

Interleukin 10 Inhibits Lipopolysaccharide-induced Survival and Cytokine Production by Human Peripheral Blood Eosinophils

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Summary

In this study we have investigated the effects of interleukin 10 (IL-10) on human peripheral blood eosinophils stimulated with granulocyte/macrophage colony stimulating factor (GM-CSF) and lipopolysaccharide (LPS). We show that LPS was able to enhance eosinophil survival in a dose-dependent manner, as well as release of the cytokines GM-CSF, tumor necrosis factor α , and IL-8. LPS-induced eosinophil survival was largely inhibited by an anti-GM-CSF neutralizing antibody and completely blocked by polymyxin B, suggesting GM-CSF involvement in the survival enhancing mechanism and LPS specificity, respectively. IL-10 significantly inhibited survival of, and cytokine production from, eosinophils induced by LPS, but did not inhibit the survival induced by GM-CSF. These observations suggest a novel activation mechanism of eosinophils and, also, that IL-10 may participate in the regulation of diseases characterized by eosinophil infiltration.

Human eosinophils, like other granulocytes, are terminally differentiated defense effector cells that are recruited from the bloodstream into tissue sites during inflammatory, particularly allergic, reactions (1, 2). Recent evidence from us (3, 4) and others (5–10) indicates that in addition to their capacity to release lipid mediators (11) and granule proteins (12), eosinophils may contribute to the inflammatory process through expression and synthesis of cytokines including TGF- α/β , TNF- α , MIP-1 α , IL-1, IL-5, IL-8, GM-CSF, and IL-6. While stimuli such as calcium ionophore and ionomycin have been found capable of eliciting cytokine expression in eosinophils in vitro (9, 13, 14), which physiologic stimuli, and which underlying mechanisms are involved in the regulation of eosinophil cytokine responses still remain largely unknown.

IL-10 is a recently identified cytokine primarily produced by mononuclear cells (15), with potent antiinflammatory activities. This molecule has been shown to inhibit the release of cytokines from monocytes (16), T lymphocytes (17) and neutrophils (18), but its effect on effector activities of eosinophils remains to be elucidated.

In this report, we demonstrate that IL-10 is a potent inhibitor of both eosinophil survival and secretion of the proinflammatory cytokines GM-CSF, TNF- α , and IL-8 induced by an infectious agent, LPS, thus providing potential novel mechanisms for activation and regulation of functions of human eosinophils.

Materials and Methods

Reagents. Recombinant human (rh) GM-CSF (1 U = 40 pg) was kindly provided by Dr. John Abrams (DNAX Research Institute, Palo Alto, CA). Recombinant human IL-10 was purchased from Pepro Tech Inc. (Rocky Hill, NJ). LPS (from 055:B5 *Escherichia coli*), polymyxin B, and mouse IgG₁ were purchased from Sigma Chemical Co. (St. Louis, MO). The specific mouse anti-human GM-CSF monoclonal antibody was purchased from Genzyme Corp. (Cambridge, MA).

Isolation and Culture of Human Eosinophils. Blood was drawn from healthy nonatopic volunteers (skin test negative). Eosinophils were isolated by negative-immunomagnetic selection using an antibody against CD16, a FcRIII receptor found on neutrophils but not eosinophils (19). Briefly, red blood cells were removed from 100 ml of the whole blood by dextran (clinical grade; ICN Biomedicals Inc., Costa Mesa, CA) sedimentation. A granulocyte layer was obtained by density centrifugation on a Percoll gradient (1.079; 1.100, prepared in HBSS) and was resuspended in Ca²⁺ free HBSS. The residual contaminating erythrocytes were eliminated by hypotonic lysis. Granulocytes were resuspended in 2% FCS in HBSS and incubated for 40 min at 4°C with anti-CD16 conjugated with micromagnetic beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Upon passing through the magnetic column (MACS: Miltenyi Biotec GmbH), neutrophils bound to the beads were retained within the column, whereas eosinophils were eluted out and collected. This purification procedure resulted in a highly pure eosinophil population (>99%). Eosinophils were cultured in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin and

100 mg/ml streptomycin with or without addition of LPS, cytokines or specific inhibitors.

Eosinophil Survival. Eosinophils were cultured in 96-well plates (2×10^5 /well in 0.2 ml medium). Cells were removed from each well after gentle pipetting and viability was assessed at days 2 and 4 by Trypan blue exclusion using a hemocytometer.

Cytokine Assays. Eosinophils were cultured in the presence of LPS with or without IL-10 in 24-well plates and supernatants collected at 24 h (2×10^5 /well in 0.5 ml of medium). GM-CSF, TNF- α , and IL-8 contents in supernatants were measured by ELISA (Amersham International, Little Chalfort, UK). The sensitivity of these assays was 1.5 pg/ml, 4.4 pg/ml, and 4.7 pg/ml for GM-CSF, TNF- α , and IL-8, respectively.

Data Analysis. Data were expressed as mean \pm SEM. Whenever applied, the significance of differences in results was analyzed by analysis of variance. The differences were considered statistically significant when $p < 0.05$.

Results and Discussion

Effect of IL-10 on Eosinophil Survival Induced by GM-CSF. We and others have previously shown that GM-CSF promotes the survival of human peripheral blood eosinophils (20, 21). To investigate whether IL-10 modulated this effect, human peripheral blood eosinophils were incubated with different amounts of rhGM-CSF in the presence of rhIL-10 (5 or 20 ng/ml), and viability assessed at day 4. Consistent with our previous findings (20), the viability of eosinophils cultured in RPMI alone was $<20\%$ at day 4, whereas GM-CSF enhanced survival in a dose-dependent manner with maximal stimulation observed at 1 U/ml ($88 \pm 2.4\%$) (Fig. 1). Coincubation with either 5 or 20 ng/ml of IL-10 had no effect on eosinophil survival (Fig. 1). Similar results were observed when eosinophils were pretreated with IL-10 for 4 h before exposure to GM-CSF (data not shown).

Effect of LPS on Eosinophil Survival. LPS is a major component of the outer membrane of Gram negative bacteria and it has been shown to induce many cellular responses including survival and cytokine production by monocyte/macrophages, neutrophils and some tissue stromal cells (22–24). To our

knowledge, the only evidence of LPS stimulation of eosinophils is the work by Pozo et al. (7) showing IL-1 release from LPS-stimulated peritoneal eosinophils from parasite infected mice. The a priori criterium used to determine responsiveness to LPS was as follows: when day 4 survival of eosinophils exposed to LPS was within the range of mean \pm 2 SD of day 4 survival of untreated (control) eosinophils, we considered this “nonresponsiveness.” Interestingly, we found that responsiveness to LPS of eosinophil preparations from healthy donors followed a rather bimodal pattern. Indeed, close to 40% of preparations showed a day 4 survival of $13.6 \pm 9.6\%$ (mean \pm SD). In contrast, over 60% of preparations showed a day 4 survival of $72.7 \pm 14.5\%$ (mean \pm SD). The reasons for this distinct response are not immediately clear, and samples from the nonresponder group were excluded from further experimentation. In the remaining samples, LPS markedly enhanced eosinophil survival in a dose-dependent fashion, with maximal stimulation seen with as low as 0.1 ng/ml of LPS (Fig. 2). To demonstrate the specificity of LPS action on eosinophil survival, LPS (10 ng/ml) was preincubated with a specific LPS inhibitor, polymyxin B (50 U/ml), that has been shown to form a stable complex with the lipid A moiety of LPS (25). As shown in Fig. 3, pretreatment of the LPS sample with polymyxin B entirely abolished the LPS-induced eosinophil survival ($66.5 \pm 2.8\%$ by LPS vs. $6.6 \pm 2.9\%$ by LPS pretreated with polymyxin B). To rule out the possibility that polymyxin B had a direct effect on eosinophil survival, we exposed untreated and GM-CSF (1 U/ml)-treated eosinophils to polymyxin B (50 U/ml) and examined survival at day 4. Survival of untreated eosinophils with and without polymyxin B was 3.0 and 3.4%, respectively. Survival of GM-CSF-treated eosinophils with and without polymyxin B was 88.0 and 89.3%, respectively ($n = 2$). Thus, polymyxin B had little, if any, effect on eosinophil survival of itself. Together, these data indicate the specificity of LPS action on eosinophils. The molecular mechanisms underlying LPS-eosinophil interaction remain speculative at this time. Both CD14 and CD11b/CD18 have been shown or proposed to be receptors for LPS on monocyte/macrophages and neu-

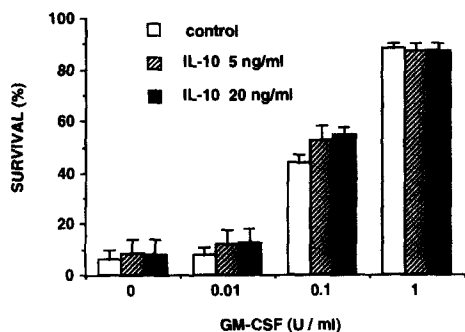


Figure 1. Effect of rhIL-10 on eosinophil survival induced by rhGM-CSF. Freshly isolated eosinophils (2×10^5) were cultured in the presence of increasing concentrations of GM-CSF with or without rhIL-10. Survival was assessed at day 4 by Trypan blue dye exclusion. Results are expressed as mean \pm SEM from five independent experiments.

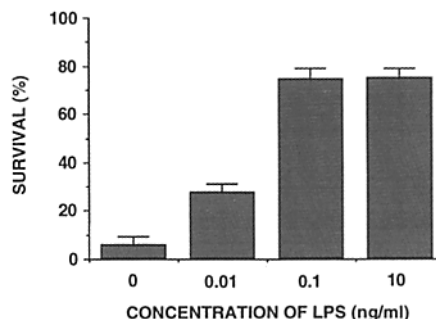


Figure 2. Effect of LPS on eosinophil survival. Freshly isolated eosinophils (2×10^5) were cultured with increasing concentrations of LPS and eosinophil survival was assessed at day 4 by Trypan blue dye exclusion. Results represent mean \pm SEM from three independent experiments.

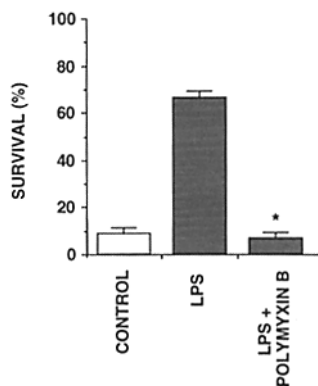


Figure 3. Abrogation of LPS-induced eosinophil survival by polymyxin B. Freshly isolated eosinophils (2×10^5) were cultured with LPS which had been preincubated with polymyxin B (50 U/ml) for 1 h at 37°C. Survival was assessed at day 4 by Trypan blue dye exclusion. Results are expressed as mean \pm SEM from three independent experiments. (Asterisk) statistically significant difference compared with LPS alone ($p < 0.01$).

trophils (22, 26, 27). Our data by immunocytochemistry suggest that eosinophils do not possess CD14 molecules on their surface (data not shown). However, expression of CD11b/CD18 by eosinophils (28) suggests the presence of potential LPS-binding sites on these cells.

Effect of IL-10 on Eosinophil Survival Induced by LPS. Fig. 4 shows that exposure of eosinophils to rhIL-10 significantly suppressed LPS-induced eosinophil survival in a dose-dependent fashion at days 2 and 4 of culture. The minimum inhibitory concentration of IL-10 appeared to be 0.2 ng/ml and complete inhibition was observed with 5 ng/ml of IL-10 ($12.8 \pm 4.6\%$ at day 4). The observation that IL-10 inhibited eosinophil survival induced by LPS but not GM-CSF implies different mechanisms underlying enhancement of eosinophil survival by these two signals.

We and others have previously shown that glucocorticosteroids prevent eosinophil survival induced by GM-CSF (29, 30). It was therefore appropriate to investigate whether glucocorticosteroids would also inhibit LPS-induced eosinophil survival. To this end, eosinophil cultures were treated with budesonide (10^{-6} M), a potent synthetic steroid, and exposed 1 h later to LPS (10 ng/ml). We found that budesonide,

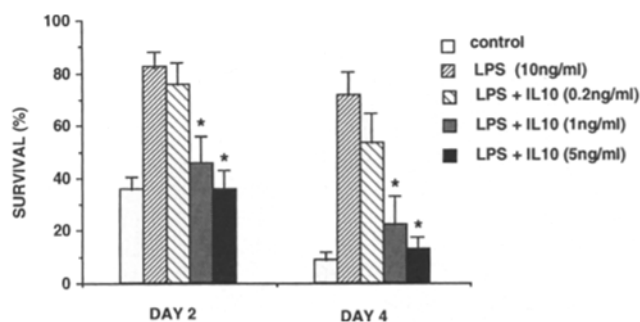


Figure 4. Effect of rhIL-10 on eosinophil survival induced by LPS. Freshly isolated eosinophils (2×10^5) were cultured with LPS in the presence of different concentrations of IL-10. Survival was assessed at days 2 and 4 by Trypan blue dye exclusion. Results represent mean \pm SEM from six independent experiments. (Asterisk) statistically significant difference compared with LPS alone ($p < 0.001$).

similar to IL-10, markedly prevented also LPS-induced survival enhancement, $21 \pm 2.9\%$ at day 2 and 4.2 ± 1.4 at day 4 ($n = 3$).

Effect of IL-10 on Eosinophil Cytokine Secretion Induced by LPS. Having established the inhibitory effect of IL-10 on LPS-induced eosinophil survival, we proceeded to examine whether LPS could stimulate eosinophils to release proinflammatory cytokines and the modulatory effect of IL-10 in these responses. Eosinophils stimulated by 10 ng/ml of LPS were cultured with or without 5 ng/ml of IL-10 for 24 h and the resulting supernatants were assayed by ELISA for GM-CSF, TNF- α , and IL-8. Table 1 shows that unstimulated eosinophils did not release detectable levels of GM-CSF and released only very low levels of TNF- α and IL-8. LPS challenge resulted in a ~ 10 – 20 -fold increase in cytokine release (23.0 ± 8.4 , 44.2 ± 14.4 , and 2037 ± 1214 pg/ml of GM-CSF, TNF- α , and IL-8, respectively). Coincubation with IL-10 markedly inhibited LPS-induced secretion of these three cytokines by eosinophils.

Since the eosinophil preparations that we used were highly purified ($>99\%$), the possibility that these increased amounts of cytokines were contributed by other cell types such as mononuclear cells and neutrophils, seemed very unlikely. To completely rule out this option, separate cultures containing the maximum number of potentially contaminating mononuclear cells or neutrophils (10,000/ml) were stimulated with 10 ng/ml of LPS for 24 h and the resulting supernatants assayed for GM-CSF by ELISA. Only minimal amounts of GM-CSF were detected in supernatants from LPS-stimulated mononuclear cells (1.4 ± 0.7 pg/ml; $n = 5$) or neutrophils (1.5 ± 1.2 pg/ml; $n = 5$) indicating that the contribution from these cell populations to cytokine content in the eosinophil supernatants was indeed negligible. Together these data demonstrate that human peripheral blood eosinophils can be activated to release substantial amounts of cytokines directly by LPS.

Modulation of LPS-induced Survival by Anti-GM-CSF Antibodies. The finding that both rhGM-CSF and LPS enhance eosinophil survival and that LPS stimulates these cells to release GM-CSF, led us to hypothesize that LPS-induced eosinophil survival was secondary to the induction of GM-CSF in eosinophils by LPS. To address this issue, eosinophils were cultured in the presence of LPS with $0.4 \mu\text{g/ml}$ of a mouse

Table 1. Cytokine Release by Eosinophils

	GM-CSF	TNF- α	IL-8
Control	0	5.7 ± 1.7	277 ± 119
LPS	23.0 ± 8.4	44.2 ± 14.4	$2,037 \pm 1,214$
IL-10 + LPS	0.9 ± 0.4	6.3 ± 5.0	251 ± 93

Freshly isolated eosinophils (5×10^5) were cultured for 24 h with or without IL-10 (5 ng/ml) in the presence of LPS (10 ng/ml). Results represent means \pm SEM from three independent experiments.

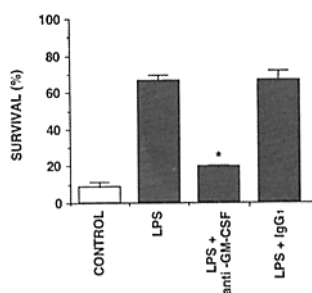


Figure 5. Abrogation of LPS-induced eosinophil survival by anti-GM-CSF. Freshly isolated eosinophils (2×10^5) were cultured with LPS in the presence of 0.4 $\mu\text{g}/\text{ml}$ mouse anti-human GM-CSF or 0.4 $\mu\text{g}/\text{ml}$ mouse IgG₁. Survival was assessed at day 4 by Trypan blue dye exclusion. Results are expressed as mean \pm SEM from three independent experiments. (Asterisk) statistically significant difference compared with LPS alone ($p < 0.01$).

monoclonal neutralizing antibody specifically against human GM-CSF, or with the same concentration of normal mouse IgG₁ as a control, and survival was assessed at day 4. As shown in Fig. 5, the presence of anti GM-CSF but not IgG₁ markedly blocked LPS-induced eosinophil survival ($19.9 \pm$

0.3%). This indicates that LPS increases eosinophil survival, at least in a substantial part, through stimulation of GM-CSF release from eosinophils thus suggesting an interesting mechanism of self-activation. Moreover, it further supports the notion that IL-10 suppresses LPS-induced eosinophil survival through blockade of LPS-induced GM-CSF production.

Our data provide evidence that IL-10 inhibits effector activities of eosinophils in vitro. This, together with other published evidence on monocytes (16), neutrophils (18), and lymphocytes (17) suggests that IL-10 is a potent antiinflammatory cytokine. The inhibitory effect of IL-10 on eosinophils appears to be selective since it only inhibits LPS-induced survival and cytokine production but not GM-CSF-mediated survival. While the molecular mechanisms underlying LPS stimulation and suppressive action by IL-10 on cytokine responses in eosinophils remain to be fully elucidated, our findings suggest a regulatory role for IL-10 in inflammatory diseases characterized by eosinophilic infiltration.

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