

## Lipopolysaccharide-induced interleukin-8 gene expression in human granulocytes: transcriptional inhibition by interferon- $\gamma$

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We recently showed that lipopolysaccharide (LPS) is a potent inducer of interleukin-8 (IL-8) expression in human polymorphonuclear leucocytes (PMN), at the level of both mRNA and protein, and that interferon- $\gamma$  (IFN $\gamma$ ) inhibits IL-8 mRNA accumulation in stimulated PMN. To further define the molecular basis of the regulation of IL-8 gene expression in PMN, we investigated the effects of LPS and IFN $\gamma$  at both the transcriptional and post-transcriptional levels. As determined by Northern blot analysis, new protein synthesis was not required for the induction of IL-8 mRNA expression by LPS. Neither did the half-life of IL-8 mRNA in LPS-treated PMN differ from that

observed in untreated cells. However, nuclear run-on analysis revealed that LPS increased the transcription of the IL-8 and IL-1 $\beta$  genes and that, in LPS-activated cells, IFN $\gamma$  markedly inhibited the rate of IL-8 gene transcription, but not that of IL-1 $\beta$ . IFN $\gamma$  did not affect IL-8 mRNA stability in LPS-treated PMN, indicating that the cytokine does not regulate LPS-induced IL-8 gene expression through post-transcriptional events. These results provide the first evidence that human granulocytes can actively transcribe the IL-8 gene, and that transcriptional inhibition is the mechanism by which IFN $\gamma$  inhibits IL-8 gene expression in PMN.

### INTRODUCTION

In recent years it has become evident that human polymorphonuclear leucocytes (PMN) have the ability to synthesize and secrete a number of proteins [1], including several inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 $\alpha$  and -1 $\beta$  (IL-1 $\alpha/\beta$ ), the IL-1 receptor antagonist (IL-1ra), IL-8, IL-12, macrophage inflammatory protein-1 $\alpha$  and -1 $\beta$  and transforming growth factor- $\beta$  [1,2]. Although on a per cell basis PMN produce lower amounts of cytokines than do monocytes, the contribution of PMN-derived cytokines is likely to be of considerable importance at sites of acute inflammation, where they clearly predominate over other cell types [2].

IL-8, a member of the recently discovered family of chemotactic cytokines (reviewed in [3]), was originally described as a neutrophil chemotactic and activating peptide [4]. Various cell types are known to express IL-8 mRNA and to secrete the protein. These include monocytes, keratinocytes, epithelial cells, fibroblasts, endothelial cells, synoviocytes and T cells [3]. In addition, we [5,6] and other groups [7–9] have shown that activated PMN also have the ability to secrete large quantities of IL-8. Recent studies addressing the regulation of IL-8 gene and protein expression in PMN have revealed that these responses can be modulated by immunoregulatory cytokines such as interferon- $\gamma$  (IFN $\gamma$ ) [10,11], IL-4 [8,12] and IL-10 [8,13,14]. Collectively, these observations suggest that the ability of PMN to release IL-8, and thus to potentially amplify inflammatory responses by promoting the recruitment of further PMN to inflammatory foci, is tightly controlled.

Little is known, however, about the molecular mechanisms by which IL-8 gene expression is regulated in PMN. This probably owes a lot to the difficulties encountered in attempting to detect the low transcriptional activity of these cells [8,15]. In the present

study, we investigated the inhibitory action of IFN $\gamma$  on lipopolysaccharide (LPS)-induced IL-8 gene expression in PMN at the transcriptional and post-transcriptional levels, by using nuclear run-on assays and Northern blot analysis respectively. We now report that the effects of LPS and IFN $\gamma$  on IL-8 mRNA are mediated at the level of transcription.

### MATERIALS AND METHODS

#### Cell purification and culture

PMN were prepared under endotoxin-free conditions and were made virtually free of monocytes (< 0.5%), as previously described [16]. Immediately following purification, PMN were resuspended at  $(5\text{--}10) \times 10^6$  cells/ml in RPMI-1640 medium supplemented with antibiotics and 10% (v/v) low-endotoxin fetal calf serum (LPS content < 0.006 ng/ml; Irvine Scientific, Santanna, CA, U.S.A.), stimulated with 1  $\mu\text{g}/\text{ml}$  LPS (from *Escherichia coli* 026:B6; purchased from Sigma, St. Louis, MO, U.S.A.) [10,11], and cultured at 37 °C in polystyrene flasks (Greiner, Nürtingen, Germany). In selected experiments, PMN were preincubated for 20 min with the following agents prior to LPS addition: 20  $\mu\text{g}/\text{ml}$  cycloheximide (CHX) (a dose which produced a > 95% decrease in the incorporation of [<sup>35</sup>S]methionine into trichloroacetic acid-precipitable material [10]), 5  $\mu\text{g}/\text{ml}$  actinomycin D or 100 units/ml recombinant human IFN $\gamma$  (specific activity  $5 \times 10^7$  units/mg; endotoxin free; kindly provided by Dr. G. Garotta of Hoffman-LaRoche, Basel, Switzerland) [10,11]. In other experiments, PMN were exposed to IFN $\gamma$  or its diluent and then to LPS, and were finally incubated with actinomycin D for various intervals of time after stimulation. All reagents used were dissolved in clinical pyrogen-free water [16,17].

Abbreviations used: PMN, polymorphonuclear leucocytes; IL, interleukin; LPS, lipopolysaccharide; IFN $\gamma$ , interferon- $\gamma$ ; TNF $\alpha$ , tumour necrosis factor- $\alpha$ ; DTT, dithiothreitol; CHX, cycloheximide; NF, nuclear factor.

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### RNA isolation and Northern blot analysis

Total RNA was isolated, analysed and quantified as previously described [17]. Samples of 10  $\mu\text{g}$  of total RNA were loaded on each gel lane. Human IL-8, IL-1 $\beta$ , IL-6, 47 kDa cytosolic component of the NADPH oxidase (p47-phox) and actin mRNAs were detected by hybridization of the filters with denatured  $^{32}\text{P}$ -labelled cDNA fragments (Ready-to-go Kit; Pharmacia, Uppsala, Sweden). The extent of hybridization was quantified by laser densitometry (Ultrascan XL; LKB Instruments Inc., Gaithersburg, MD, U.S.A.) of the autoradiograms and results were plotted after normalization for loading on the basis of hybridization to actin. The absence of monocyte RNA contamination was confirmed by lack of hybridization with an IL-6 cDNA probe [8,16].

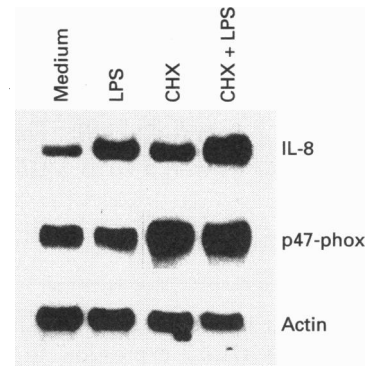
### Nuclear run-on assays

Nuclear run-on experiments were performed as previously described [18]. Briefly, after preincubation with IFN $\gamma$  for 15 min followed by addition of LPS for a further 4 h, PMN ( $150 \times 10^6$  per condition) were recovered, washed twice with ice-cold PBS, resuspended in 2 ml of lysis buffer [10 mM Tris/HCl, pH 7.4, 3 mM MgCl $_2$ , 10 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 0.85 mM dithiothreitol (DTT)], vortexed vigorously and kept on ice for 5 min. Polypropylene tubes were centrifuged at 440  $g$  (4  $^{\circ}\text{C}$ ), and the nuclear pellets were washed again with 2 ml of lysis buffer. The nuclei were then resuspended in 295  $\mu\text{l}$  of ice-cold freezing buffer (50 mM Tris/HCl, pH 8.3, 40% glycerol, 5 mM MgCl $_2$ , 0.1 mM EDTA, pH 8, 1 mM PMSF, 0.85 mM DTT). Immediately, 80  $\mu\text{l}$  of 5 $\times$  run-on buffer (25 mM Tris/HCl, pH 8, 12.5 mM MgCl $_2$ , 750 mM KCl, 0.5 mM PMSF, 6 mM DTT and 1.25 mM each of dGTP, dCTP and dATP) and 167.5  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]UTP (3000 Ci/mmol; Du Pont-New England Nuclear) were added to the nuclear suspensions, which were then incubated for 30 min at 30  $^{\circ}\text{C}$ . Reactions were stopped by adding 50  $\mu\text{g}$  of yeast tRNA and 2 ml of guanidine isothiocyanate solution to each tube. The nuclear lysates were then vortexed, drawn into a syringe fitted with a 23-gauge needle, layered on to a cushion of 5.7 M CsCl and centrifuged at 150000  $g$  for 18 h. The RNA pellets were then resuspended in 180  $\mu\text{l}$  of ice-cold TNE (0.5 M Tris/HCl, pH 8, 1.5 M NaCl), denatured on ice for 10 min with 20  $\mu\text{l}$  of 2 M NaOH, neutralized by the addition of 200  $\mu\text{l}$  of 0.48 M HEPES, pH 7.2, and ethanol-precipitated. Each RNA pellet was resuspended in 100  $\mu\text{l}$  of hybridization solution (10 mM TES, 0.2% SDS, 10 mM EDTA, 300 mM NaCl), checked for radioactivity in a  $\beta$ -counter, and hybridized at 65  $^{\circ}\text{C}$  for 96 h with 10  $\mu\text{g}$  of cDNA plasmids, previously spotted on to a nylon filter using a slot blot apparatus (Schleicher & Schuell) and subjected to prehybridization at 65  $^{\circ}\text{C}$  for 18 h. The plasmids used were human IL-8 cDNA, human IL-1 $\beta$  cDNA and pB $_{AB}$  probe for 28 S ribosomal RNA, and their respective vectors pUC119, pXM and pBR322. Filters were then washed for 3  $\times$  10 min in 0.2  $\times$  SSC at 65  $^{\circ}\text{C}$ , incubated for 30 min at 37  $^{\circ}\text{C}$  in 0.2  $\times$  SSC containing 2  $\mu\text{g}/\text{ml}$  RNase A and then exposed for autoradiography for 42 days. Filters were also scanned using a Phospho-Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) in order to determine the absolute c.p.m. in each condition.

## RESULTS

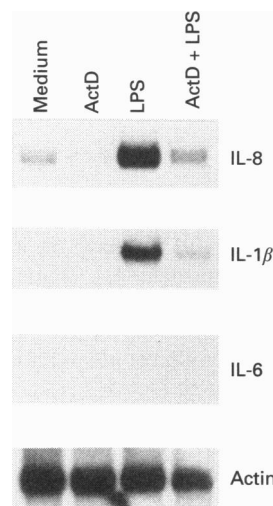
### Effects of cycloheximide and actinomycin D on LPS-induced IL-8 expression

To elucidate the molecular mechanisms by which LPS increases the accumulation of IL-8 transcripts in PMN, cells were treated



**Figure 1** Effect of CHX on LPS-induced expression of IL-8 transcripts

PMN were incubated with or without 1  $\mu\text{g}/\text{ml}$  LPS for 3 h in the presence or absence of 20  $\mu\text{g}/\text{ml}$  CHX. Total RNA was then extracted and processed for Northern analysis using IL-8 and p47-phox cDNAs. Results are from one experiment representative of two.

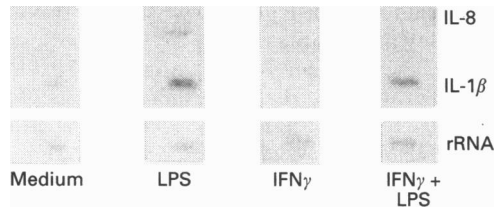


**Figure 2** Effect of actinomycin D on LPS-induced expression of IL-8 transcripts

PMN were incubated with or without LPS for 1 h in the presence or absence of 5  $\mu\text{g}/\text{ml}$  actinomycin D (ActD). Total RNA was then extracted and processed for Northern analysis using IL-8 and IL-1 $\beta$  cDNAs. Results are from one experiment representative of two.

with LPS in the presence of metabolic inhibitors. Figure 1 shows that inhibition of protein synthesis by CHX not only augmented, as already described [7,10], the basal levels of IL-8 transcripts, but further increased the IL-8 mRNA accumulation induced by LPS. In contrast, while the basal levels of the mRNA encoding the 47 kDa cytosolic component of the NADPH oxidase system (p47-phox) were also increased by CHX [18], they were not influenced by LPS. These results indicated that the stimulatory effect of LPS towards IL-8 mRNA steady-state levels is independent of *de novo* protein synthesis.

Figure 2 shows that treatment of PMN with the transcriptional inhibitor, actinomycin D, reduced the LPS-induced accumulation of both IL-8 and IL-1 $\beta$  mRNAs to baseline levels, suggesting that the induction of these genes might take place at the transcriptional level. Importantly, no hybridization with an IL-6 cDNA probe was detectable in RNA preparations from PMN (Figure 2). In contrast, RNA preparations from



**Figure 3** Nuclear run-on assays of IL-8 and IL-1 $\beta$  gene transcription in human PMN

Nuclei were prepared from PMN cultured for 4 h in the presence of LPS and/or IFN $\gamma$ , as indicated. After *in vitro* transcription, purified  $^{32}$ P-labelled nuclear RNAs from each sample were hybridized to IL-8, IL-1 $\beta$  and ribosomal slot-blotted cDNAs. Full-length plasmid DNAs were also used as negative controls and did not give detectable signals (not shown). Hybridized filters were processed as described in the Materials and methods section and exposed for 46 days.

**Table 1** Transcriptional activities of the IL-8, IL-1 $\beta$  and actin genes in human PMN

Results were determined by Phospho-imager analysis after 8 days of exposure, as described in the Materials and methods section.

	Activity (c.p.m.)			
	Medium	LPS	IFN $\gamma$	IFN $\gamma$ + LPS
IL-8	92	3262	0	382
IL-1 $\beta$	680	6194	563	5024
Actin	2200	3193	1559	2244

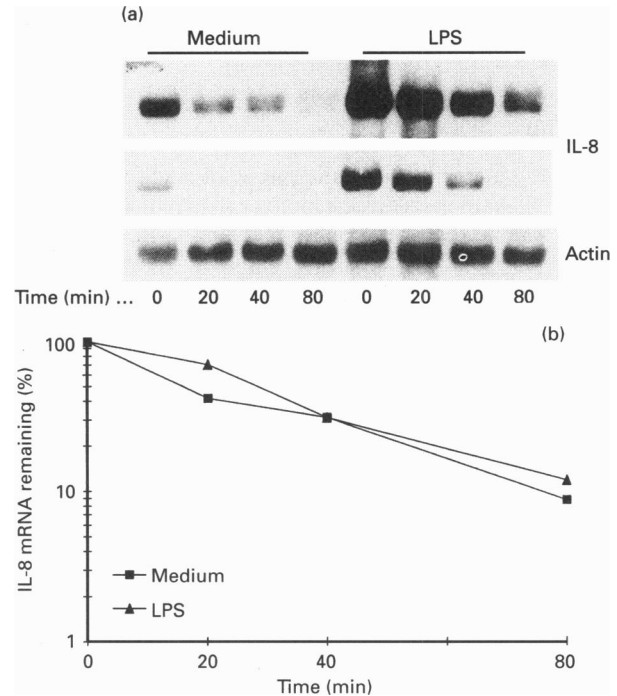
LPS-stimulated monocytes isolated in the same experiments yielded a strong signal for IL-6 mRNA (results not shown).

### Transcriptional regulation of IL-8 expression in PMN

To directly determine whether IL-8 mRNA was induced by LPS at the transcriptional level, nuclear run-on assays were performed in PMN stimulated with LPS. In the same experiments we also investigated the effect of IFN $\gamma$ , which is known to down-regulate the enhancement by LPS of IL-8 mRNA expression [10,11]. Whereas unstimulated PMN exhibited little or no IL-8 mRNA synthesis, LPS significantly enhanced the transcription of the IL-8 gene (Figure 3), and this enhanced transcription was dramatically inhibited by IFN $\gamma$  (see also Table 1 for a quantitative representation). Figure 3 also shows that the transcriptional activity of the IL-1 $\beta$  gene was almost undetectable in resting PMN, but that it was strongly induced by LPS. IFN $\gamma$  did not significantly modify the up-regulatory effect of LPS on IL-1 $\beta$  transcription (see also Table 1).

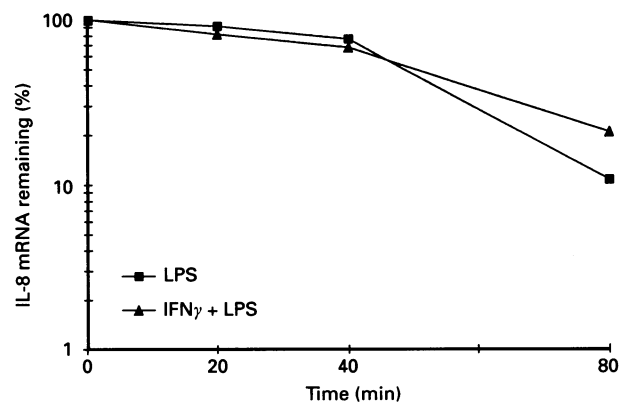
### Effects of LPS and IFN $\gamma$ on IL-8 mRNA stability

Since mRNA accumulation can be affected by actions exerted at the level of mRNA stabilization, we examined the half-life of IL-8 mRNA in our system. Cells were treated with or without LPS for 3 h and then exposed to actinomycin D for various times. As shown in Figure 4, the half-life of IL-8 mRNA in control PMN averaged  $53 \pm 7$  min in four independent experiments, and was not significantly modified by LPS treatment of the cells ( $66 \pm 8$  min;  $n = 3$ ). Under the same conditions, the half-life of actin mRNA was not altered (Figure 4). Additionally, PMN were stimulated with LPS for 2 h in the presence or absence of



**Figure 4** Effect of LPS on IL-8 mRNA stability

(a) PMN were incubated for 3 h with or without LPS prior to actinomycin D addition; total RNA was then isolated at the indicated time points and Northern blot analysis of IL-8 and actin mRNAs was performed. To allow direct comparison of the results obtained for IL-8 mRNA, the Figure also shows a further autoradiogram in which the LPS condition has an intensity of hybridization at the zero time point equivalent to that of untreated PMN. (b) Quantitative representation of IL-8 mRNA expression after densitometric analysis of the corresponding blots. Half-lives were calculated by regression analysis. This experiment is representative of two.



**Figure 5** Effect of IFN $\gamma$  on the turnover rate of IL-8 mRNA in LPS-stimulated PMN

PMN were pretreated with IFN $\gamma$  or its diluent for 20 min at 37 °C and then cultured with LPS. After 2 h, the cells were treated with actinomycin D. PMN were harvested at the indicated times, and total RNA was prepared and analysed by Northern blotting with IL-8 and actin cDNA probes. The Figure shows a quantitative representation of the data obtained as described in the legend to Figure 4. This experiment is representative of three.

IFN $\gamma$ , and mRNA synthesis was then stopped by adding actinomycin D. Total RNA was isolated at specific time points following actinomycin D addition and processed for Northern blot analysis. As shown in Figure 5, IFN $\gamma$  had little, if any, effect

on the half-life of IL-8 transcripts in PMN treated with LPS ( $63 \pm 10$  min;  $n = 3$ ). Similar results were obtained if PMN were stimulated with LPS for 5 h (not shown).

## DISCUSSION

IL-8 is a key factor in the pathogenesis of acute inflammatory diseases [19]. Among its diverse biological activities [19], chemotaxis as well as activation and regulation of the adhesive properties of neutrophils represent potent functions of this chemokine [3,4]. Interestingly, while PMN appear to be the principal targets of IL-8 action, they also represent a significant source of IL-8 in response to various stimuli [5–14,20–26]. In the present study, we provide the first direct evidence that peripheral blood PMN have the ability to actively transcribe the IL-8 gene in response to LPS. This finding is consistent with the fact that other cell types are known to produce IL-8 through increased transcriptional activities [27–31]. In contrast, endotoxin failed to alter the stability of IL-8 transcripts. Furthermore, the IL-1 $\beta$  gene was also found to be transcriptionally activated by LPS, in agreement with previous observations made in PMN stimulated with TNF and/or IL-1 [32].

In the current study we also demonstrate that the mechanism underlying the inhibitory action of IFN $\gamma$  towards IL-8 mRNA accumulation and protein production is a transcriptional inhibition of IL-8 gene expression. This inhibitory effect of IFN $\gamma$  was specific for IL-8, in that under the same conditions the LPS-induced enhancement of IL-1 $\beta$  gene transcription was not influenced by IFN $\gamma$ . Moreover, we showed that IFN $\gamma$  did not significantly affect IL-8 mRNA stability in LPS-stimulated cells, in agreement with our previous finding that IL-8 mRNA stability was not influenced by IFN $\gamma$  in resting PMN [10]. Thus IFN $\gamma$  appears to modulate IL-8 gene expression by acting at the transcriptional level. Nevertheless, the control of IL-8 gene expression in granulocytes can be quite complex as, under some circumstances, IL-8 mRNA accumulation can be modulated at the level of mRNA stability. For instance, Wang et al. [8], as well as Kasama et al. [14], recently reported that a significant portion of the inhibitory effect of IL-10 towards LPS-induced IL-8 mRNA accumulation in PMN was correlated with an enhancement of IL-8 mRNA degradation. In this regard, we found that IL-10 also inhibits the rate of LPS-stimulated IL-8 gene transcription in PMN (M. A. Cassatella, S. Gasperini, F. Calzetti, P. P. McDonald and G. Trinchieri, unpublished work). Thus, in contrast to IFN $\gamma$ , IL-10 appears to inhibit LPS-induced IL-8 mRNA accumulation through both inhibition of gene transcription and enhanced mRNA degradation, as described for mononuclear cells [8]. Moreover, in freshly isolated monocytes, IFN $\gamma$  had suppressive effects on basal as well as induced IL-8 mRNA accumulation by acting at the transcriptional level [27], whereas in the U937 human monocytic cell line, IFN $\gamma$  augmented IL-8 mRNA steady-state levels by enhancing mRNA stability [33]. The above considerations emphasize the complexity of the regulation of IL-8 gene expression by IFN $\gamma$ , as the molecular mechanisms involved not only differ depending upon the stimulus used, but also vary depending on the cell type that an individual stimulus acts upon.

Finally, we also report that treatment of PMN with CHX superinduced the LPS-stimulated mRNA expression, indicating that the enhancement of IL-8 mRNA by LPS did not require *de novo* protein synthesis. These results are consistent with previous data from this laboratory on the ability of IFN $\gamma$  to down-regulate the constitutive expression of IL-8 transcripts in PMN without a requirement for ongoing protein synthesis [10]. Taken together, our results suggest that, in granulocytes, the tran-

scriptional regulation of IL-8 gene expression by IFN $\gamma$  is likely to be mediated by pre-existing factors. In this respect, studies on the *cis*-acting elements involved in the transcriptional induction of the IL-8 gene by TNF, IL-1 and phorbol esters [34], or by hepatitis B virus X protein [35], have identified nuclear factor (NF)- $\kappa$ B-like and NF/IL-6-like sites as necessary and sufficient to confer inducibility. Similarly, studies in which treatment of activated fibroblasts with IFN $\beta$  inhibited IL-8 gene transcription induced by TNF $\alpha$  [36] revealed that the NF- $\kappa$ B-like element in the IL-8 promoter was sufficient to mediate the inhibitory effect of IFN $\beta$  [37]. Furthermore, in a gastric cell line which was synergistically induced by TNF and IFN $\gamma$  to produce IL-8, both AP-1- and NF- $\kappa$ B-like promoter elements were presumed to be involved in conferring responsiveness to TNF + IFN $\gamma$  [38]. These observations, therefore, stress the need to investigate whether DNA-binding factors might be involved in the modulation by LPS and IFN $\gamma$  of IL-8 gene transcription in PMN.

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