

Interleukin-10 inhibits neutrophil phagocytic and bactericidal activity

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Abstract

Effective host defense against bacterial invasion is characterized by the vigorous recruitment and activation of inflammatory cells, which is dependent upon the coordinated expression of both pro- and anti-inflammatory cytokines. Interleukin-10 (IL-10) is a recently described cytokine with potent anti-inflammatory properties *in vivo* and *in vitro*. In this study we investigated whether IL-10 could directly regulate the ability of neutrophils (PMN) to phagocytose and kill bacteria. Initial studies demonstrated that human recombinant IL-10 (hrIL-10) inhibited the ability of PMN to phagocytose *Escherichia coli* *in vitro*. Inhibition of phagocytosis occurred in the absence of changes in CR1 (C3b) or Fc receptor expression, as treatment of PMN with IL-10 failed to induce significant changes in Fc γ IIR, Fc γ IIIR or CR1 cell surface expression. However, incubation of PMN with IL-10 resulted in a dose-dependent decrease in CD11b (Mac-1) expression. In addition to effects on PMN phagocytosis, hrIL-10 significantly attenuated PMN microbicidal activity, as bactericidal assays revealed that co-incubation of PMN with hrIL-10 resulted in a marked decrease in killing of phagocytosed bacteria. Furthermore, IL-10 inhibited the production of superoxide from PMA-stimulated PMN, suggesting that the detrimental effects of IL-10 on PMN microbicidal activity were due, in part, to suppression of respiratory burst. In summary, our studies indicate that IL-10 inhibits PMN-dependent phagocytosis and killing of *E. coli* *in vitro*, and suggest that this cytokine may impair effective antibacterial host defense *in vivo*.

Keywords: Interleukin-10; Phagocytosis; Cytokine; Bacteria; *Escherichia coli*

1. Introduction

Effective host defense against bacterial invasion is dependent upon the vigorous recruitment and activation of inflammatory cells. In the setting of bacterial

infection, the neutrophil is instrumental in both the phagocytosis and killing of bacterial organisms [1]. The efficacy of phagocytosis is greatly enhanced by opsonization of bacteria with specific (immunoglobulin) and non-specific (complement) opsonins, as well as the expression of Fc receptors (Fc γ I, Fc γ II, and Fc γ III) and C3b receptors (CR1) on the surface of PMN [2]. In addition, PMN phagocytic activity has recently been shown to be regulated by

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cell surface adhesion molecules, in particular the β_2 -integrin CD11b (Mac-1) [3,4]. Once internalized, the killing of bacteria by PMN occurs by oxidative and nonoxidative pathways [5]. Oxidative pathways involve the generation of reactive oxygen intermediates, whereas nonoxidative pathways are mediated through degranulation of primary and secondary granules containing hydrolytic and proteolytic enzymes, lactoferrin, and defensins.

While resting PMN display innate phagocytic and bactericidal activity, this activity can be substantially regulated by several host-derived cytokines. Specifically, tumor necrosis factor (TNF), interferon-gamma (IFN- γ), granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) have been shown to augment the phagocytic and/or bactericidal activities of PMN [6–9]. In addition, we have found that specific C-X-C chemokines can enhance PMN antimicrobial activities (unpublished observations). In contrast, mounting evidence suggests that other host-derived cytokines, such as interleukin-10, may have detrimental effects on antibacterial host defense, which may be due to direct inhibitory effects on phagocytic cells [10–12].

Interleukin-10 (IL-10) is a cytokine that was initially identified as an important mediator of Th2-driven immune responses [13]. More recently, this protein has been shown to exert anti-inflammatory properties, in part, by down-regulating the expression of TNF, IL-1, and members of both the C-X-C and C-C chemokine families [14–16]. We and others have shown that IL-10 inhibits the overzealous production of proinflammatory cytokines in states of systemic immune cell activation, such as sepsis [17,18]. However, IL-10-mediated suppression of activating and chemotactic cytokines may be detrimental to the host in the setting of bacterial infection, where a vigorous inflammatory response is essential for effective microbial clearance. In support of this premise, IL-10 inhibits macrophage antimicrobial activity *in vitro* [11]. In addition, the exaggerated expression of IL-10 in patients with leprosy has been associated with persistent and chronic infection [12], and the inhibition of IL-10 bioactivity *in vivo* promotes effective clearance of *M. avium-intracellulare* in mice [9]. Finally, we have recently identified the compartmentalized expression of murine IL-10 within

the lungs of mice with *Klebsiella pneumoniae*, and inhibition of IL-10 bioactivity *in vivo* resulted in a substantial increase in the clearance of *K. pneumoniae* and significant improvements in both short- and long-term survival [19]. The mechanism(s) by which endogenous IL-10 inhibits antimicrobial responses has not yet been completely defined.

Because PMN are principal cellular participants in the effective clearance of Gram-positive and Gram-negative bacterial organisms, the current study was performed to determine if hrIL-10 can directly influence the ability of PMN to phagocytose and kill *E. coli* *in vitro*.

2. Materials and methods

2.1. Reagents

Human recombinant IL-10 was purchased from R&D Systems (Minneapolis, MN). Stock fMLP was prepared in DMSO at concentration of 10^{-2} M.

2.2. Bacteria

The bacteria used in all studies was *E. coli*, rough strain NCTC 86 (ATCC 4157, Rockville, MA). Bacteria were grown in tryptic soy broth (Difco, Detroit, MI) for 18 h at 37°C, centrifuged at $2000 \times g$ for 10 min, washed in HBSS twice, then resuspended in HBSS at the desired concentration, as determined by the spectrophotometric OD reading at 600 nm.

2.3. Recovery and isolation of human PMN

Heparinized blood was collected by venous puncture and diluted 1:2 with 0.9% saline and mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. PMN were then isolated by sedimentation in 5% dextran/0.9% saline (Sigma) and separated from erythrocytes by hypotonic lysis. The resultant population was > 95% PMN as confirmed by cytospin differentials using Diff-Quik (Baxter, Miami, FL) staining. After washing twice, PMN were suspended in Hank's balanced salt solution with calcium/magnesium (Gibco, Grand Island, NY) at a concentration of 1×10^6 cells/ml. PMN

were > 95% viable as determined by Trypan blue exclusion.

2.4. Phagocytic assay

Human neutrophils (1×10^5 cells) were incubated with 10% human serum for 5 min at 37°C in 8-well labteks (Nunc, Inc., Naperville, IL) *E. coli* (1×10^6 bacteria) and a stimulant or equal volume of HBSS were added and incubated for 30 min at 37°C. The supernatants were removed and the cells were washed 3 times with HBSS. The gasket was removed and slides allowed to air dry. Diff-Quick stain was performed and 200 cells per well counted to determine number of intracellular *E. coli* and percent of PMN containing bacteria. Phagocytic index was determined by the following formula [20]:

Phagocytic Index (PI)

$$= \text{mean\% PMN containing } E. coli \\ \times \text{mean number } E. coli / \text{PMN}$$

2.5. Bactericidal assay

Human PMN (1×10^5 cells) were incubated with 10% human serum for 5 min at 37°C in 35 mm plastic culture dishes. *E. coli* (1×10^6 bacteria) and a stimulant or equal volume of HBSS were added and incubated for an additional 1 h at 37°C. The supernatants were removed and the cells were washed 3 times with HBSS. The cells were then lysed by adding 1 ml 0°C sterile H₂O, disrupting the cells with a cell lifter, and incubating them on ice for 10 min. One ml 2 × HBSS was added per well, then serially diluted on blood agar plates. Plates were incubated for 18 h at 37°C, and colony counts performed. Percent survival of intracellular bacteria was calculated by the following formula:

$$\% \text{ survival} = \frac{\text{number } E. coli \text{ cfu/ml PMN lysate}}{\text{total number intracellular } E. coli} \\ \times 100$$

Total intracellular *E. coli* is the product of total number of PMN × % PMN containing intracellular bacteria × mean number of intracellular *E. coli* per PMN.

2.6. Immunofluorescence staining and flow cytometry

The following monoclonal antibodies were obtained from PharMingen (San Diego, CA) as directly-fluorescein-isothiocyanate (FITC)-labeled conjugates: anti-LFA-1 (CD11a), mouse IgG₁; anti-L-selectin (LECAM-1), mouse IgG₁; anti-Mac-1 (CD11b), mouse IgG₁; anti-FcγRII (CD32), mouse IgG₂; anti-FcγRIII (CD16), mouse IgG₁; anti-CR1 (C3b (or CD35)), mouse IgG₁; and control mouse IgG₁ and IgG₂. Aliquots of purified PMN (5×10^5 cells in 0.1 ml) were stained with 1 μg of monoclonal antibody for 30 min on ice, washed, fixed in 2% paraformaldehyde in PBS, and stored at 4°C in the dark until analyzed. Samples were analyzed on an EPICS C flow cytometer with accompanying software (Coulter Corporation, EPICS Division, Hialeah, FL), examining at least 20 000 events per sample.

2.7. Superoxide assay

Superoxide production was determined by assessing the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C as previously described [21]. Briefly, 100 μl HBSS plus 8% albumin, and 50 μl ferricytochrome C (2.7 mg/ml) were mixed in quadruplicate wells of a 96-well plate ± 50 μl SOD (1 mg/ml) ± 10 μl PMA (10 μg/ml) to achieve a final volume of 0.2 ml per well. The plate was then incubated at 37°C for 10 min. Fifty microliters of warmed PMN (1×10^7 /ml) were added to each well and incubated for 20 min at 37°C on a microshaker. Plates were then read at 550 nm and nmol O₂⁻ calculated using the following formula:

$$\text{nmol produced} / 5 \times 10^5 \text{ PMN} \\ = (\text{OD without SOD} - \text{OD with SOD}) \times 26.5$$

2.8. Statistical analysis

Data were analyzed by a Macintosh 7100/80 computer using Statview 4.0 statistical package (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as mean ± S.E.M. Data was analyzed by

one-way or two-way ANOVA, as appropriate. Comparisons were made using the Student-Neuman-Keuls pairwise multiple comparison procedure. Data were considered statistically significant if *P* values were less than 0.05.

3. Results

3.1. IL-10 inhibits PMN phagocytic activity

Initially, studies were performed to determine the effect of hrIL-10 on the phagocytosis of *E. coli* by PMN. Treatment of human PMN with hrIL-10 for 1 h resulted in only modest and statistically insignificant changes in the percentage of PMN containing intracellular *E. coli* (Table 1). However, incubation of PMN with 10 and 50 ng/ml hrIL-10 resulted in a significant decrease in the mean number of *E. coli* per cell, with a maximal 37% decrease in intracellular bacteria, respectively, as compared to unstimulated PMN (Table 1). Furthermore, the phagocytic index of IL-10-challenged PMN was maximally 1.6-fold less than control PMN.

3.2. IL-10 inhibits cell surface expression of CD11b

Having demonstrated that IL-10 attenuated PMN phagocytic activity, we next wanted to determine if this cytokine did so by altering the cell-surface expression of complement, immunoglobulin, or adhesion molecule receptors. Incubation of PMN with IL-10 did not influence the cell surface expression Fc γ IIIR, Fc γ IIIIR, or CR1 (data not shown). How-

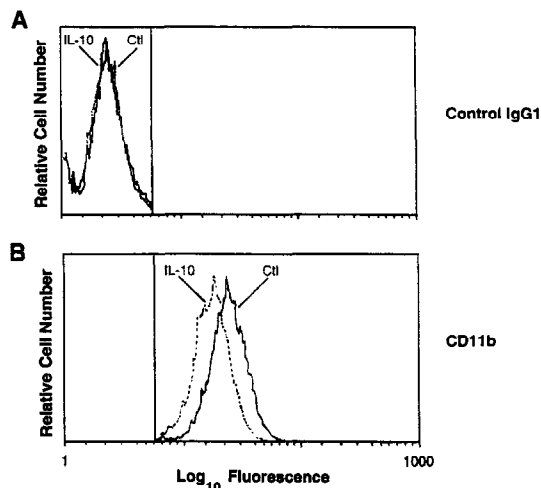


Fig. 1. Effect of IL-10 on PMN cell surface expression of CD11b. Representative flow cytometric analysis depicting effect of IL-10 on cell surface expression of CD11b. (A) control IgG₁; (B) CD11b. Ctl = unstimulated PMN (solid line), IL-10 = PMN incubated with IL-10 (50 ng/ml, dashed line) \times 30 min. Relative PMN number is expressed on the vertical axis and log₁₀ fluorescence expressed on the horizontal axis. Representative of 6 separate experiments.

ever, incubation of PMN with IL-10 resulted in a dose-dependent and selective decrease in CD11b expression (Fig. 1), without significant changes in the cell-surface expression of L-selectin or CD11a (Table 2).

3.3. IL-10 attenuates PMN bactericidal activity

Additional studies were performed to determine the effect of interleukin-10 on PMN bactericidal activity in vitro. As shown in Fig. 2, incubation of

Table 1
Effect of IL-10 on phagocytosis of *E. coli* by PMN

Treatment	<i>E. coli</i> /PMN	% PMN	PI
Control	1.45 \pm 0.10	69.9%	0.97
fMLP (10 ⁻⁷ M)	2.17 \pm 0.13 *	78.5%	1.63
hrIL-10			
10 ng/ml	1.07 \pm 0.09 *	58.5%	0.62
50 ng/ml	0.91 \pm 0.06 *	65.0%	0.59

%PMN = percentage of PMN containing intracellular *E. coli*.

PI = phagocytic index (calculated as described in Section 2, Materials and methods).

Values represent mean \pm S.E.M. of 12 separate experiments.

* *P* < 0.01 as compared to unstimulated control.

Table 2
Effect of IL-10 on the PMN cell-surface expression of CD11a, CD11b and L-selectin

Treatment	CD11a	CD11b	L-Selectin
Resting	2.22 \pm 0.03	6.21 \pm 0.25	6.34 \pm 0.14
hrIL-10			
1 ng/ml	2.13 \pm 0.07	5.59 \pm 0.15	6.11 \pm 0.14
10 ng/ml	2.25 \pm 0.04	5.23 \pm 0.43 *	6.43 \pm 0.12
50 ng/ml	2.35 \pm 0.05	5.14 \pm 0.24 *	6.89 \pm 0.15

Values represent mean log₁₀ fluorescence minus log₁₀ fluorescence of control IgG (*n* = 8 separate experiments).

* *P* < 0.05 as compared to unstimulated PMN.

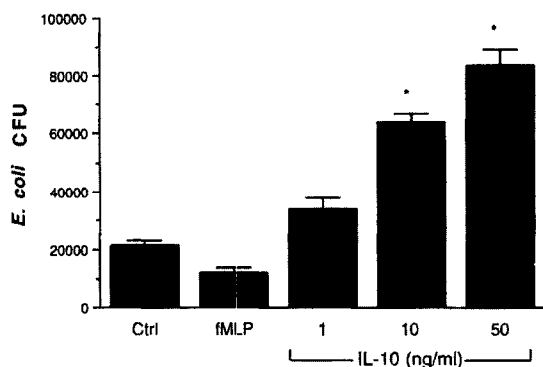


Fig. 2. Effect of IL-10 on killing of intracellular *E. coli* by PMN. Resting or cytokine-stimulated PMN (1×10^5) were incubated with 10% human serum and *E. coli* (1×10^6) in HBSS for 1 h, then the number of viable bacteria in cell lysates determined. * $P < 0.05$ compared to untreated PMN. Experimental $n = 18$ per condition.

PMN with hrIL-10 resulted in a substantial decrease in killing of *E. coli*, with a maximal 3.9-fold increase in viable intracellular *E. coli* in PMN treated with hrIL-10 at a concentration of 50 ng/ml. Furthermore, hrIL-10 dose-dependently impaired the ability of PMN to kill ingested bacteria (Table 3). The IL-10-mediated inhibition of bacterial killing was PMN-dependent, as incubation of *E. coli* with IL-10 alone did not alter *E. coli* growth (data not shown).

3.4. IL-10 suppresses the production of superoxide by PMA-stimulated PMN

To determine if the inhibitory effect of IL-10 on PMN bactericidal activity was attributable to sup-

Table 3
Effect of IL-10 on intracellular killing of *E. coli* by PMN

Treatment	% Survival
Control	8.9 ± 0.6
fMLP (10^{-7} M)	2.9 ± 0.3 *
hrIL-10	
1 ng/ml	15.6 ± 3.3
10 ng/ml	42.6 ± 2.9 **
50 ng/ml	59.2 ± 3.5 **

% survival of intracellular *E. coli* is calculated as described in Section 2, Materials and methods.

* $P < 0.05$, ** $P < 0.01$ as compared to unstimulated PMN.

Table 4

Effect of IL-10 inhibits on the production of superoxide by unstimulated and PMA-stimulated PMN

Treatment	nM Superoxide
Control	0.52 ± 0.43
IL-10 (1 ng/ml)	0.30 ± 0.16
IL-10 (10 ng/ml)	0.16 ± 0.13
IL-10 (50 ng/ml)	0.25 ± 0.12
PMA	6.53 ± 0.16
PMA + IL-10 (1 ng/ml)	5.96 ± 0.37
PMA + IL-10 (10 ng/ml)	5.53 ± 0.22 *
PMA + IL-10 (50 ng/ml)	5.42 ± 0.32 *

Values represent mean \pm SEM nmol superoxide production per 5×10^5 PMN.

* $P < 0.05$.

Experimental $n = 12$ per condition.

pression of respiratory burst, we next assessed the effect of hrIL-10 on the production of superoxide from resting and phorbol myristate acetate (PMA)-stimulated PMN. As shown in Table 4, unstimulated PMN produced minimal amounts of superoxide, which was not significantly altered by co-incubation with hrIL-10. However, treatment of PMN with PMA (10 μ g/ml) resulted in a substantial increase in superoxide production. Furthermore, incubation of PMN with IL-10 at concentrations of 10 and 50 ng/ml resulted in modest but significant reductions in PMA-induced superoxide production.

4. Discussion

The current study indicates that IL-10 has an important immunoregulatory effect on PMN phagocytic and microbicidal activities. Interleukin-10 has previously been shown to regulate the effector cell activities of a variety of immune and non-immune cells, including macrophages and PMN [11,14–16,23,24]. Similar to effects on various macrophage populations, IL-10 exerts predominant anti-inflammatory effects on PMN function. Specifically, IL-10 inhibits the expression of TNF, IL-1, interleukin-8 (IL-8) and macrophage inflammatory protein-1 alpha (MIP-1 α) and beta (MIP-1 β) from LPS-stimulated PMN [14,15,24], which is partially mediated through a decrease in tyrosine phosphorylation of lipopolysaccharide-induced phosphoproteins.

In contrast, IL-10 has been shown to enhance the production of the anti-inflammatory cytokine IL-1 receptor antagonist from PMN [22,24]. The suppressive influence of IL-10 on PMN phagocytic and antimicrobial properties further illustrates the role of this cytokine as an endogenous inhibitor of acute inflammatory responses.

The mechanism(s) by which IL-10 inhibits PMN phagocytic activity has not been completely defined. Our studies indicated that IL-10 failed to significantly alter the cell surface expression of Fc γ RII, Fc γ RIII, or CR1. Recently, novel non-opsonic mechanisms of phagocytosis have been described. In particular, PMN β_2 -integrins have been shown to mediate attachment of microbes or foreign substances to PMN, facilitating efficient phagocytosis [3,4]. Interestingly, our studies indicate that IL-10 selectively inhibits CD11b expression on the surface of PMN, which may partially account for the inhibitory effects of IL-10 on PMN phagocytosis. Interleukin-10 has previously been shown to inhibit the expression of intracellular adhesion molecule-1 (ICAM-1) on endothelial cells and blood monocytes [25,27], but failed to inhibit the expression of CD11a, CD11b, and CD18 on THP-1 [25], or CD11a, CD18, or ICAM-1 on B-cells [26]. This is the first study to demonstrate that IL-10 selectively regulates the expression of β_2 integrins on phagocytic cells, in particular PMN.

Our studies indicate that IL-10 not only inhibited PMN phagocytosis of bacteria, but also inhibited the ability of PMN to kill internalized *E. coli*. The mechanism of IL-10-mediated suppression of PMN bacterial killing has not yet been fully elucidated. However, treatment of PMN with IL-10 resulted in a modest, but significant dose-dependent decrease in superoxide production. Our findings extend the findings of Bogdan and colleagues [10], who demonstrated that murine IL-10 inhibited the generation of reactive oxygen intermediates from stimulated mouse peritoneal macrophages. The suppressive effect of IL-10 on PMN antimicrobial activity cannot be completely explained by these modest observed effects on respiratory burst alone, suggesting that IL-10 is also likely to have inhibitory effects on non-oxidative pathways of microbial killing.

We have previously shown that murine IL-10 mRNA and protein is expressed during the evolution

of *Klebsiella pneumoniae* in mice, and the passive immunization of animals with rabbit antimurine IL-10 polyclonal antibodies resulted in improved bacterial clearance, and increased early and late survival as compared to control animals receiving *K. pneumoniae* i.t. [18]. Importantly, anti-IL-10 antibodies did not alter lung PMN influx, as determined by lung myeloperoxidase activity. These observations, taken together with our current findings, suggest that the direct inhibitory effects of IL-10 on both PMN and macrophage phagocytic and antimicrobial activities may contribute greatly to the detrimental effects of IL-10 on innate immune responses against bacterial organisms. Moreover, our findings suggest that immunotherapy directed against endogenous IL-10 may serve as a potentially useful adjuvant therapy in the treatment of patients infected with virulent and/or multi-drug resistant bacterial organisms.

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