Brief Definitive Report

Interleukin 10 (IL-10) Inhibits the Release of Proinflammatory Cytokines from Human Polymorphonuclear Leukocytes. Evidence for an Autocrine Role of Tumor Necrosis Factor and IL-1β in Mediating the Production of IL-8 Triggered by Lipopolysaccharide

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Summary

In this study we have examined the effects of interleukin 10 (IL-10) on polymorphonuclear leukocytes (PMN), and found that it is a potent inhibitor of tumor necrosis factor (TNF), IL-1β, and IL-8 secretion triggered by lipopolysaccharide (LPS). Cytokine production by phagocytosing PMN was also inhibited by IL-10, but to a lesser extent than the LPS-induced production. As shown by Northern blot analysis, IL-10 diminished the levels of TNF, IL-1β, and IL-8 mRNAs late after the onset of stimulation of PMN with LPS. In addition, we provide evidence that the kinetics of LPS-induced IL-8 production by PMN is composed of two distinct phases. Specifically, our experiments demonstrated that in the first phase, the production of IL-8 is a process directly induced by LPS that lasts for some hours. After this early wave, a second phase begins that is sustained and leads to an elevated production of IL-8 that appears to be due to the endogenous release of TNF and IL-1β. This second wave can in fact be blocked by anti-TNF and anti-IL-1β neutralizing antibodies, and by IL-10 as the consequence of its downregulatory effects on TNF and IL-1β release. Taken together, these findings identify novel biological actions of IL-10 as a suppressor of the inflammatory response.

Accumulation of PMN in tissues is a characteristic feature of inflammation. PMN stimulates and amplifies additional migration of cells, and one possible mechanism by which this occurs is the production of cytokines by the same PMN. Evidence from a number of laboratories (for a review see reference 1), has shown that PMN can synthesize a selective range of cytokines when given the appropriate stimuli. Our own in vitro studies demonstrated that, upon stimulation with LPS, or after phagocytosis of yeast particles opsonized with IgG (Y-IgG), PMN are capable of expressing the genes and secreting newly synthesized IL-8 (2), TNF (3), and IL-1β (4). These three cytokines have a critical role in the regulation of PMN recruitment, and also of many PMN functions directed against the offending agent to be eliminated (5–7).

IL-10 is a recently characterized cytokine (8, 9) that has been implicated in the regulation of the functions of lymphoid and myeloid cells (10) because of its ability to suppress the synthesis of proinflammatory cytokines from T cells (9) and monocytes/macrophages (11–13). In this study, we show that IL-10 is a potent inhibitor of proinflammatory cytokine release by PMN stimulated with LPS, suggesting that IL-10 may also have an important regulatory role in limiting the duration and extent of acute inflammatory response.

Materials and Methods

Cell Purification and Culture. Highly purified (>99.5%) PMN were isolated under endotoxin-free conditions from buffy coats of healthy donors, as previously described (2–4). Immediately after purification, PMN were suspended in RPMI-1640 medium supplemented with 10% low endotoxin FCS (<0.006 ng/ml, Irvine Scientific, Santa Ana, CA) in the presence or not of purified recombinant human IL-10 (kindly provided by Dr. K. Moore, DNAX and Schering-Plough Corporation, Palo Alto, CA), and were then cultured at 37°C, either in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at 10^6/300 μl, or in polystyrene flasks

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(Greiner, Nurtingen, FRG) at 5–10^6/ml. Stimuli were usually added to the cells at the following doses: 1 μg/ml LPS (from *Escherichia coli* 026:B6, purchased from Sigma Chemical Co., St. Louis, MO); 5 ng/ml TNF (purchased from Bachem Inc., Hannover, Germany); 20 ng/ml IL-1β (obtained from Hazleton Laboratories, Vienna, VA); and heat-killed yeast particles opsonized with IgG (Y-IgG) at a particle/cell ratio of 2:1 (2–4). In selected experiments, neutralizing mAbs against TNF (B.154.2) (14) and IL-1β (609.9) (15), and an isotype-matched control mAb for B.154.2 and 609.9, 2B10 (16), were used. Cell-free supernatants for cytokine determination were harvested at predetermined times, spun at 2,000 rpm for 30 s, and stored at −70°C.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from PMN and analyzed as already described (2–4). mRNAs for TNF, IL-1β, IL-8, and actin, were detected by autoradiography after hybridization of nylon filters with ^{32}P-labeled cDNA fragments (Ready to go-DNA labelling kit; Pharmacia, Uppsala, Sweden).

Cytokine Assays. Antigenic TNF was determined in the cell-free supernatants by using a double-ligand immunoassay (50 pg/ml detection limit) (4). Extracellular antigenic IL-8 was measured by a specific ELISA, having a detection limit of 20 pg/ml (2). IL-1β was determined by a double-determinant RIA developed with mAbs 609 and 206 (50 pg/ml detection limit) (4, 15).

Superoxide Anion (O_2^-) Production. This was performed exactly as described previously (17).

Statistical Analysis. Data are expressed as means ± SEM. Statistical evaluation was performed by the Student's *t* test for paired data and considered significant if *p* values were <0.05.

Results and Discussion

IL-10 Inhibits the Production of TNF, IL-1β, and IL-8 by Human PMN Stimulated with LPS. To determine whether IL-10 had any effect on the neutrophil production of IL-8, TNF, and IL-1β, PMN were preincubated with IL-10 (100 U/ml) for 45 min and then stimulated with LPS for up to 18 h (Fig. 1). The accumulation of IL-8 upon LPS stimulation was already significantly elevated after 2 h, increased slightly over the next 4–6 h, and then rose dramatically up to 18 h. TNF levels in response to LPS were detectable after 2 h, peaked at the 6-h time point, and thereafter decreased. The first significant levels of IL-1β were detected at 3–4 h and then continued to rise up to 18 h. This pattern of cytokine secretion by PMN was consistent with results previously shown by us (2–4, 17), and by other investigators (18–21). Even though after 2–3 h of LPS stimulation IL-10 did not influence IL-8 release (5 ± 3% inhibition, $n = 8$), at 6 h it markedly suppressed the accumulation of IL-8 (by 44 ± 23%, $p < 0.05$, $n = 9$), and even more at 18 h (by 69 ± 13%, $p < 0.05$, $n = 8$). Inhibition by IL-10 was greater if calculated on the basis of the increased accumulation of IL-8 from 2 to 6 h (79 ± 20% inhibition, $p < 0.05$, $n = 7$), or from 6 to 18 h (86 ± 10% inhibition, $p < 0.05$, $n = 7$). The production of TNF and IL-1β was almost completely abrogated by IL-10 at all time points tested (Fig. 1). Results similar to those just described were obtained in five other experiments in which IL-10 was added to PMN at the same time as LPS, and in two more in which LPS was used at 10 and 100 ng/ml (data not shown). Other studies indicated that the inhibitory effects were dependent on the concentration of IL-10 used: for IL-1β, maximal inhibition was reached upon exposure of LPS-stimulated PMN to 10 U/ml IL-10, whereas for TNF and IL-8, maximal inhibition was reached at 100 U/ml IL-10 (data not shown). To understand whether the inhibitory effect of IL-10 was selective for cytokine generation, we investigated the effect of IL-10 on neutrophil respiratory burst. PMN were incubated for 4 and 18 h with or without 100 U/ml IL-10, in the presence or not of IFN-γ and LPS, which are known to potentiate PMN respiratory burst (22, 23). In these conditions, IL-10, contrary to what has been observed with PMA-stimulated mouse macrophages (13), did not significantly influence either the constitutive or the enhanced capability of PMN to produce O_2^- in response to FMLP. Similar results were obtained if TNF was used as an activating agent, or if PMA was used as a triggering stimulus (data not shown).

Autocrine Role of TNF and IL-1β for the LPS-stimulated IL-8 Production. Since IL-10 started to suppress the accumulation of IL-8 only after 5–6 h (Fig. 1), we evaluated the possibility that the inhibitory effect was the result of the suppression of TNF and IL-1β secretion. Fig. 2 *A* shows that PMN were able to release IL-8 after stimulation with TNF, and much less with IL-1β (Fig. 2 *B*). However, when TNF and IL-1β were added to PMN in combination, at any concentrations, including those observed to be released by LPS-stimulated PMN, they produced an additive, if not often a synergistic, effect on the release of IL-8 measured from 2 and up to 18 h

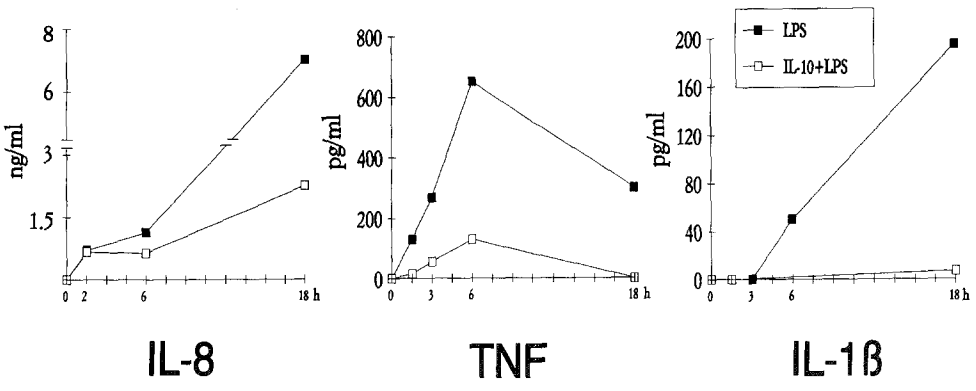

Figure 1. Effect of IL-10 on LPS-induced release of IL-8, TNF, and IL-1β from PMN. PMN (10^6/300 μl) were preincubated with or without 100 U/ml IL-10 for 45 min and then cultured for up to 18 h after the addition of 1 μg/ml LPS. Released cytokines into the cell-free supernatants were measured by immunoassays as described in Material and Methods. Shown are the mean values of duplicate assays from a single representative experiment out of nine performed with similar results.

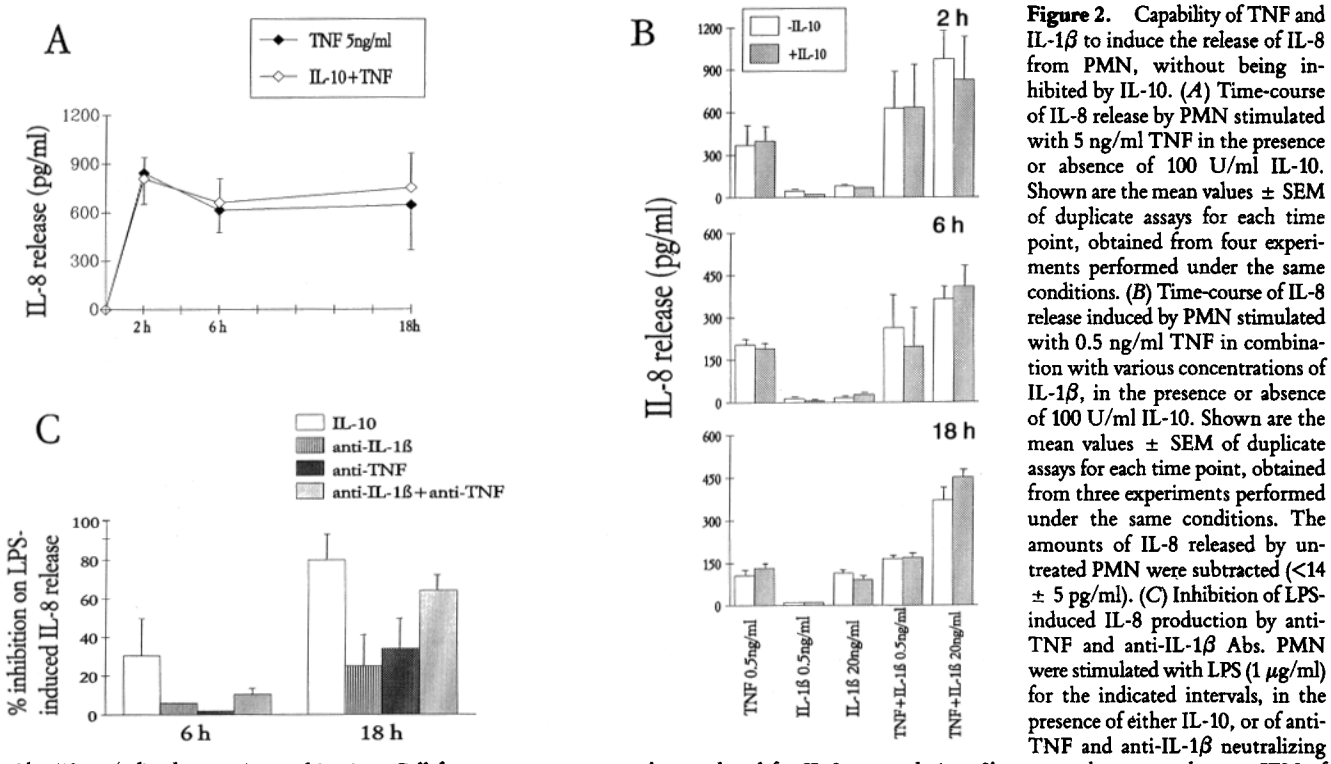

Figure 2. Capability of TNF and IL-1β to induce the release of IL-8 from PMN, without being inhibited by IL-10. (*A*) Time-course of IL-8 release by PMN stimulated with 5 ng/ml TNF in the presence or absence of 100 U/ml IL-10. Shown are the mean values ± SEM of duplicate assays for each time point, obtained from four experiments performed under the same conditions. (*B*) Time-course of IL-8 release induced by PMN stimulated with 0.5 ng/ml TNF in combination with various concentrations of IL-1β, in the presence or absence of 100 U/ml IL-10. Shown are the mean values ± SEM of duplicate assays for each time point, obtained from three experiments performed under the same conditions. The amounts of IL-8 released by untreated PMN were subtracted (<14 ± 5 pg/ml). (*C*) Inhibition of LPS-induced IL-8 production by anti-TNF and anti-IL-1β Abs. PMN were stimulated with LPS (1 μg/ml) for the indicated intervals, in the presence of either IL-10, or of anti-TNF and anti-IL-1β neutralizing Abs (10 μg/ml), alone or in combination. Cell-free supernatants were then analyzed for IL-8 accumulation. Shown are the mean values ± SEM of percent inhibition exerted by the different agents on LPS-induced IL-8 production. Values at 18 h were calculated on the basis of the increased accumulation of IL-8 from 6 to 18 h. Data derive from these experiments.

of incubation (Fig. 2 *B*). Remarkably, accumulation of IL-8 triggered by TNF, used alone (Fig. 2 *A*) or in combination with IL-1β (Fig. 2 *B*), was unaffected by IL-10. The effect on IL-8 release of anti-TNF and anti-IL-1β neutralizing Abs added to LPS-stimulated PMN either alone or in combination, was then investigated and compared with that of IL-10. After 2 (data not shown) or 6 h (Fig. 2 *C*), anti-TNF and/or anti-IL-1β Abs did not significantly influence the accumulation of IL-8 stimulated by LPS. However, over the remainder of the time course, the rise of IL-8 induced by LPS was significantly reduced by anti-IL-1β and anti-TNF Abs alone (by 25 ± 13% and 39 ± 11%, n = 3, respectively), and especially by the combination of the two Abs (by 64.5 ± 12%, n = 3). This latter inhibition was comparable with that of IL-10 (81 ± 7%, n = 3). Although we cannot exclude the role of other endogenous factors produced in response to LPS, for example IL-1α (21), or of the same IL-8 that induces itself in an autoregulatory fashion (2), collectively, these studies strongly indicate that the stimulus for IL-8 release provided by LPS to PMN is divided in two phases: an early one, directly induced by LPS itself and that can be inhibited by IFN-γ (24), and a late, prolonged one, due to LPS-stimulated release of endogenous mediators, including TNF and IL-1β which play a fundamental role. Earlier observations in which IL-8 release by LPS-stimulated whole blood was examined, also identified a biphasic pattern of IL-8 production in that system, and attributed a mediating role to endogenous TNF and IL-1 (25). The data reported herein identify the PMN as one of the cell types responsible for this

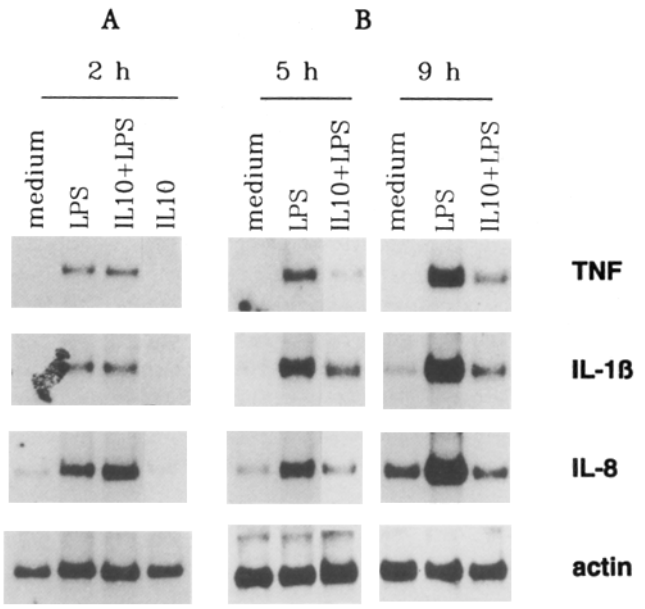

Figure 3. Effects of IL-10 on the modulation of cytokine gene expression induced by LPS on PMN. PMN were preincubated with or without IL-10 for 45 min, before addition of LPS for the times indicated. Total RNA was extracted and analyzed by Northern blot analysis. (*A* and *B*) Two experiments performed with different donors, each representative of three with similar results.

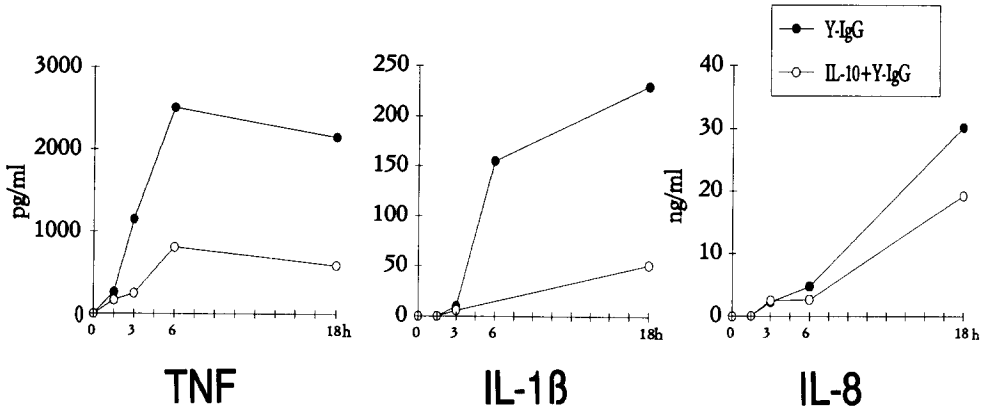

Figure 4. Effect of IL-10 on Y-IgG phagocytosis–induced release of IL-8, TNF, and IL-1β. PMN (10^6/300 μl) were preincubated with or without IL-10 for 45 min and then cultured for up to 18 h in the presence of Y-IgG at a particle/cell ratio of 1:2. Shown are the mean values of duplicate assays from a single representative experiment out of five performed with similar results.

production, and strengthen the relationship between the endogenous release of TNF and IL-1β and the late wave of IL-8 release. Importantly, in conditions in which production of TNF or IL-1β does not occur, as observed in the case of FMLP-stimulated PMN, the release of IL-8 peaks at 2–3 h and then rapidly decreases to basal levels (17).

Effect of IL-10 on Cytokine mRNA Accumulation Induced by LPS. To determine at which level IL-10 inhibited the production of cytokines, Northern blot analyses were performed on total RNA isolated from PMN preincubated with IL-10 and stimulated for different times with LPS. As shown in Fig. 3, and in agreement with previous observations (2–4, 17–21), stimulation of PMN with LPS resulted in enhanced mRNA expression for TNF, IL-1β, and IL-8. To our surprise, IL-10 did not inhibit the LPS-induced upregulation of cytokine mRNAs at their onset (2 h), but was effective at later time points (>4 h). These findings would suggest that the negative action of IL-10 on PMN cytokine production could be attributed to different mechanisms, not necessarily mutually exclusive: some acting, for example, at the level of cytokine mRNA stability, or others acting at the level of cytokine translation or secretion. A recently published (26) extensive analysis on the mechanisms of the suppressive effects of IL-10 on the capacity of mouse macrophages to produce TNF and IL-1β, would support our speculations.

Effects of IL-10 on TNF, IL-8, and IL-1β Produced after Y-IgG Phagocytosis. Fig. 4 shows that IL-10 also decreased Y-IgG–induced cytokine accumulation. The most suppressive effect at 18 h was exerted on IL-1β (69 ± 12% inhibition, n = 4), whereas inhibitions of TNF (54 ± 18% inhibition, n = 5) and IL-8 (27 ± 18% inhibition, n = 4) were less pronounced, especially if compared with those obtained upon LPS stimulation. At the level of gene expression, the effects of IL-10 on the mRNA steady state levels of the cytokines under investigation were very similar to those described for LPS (data not shown). Together with the observations previously described (2–4), it is once again evident that LPS and Y-IgG phagocytosis affect PMN cytokine production by distinct pathways, and, in the case of Y-IgG–stimulated IL-8 production, the autocrine role played by TNF and IL-1β is apparently not as determinant as for LPS.

In conclusion, the findings that IL-10 has a potent selective inhibitory effect on LPS-induced TNF, IL-1β, and IL-8 production by human PMN, add new important properties to the numerous biological effects exerted by IL-10 on leukocytes (10), which could have clinical relevance. Recent data (27, 28) indicated that pretreatment with IL-10 prevents the toxicity of LPS in a murine model of endotoxic shock. Our results would suggest that inhibition of PMN cytokine production, in addition to the suppression of monokines, could presumably contribute to IL-10–mediated protection from lethal endotoxemia.

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