Tumour necrosis factor-α production in human alveolar macrophages: modulation by inhaled corticosteroid

B.G. Marshall*, A. Wangoo*, L.I. Harrison***, D.B. Young**, R.J. Shaw*

Tumour necrosis factor-α production in human alveolar macrophages: modulation by inhaled corticosteroid. B.G. Marshall, A. Wangoo, L.I. Harrison, D.B. Young, R.J. Shaw. ©ERS Journals Ltd 2000.

ABSTRACT: Using an *ex vivo* alveolar macrophage model, the hypothesis that inhaled preparations of corticosteroids might have important anti-inflammatory effects on cells of the peripheral airway was tested.

The tumour necrosis factor (TNF)-α-inducing potential of three glycolipid preparations from nonpathogenic (arabinofuranasyl lipoarabinomannan (LAM (AraLAM)) and virulent (mannase LAM (ManLAM)) mycobacteria and Gram-negative bacteria (lipopolysaccharide (LPS)), in primary alveolar macrophage preparations was investigated. A novel inhaled chlorofluorocarbon (CFC)-free preparation of beclomethasone dipropionate (hydrofluoroalkane 134a (HFA)-BDP) with increased peripheral lung deposition was investigated for its ability to modulate glycolipid-induced TNF-α production by human alveolar macrophages, in comparison with a CFC-containing preparation and placebo.

Compared to the basal TNF-α bioactivity of 0.72 ng·mL^{-1} (geometric mean), the TNF-α bioactivity in the macrophage preparation increased following incubation with LPS (138 ng·mL^{-1}, p<0.001), AraLAM (12.6 ng·mL^{-1}, p<0.001) and ManLAM (1.42 ng·mL^{-1}, p=0.02). HFA-BDP, administered *in vivo*, significantly reduced LPS- and ManLAM-induced TNF-α production by alveolar macrophages cultured *ex vivo*. No change in glycolipid-induced TNF-α production was observed following *in vivo* administration of CFC-BDP or HFA-placebo.

This is the first demonstration of an immunomodulatory effect on alveolar cells of corticosteroid delivered *via* metered dose inhaler. The present findings suggest that alveolar deposition of beclomethasone dipropionate is capable of modulating the inflammatory potential of the alveolar macrophage population.
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Tumour necrosis factor-α (TNF-α), a key inflammatory mediator, is released from cells of macrophage or monocyte lineage, such as alveolar macrophages. TNF-α has been implicated in the pathogenesis of asthma [1] and interstitial lung diseases [2]. It also plays an important role in both protective [3] and pathological immune responses to Gram-negative bacteria and mycobacteria [4].

The major antigenic lipoglycans of Gram-negative bacteria and mycobacteria are lipopolysaccharide (LPS) and lipoarabinomannans (LAMs), respectively. LAM isolated from pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *M. bovis* is capped with mannose residues at the nonreducing arabinofuranosyl termini (ManLAM), whereas LAM from rapidly growing a virulent mycobacteria lacks mannose caps (AraLAM) [5].

Alveolar macrophages harvested from patients with pulmonary infections, such as tuberculosis [6], release TNF-α spontaneously in culture. However, there has not as yet been a demonstration of TNF-α release by human alveolar macrophages from healthy subjects in response to these mycobacterial preparations, showing that the release of TNF-α by alveolar macrophages in response to purified LAMs would be beneficial in understanding the pathogenesis of mycobacterial diseases.

The aims of this study were two-fold. First, the intention was to test the stimulatory capacity of purified mycobacterial preparations in human alveolar macrophage cultures. The inclusion of LPS in the study served as a positive control, since LPS has been shown to be a potent stimulator of alveolar macrophages to produce TNF-α. Secondly, the ability of inhaled preparations of corticosteroid to inhibit TNF-α release from alveolar macrophage populations, cultured *ex vivo*, was investigated. One of the inhaled corticosteroid preparations under test was a new chlorofluorocarbon (CFC)-free formulation of beclomethasone dipropionate (BDP), which utilized the propellant hydrofluoroalkane 134a (HFA) (QVAR^{TM} (3M Pharmaceuticals, St Paul, MN, USA), HFA-BDP). HFA-BDP has been shown to have a finer particle size distribution, resulting in greater alveolar deposition than obtained using current CFC-BDP products [7]. It was predicted that HFA-BDP would show greater inhibition of TNF-α release and higher bronchoalveolar lavage fluid levels than would CFC-BDP.

Subjects and methods

Study patients and subjects

Six patients with inflammatory pulmonary diseases, who were undergoing routine diagnostic bronchoscopic examination, were included in a preliminary study. These patients were aged 34–72 yrs. Twenty-eight healthy non-smoking volunteers, aged 21–42 yrs, were then studied. The project was approved by the St. Mary's Hospital Local Research Ethics Committee.

Bronchoalveolar lavage. Fibreoptic bronchoscopy and bronchoalveolar lavage (BAL) were performed by the same operator on the control subjects with their full informed consent, using an Olympus BF P20D fibreoptic broncho-scope (Keymed, Southend, UK). Subjects were premedi-cated using midazolam and fentanyl given intravenously. Topical lignocaine was applied to the vocal cords, trachea and right main bronchus. The bronchoscope tip was wedged in a segmental orifice of the right middle lobe and five 50-mL aliquots of sterile saline instilled in an identical fashion in each volunteer. At least 60% of this fluid was reaspirated and collected in a 500-mL polypropylene bottle, placed on ice and immediately transferred for *ex vivo* cul-ture. Each subject underwent two fibreoptic broncho-scopic 14 days apart at either end of the course of inhaled medication. The second procedure was carried out 90–120 min after the last dose of inhaled medication was taken.

Preparation of alveolar macrophages. Alveolar macro-phages were separated and cultured as described previously [8]. Briefly, cells were counted, centrifuged ($600 \times g$, 10 min) at room temperature ($20°C$) and washed once in phos-phate-buffered saline before resuspension in RPMI 1640 medium supplemented with 5 mM *N*-2-hydroxyethylpiper-azine-*N*-2 ethanesulphonic acid HEPES, 10% foetal calf serum, 2 mM glutamine and $100 \, \text{UmL}^{-1}$ penicillin and $100 \, \mu\text{M}$ streptomycin at a density of 10^6 cells·well^{-1}. The via-bility of the alveolar macrophages was determined in rep-resentative samples by means of trypan blue exclusion. After 1 h of incubation, nonadherent cells were washed off and the adherent population resuspended in 1 mL of med-ium containing either $10 \, \mu\text{g·mL}^{-1}$ LPS *Escherichia coli* 026:B6, Sigma Chemical Co., Dorset, UK), $10 \, \mu\text{g·mL}^{-1}$ AraLAM, $10 \, \mu\text{g·mL}^{-1}$ ManLAM or medium alone in sep-arate tissue culture dishes for 24 h. ManLAM isolated from *M. tuberculosis* strain H37Rv and AraLAM from a rapid growing *Mycobacterium* species were provided by J. Belisle (Colorado State University). Endotoxin contamination in these preparations was $1.38 \, \text{ng·mg}^{-1}$ for both ManLAM and AraLAM, as determined by the *Limulus* amoebocyte Assay (personal communication, J. Belisle). The adherent population of cells was found to be 90–95% alveolar mac-rophages, by both cytological examination and immuno-fluorescent staining using antihuman CD68 (Dako, High Wycombe, UK).

Measurement of beclomethasone 17-monopropionate con-centration in bronchoalveolar lavage fluid supernatants. Supernatants from centrifuged unstimulated bronchoal-veolar lavage (BAL) specimens from each volunteer were stored at $-70°C$ and tested in one batch. After solid-phase extraction, BAL fluid concentrations of beclomethasone 17-monophosphate (17-BMP) were measured using a liquid chromatography/mass spectrometry method developed at 3M Pharmaceuticals. This method was automated with the MultiProbe (Packard, Chicago, IL, USA) using the Empore C1896-wells format (3M Pharmaceuticals) for extraction. The linear range was 75–2000 pg·mL^{-1} for 17-BMP in BAL. The lavage concentrations were calculated using standard curves that were fitted to a weighted linear regression with correlations ranging 0.9958–0.9997. The signal-to-noise ratio at the limit of quantification was >10:1.

Measurement of tumour necrosis factor-α bioactivity. The biological activity of TNF-α in the culture supernatants was measured in triplicate, as previously described [9], using murine L929 cells to assay for cytotoxicity in the presence of actinomycin D. Confirmation of the TNF-α concentration in selected samples was obtained using an enzyme-linked immunosorbent assay kit (Human TNF-α Quantikine; R&D Systems, Abingdon, UK) (results not shown).

Inhaled steroid regime. Following the first bronchoscopic examination, control subjects were started on a course of either inhaled BDP or placebo, administered *via* a press-and-breathe metered dose inhaler, randomized in an open-labelled manner. BDP was given at a dose of 400 μg (four puffs) twice daily as either HFA-BDP (QVAR^{TM}) or CFC-BDP (FILAIR^{TM}, 3M Pharmaceuticals). The placebo was an aerosolized preparation using the HFA propellant (HFA-placebo). Subjects on placebo followed the same protocol as those on the inhaled steroid regimen (*i.e.* four puffs taken morning and evening). Inhaler technique was checked and monitored, and compliance was supervised by an exper-ienced asthma nurse.

Statistical analysis. Since the raw data included at least triplicate values for each culture supernatant and standard curve sample, a statistical test was used which took advantage of both the mean and standard error of the mean for each data point. In order to test for differences in TNF-α levels before and after the two active BDP treatments compared to placebo, a linear model allowing for fixed patient effects was fitted to the logarithms of the TNF-α concentrations. A fixed effects term for the three *ex vivo* treatments was also included. As the TNF-α bioassay reports only mean and standard error of the three measured TNF-α concentrations, it was necessary to simulate three data points having the appropriate mean and standard error for each subject's TNF-α concentrations. The results show the change in log TNF-α concentration post-inhaler com-pared to placebo for each of the three *ex vivo* treatments. A p-value of <0.05 was considered significant. The analysis was performed by fitting linear models in Splus (Cambridge, MA, USA), each of the three drug groups, HFA-placebo, HFA-BDP and CFC-BDP, being fitted simultaneously to the model. The BAL fluid 17-BMP concentration data were analysed using Fisher's exact test.

Results

Production of tumour necrosis factor-α by alveolar mac-rophages in response to lipopolysaccharide and lipoara-binomannan

Preliminary experiments on alveolar macrophages from six hospital patients undergoing diagnostic bronchoscopy were performed to characterize the titration of LPS and

Table 1. – Tumour necrosis factor-α (TNF-α) concentrations from initial titration experiments to characterize the optimal concentrations of glycolipid preparation and duration of stimulation required

Ex vivo stimulant	TNF-α ng·mL^{-1}	
	6-h culture	24-h culture
Medium alone	5.16±1.68	2.02±0.98
LPS 10 µg·mL^{-1}	>12	>12
AraLAM 1 µg·mL^{-1}	5.66±2.59	2.87±2.23
AraLAM 3 µg·mL^{-1}	6.65±3.04	3.47±2.92
AraLAM 10 µg·mL^{-1}	7.74±3.38	8.96±7.54
ManLAM 1 µg·mL^{-1}	4.83±2.63	2.30±1.81
ManLAM 3 µg·mL^{-1}	5.28±2.75	3.11±2.41
ManLAM 10 µg·mL^{-1}	6.16±2.87	8.88±2.83

Data are presented as mean±SEM. TNF-α concentrations were measured by means of enzyme-linked immunosorbent assay of the alveolar macrophage culture supernatants from the bronchoalveolar lavage specimens of six patients. LPS: lipopolysaccharide; AraLAM: arabinofuranosyl lipoarabinomannan (LAM); ManLAM: mannose LAM.

LAM and also the optimal length of time of cell culture. Results indicated that a 24-h culture time resulted in the induction of maximal TNF-α bioactivity by LPS and both LAM preparations at a concentration of 10 µg·mL^{-1}. Not surprisingly, alveolar macrophages incubated with lower concentrations of LAMs produced lower but statistically nonsignificant concentrations of TNF-α (table 1).

The geometric mean and 95% confidence interval of TNF-α concentrations in alveolar macrophage culture supernatants were calculated for the group of 28 control subjects, for the pre-inhaler 24-h ex vivo cultures (table 2). In the combined pretreatment groups, TNF-α concentrations in alveolar macrophage culture supernatants were significantly higher when culture was performed in the presence of LPS (p<0.001) and in the presence of AraLAM (p<0.001) (fig. 1a and c). The difference was less marked, although still significant, when culture was performed in the presence of ManLAM (p=0.02) compared to medium alone (fig. 1b).

Beclomethasone 17-monopropionate concentrations in cell-free bronchoalveolar lavage supernatants

No measurable 17-BMP was detectable in any of the pretreatment BAL supernatants or in any of the post-treatment BAL fluid supernatants from the HFA-placebo

Table 2. – Tumour necrosis factor-α (TNF-α) concentrations in alveolar macrophage culture supernatants stimulated with one of the three glycolipids, lipopolysaccharide (LPS), arabinofuranosyl lipoarabinomannan (LAM) (AraLAM) and mannose LAM (ManLAM), from 28 healthy volunteers*

Ex vivo stimulant	TNF-α ng·mL^{-1}	95% confidence interval	p-value
Medium alone	0.72	0.48–1.07	
LPS 10 µg·mL^{-1}	138	110–173	<0.001
ManLAM 10 µg·mL^{-1}	1.42	0.94–2.14	0.02
AraLAM 10 µg·mL^{-1}	12.6	8.9–17.7	<0.001

*: taken from placebo, chlorofluorocarbon and hydrofluoroalkane groups prior to any inhaled steroid preparation. Data are presented as geometric mean.

group. There was marked variability in the post-treatment values from the HFA-BDP and CFC-BDP groups (table 3). However, seven of the 10 post-treatment BAL fluid supernatants harvested from subjects who were randomized to CFC showed measurable BAL fluid 17-BMP concentrations ranging 77.4–3165 pg·mL^{-1}. Only one of 10 post-treatment BAL fluid supernatants from the HFA-BDP group had a measurable BAL fluid concentration. There was a significantly greater chance of the 17-BMP concentration being higher in the CFC-BDP group than in the HFA-BDP group. This reached statistical significance (p=0.02).
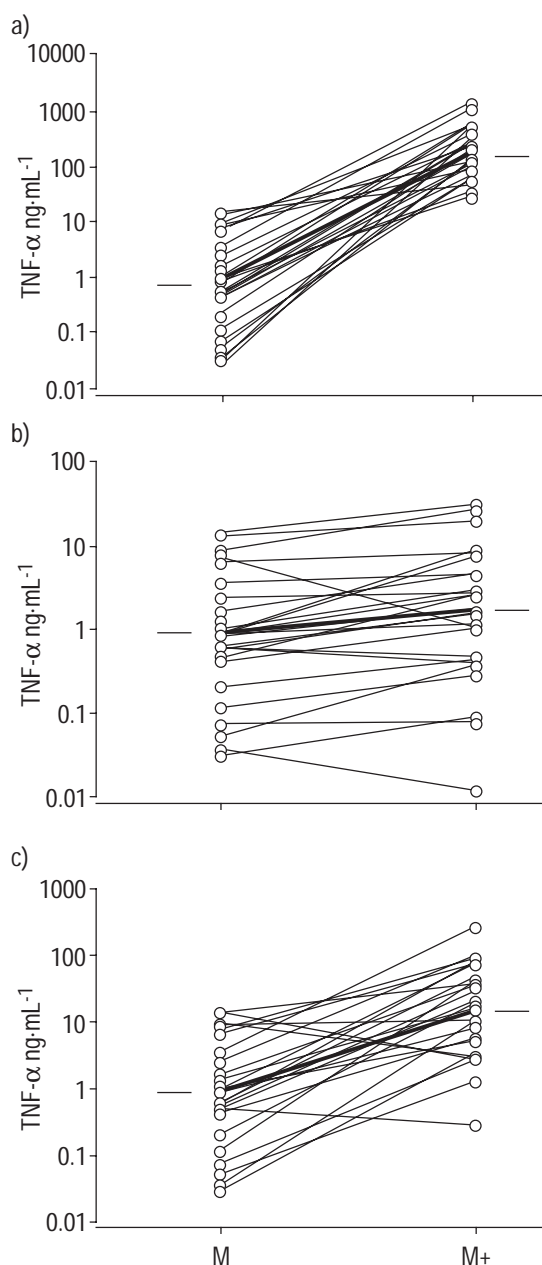

Fig. 1. – Tumour necrosis factor (TNF-α) bioactivity in 24-h culture supernatants of alveolar macrophages from healthy nonsmoking volunteers incubated with: a) lipopolysaccharide (10 µg·mL^{-1}) (p<0.001); b) mannose lipoarabinomannan (LAM) (10 µg·mL^{-1}); and c) arabirofuranosyl LAM (10 µg·mL^{-1}) (M+) (p<0.01) compared to culture supernatants from unstimulated alveolar macrophages (medium alone (M)). The horizontal bars and thick lines represent the geometric mean.

Table 3. – Beclomethasone 17-monopropionate (17-BMP) concentrations in bronchoalveolar lavage fluid supernatants from healthy volunteers following a 14-day course of either inhaled chlorofluorocarbon (CFC)-beclomethasone diproprionate (BDP) or hydrofluoroalkane 134a (HFA)-BDP at a dose of 800 µg·day^{-1}*

	17-BMP pg·mL^{-1}	
Volunteer No.	HFA-BDP	CFC-BDP
1/11	<75	<75
2/12	<75	86.3
3/13	<75	<75
4/14	<75	76.1
5/15	<75	100.6
6/16	<75	80.6
7/17	<75	452.3
8/18	<75	3165
9/19	122.8	77.4
10/20	<75	<75

*: these results correspond to the same groups of volunteers as used in figures 2 and 3.

Tumour necrosis factor-α production by stimulated cultured alveolar macrophages following in vivo *administration of inhaled hydrofluoroalkane 134a-beclomethasone dipropionate preparation*

Administration of HFA-BDP (800 µg·day^{-1}) resulted in a significant reduction in TNF-α production in the group of 10 subjects randomized to inhaled HFA-BDP. Log TNF-α concentrations in culture supernatants of LPS-stimulated and ManLAM-stimulated alveolar macrophages decreased significantly compared to those in the group taking inhaled placebo (p<0.001 and p<0.02 respectively) (fig. 2b and c). However, the decrease in mean alveolar macrophage culture supernatant TNF-α concentrations following Ara-LAM stimulation did not reach significance (fig. 2d). TNF-α levels in unstimulated alveolar macrophage culture supernatants from this group did not fall significantly following 2 weeks of inhaled HFA-BDP (fig. 2a).

Tumour necrosis factor-α production by stimulated cultured alveolar macrophages following in vivo *administration of inhaled chlorofluorocarbon-beclomethasone dipropionate preparation*

There was no statistically significant change in TNF-α production by alveolar macrophages stimulated with LPS, ManLAM, or AraLAM from the 10 patients studied prior to and following 14 days' treatment with inhaled CFC-BDP at a dose of 800 µg·day^{-1}, compared to placebo (fig. 3).

Tumour necrosis factor-α production by stimulated cultured alveolar macrophages following in vivo *administration of inhaled hydrofluoroalkane134a-placebo preparation*

In the group of eight subjects randomized to the inhaled placebo preparation, there was no significant change in logTNF-α concentration pre- and post-inhaler in LPS-, AraLAM- and ManLAM-stimulated alveolar macrophage culture supernatants. When supernatants of cells cultured
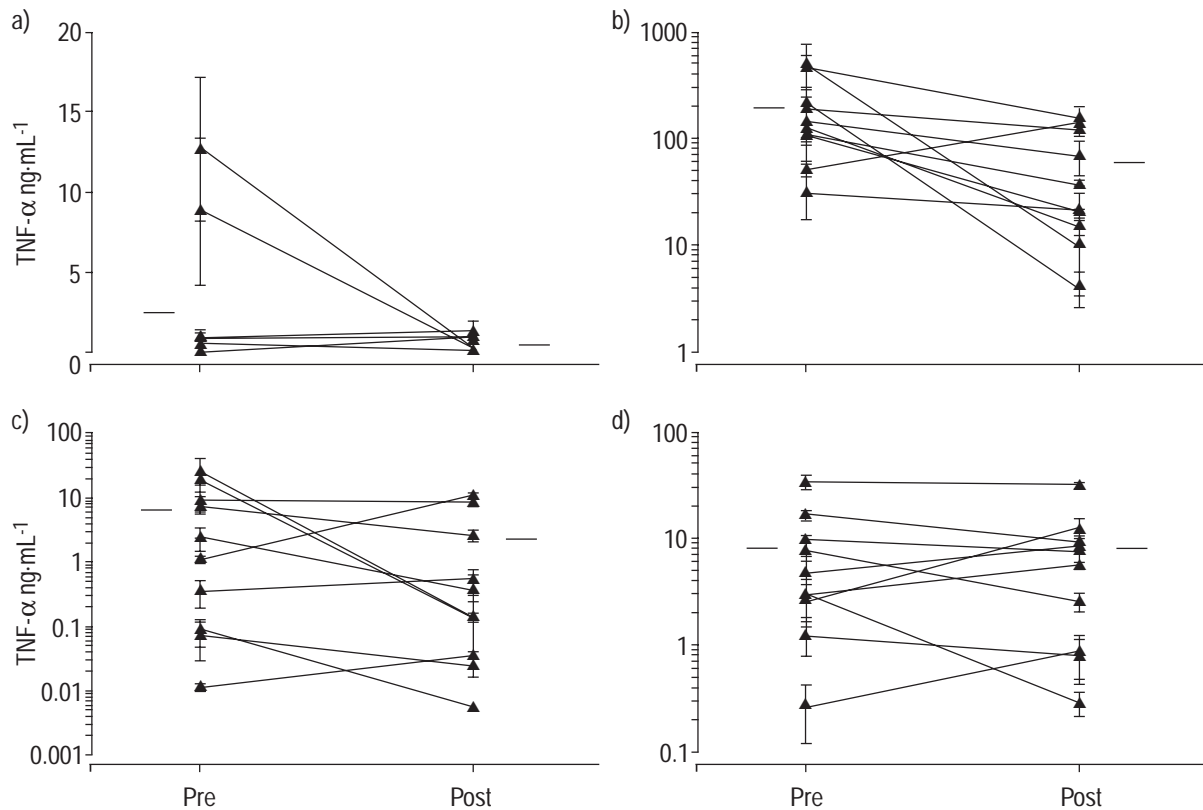

Fig. 2. – Tumour necrosis factor-α (TNF-α) bioactivity of alveolar macrophage culture supernatants from the inhaled hydrofluoroalkane 134a-beclomethasone dipropionate volunteer group stimulated with: a) medium alone (NS); b) lipopolysaccharide (10 µg·mL^{-1}) (p<0.001); c) mannose lipoarabinomannan (LAM) (10 µg·mL^{-1}) (p=0.02); and d) arabinofuranosyl LAM (10 µg·mL^{-1}) (NS). Each symbol corresponds to one volunteer. The horizontal bars represent the geometric mean. Pre: pre-steroid; Post: post-steroid.
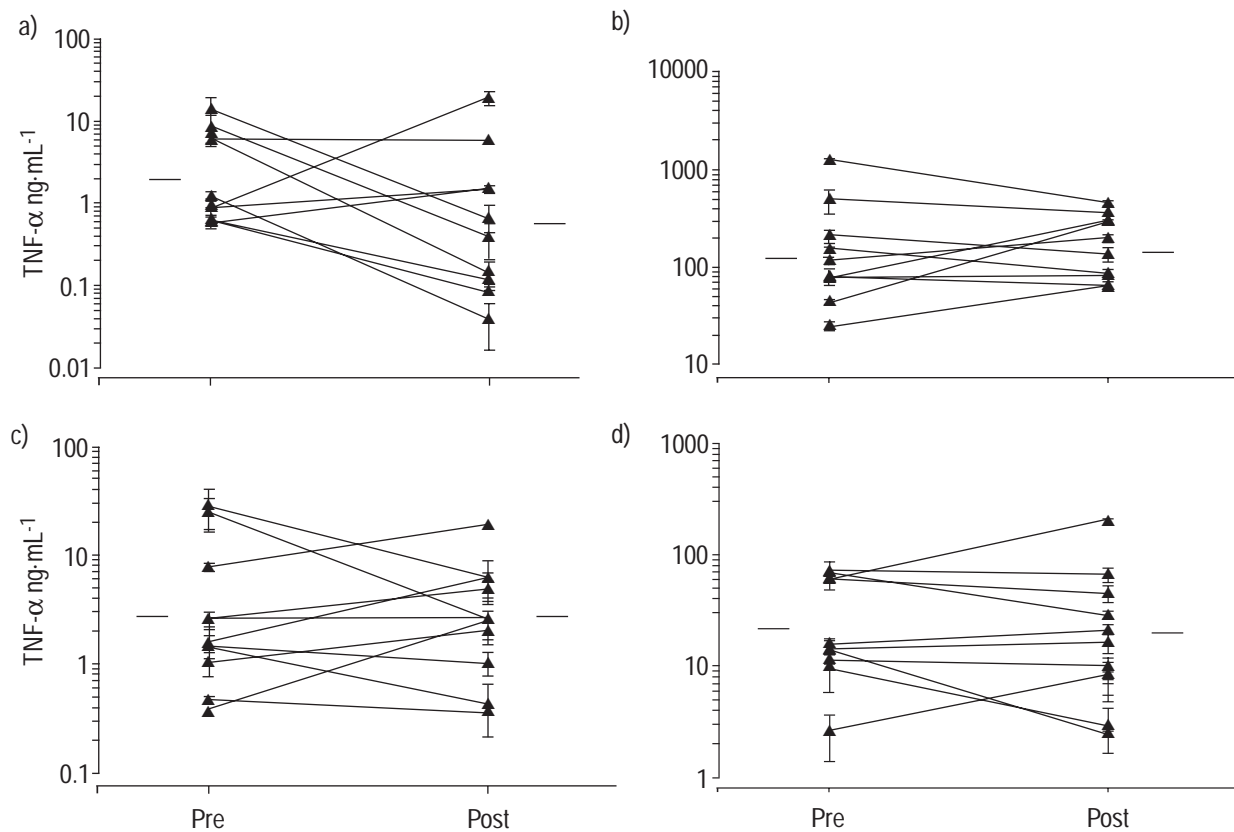
Fig. 3. – Tumour necrosis factor-α (TNF-α) bioactivity of alveolar macrophage culture supernatants from the inhaled chlorofluorocarbon-bec-lomethasone dipropionate volunteers stimulated with: a) medium alone (NS); b) lipopolysaccharide (10 μg·mL^{-1}) (NS); c) mannose lipoarabinomannan (LAM) (10 μg·mL^{-1}) (NS); and d) arabinofuranosyl LAM (10 μg·mL^{-1}) (NS). Each symbol corresponds to one volunteer (11-20). The horizontal bars represent the geometric mean. Pre: pre-steroid; Post: post-steroid.

in medium alone collected prior to and following a 2-week course of placebo inhaler were compared, there was a small but statistically significant reduction (p=0.04) in TNF-α release (fig. 4).

Discussion

In this study, the first clear evidence is provided that a novel inhaled CFC-free preparation of BDP (HFA-BDP), with increased peripheral lung deposition, can modulate glycolipid-induced TNF-α production by human alveolar macrophages, in comparison with CFC-BDP and HFA-placebo. A dose of 400 μg HFA-BDP, administered twice daily for 2 weeks, significantly reduced glycolipid-induced TNF-α production by alveolar macrophages cultured *ex vivo*. However, following the administration of CFC-BDP or HFA-placebo at an identical dose, no change in TNF-α production in response to the same preparations was obser-ved. As physiological and pathological evidence suggests that the entire pulmonary airway, including peripheral elements and lung parenchyma, is involved in the inflam-matory processes in asthma [10, 11], these findings imply that inhaled corticosteroid drug distribution to the alveoli might be beneficial for the treatment of asthma.

This report provides the first evidence that two preparations of LAM, AraLAM and ManLAM, the major cell wall glycolipids of mycobacteria, are able to elicit TNF-α production from human alveolar macrophages ob-tained from healthy human volunteers. This study indicates

that ManLAM from virulent *M. tuberculosis* has a lesser capacity to activate human alveolar macrophages to pro-duce TNF-α than the structurally similar AraLAM, puri-fied from rapidly growing nonpathogenic mycobacteria. These data corroborate two recent studies which have demonstrated that live preparations of two laboratory strains of *M. tuberculosis* induce TNF-α production in primary human alveolar macrophages [12, 13]. The results are also consistent with the differential responses to my-cobacterial LAMs of human and murine mononuclear phagocytes, reported by several other investigators [14–18]. The differences in cytokine induction by the two preparations has been attributed to variations in the non-reducing termini of the two molecules [5, 19]. The results in this human volunteer study, however, showed more interindividual variation in the ability of ManLAM to induce TNF-α production in alveolar macrophages, com-pared to AraLAM (fig. 1b c). Overall, the mean increase in TNF-α concentration in supernatants of AraLAM-stimulated alveolar macrophages was highly significant (p<0.001) when compared to the TNF-α bioactivity from unstimulated alveolar macrophage cultures; the increase was less marked in supernatants of ManLAM-stimulated cells (p=0.02). The TNF-α release induced by Gram-neg-ative bacterial LPS in the present study was substantial and an order of magnitude greater than that from AraLAM-induced alveolar macrophages. This finding of LPS-induced TNF-α production was similar to that in previous studies [12, 20, 21].
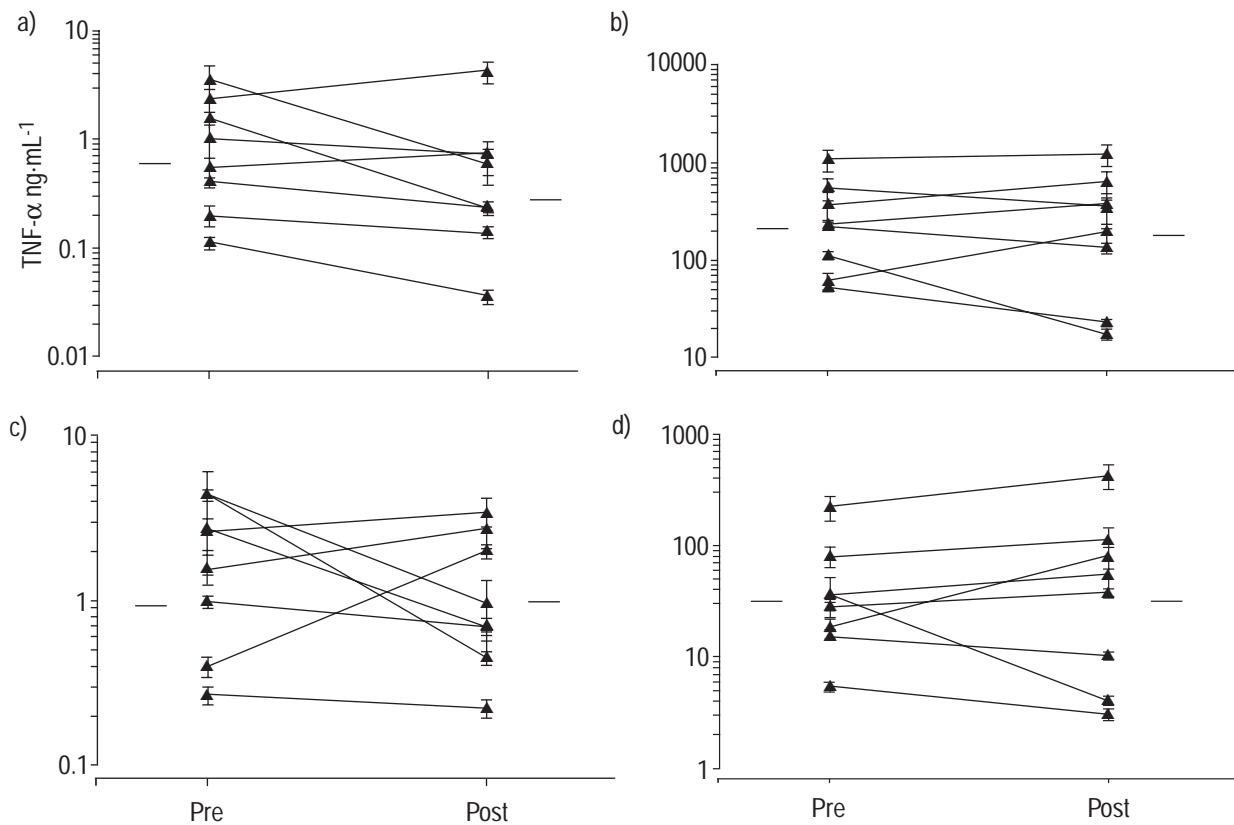
Fig. 4. – Tumour necrosis factor-α (TNF-α) bioactivity of alveolar macrophage culture supernatants from the hydrofluoroalkane-placebo volunteer group stimulated with: a) medium alone (p=0.04); b) lipopolysaccharide (10 μg·mL^{-1}) (NS); c) mannose lipoarabinomannan (LAM) (10 μg·mL^{-1}) (NS); and d) arabinofuranosyl LAM (10 μg·mL^{-1}) (NS). Each symbol corresponds to one volunteer (21-28). The horizontal bars represent the geometric mean. Pre: pre-placebo; Post: post-placebo.

It was intriguing that TNF-α production by AraLAM-stimulated alveolar macrophages was not significantly modulated (p=0.2) by a course of HFA-BDP. Since AraLAM binds to the same high-affinity CD14 macrophage receptor as LPS [22], leading to transduction of an activation signal for TNF-α production [21], it might be expected that corticosteroids would modulate the effects of AraLAM and LPS similarly, as has been demonstrated previously [23]. However, the signal transduction pathway initiated by AraLAM binding to CD14 is not well characterized [24], and recent studies have demonstrated that, although LPS and AraLAM share some CD14-dependent responses, the signal transduction pathway for AraLAM requires one or more components that are at least partially distinct from that initiated by LPS [16, 25], raising the possibility of different regulatory steps. By contrast, ManLAM is able to signal through a different receptor, the macrophage mannose receptor [26], and thus might also be subject to differing mechanisms of immunomodulation by corticosteroid. This might explain the significant modulatory effect of BDP on TNF-α induction by ManLAM-stimulated alveolar macrophages.

The results of the 17-BMP concentration measurements in BAL fluid supernatants from volunteers were unexpected. Although an *in vivo* course of inhaled CFC-BDP had no significant modulatory effect on alveolar macrophage function, BAL fluid levels of its active metabolite, 17-BMP, were significantly increased compared to 17-BMP levels following HFA-BDP. A key to understanding this observation can be found in the substantial particle size differences between CFC-BDP and HFA-BDP [7]. Whereas CFC-BDP delivers an aerosol with particles of mass medium aerodynamic diameter (MMAD) of 3.5–4.0 μm, HFA-BDP delivers particles with a MMAD of 1.1 μm. The present findings suggest that the smaller-sized particles from HFA-BDP result in greater lung distribution and greater alveolar uptake, which in turn results in a higher drug concentration at the receptor site than obtained with the larger drug particles from CFC-BDP. The high concentrations of 17-BMP in BAL fluid resulting from CFC-BDP administration probably reflects the poorer drug distribution in the lungs and the reduced absorption of the large particles. It is interesting to note that BAL fluid levels appear to measure drug which is not absorbed and presumably not efficacious.

In summary, this study demonstrates that human alveolar macrophages from healthy volunteers activated *ex vivo* by glycolipids of Gram-negative bacteria and mycobacteria respond by upregulating tumour necrosis factor-α release. This inflammatory response can be modulated by the peripheral deposition of inhaled corticosteroid *in vivo*. It was observed that inhaled corticosteroids must reach the distal airways to be effective. These findings may have important implications in the development of new immunotherapeutic agents designed to improve treatment of inflammatory pulmonary disorders.

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