Reduced production of interleukin 12 by interferon γ primed alveolar macrophages from atopic asthmatic subjects

M J Plummeridge, L Armstrong, M A Birchall, A B Millar

Abstract

Background—Asthma is characterised pathologically by an inflammatory pulmonary infiltrate rich in T helper (Th) 2 cells and eosinophils. Interleukin (IL)-12 is a heterodimeric cytokine critical for driving the development of uncommitted Th cells to express a Th 1 phenotype. Reduced pulmonary production of IL-12 may therefore play a role in the pathogenesis of asthma by contributing to the pulmonary cytokine imbalance seen in asthma.

Methods—IL-12 p70 protein levels in bronchoalveolar lavage fluid and p70 protein levels and IL-12 messenger RNA in alveolar macrophage cultures from normal and atopic asthmatic subjects were measured.

Results—There was a significant difference between the mean IL-12 p70 protein level in the bronchoalveolar lavage fluid from asthmatic subjects (37.5 pg/ml) and from normal subjects (131 pg/ml, p = 0.04). Alveolar macrophages from asthmatic subjects produced significantly less IL-12 protein (30 pg/ml) and messenger RNA than those from normal subjects (69.5 pg/ml, p<0.005). These differences were not caused by inhibition of IL-12 production by IL-10 nor to generalised hyporesponsiveness of asthmatic alveolar macrophages from subjects to the effects of interferon (IFN)-γ.

Conclusions—Pulmonary IL-12 production is lower in asthmatic subjects. This reduction is not the result of generalised hyporesponsiveness to IFN-γ. Reduced IL-12 levels may contribute to the development of asthmatic pulmonary inflammation through dysregulation of Th cell development.

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Keywords: asthma; alveolar macrophages; interleukin 12; interferon γ

Asthma is characterised by an inflammatory infiltrate of the bronchial mucosa that is rich in T helper 2 (Th2) lymphocytes and cytokines which in turn both attract eosinophils and prolong their survival. Isotype switching to IgE production is initiated and the “allergic pattern” of inflammation seen in asthma is perpetuated. It is possible to relate the levels of Th2 cytokines to both the severity of clinical disease and the response to treatment.
The production of IL-10, a potentially anti-inflammatory cytokine, by AMs from asthmatic subjects is reduced and this deficiency can be reversed by treatment with inhaled steroids.\textsuperscript{16, 17} AMs from subjects with atopic asthma but not from normal subjects enhance the production of the Th2 cytokine IL-5 by CD4+ T cells.\textsuperscript{18} We hypothesised that there is abnormal production of IL-12 by the AM in asthma which may contribute to the pulmonary Th1/Th2 imbalance seen in this disease. The aims of the current study were to assess intrapulmonary IL-12 levels and spontaneous IL-12 production by AMs in asthmatic versus normal subjects. In addition, we looked at lipopolysaccharide (LPS)- and IFN-γ-induced expression of IL-12 in AMs to determine whether abnormal responses to these stimuli could account for altered IL-12 expression in these subjects.

### Methods

#### SUBJECTS

Asthmatic subjects with a clinical history of asthma, reversible airflow obstruction with at least a 15\% improvement in forced expiratory volume in one second (FEV₁), either spontaneously or after administration of inhaled β₂ agonists (200 μg albuterol), and increased airway responsiveness to histamine (PD₂₀ <8 mg/ml) were included in the study. All were taking either β₂ agonists or received no medication. None had receivedinhaled or systemic corticosteroids for at least three months before the study or had ever undergone a course of immunotherapy. Skin prick responses to a panel of common Aeroallergens were assessed with all atopic subjects exhibiting at least two positive responses. Patient characteristics are given in table 1. Normal subjects had no history of asthma or other respiratory or allergic disease, normal pulmonary function, and were not atopic. All subjects were non-smokers or ex-smokers with a smoking history of <3 pack years. None had a history of respiratory tract infection in the two months preceding the study.

Ethical approval for the study was given by Southmead NHS Trust research ethics committee and written informed consent was obtained from all subjects.

#### BRONCHOSCOPY

Bronchoscopy was performed according to standard guidelines. Premedication with 2.5 mg nebulised albuterol was given. Intravenous midazolam (2.5–10mg) with or without alfentanil (250–500 μg) was used as sedation. Nasal oxygen was administered throughout, and pulse oximetry performed continuously. After topical anaesthesia with 2\% lidocaine, bronchoalveolar lavage (BAL) with four 60 ml aliquots of buffered saline was performed into the (right) middle lobe. The BAL fluid was aspirated into a siliconised glass bottle and stored on ice until processing. Samples were processed within 15 min of collection. BAL samples were collected from a total of 10 normal subjects and eight atopic asthmatics. BAL details are given in table 1.

#### ISOLATION OF ALVEOLAR MACROPHAGES

The chilled BAL fluid was strained through a single layer of coarse gauze to remove mucus clumps and then spun at 400g for 5 min to recover cells. Filtered BAL fluid was collected and stored at −80°C until analysis. The cell pellet was resuspended in serum-free RPMI 1640 medium (Sigma, Poole, UK) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) and adjusted to 2 × 10⁶ AMs/ml as defined by morphology. 100 μl aliquots of the cell suspension were then plated out onto sterile 96 well plates (Gibco, Nunc, Denmark) for two hours. The non-adherent population was then removed by aspiration and 100 μl of fresh RPMI added to each well. The resulting AM populations were >95\% pure in both asthmatic and normal populations with a viability >90\% as assessed by Diff-Quik and trypan blue staining.

#### CELL CULTURES

Preincubation with interferon γ (IFN-γ) has previously been shown to increase macrophage IL-12 production.\textsuperscript{4} To investigate the effect of IFN-γ priming on AM IL-12 production, half of the AMs from each sample were preincubated overnight with 1000 units/ml IFN-γ (PeproTech EC Ltd, London, UK) for 18 hours whilst the remaining half were cultured in medium alone. At the end of this period all cells were then cultured for a further 24 hours. For each experiment, to both the IFN-γ primed cells and the non-primed cells were added either (1) medium alone (unstimulated), (2) LPS (E coli 0111:B4, 10 μg/ml) or (3) anti-IL-10 antibody (JE53–19F1, 10 μg/ml). Anti-IL-10 antibody was added to neutralise the potential inhibitory effects of endogenous IL-10 on IL-12 production. The supernatants were then harvested and stored at −80°C until analysis by ELISA.

#### RNA STUDIES

For RNA studies, purified AMs were cultured at 1 × 10⁶/ml in 24 well culture plates (Costar, Cambridge, USA). After preincubation with IFN-γ as above, lipopolysaccharide (LPS) and anti-IL-10 antibodies were added as described above. RNA was harvested at six hours as determined by time course experiments.

#### RNA EXTRACTION

Total cellular RNA was extracted from cells using RNAzol B (AMS Biotechnology, Oxon, UK) according to manufacturer’s instructions. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was

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**Table 1 Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Atopic asthma</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35 (20–64)</td>
<td>35 (23-70)</td>
</tr>
<tr>
<td>M:F</td>
<td>5:3</td>
<td>5:5</td>
</tr>
<tr>
<td>BAL recovery (ml)</td>
<td>127.5 (90–180)</td>
<td>125 (100–150)</td>
</tr>
<tr>
<td>Total cell count (x10⁶/ml)</td>
<td>5.54 (1.3–10)</td>
<td>6.4 (2.5–12.2)</td>
</tr>
<tr>
<td>Differential cell count (%)</td>
<td>AM 90 (80–95%); Eo 5.7</td>
<td>AM &gt;96%</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>82 (72–100)</td>
<td>ND</td>
</tr>
<tr>
<td>Reversibility (%)</td>
<td>17.75 (15–27)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean (range). AM = alveolar macrophages; Eo = eosinophils; Lym = lymphocytes, ND = not done.
added. 1 μl glycogen (Boehringer Mannheim, Germany) was added and the RNA precipitated at –20°C overnight. The following morning samples were centrifuged at 12 000g for 15 min (4°C). The supernatant was removed and the RNA washed once with 75% ethanol by vortexing and subsequent centrifugation for 8 min at 7500g (4°C). The RNA pellet was then air dried, resuspended in nuclease free water, and stored at –80°C.

REVERSE TRANScription POLYMERASE CHAIN REACTION
RNA from cell samples was measured by densitometry at optical density (OD) 260 nm using a GeneQuant II (Pharmacia Biotech Ltd, Cambridge, UK). Reverse transcription was performed using a commercial kit (Promega Corp, Madison, USA). 1 μg RNA was reverse transcribed according to manufacturer’s instructions. IL-12 p35 and p40 primers were obtained from Oswel DNA Service (Southampton, UK) using published sequences designed to amplify cDNA but not genomic DNA. GAPDH primers were obtained from the same supplier. The sequences used are provided in table 2. cdNA (1 μl for GAPDH, 3 μl for IL-12) was added to a 25 μl reaction mixture comprising PCR buffer, MgCl2 (1.5 mM), dNTP (0.2 mM), primers (40 ng), and 0.75 U Taq polymerase. A thermocycler (GRI, UK) ran 30 cycles for IL-12 p35 and p40 and for GAPDH. Programmes used were 30 s denaturation at 94°C, 45 s annealing at 62°C, and 45 s extension at 72°C for GAPDH, and 30 s denaturation at 94°C, 45 s annealing at 60°C, and 45 s extension at 72°C for IL-12 p35 and p40. The linearity of the system has been previously validated by serial dilution. The PCR products were then visualised using electrophoresis on 1.8% agarose gel stained with ethidium bromide.

ELISA
IL-12 p70 Supernatants were analysed for IL-12 p70 using an ELISA matched pair (Pharmingen, San Diego, CA, USA) according to manufacturer’s instructions. This ELISA is specific for the potentially bioactive p70 heterodimer and its activity was not affected in the presence of human plasma or serum. The plates were then washed three times and blocked with PBS containing 0.5% BSA for one hour at 37°C. The plates were washed a further three times and samples were added, together with the recombinant p75 standards (100 μl per well) and the plates incubated for two hours at 37°C. The plates were washed four times in wash buffer and 100 μl of biotinylated TNF-α (50 ng/ml) was added to each well. The plates were incubated for one hour at 37°C and then washed four times with wash buffer.

STATISTICAL ANALYSIS
The data were non-parametric, with the exception of the TNF-sR75 data. Assessment of significant differences within and between groups was therefore carried out by Wilcoxon signed rank and Mann-Whitney U analysis, respectively, using a Minitab for Windows package. The TNF-sR75 data were compared using Student’s t test (paired for analysis within a group, unpaired for analysis between groups). A p value of <0.05 was considered significant.

Results
IL-12 P70 PROTEIN LEVELS
BAL fluid
Significantly higher levels of IL-12 p70 protein were detected in the BAL fluid of normal subjects than in asthmatic subjects (median values 131 pg/ml and 37.5 pg/ml, respectively (95% CI 30.15 to 168.2), p = 0.04; fig 1).

Table 2 Primer pairs for RT-PCR of alveolar macrophage mRNA

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primer pair</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 p35</td>
<td>5'-CCCTCAAGAGTTGGCGCAGAACCC</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>5'-GGTTTTCTGAGGCGAGGAGCC</td>
<td>355</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>5'-CCAGGGACCTGGCCAGTGAGAG</td>
<td>288</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GCCAAAAGGGTCTCATGCTC</td>
<td>355</td>
</tr>
<tr>
<td></td>
<td>5'-GTAGGAGCGGATTGAGTGT</td>
<td>288</td>
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AM cultures

Normal subjects: In AM cell culture experiments there was a significant increase in the production of IL-12 only in those cells pre-stimulated with IFN-\(\gamma\). In AM cultures from normal subjects IL-12 production was upregulated significantly by LPS following preincubation with IFN-\(\gamma\) (control = 30 pg/ml, IFN-\(\gamma\) + LPS = 69.5 pg/ml; p<0.006, fig 2).

Asthmatic subjects: In contrast, there was no significant increase in IL-12 production by AMs from asthmatic subjects in response to LPS, either with or without IFN-\(\gamma\) priming (fig 2). Production of IL-12 in response to LPS stimulation in cultures primed with IFN-\(\gamma\) was significantly lower in asthmatic subjects than in normal subjects (asthmatics = 30 pg/ml, normal subjects = 69.5 pg/ml (95% CI 14.0 to 61.0), p<0.005).

Effect of blocking endogenous IL-10: To determine whether the lower IL-12 levels in asthmatic subjects resulted from increased endogenous production of IL-10 we stimulated AMs with LPS in the presence of anti-IL-10 antibody. This antibody had no significant effect on IL-12 production in either group (fig 2).

TNF-\(\alpha\)R75
To determine whether the reduced response to IFN-\(\gamma\) in the asthmatic AMs was generalised hyporesponsiveness or specific to IL-12 induction, we measured shedding of TNF-\(\alpha\)R75 which is a recognised consequence of IFN-\(\gamma\) stimulation of AMs.\(^9\) There was a significant increase in production of TNF-\(\alpha\)R75 in response to IFN-\(\gamma\) by AMs from asthmatic subjects (mean (SE) 228 (61) pg/ml unstimulated, 641 (53) pg/ml + IFN-\(\gamma\), p = 0.008; fig 3). AMs from asthmatic subjects were not therefore refractory to stimulation by IFN-\(\gamma\) and, in fact, produced significantly more TNF-\(\alpha\)R75 in response to IFN-\(\gamma\) than those from normal subjects (311 (53) pg/ml in normal subjects, 641 (53) pg/ml in atopic asthmatic subjects, p = 0.03).

RNA
The PCR gels for GAPDH and IL-12 p35 and p40 are shown in fig 4. In normal subjects mRNA production for IL-12 p40 was detectable following stimulation with LPS only after preincubation with IFN-\(\gamma\). There was no detectable mRNA in unstimulated cells or in cells stimulated with either LPS or IFN-\(\gamma\) alone in either group.

Discussion
This study is the first to show reduced IL-12 p70 production by AMs from asthmatic subjects. The reduction in both IL-12 p70 protein and mRNA for the IL-12 p35 and p40 subunits in subjects with asthma suggests altered regulation at the level of transcription. In this study, mRNA and subsequent protein...
production required priming with IFN-γ before the addition of LPS, previously shown to be necessary for IL-12 production by normal human monocytes in vitro.\(^1\) There were significantly lower levels of mRNA for the p40 subunit compared with p35, consistent with the comparative excess in p40 production which is known to occur.\(^2\)

The dependency on IFN-γ priming which we observed in both subject groups contrasts with a recent study which showed IL-12 mRNA production by AMs in the presence of LPS alone.\(^3\) In that study AMs were collected from carcinoma resection specimens which may not necessarily have had normal characteristics. The AMs were also cultured with 10% FCS, a potential influence on cellular protein production. This may also explain the high IL-10 production in AMs and consequent effect of anti-IL-10 in comparison with this and previously published studies.\(^4\) More importantly, however, they were unable to detect the active p70 protein under these conditions. Another study has reported higher levels of IL-12 p40 production by AMs from untreated asthmatic subjects compared with normal AMs.\(^5\) It is known that secretion of IL-12p40 is increased when IL-12p35 is present, indicating a requirement for both subunits for IL-12p70 production.\(^6\) The IL-12p40 component of IL-12p70 cytokine, but not the p40 component. IL-12 p40 production may be 100 times that of IL-12 p70 and may not accurately reflect p70 production.\(^7\)

Study of the pulmonary compartment in asthma is important because cell and organ specific immune responses may differ from those observed in other cell types and cell lines. Our findings demonstrate an abnormality in IL-12 p70 production by cells within the pulmonary compartment of asthmatic subjects for the first time, and are consistent with reports highlighting differences in peripheral blood production of IL-12 in both atopic and other conditions.\(^8\) In the current study IL-12 was detected in the BAL fluid of both groups. Isolated AMs, however, did not produce IL-12 in vitro unless stimulated. It is therefore possible that AMs are not the main source of this cytokine in the BAL fluid. The possibility that non-adherent cells (including possible non-adherent AMs) discarded during purification had the potential to contribute significantly to in vitro IL-12 production is unlikely. However, to investigate this possibility we cultured mixed BAL cells from three normal subjects. Non-purified mixed BAL cells in culture do not produce IL-12 unless stimulated. Dendritic cells may also be a source of IL-12, and the isolation and culture of these cells from human airways is the subject of ongoing research in our laboratory. Their ability to influence significantly BAL fluid cytokine levels is currently under investigation.

It is also possible that the collection, processing, and purification of AMs reduces their capacity to produce IL-12 in vitro, unless stimulated, to below the limits of detection used in these experiments. This possibility is supported by the observation that the purification of peripheral blood mononuclear cells (PBMCs) significantly impairs IL-12 production compared with that seen by PBMCs in whole blood, by a mechanism that is not as yet understood.\(^9\)

AMs from asthmatic subjects show generally increased activation.\(^10\) Despite this, we considered the possibility that the lower level of IL-12 production by cultured AMs from asthmatic compared with normal subjects was due specifically to an impaired response to IFN-γ during the initial priming period. We therefore measured supernatant levels of TNF-sR75 (CD 120b), which has previously been shown by ELISA to be specifically increased in AM cultures in response to IFN-γ.\(^11\) AMs from asthmatic subjects were responsive to IFN-γ, suggesting that global refractoriness to IFN-γ stimulation could not explain our results. In keeping with previous reports of generally increased AM activation in asthma,\(^12\) AMs from asthmatic subjects produced significantly more TNF-sR75 than those from normal subjects. There are several possible explanations for this finding. One possibility is that sensitivity to IFN-γ stimulation is different for TNF-sR75 and IL-12. IFN-γ signal transduction has been very well characterised\(^13\) and it is known that there is only one signalling pathway for IFN-γ inducible genes, which is mediated through JAK1 and JAK2 activation and STAT1 phosphorylation. Activation of the intracellular signal requires the presence of both the α and β subunit. The α subunit is involved with ligand binding and contains the JAK1 and STAT1 binding sites, whereas the β subunit plays no part in lig and binding but is required to stabilise the receptor binding complex. The β subunit also contains the JAK2 binding site. Although different transduction pathways cannot account for the altered responsiveness to IFN-γ in asthmatic subjects, it is possible that the signalling thresholds for TNF-sR75 and IL-12 induction are not the same. This threshold could be influenced by altered expression of either the α or β subunits, or altered expression of the intracellular signal...
transduction proteins. Recent studies have shown that the phenotype of AMs and macrophage-derived monocytes from asthmatic subjects differs from normal subjects27 and although IFN-γ receptor expression has not been explored in this context, such investigation is warranted.

Since IL-10 is an IFN-γ-inducible gene, we explored the possibility that endogenous IL-10 could account for low IL-12 levels in our asthmatic subjects. However, inhibition of endogenous IL-10 activity resulted in no significant effect on IL-12 production. This was not an unexpected finding since it has previously been observed that the levels of IL-10 in AM cultures from asthmatic subjects are lower than in normal subjects.17

AM mediated suppression of the immune response, and maintenance of a dynamic equilibrium between pro-inflammatory and anti-inflammatory mediators by the AM, are likely to be important in maintaining immunological homeostasis in the lung.28 29 In asthma, inappropriately reduced IL-12 production by the AM in response to a stimulus may play a part in the initiation and maintenance of Th2 dominated airway inflammation seen in this disease. A recent study has shown enhanced IL-5 production by CD4+ T cells by AMs from patients with atopic asthma.18 In a murine model of asthma both airway hyperresponsiveness and pulmonary eosinophilia following provocation are CD4+ T cell dependent.30 Factors controlling CD4+ T cell development, such as IL-12, are therefore likely to be important in asthma. Our finding of lower IL-12 production by AMs from asthmatic subjects suggests a direct mechanism by which the AM may influence the local microenvironment, potentially controlling both the development of CD4+ T lymphocytes and their subsequent responses to re-stimulation, thus upsetting the balance that usually exists between T helper cell subsets. Furthermore, pre-existing differentiated Th1 cells could both proliferate and produce cytokines suboptimally as IL-12 is required for this function during antigen presentation to T cells.31

In conclusion, this study has shown lower IL-12 protein and mRNA production by AMs from asthmatic subjects than from healthy subjects. Our observations support further the potential role of the AM in the development of the pulmonary inflammation associated with asthma, and suggest one mechanism to explain why the production of Th2 cytokines by lymphocytes in asthma is increased.

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