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# Neutrophils are significant producers of IL-10 during sepsis ☆

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ABSTRACT

Sepsis is a syndrome involving systemic inflammation as well as an infectious focus. Accordingly, the host immune response to sepsis involves complex leukocyte interplay that is incompletely understood. It is known that the immunoregulatory cytokine, IL-10, is rapidly expressed during the early stages of sepsis. In a murine model of sepsis, we sought to elucidate which leukocytes are early IL-10 producers. Using a novel IL-10 transcriptional reporter mouse, we observed that splenic leukocytes produced little IL-10. At the site of infection, peritoneal neutrophils produced the highest levels of IL-10 among leukocytes. Using cytokine antibody labeling, we further show that peritoneal neutrophils had high amounts of intracellular IL-10. We next depleted neutrophils and found a 40% decrease in peritoneal IL-10 levels. Altogether, this report demonstrates that among leukocytes, neutrophils are significant contributors of IL-10 at the site of infection during sepsis.



## Introduction

Sepsis is defined as the systemic inflammatory response directed against an infectious focus. It remains a difficult syndrome to treat, in part, due to the complexity of the immune response. Among the numerous cytokines and chemokines produced during sepsis, IL-10 is a potent immunoregulatory molecule. The consequences of IL-10 on the immune response include: the down-regulation of key signaling receptors on antigen presenting cells such as CD40, CD80, CD86 and MHC II, decreased Mac-1 expression [1] and inhibition of neutrophil oxidative burst [2], suppression of T cell proliferation and IL-2, IL-6 and IFN-γ production, the maintenance of FoxP3 expression in regulatory T cells, and suppression of NK cell function [3].

During sepsis, blockade of IL-10 by neutralizing antibodies was shown to decrease survival and increase neutrophil accumulation. Additionally, it was found that IL-10 reduced NK activation and IFN-γ production [3]. Another study investigated the use of the nontoxic immunomodulator, AS101, which is known to inhibit IL-10 expression [4]. When AS101 was administered 12 h following sepsis induction, survival was improved. Consistent with this, AS101 increased MHC II expression on APCs, T cell IFN-γ production, and bacterial clearance, while decreasing tissue damage. Thus, the timing for IL-10 neutralization in order to improve the host response during sepsis is critical. Finally, it was found that during sepsis, the combination of infectious focus removal and administration of recombinant IL-10 decreased mortality and serum IL-6, while increasing T cell responsiveness [5].

A large number of leukocytes, including monocytes/macrophages, dendritic cells, neutrophils, natural killer cells, eosinophils, T and B cells, as well as nonimmune cells such as keratinocytes and hepatocytes, can express IL-10 [6–11]. However, despite the importance of IL-10 during sepsis, leukocyte contributions towards IL-10 production and accumulation are not completely understood. Here, we utilized a novel IL-10 transcriptional reporter mouse and antibody labeling to determine that significant numbers of neutrophils produce IL-10 at the site of infection. Additionally, using neutrophil depleting antibodies, we determined the net neutrophil contribution to IL-10 concentrations at the site of infection.

## Materials and methods

*Cecal ligation and puncture.* Homebred male C57BL/6 (WT) and IL-10 transcriptional reporter (Vert-X) [12] mice between 6 and 8 weeks of age were utilized. The Vert-X mice were a generous gift from the Christopher L. Karp laboratory. All experiments involving animals were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati. Polymicrobial sepsis was induced with an 80% cecal ligation and a single puncture using a 23 gauge needle as previously described [13].

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*Neutrophil depletion.* Mice were injected intraperitoneally (i.p.) with 150 μg RB6-8C5 mAb or rat IgG as a control 36 h prior to CLP [14]. Pilot experiments demonstrated depletion of blood neutrophils after 36 h (data not shown).

*Flow cytometry for surface and intracellular staining.* Analyses of cell surface antigen expression and *in situ* cytokine expression were performed as previously described on the peritoneal lavage samples [15]. Flow cytometry data acquisition and analysis were performed on LSR II using FACS Diva software (Becton Dickinson, Mountain View, CA). After blockade of Fc receptors with CD16/32 blocking Ab (BD Pharmingen, San Diego, CA), leukocytes were labeled using mAbs to the following antigens: CD11b (BD Pharmingen), Gr-1 (Clone: RB6-8C5, BD Pharmingen), Ly-6G (Clone: 1A8, BD Pharmingen), CD4 (Clone: RM4-5, BioLegend, San Diego, CA), CD8 (Clone: 53-6.7, BD Pharmingen), F4/80 (clone: 6F12, BD Pharmingen), IL-10 (Clone: JES5-16E3, BioLegend) and anti-neutrophil (Clone: 7/4, AbD Serotec, Raleigh, NC).

*IL-10 measurement by ELISA.* Peritoneal lavage was obtained by injection of 9 ml of 0.9% normal saline intraperitoneally and removal via syringe. IL-10 (BD Pharmingen) levels were analyzed using the manufacturer's protocol [16].

*Statistical analyses.* Statistical comparisons were performed using Student's *t* test using StatView 3.5 (SAS Institute, Cary, NC). The mean and standard error of the mean were calculated in experiments containing multiple data points. A value of $p \leqslant 0.05$ was considered statistically significant.

## Results

In order to determine IL-10 producing cells during sepsis, Vert-X mice underwent either sham- or CLP-surgeries. Peritoneal and splenic leukocytes isolated from sham-operated mice did not express GFP (data not shown). In septic Vert-X mice, we analyzed splenic macrophages, CD4, CD8 and B cells (Fig. 1A) as well as peritoneal neutrophils, macrophages, and T cells (Fig. 1B) for GFP expression 24 h after CLP. Of these cells, peritoneal neutrophils exhibited the highest levels of GFP, while macrophages exhibited moderate levels of GFP. Alternatively, we collected peritoneal leukocytes from CLP-operated wild type mice and determined IL-10 production by intracellular cytokine labeling. By this technique, we observed that peritoneal neutrophils from septic mice exhibited significant IL-10 production using this alternative method (Fig. 1C). Altogether, using two distinct methodologies, we demonstrate that neutrophils are a significant producer of IL-10 during the first 24 h after CLP.

In order to define the amount of IL-10 produced by neutrophils during early sepsis, we utilized anti-Gr-1 antibodies to deplete neutrophils prior to CLP. Isotype- and anti-Gr-1-treated mice then underwent CLP. Using two distinct neutrophil-labeling panels, we observed that 24 h after CLP the proportion of peritoneal neutrophils is decreased between the two treatments (Fig. 2A–D). Enumeration of flow cytometric data 6 and 24 h after CLP demonstrate a greater than 95% depletion of neutrophils in the Gr-1-treated mice compared to the isotype-treated mice (Fig. 2E). Finally, 24 h after CLP, we observed an approximate 40% decrease of peritoneal IL-10 concentrations isolated from neutrophil depleted septic mice. Thus, neutrophils are significant producers of IL-10 during the first 24 h of sepsis.

## Discussion

IL-10 is an important immunoregulatory cytokine involved in controlling inflammation during sepsis. In this report, we utilized the CLP model, which is currently considered the "gold standard", to induce sepsis [17]. During sepsis, we observed little IL-10 production by splenic and peritoneal lymphocytes, and significant IL-10 production by myeloid cells, particularly neutrophils (Fig. 1).

Previously, it has been shown that neutrophils isolated from burn patients could express IL-10, while neutrophils isolated from

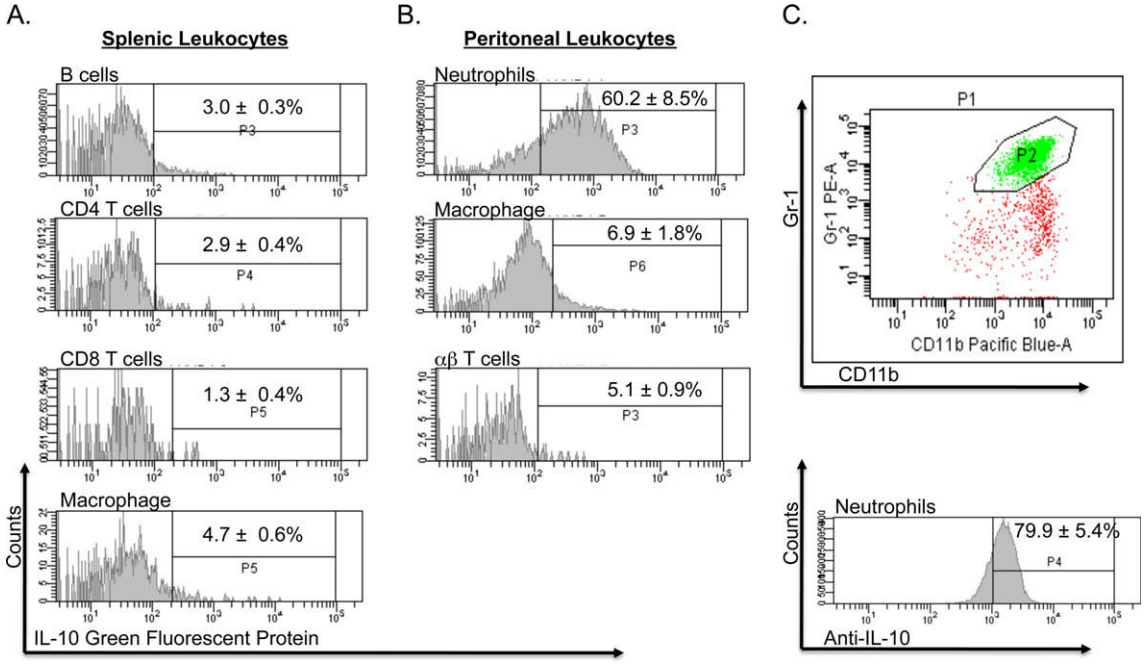


**Fig. 1.** Peritoneal neutrophils are observed to produce IL-10 by two methodologies. Using septic IL-10 GFP reporter or wild type mice 24 h after CLP, leukocytes were isolated and analyzed by flow cytometry as described in Materials and methods. After gating for viability, cells were again gated, identified as indicated, and analyzed for (A) splenic leukocyte IL-10 production and (B) peritoneal leukocyte IL-10 production. Non-GFP expressing cells from wild type mice were used to determine the gates for autofluorescence specifically for each indicated cell type. Using septic wild type mice 24 h after CLP, peritoneal neutrophils were isolated and analyzed by flow cytometry as described in Materials and methods. (C) Neutrophils are identified by CD11b and Gr-1 expression and IL-10 production determined by anti-IL-10 intracellular labeling. An IL-10 isotype control was used to determine the gate. The data are representative of four individual experiments. Data are expressed as means ± SEM.

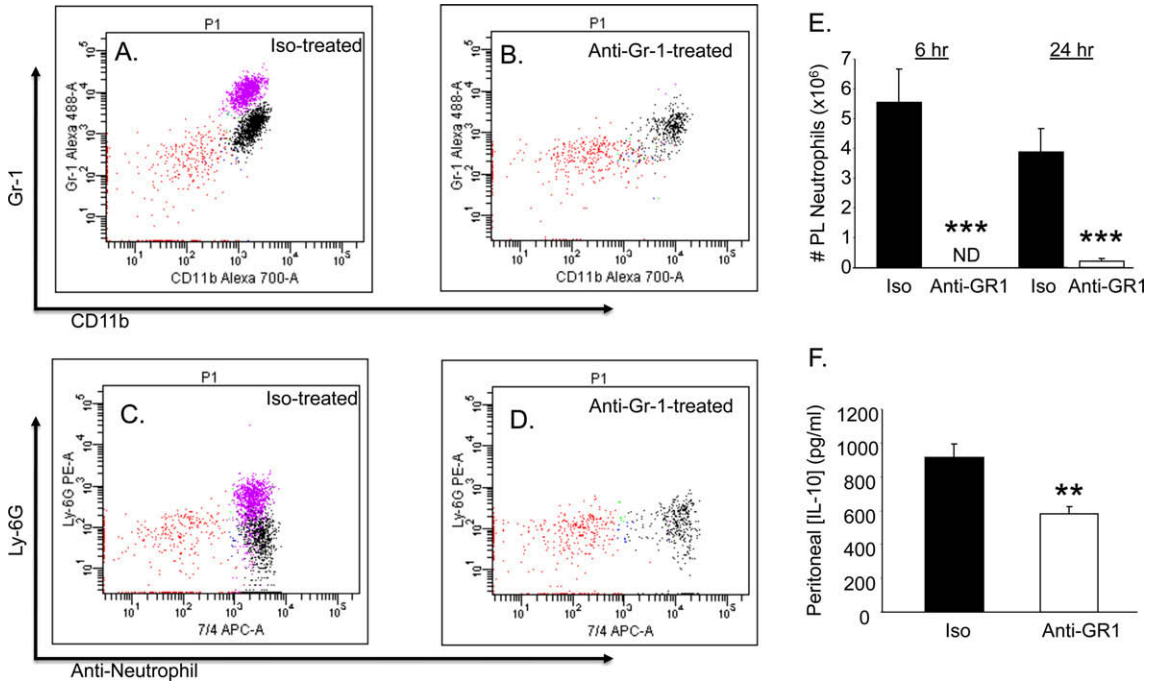


**Fig. 2.** Neutrophil depleted mice accumulate decreased peritoneal IL-10. Wild type mice were treated with neutrophil-depleting (anti-Gr-1) antibody or isotype control (Iso) prior to CLP as described in Materials and methods. Twenty-four hours following CLP, peritoneal cells were isolated and analyzed by flow cytometry using two different neutrophil panels. Representative dot plots using Gr-1 and CD11b antibodies to determine peritoneal neutrophil proportions isolated from (A) isotype-treated or (B) anti-Gr-1 treated mice. Representative dot plots using Ly-6G and anti-neutrophil antibodies to determine peritoneal neutrophil proportions isolated from (C) isotype-treated or (D) anti-Gr-1 treated mice. (E) Enumeration of peritoneal neutrophils isolated at the indicated time. (F) Peritoneal IL-10 concentrations 24 h after CLP. The sample size was 5 per group. Data are expressed as means ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$ as compared to isotype control.

healthy controls could not [18]. As burn patients are more susceptible to infections, this suggests that burn-trauma increases numbers of neutrophils with potentially pathogenic immunosuppressive properties. Signaling mechanisms responsible for this altered phenotype have not been fully elucidated, but may include inflammation-induced mediators such as GM-CSF and TNF-α. It has been reported that when treated with TNF-α, neutrophils from wild type mice, but not TNF-α receptor deficient mice, had significantly increased p38 MAPK phosphorylation [19]. It is well established that TNF-α is increased during sepsis and this likely results in increased active p38 in neutrophils. Additionally, active p38 is known to control IL-10 production [20]. Thus, increased inflammation may enhance IL-10 production through a p38-dependent mechanism.

High systemic levels of IL-10 have been reported as predictive for increased mortality, while moderate levels of IL-10 are associated with low mortality [21]. At the site of infection, one of the most abundant leukocytes is the neutrophil and we demonstrate that these cells are significant IL-10 producers (Fig. 2). It has been demonstrated that depleting neutrophils at different time points produces significantly divergent results [22]. When neutrophils were depleted prior to CLP, there were substantial increases in bacteremia, along with increases in ALT, AST, and BUN that suggested increased liver and kidney tissue injury. In contrast, when neutrophils were depleted 12 h after CLP, there were dramatic reductions in levels of bacteremia, reduced liver and renal dysfunction, and decreased mortality [22]. These data suggest that initial neutrophil activity is protective for the host response to sepsis, while later actions are pathogenic. We speculate that during the intense sepsis-associated inflammation the environment alters or switches the neutrophil phenotype such that the cells start to produce IL-10. Experiments to determine: (1) the time point when neutrophils start to produce IL-10, (2) whether neutrophil IL-10 production can be used as a clinical prognostic indicator, and (3) the role of p38 in neutrophil IL-10 production are in progress.

In summary, our data indicate that among leukocytes in the spleen or at the site of infection, neutrophils are significant producers of IL-10 during sepsis. As IL-10 is a potential therapeutic target for sepsis, this report provides significant insight into its mode of production.

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