Interleukin 10 (IL-10) regulation of tumour necrosis factor α (TNF- α) from human alveolar macrophages and peripheral blood monocytes

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Abstract

Background - Regulation of the inflammatory response within the human lung is essential to prevent this important part of the normal host defence response becoming a pathological process. Tumour necrosis factor α (TNF- α) is a cytokine involved in the pathogenesis of shock and in granuloma formation, tissue necrosis, and fibrosis in many organ systems including the lung. Interleukin 10 (IL-10) has been proposed as having an inhibitory effect on the production of several inflammatory cytokines including TNF- α .

Methods - The effect of IL-10 administration on TNF- α production was explored in human alveolar macrophages and peripheral blood monocytes from matched individuals. The effects of IL-10 on TNF- α protein production were determined by sandwich enzyme linked immunosorbant assay (ELISA), whereas the TNF- α mRNA response was established by Northern blotting using a TNF- α specific oligonucleotide probe. The protein synthesis inhibitors actinomycin D and cyclohexamide were utilised to monitor IL-10 effects on mRNA degradation and de novo protein synthesis, respectively.

Results - The lipopolysaccharide-mediated TNF- α production in alveolar macrophages was reduced from 3.508 (0.629) to 2.035 (0.385) ng/ml by 100 U/ml IL-10. Lipopolysaccharide-induced TNF- α production in peripheral blood monocytes was reduced from 2.035 (0.284) to 0.698 (0.167) ng/ml. TNF- α gene expression was also inhibited in both alveolar macrophages and peripheral blood monocytes; lipopolysaccharide-induced TNF- α mRNA was reduced by 47.8 (15.2)% and 83.1 (4.2)%, respectively, by IL-10. The IL-10 mediated suppression of TNF- α mRNA was unaffected by addition of cyclohexamide, suggesting that de novo protein synthesis was not required for TNF- α inhibition. mRNA stability experiments indicated no acceleration in lipopolysaccharide-induced TNF- α mRNA degradation in response to IL-10.

Conclusions - These findings suggest that IL-10 is a potent inhibitor of TNF- α expression and release from alveolar macrophages and peripheral blood monocytes, and thus it may have an important role in

the cytokine network of the pulmonary immune response.

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Keywords: interleukin 10, tumour necrosis factor regulation, alveolar macrophage.

The inflammatory response is a crucial part of the normal host defence mechanism and, as such, has a protective role. However, when this response is excessive or uncontrolled, pathological consequences may result. Inappropriate inflammatory responses have been implicated as the causal mechanism in many pulmonary diseases such as the adult respiratory distress syndrome (ARDS),¹⁻³ idiopathic pulmonary fibrosis, and sarcoidosis.⁴⁻⁶ Tumour necrosis factor α (TNF- α) is an important proximal mediator of the inflammatory response directly inducing neutrophil influx and degranulation,⁷ vascular endothelial cell permeability,^{8,9} and fibroblast proliferation.¹⁰ TNF- α also has an indirect role by eliciting the release of other proinflammatory cytokines from human alveolar macrophages and neutrophils including interleukin 1 (IL-1),¹¹ interleukin 6 (IL-6),¹² and interleukin 8 (IL-8).¹³ The pivotal role of TNF- α in inflammation leads to interest in the regulatory mechanisms controlling its production and activity which may have a significant part to play in the modulation of the inflammatory response.

Interleukin 10 (IL-10), formerly known as cytokine synthesis inhibitory factor (CSIF), was originally described as a murine Th2 cellular clone product which inhibited gamma interferon production by Th1 clones.¹⁴ A human form was soon identified¹⁵ and has been shown to have a regulatory role on the inflammatory response due to its pleiotropic effects on the leucocyte population.¹⁶ These include inhibition of antigen presenting cell function, downregulation of class II major histocompatibility complex (MHC) and B7, and cytokine inhibition.¹⁷⁻²² The current study was undertaken to investigate the role of IL-10 in the regulation of TNF- α production by alveolar macrophages as a potential part of the cytokine network regulating the inflammatory response within the lung.

Methods

Human recombinant IL-10 (specific activity 1.5×10^7 U/mg protein) and JES3-19F1 rat anti-human IL-10 antibody were gifts from Dr

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K W Moore, DNAX (Schering-Plough), Palo Alto, USA. Stock lipopolysaccharide (*Escherichia coli* 0111:B4; Sigma) was prepared at a concentration of 1 mg/ml in a complete medium consisting of sterile RPMI-1640 (Gibco, UK) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.5 mg/ml fungizone. Stock cyclohexamide and actinomycin D were prepared in complete medium and dimethyl sulphoxide (Sigma), respectively, and used at final concentrations of 5 µg/ml. Tissue culture plastics (NUNC) were obtained from Life Technologies (GIBCO), Paisley, UK. The TNF-α mRNA probe was purchased from R & D Systems, Abingdon, UK.

Alveolar macrophages for this study were obtained from six non-smoking healthy volunteers (mean age 25.3 (1.2) years) consenting to fiberoptic bronchoscopy and broncho-alveolar lavage (BAL) by standard techniques. Subjects were injected intramuscularly with 0.6 mg atropine and sedated with 0–2 mg alfentanil and 0–10 mg midazolam intravenously. Topical lignocaine was administered to anaesthetise the upper airway. Aliquots of sterile 0.9% saline buffered with 8.4% sodium bicarbonate (3 × 60 ml) were instilled into the right middle lobe in 60 ml aliquots and then aspirated into a siliconised bottle kept on ice. The BAL fluid was strained through a single layer of coarse gauze to remove mucus clumps and the filtrate washed twice at 500 g in complete media. The cell pellet was resuspended in complete medium and viability assessed with 0.1% trypan blue was >90%. The cells were resuspended at a concentration of 3 × 10⁶/ml and incubated in tissue culture petri dishes for two hours at 37°C. The non-adherent cells were discarded and the plate gently rinsed to remove residual non-adherent cells. The adherent population was scraped off gently with a sterile cell scraper (Costar, UK) and differential staining with Diff-quick (Baxter, Thetford, UK) revealed >98% pure alveolar macrophages. Cells were resuspended in complete medium at a concentration of 1 × 10⁶ cells/ml.

Venepuncture was performed on all bronchoscopy subjects prior to the procedure. Blood was collected into heparinised tubes. Plasma was removed by centrifugation at 1000 g for five minutes, and the remaining blood was then diluted with complete medium and carefully layered on to a Ficoll-Hypaque density gradient (Pharmacia, St Albans, UK). After 30 minutes centrifugation at 400 g, the resultant interface of mononuclear cells was removed, washed, and resuspended in culture medium supplemented with 10% fetal calf serum (FCS). Following one hour of adherence in a tissue culture flask at 37°C the adherent cells were isolated by gentle scraping. Cells were >95% viable as assessed by trypan blue exclusion and >95% pure peripheral blood monocytes as determined by morphology and staining.

Alveolar macrophages and peripheral blood monocytes were incubated at 1 × 10⁶ cells/ml in culture medium, 100 µl per well in 96 well tissue culture microtitre plates. Preliminary studies had determined that optimum in-

hibition of TNF-α protein occurred when alveolar macrophages or peripheral blood monocytes were pretreated with IL-10 for two hours before addition of 10 µg/ml lipopolysaccharide to some of the cultures. Cells were then incubated for a further 24 hours. Supernatants were harvested and stored at -70°C until TNF-α determination by ELISA.

Ninety six well microtitre plates (Nunc Maxisorp) were coated with 8 µg/ml CB006 monoclonal antibody (courtesy of Celltech, Slough, UK) and left overnight at 4°C. Samples and the TNF-α standard (NIBSC, Potters Bar, UK) were diluted in phosphate buffered saline (PBS) containing 2% normal mouse serum (Sigma). Following a one hour incubation, plates were washed three times with PBS containing 0.1% Tween (Sigma). Rabbit anti-human polyclonal antibody WBRA-pg2 was added at a dilution of 1:500 and incubated for a further hour. Following a further three washes the polyclonal was captured by a goat anti-rabbit IgG peroxidase conjugate (Sigma) which gave a colour reaction upon addition of tetramethylbenzidine substrate. The reaction was stopped with 1 M sulphuric acid and the product read at 405 nm on a plate reader (Titertek, Flow Laboratories, Herts, UK). This assay has a detection limit of 30 pg/ml TNF-α.

Total cellular RNA was obtained from peripheral blood monocytes or alveolar macrophages by the methods described by Jonas *et al.*²³ and Chomczynski and Sacchi²⁴ and as modified by Strieter *et al.*²⁵ In brief, the cells were lysed into a guanidinium thiocyanate buffer and frozen at -70°C overnight. Total RNA was then extracted using phenol chloroform and chloroform isoamyl alcohol. RNA was ethanol precipitated and dissolved in water, and the concentration was determined by measuring the absorbance at 260 nm. RNA (10 µg per lane) was separated by electrophoresis through a 1% agarose/formaldehyde gel. Ethidium bromide was included in each sample, enabling equal loading to be assessed by monitoring the 18S and 28S ribosomal RNA. RNA was transblotted overnight to nylon membrane (Boehringer Mannheim) and fixed by baking at 120°C for 20 minutes. The membranes were prehybridised at 42°C, then hybridised overnight with digoxigenin-labelled oligonucleotide probe for TNF-α at 10 ng/ml (Boehringer Mannheim). Membranes were washed, blocked with blocking buffer (Boehringer Mannheim), and incubated for 30 minutes with alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:10000. Lumigen PPD (Boehringer Mannheim) diluted 1:100 was added as a chemiluminescent substrate. Blots were exposed to x ray film (Kodak Omat AR5) for 1–2 hours at room temperature. The autoradiographs were quantitated by a scanning laser densitometer (GS-670, Biorad, Hemel Hempstead, UK). Equivalent amounts of total RNA were assessed by monitoring 28S and 18S ribosomal RNA.

DATA ANALYSIS

Data were analysed using the Minitab package for Windows. Supernatant data are expressed as

Figure 1 Suppression of TNF- α protein release in (A) in alveolar macrophage supernatants and (B) from peripheral blood monocytes. These ELISA data demonstrate the effect of a dose range of IL-10 on lipopolysaccharide induced TNF- α release by alveolar macrophages and peripheral blood monocytes after 24 hours in culture. Lane 1: medium control; lane 2: 10 μ g/ml lipopolysaccharide; lanes 3–7: 1, 10, 50, 100 and 200 units/ml IL-10 + 10 μ g/ml lipopolysaccharide. Representative of six experiments.

mean (SE) and the significance was determined using a two-way analysis of variance with Dunnett's correction. *p* values of <0.05 were considered statistically significant.

Results

Adherence purified alveolar macrophages and peripheral blood monocytes (*n* = 6) were plated on to 96 well microtitre plates and incubated for 24 hours with 10 μ g/ml lipopolysaccharide alone and with the addition of recombinant IL-10 (1–200 U/ml). Extracellular TNF- α in the supernatants was measured with a specific enzyme linked immunosorbant assay (ELISA). Previous work had determined that recombinant IL-10 had no effect on basal TNF- α production in normal alveolar macrophages and peripheral blood monocytes (data not shown). Constitutively, alveolar macrophages and peripheral blood monocytes produced low levels of TNF- α protein (0.641 (0.160) ng/ml and 0.058 (0.018) ng/ml, respectively) which was increased to 3.508 (0.629) ng/ml and 2035 (0.284) ng/ml, respectively, when maximally stimulated with lipopolysaccharide (fig 1A). The lipopolysaccharide-mediated increase in TNF- α protein was significantly abrogated by IL-10 at doses of 50 U/ml and above; 50 U/ml IL-10 reduced alveolar macrophage TNF- α protein by 37% to give 2.035 (0.284) ng/ml (*p* < 0.01), whereas peripheral blood monocytes gave a more than 60% reduction which translated to 0.698 (0.167) ng/ml (*p* < 0.01) (fig 1B).

Figure 2 Suppression of TNF- α mRNA in alveolar macrophages by IL-10. Representative Northern analysis showing IL-10 mediated inhibition of TNF- α mRNA. Lane 1: medium control; lane 2: 10 μ g/ml lipopolysaccharide; lane 3: 100 U/ml IL-10 + lipopolysaccharide; lane 4: IL-10 + lipopolysaccharide + 10 μ g/ml JES3-19F1 antibody. Representative of three experiments. The top panel is an autoradiograph of the Northern blot, the middle section is the densitometry, and the lower panel is the 18S and 28S mRNA. Representative of six experiments.

These data demonstrate that IL-10 significantly suppresses lipopolysaccharide-induced extracellular TNF- α production from alveolar macrophages and peripheral blood monocytes.

Having established a decrease in TNF- α extracellular protein secretion by alveolar macrophages, we wanted to assess the effect of IL-10 administration on TNF- α mRNA levels. Alveolar macrophages (1×10^7 per point) were pretreated for two hours with 100 U/ml IL-10 followed by incubation for a further one hour with 10 μ g/ml lipopolysaccharide. Northern analysis of alveolar macrophage extracted mRNA revealed a reduction of 47.8 (15.2)% (*p* < 0.05) in the level of TNF- α message in three cultures containing 100 U/ml IL-10 (fig 2).

To establish the temporal window of IL-10 mediated suppression of TNF- α mRNA, peripheral blood monocytes were treated with 100 U/ml IL-10 either one or two hours before lipopolysaccharide challenge, simultaneously, and 30 minutes after lipopolysaccharide. Total RNA was extracted one hour after lipopolysaccharide administration. Pretreatment for two hours and one hour with IL-10 ensured substantial suppression of lipopolysaccharide-induced TNF- α message (91.3 (7.3)% and

Figure 3 Effect of pretreatment and post-treatment with IL-10 on TNF- α mRNA expression from peripheral blood monocytes. Lane 1: medium control; lane 2: 10 μ g/ml lipopolysaccharide, lanes 3 and 4: preincubation for two and one hour, respectively, with 100 U/ml IL-10; lane 5: simultaneous addition of IL-10 and lipopolysaccharide; lane 6: addition of IL-10 30 minutes after addition of lipopolysaccharide. Representative of four experiments.

Figure 4 Suppression of TNF- α mRNA in peripheral blood monocytes by IL-10. Representative Northern analysis showing the effect of a dose range of IL-10 on TNF- α mRNA expression. Lane 1: medium control; lane 2: 10 μ g/ml lipopolysaccharide alone; lanes 3–7: 100, 50, 10, 1, and 0.1 U/ml IL-10, respectively, co-incubated with 10 μ g/ml lipopolysaccharide.

84.4 (6.6)%, respectively) as shown in fig 3 (representative of three experiments). A reduction in the inhibitory effect of IL-10 of 27.6 (11.4)% was shown at an interval as short as 30 minutes after lipopolysaccharide stimulation.

Having established a decrease in TNF- α extracellular protein secretion by peripheral blood monocytes, we wanted to assess the effect of IL-10 administration on TNF- α mRNA levels. Cells were pretreated for two hours with IL-10 (dose range 0.1–100 U/ml) before addition of 10 μ g/ml lipopolysaccharide. Cells were lysed after a further hour of incubation and Northern analysis was performed to determine mRNA levels. The data show that 100 U/ml IL-10 is the optimum dose for TNF- α mRNA suppression, giving a reduction of 83.1 (4.2)% ($p < 0.01$) (see fig 4 which represents six experiments). No loss of cell viability was observed at any of the doses of IL-10 used up to 500 U/ml.

To determine if de novo protein synthesis was involved in IL-10 induced suppression of TNF- α , peripheral blood monocytes were treated with and without IL-10 plus 5 μ g/ml cyclohexamide two hours before addition of lipopolysaccharide. Cyclohexamide at this dose has been shown to have an inhibitory effect on translation.²⁶ Total RNA was extracted one hour later. TNF- α mRNA with and without lipopolysaccharide treatment was increased by cyclohexamide treatment (fig 5), but addition in the presence of 100 U/ml IL-10 led to a decrease in lipopolysaccharide and cyclohexamide-induced TNF- α message by 83.4 (11.7)%. These data, which are representative of three experiments, indicate that IL-10 can directly suppress the transcription of TNF- α

mRNA without the requirement for de novo protein synthesis.

The effect of IL-10 on mRNA half life was assessed by mRNA stability experiments. Peripheral blood monocytes were cultured with and without pretreatment with 100 U/ml IL-

Figure 5 Effect of cyclohexamide on IL-10 induced suppression of TNF- α mRNA from peripheral blood monocytes. Lane 1: medium control; lane 2: 100 U/ml IL-10 alone; lane 3: 5 μ g/ml cyclohexamide alone; lane 4: 10 μ g/ml lipopolysaccharide; lane 5: IL-10 + cyclohexamide; lane 6: lipopolysaccharide + cyclohexamide; lane 7: lipopolysaccharide + IL-10; lane 8: lipopolysaccharide + cyclohexamide + IL-10. Representative of three experiments.

Figure 6 Effect of IL-10 on TNF- α mRNA stability. TNF- α mRNA was quantified in lipopolysaccharide (LPS)-treated peripheral blood monocytes in the presence and absence of 100 U/ml IL-10 following treatment with 5 μ g/ml actinomycin D. Representative of four experiments.

10 before the addition of 10 μ g/ml lipopolysaccharide. Following incubation with lipopolysaccharide for one hour, 5 μ g/ml actinomycin D was added and cells were lysed at one, two, four, and eight hours following the addition of actinomycin D. Actinomycin D at this dose has been shown to inhibit translation.²⁷ No differences were seen in the rate of TNF- α mRNA decay between the IL-10 treated group and the group treated with lipopolysaccharide alone. Both groups exhibited 100% mRNA degradation by eight hours (fig 6, representative of four experiments).

Discussion

The inflammatory response is a crucial part of the normal host defences within the lungs as well as other organ sites. The exposure of the lungs to the environment makes it vital to have an effective host defence system. However, it must be closely regulated so that this protective mechanism does not become pathological. To date, the homeostatic mechanisms by which the inflammatory response is regulated within the lung are poorly understood. The alveolar macrophage is regarded as a pivotal influence in orchestrating the pulmonary inflammatory response. Many studies have focused on the increased production of potentially inflammatory mediators such as TNF- α by alveolar macrophages in lung disease.¹⁻⁶ Furthermore, several studies of differing inflammatory models have shown abolition or amelioration by the use of TNF- α neutralising antibodies including animal models of acute and chronic lung injury.^{28,29}

The production of TNF- α is also part of the protective response, and similar experiments have shown that the timing of TNF- α pro-

duction in relation to the initiating event is crucial in determining the outcome.³⁰ In addition, the effect of TNF- α on a target cell may alter in the presence of other cytokines.³¹ This has led to considerable interest in agents which might provide checks and balances within this complex network. Indeed, a recent paper has described a TNF- α mediated induction of IL-10 mRNA (and to a lesser extent protein) in human peripheral blood mononuclear cells.³² This demonstrates that TNF- α is capable of inducing at least one cytokine which may be involved in its regulation.

IL-10 has previously been shown to inhibit the release of IL-1 and IL-6 and IL-8 production from human blood monocytes.²¹ Specific studies on the regulatory role of IL-10 in TNF- α synthesis have shown an inhibitory effect in macrophage cell lines¹⁸ and human peripheral blood monocytes.²¹ This study was conducted in order to determine the relevance of IL-10 to TNF- α production in the arena of pulmonary inflammation, specifically its effects on the alveolar macrophage. Our findings suggest that both peripheral blood monocytes and alveolar macrophages are susceptible to inhibition of lipopolysaccharide-induced TNF- α production by IL-10, with peripheral blood monocytes perhaps showing the greater sensitivity as previously reported.^{33,34} It has been suggested that the more terminally differentiated alveolar macrophages are appropriately less sensitive to immunoregulation because of their continuous exposure to potential allergen and antigen. Both cell types demonstrate a reduction in lipopolysaccharide-induced TNF- α production by IL-10 in terms of released protein and at the mRNA level.

Our results indicate that the inhibitory effects of IL-10 on peripheral blood monocytes and alveolar macrophages are seen at the level of TNF- α mRNA expression when stimulated by lipopolysaccharide. IL-10 had no demonstrable effect on basal levels of TNF- α production. Previous studies have examined in some detail the regulation of TNF- α gene transcription and translation³³⁻³⁹ and the specific role of IL-10 inhibition.^{17,21,22} Data on the mechanisms by which lipopolysaccharide increases TNF production (and hence the possible site of IL-10 activity) are conflicting in terms of these mechanisms. Two in vitro studies have reported an increase in the transcription rate of the TNF- α gene in response to lipopolysaccharide,^{38,39} whereas Bogdan *et al*⁴⁰ found that transcription rates were unaffected in mouse peritoneal macrophages. By contrast, Han *et al*³⁹ reported that lipopolysaccharide modified TNF- α at both the transcriptional and translational level by regulating the TNF- α gene promoter and the 3' untranslated region, respectively, but that the increase in TNF- α protein was largely due to translational depression.³⁴ The current study has not addressed the possibility that IL-10 may inhibit TNF by decreasing gene transcription, nor by increased translation, but we have established that no *de novo* protein synthesis is required.

The inhibitory effect of IL-10 has been shown to be greatly reduced if added more

than two hours after lipopolysaccharide administration, as demonstrated here and elsewhere,⁴⁰ by which time TNF- α mRNA levels have peaked.³⁹ This suggests that IL-10 is important at the early stage of TNF- α induction. One possible mechanism of IL-10 activity would be an increase in the rate of transcript degradation. The TNF- α gene has, in common with other cytokines, a 3' untranslated region with a UA rich motif. Such a sequence has been shown to confer instability on the resultant mRNA transcript.⁴¹ It has been reported that lipopolysaccharide-treated murine macrophages have increased ribonuclease activity which degrades mRNA containing the UA rich motif,⁴² and it is possible that IL-10 enhances production of this or another ribonuclease. However, our mRNA stability experiments showed no increase in mRNA degradation in response to IL-10, which has been confirmed in murine peritoneal macrophages.⁴⁰ This suggests that IL-10 must be acting (at least in part) to inhibit gene transcription of the mRNA, since this is the only other mechanism by which the steady state mRNA level of TNF- α may be reduced. This contrasts with the results of a study of the inhibition of lipopolysaccharide-induced TNF- α production in human neutrophils by IL-10,²² which suggest that enhanced degradation of mRNA did occur. Conflicting results from different cell systems and species suggest that there are cell and species specific mechanisms of TNF- α inhibition by IL-10, thus underlying the importance of examining mechanisms in specific cell types such as the alveolar macrophages.

Investigation into the role of IL-10 in inflammatory disease is only just beginning.^{43,44} The data we have presented have shown an in vitro mechanism of TNF- α regulation by IL-10 following lipopolysaccharide stimulation. In many inflammatory lung diseases such as fibrosing alveolitis it has been proposed that alveolar macrophages are activated in vivo by hitherto unidentified antigens where changes in this mechanism might be observed without extraneous lipopolysaccharide stimulation. A role of IL-10 has been suggested both in septicemia and rheumatoid and osteoarthritis.^{43,44} Although, in the latter case, IL-10 production was found to occur, the use of anti-IL-10 demonstrated an additional increase in pro-inflammatory cytokines (including TNF- α levels). These data suggest that IL-10 is attempting to reduce the inflammatory response in this situation and leads to the speculation that increased levels of IL-10 would be beneficial. Indeed, one recent study has indicated that IL-10 may be important in the auto-regulation of TNF- α in human peripheral blood monocytes, and that cells of the monocyte/macrophage phenotype may be a major source of IL-10 in human systems.³² We also have preliminary data to suggest that IL-10 may be constitutively produced by peripheral blood monocytes and alveolar macrophages. We have not addressed the issue of specificity in this study, and it would be surprising if IL-10 alone had an inhibitory effect on TNF- α in view of the many examples of redundancy in the

cytokine network. This is most elegantly shown by studies of genetically engineered mice with the absence of single cytokines ("knockout mice") in which, although disease processes may be more common, fatality rarely results. Studies on the autocrine role of IL-10 and its relationship with other anti-inflammatory cytokines such as IL-4⁴⁵ and IL-13⁴⁶ may give further insight into the complex regulation of the cytokine network.

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