence of 20 ng/mL G-CSF (G-CSF-treated), 10^{-7} mol/L Dex (Dex-treated), or in combination (G-CSF— and Dex-treated) at 37°C for various time periods. The results (Fig 6) showed
that IL-1 binding to Dex-treated neutrophils was elevated by more than 2.4-fold after 4 hours and reached a plateau (>3.3-fold) after 8 hours, relative to that of control neutrophils. For G-CSF–treated neutrophils, the binding reached a plateau (>1.5 fold) after 4 hours and remained at that level even after 16 hours. A superadditive interaction was seen between G-CSF and IL-1 binding to neutrophils treated with both agents being greater than 4.0-fold after 4 hours and reaching a peak (>6.6-fold) after 8 hours.

IL-1 binding of control, Dex-treated, G-CSF–treated, and G-CSF– and Dex-treated neutrophils. Up-modulation of IL-1 binding to neutrophils may be due to an increase in receptor affinity and/or an increase in receptor numbers. To resolve this issue, neutrophils were incubated in the presence or absence of G-CSF, Dex, or a combination of the two at 37°C for 8 hours and binding assays were performed. As shown in Fig 7, IL-1 binding to control, G-CSF–treated, Dex-treated, and G-CSF– and Dex-treated neutrophils could be saturated. Scatchard analysis9 of the binding data showed that control neutrophils displayed a maximal binding site (Bmax) of 1.8 × 10² sites per cell with a dissociation constant (kd) of 150 pmol/L; G-CSF–treated neutrophils displayed a Bmax of 2.4 × 10² sites per cell displayed a kd of 160 pmol/L; Dex-treated neutrophils displayed a Bmax of 5.7 × 10² sites per cell with a kd of 120 pmol/L; and G-CSF– and Dex-treated neutrophils displayed a Bmax of 8.9 × 10² sites per cell with a kd of 90 pmol/L.

Synergistic up-modulation of IL-1 binding to neutrophils from human umbilical cord blood and human bone marrow. To examine whether Dex and G-CSF also stimulate
IL-1R expression on neutrophils from umbilical cord blood and human bone marrow, neutrophils from these sources were incubated in the presence (G-CSF-treated) or absence (control) of 20 ng/mL G-CSF, 10^{-7} mol/L Dex (Dex-treated), or in combination (G-CSF- and Dex-treated) at 37°C for 8 hours before binding assays. The data (Table 1) showed that both umbilical cord blood and bone marrow neutrophils responded to the G-CSF and Dex with a synergistic increase in IL-1 binding. As shown in Table 1, IL-1 binding to G-CSF-, Dex-, and G-CSF- and Dex-treated neutrophils from umbilical cord blood no. 1 were increased by greater than 0.3-, greater than 3.1-, and greater than 6.76-fold relative to that of control neutrophils, respectively. For neutrophils from bone marrow no. 1, IL-1 binding to G-CSF-, Dex-, and G-CSF- and Dex-treated neutrophils were increased by greater than 0.32-, greater than 3.25-, and greater than 9.89-fold, respectively.

**DISCUSSION**

Our results showed that IL-1 up-modulated IL-1 binding and down-modulated G-CSF binding to human neutrophils in vitro. Neutrophils (2 × 10^6 cells/mL in IMDM with 10% FCS) were incubated in the presence (G-CSF-treated) or absence (control) various dose of G-CSF, 10^{-7} mol/L Dex (Dex-treated), or in combination (G-CSF- and Dex-treated) at 37°C for 8 hours before 125I-IL-1 binding assays at 4°C.
philms in vivo. This clinical observation was consistent with animal studies which showed that in vivo IL-1 increased IL-1 binding and increased G-CSF binding to murine bone marrow cells.\textsuperscript{50,51} In contrast, in vitro IL-1 specifically down-modulated IL-1 binding to neutrophils but not G-CSF binding, implying that IL-1 modulation of IL-1 and G-CSF binding to neutrophils in vivo was an indirect mechanism.

The mechanism(s) for the in vivo IL-1 increase of IL-1R type II on neutrophils may involve (1) IL-1-induced secondary biological molecule(s), which subsequently increase IL-1 binding to neutrophils; and/or (2) IL-1-induced release of neutrophils from bone marrow stores into peripheral blood. It has been reported that IL-1 induces a short-term neutrophilia by mobilization of neutrophils from the bone marrow compartments to peripheral blood compartments.\textsuperscript{19,20,33,38} If bone marrow neutrophils display a higher IL-1R number than peripheral blood neutrophils, this mobilization might result in an increase in IL-1 binding to peripheral neutrophils after IL-1 administration. This latter possibility is rendered
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unlikely by our previous studies showing that peripheral neutrophils and bone marrow neutrophils from the same normal donor exhibited a similar $B_{\text{max}}$ and $K_d$.32

Administration of IL-1 to patients resulted in an increase in G-CSF, IL-6, and glucocorticoid levels in patients' plasma within 2 to 3 hours. Our preliminary study indicated that IL-6 did not increase IL-1 binding to human neutrophils nor synergize with Dex (submitted for publication), suggesting that IL-6 might not be directly involved in the up-modulation of IL-1 binding in vivo. The IL-1-induced G-CSF plasma elevation in vivo may well account for the rapid reduction in G-CSF binding to neutrophils in vivo, and extend data from previous reports showing that IL-1 stimulated endothelial cells12 and fibroblasts14 to secrete G-CSF in vitro. In vivo, IL-1 stimulation of glucocorticoid secretion in patients was in agreement with animal results showing that in vivo IL-1 stimulated the secretion of glucocorticoids.32 Further studies indicated that synthetic glucocorticoids, but not other steroids, increased IL-1 binding on neutrophils in a dose-dependent manner. This observation was consistent with the previous studies showing that glucocorticoids increased IL-1 binding B cells,54 fibroblasts,54 murine bone marrow cells, and peritoneal macrophages.35 Incubation of neutrophils with glucocorticoids and G-CSF caused a synergistic increase of IL-1 binding in a dose- and time-dependent manner. These data confirm and extend the conclusion from our previous studies showing that G-CSF and glucocorticoids up-modulated IL-1R on murine bone marrow cells in vitro.51 Kinetic studies have shown that G-CSF and Dex stimulation of IL-1 binding to neutrophils in vitro occurs within 4 hours, which correlates with the time required for IL-1 up-modulation of its own receptors in vivo. This synergistic stimulation of IL-1 binding was due to an increase in receptor numbers, rather than an increase in binding affinity. While neutrophils from various donors and sources displayed a range in IL-1 binding capacity reflecting individual physiological status, neutrophils from all sources responded to G-CSF and Dex with a superadditive increase of IL-1 binding (Table 1), implying that this synergistic modulation could occur systemically. Therefore, one of the mechanisms for IL-1 increase of type II IL-1R on human neutrophils in vivo may be due to the fact that IL-1 stimulates the secretion of G-CSF and glucocorticoids, which subsequently increase IL-1R expression on human neutrophils.

IL-1 might stimulate production of cytokines and/or metabolites other than G-CSF and glucocorticoids to increase type II IL-1R in vivo. Fasano et al56 reported that neutrophils from septic patients displayed a higher IL-1R number than those of control patients, and GM-CSF increased IL-1 binding to neutrophils in vitro. Recently, Dubois et al57 demonstrated

![Fig 7. $^{125}$I-IL-1 binding to control (□), G-CSF-treated (■), Dex-treated (○), and G-CSF- and Dex-treated (△) neutrophils. Neutrophils (2 × 10^6 cells/mL in IMDM with 10% FCS) were incubated in the presence (G-CSF-treated) or absence (control) of 20 ng/mL G-CSF, 10^-7 mol/L Dex (Dex-treated), or in combination (G-CSF- and Dex-treated) at 37°C for 8 hours before titration of IL-1 binding at 4°C. Inset shows the Scatchard analysis of binding data.]

<table>
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<th>Sources of Neutrophils</th>
<th>Control</th>
<th>G-CSF-Treated</th>
<th>Dex-Treated</th>
<th>G-CSF- and Dex-Treated</th>
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<tr>
<td>Umbilical cord blood</td>
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<tr>
<td>No. 1</td>
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<tr>
<td>No. 2</td>
<td>524 ± 61</td>
<td>654 ± 43</td>
<td>2,096 ± 27</td>
<td>4,357 ± 136</td>
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<tr>
<td>Bone marrow</td>
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<tr>
<td>No. 1</td>
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<td>602 ± 5</td>
<td>1,939 ± 104</td>
<td>4,950 ± 69</td>
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<tr>
<td>No. 2</td>
<td>711 ± 16</td>
<td>714 ± 9</td>
<td>3,569 ± 55</td>
<td>7,836 ± 519</td>
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</table>

Neutrophils (2 × 10^6 cells/mL in IMDM supplemented with 10% FCS) from two umbilical cord blood specimens and two bone marrow specimens were incubated in the presence (G-CSF-treated) or absence (control) of 20 ng/mL G-CSF, 10^-7 mol/L Dex (Dex-treated), or in combination (G-CSF- and Dex-treated) at 37°C for 8 hours before binding assays at 4°C.

In vivo, IL-1 stimulation of glucocorticoid secretion in patients was
that G-CSF, GM-CSF, IL-3, or IL-6 up-modulated type II IL-1R on murine Lin progenitor cells. Our data also showed that GM-CSF and Dex stimulated a superadditive increase of type II IL-1R on human neutrophils (submitted for publication). In addition, Spriggs et al. showed that Dex and PGE2 synergistically up-modulated type II IL-1R on primary human monocytes and neutrophils. Our preliminary studies also indicated that GM-CSF, PGE2, and Dex synergistically up-regulated IL-1 binding to murine peritoneal exudate macrophages (PEM) (unpublished results). Relevant to the above data, IL-1 was capable of inducing secretion of PGE in vitro, and Ye et al showed that IL-2 increased mRNA for IL-1R type I in murine EL-4 cells. It seems that in vivo IL-1 might induce several biological molecules to up-modulate expression of IL-1R. Further understanding of IL-1-induced mediators (such as cytokines and lipid mediators) in vivo will help to resolve this mechanism.

IL-1 up-modulation of IL-1R in vivo may play an important role in the hematopoeitic effects of IL-1. In vivo IL-1 stimulated the secretion of glucocorticoids and IL-6 and G-CSF (which either alone or in combination) elevated the expression of IL-1R on fibroblasts, B cells, monocytes, macrophages, neutrophils, immature myeloid cells, and leukemic cells in vitro. In vivo, Dubois et al. showed that administration of type I IL-1R antibody blocked the IL-1 increase of type II IL-1R, as well as the IL-1 radioprotective effects. They also showed that IL-1 increased type II IL-1R on immature myeloid cells and mature neutrophils. Relevant to this, Nicola et al. suggested that greater than 50% of the CSFR on hematopoietic progenitor cells need to be occupied to induce a maximal biological response. Our animals studies showed that G-CSF and IL-1 synergistically stimulated hematopoietic progenitor cell recovery and hematopoietic regeneration following 5-fluorouracil treatment, and that in vivo administration of G-CSF caused an increase of IL-1R on murine BMC. It is likely that in vivo up-modulation of IL-1R on immature myeloid cells and accessory cells may present a more favorable environment for the hematopoietic effects of endogenous and/or exogenous IL-1R subsequently. Because IL-1 in high doses (>0.1 μg/kg) exhibited a profound effect on human hematopoiesis but with toxic side effects, administration of agents that increase IL-1R and kinetic monitoring of IL-1 binding may allow us to set up a schedule using low doses of IL-1 to obtain maximal hematopoietic effects with minimal toxicity.

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Interleukin-1 modulation of cytokine receptors on human neutrophils: in vitro and in vivo studies

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