

Expression of pro-inflammatory cytokines by flow-sorted alveolar macrophages in severe pneumonia

U. Maus⁺, S. Rosseau⁺, U. Knies^{*}, W. Seeger⁺, J. Lohmeyer⁺

Expression of pro-inflammatory cytokines by flow-sorted alveolar macrophages in severe pneumonia. U. Maus, S. Rosseau, U. Knies, W. Seeger, J. Lohmeyer. ©ERS Journals Ltd 1998.

ABSTRACT: The aim of the present study was to further characterize the role of alveolar macrophages (AM) in acute human lung inflammation by evaluating their capacity to produce pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-6 and IL-8.

Patients with severe community-acquired pneumonia (CAP; n=12) and healthy volunteers (n=10) underwent bronchoalveolar lavage (BAL). AM were separated to high purity (>96%) using fluorescence-activated cell sorting. We determined the TNF- α , IL-6 and IL-8 cytokine gene expression in AM *ex vivo* using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). Moreover, we measured *in vitro* unstimulated, lipopolysaccharide (LPS)- and LPS/interferon- γ inducible TNF- α , IL-6 and IL-8 cytokine release and evaluated samples of BAL fluids for the same pro-inflammatory cytokines using an enzyme-linked immunosorbent assay (ELISA).

We found increased TNF- α , IL-6 and IL-8 messenger ribonucleic acid (mRNA) levels in AM from CAP patients that were significantly elevated only for IL-8. When challenged with endotoxin *in vitro*, AM obtained from CAP patients showed a strongly reduced potential to release TNF- α and IL-6 compared to healthy controls, whereas IL-8 secretion did not differ significantly between groups. Moreover, stimulation of AM from CAP patients with LPS plus IFN- γ augmented TNF- α and IL-6 cytokine release to near normal levels. Interestingly, no TNF- α protein was measured in BAL samples from CAP patients, whereas IL-6 and IL-8 protein levels were found to be significantly increased.

Together, highly purified alveolar macrophages from community-acquired pneumonia patients show relatively low *ex vivo* tumour necrosis factor- α and interleukin-6 but not interleukin-8 messenger ribonucleic acid levels that are associated with a decreased pro-inflammatory cytokine release *in vitro* which, however, can be restored by concurrent interferon- γ stimulation.

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Alveolar macrophages (AM), as the major resident immunocompetent cell population in the lower respiratory tract, protect the lungs against invading microbial agents and particle load. In addition, there is increasing evidence that AM play a crucial role in acute lung injury due to their exceptional capacity to release reactive oxygen species [1], lytic enzymes and an array of inflammatory cytokines including tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and IL-8 [2, 3]. A large body of data indicates the participation of these cytokines in mediating acute inflammatory responses [4]. At the same time, these mediators contribute to an effective host defence [3].

Hitherto, the functional state of AM in severe pneumonia, which continues to be a major cause of morbidity and mortality worldwide, has been poorly defined. Many cellular sources such as AM, polymorphonuclear neutrophils (PMN), fibroblasts and epithelial cells may contribute to the generation of pro-inflammatory cytokines such

as TNF- α , IL-6 and IL-8 within the alveolar compartment [5–10]. Thus, the characterization of AM within the context of acute inflammatory lung disease requires specialized separation techniques to discriminate these cells from other cytokine-producing inflammatory cells (*e.g.* PMN) without altering their *in vivo* activation state [11]. In the current study, we sorted AM obtained from bronchoalveolar lavage (BAL) samples from patients with severe community-acquired pneumonia (CAP) and from healthy volunteers to high purity and determined their TNF- α , IL-6 and IL-8 cytokine gene expression. Moreover, we analysed the AM potential to secrete TNF- α , IL-6 and IL-8 following *in vitro* endotoxin challenge and evaluated the modulation of the pro-inflammatory response by co-stimulating AM with interferon- γ (IFN- γ). In addition, we measured TNF- α , IL-6 and IL-8 contents in BAL fluids from CAP patients and healthy controls and compared them with corresponding *ex vivo* messenger ribonucleic acid (mRNA) data.

Materials and methods

Study population

We studied: 1) patients with severe CAP; and 2) healthy volunteers as controls.

Severe pneumonia. This study group consisted of 12 patients with severe CAP, requiring mechanical ventilation, including 10 males and two females (average age 51 yrs, range 28–77 yrs; four smokers and eight nonsmokers). All patients underwent BAL for diagnostic reasons, which was performed within 72 h after intubation. At the time that the lavage was performed, the mean inspiratory oxygen fraction (FI_{O_2}) was 0.72, and the positive end-expiratory pressure (PEEP) ranged 8–12 cmH₂O. Diagnostic criteria for severe pneumonia were fever, tachycardia, dyspnoea, characteristic chest radiographs and identification of microbiological pathogens in the lower respiratory tract (table 1). According to radiological criteria described by FRASER [12], nine patients suffered from bronchopneumonia (patchy peribronchially centred consolidations) and three from alveolar pneumonia (homogeneous, sharply demarcated consolidations with air bronchogram). All patients enrolled in this study were free of acute or chronic left heart failure (capillary wedge pressure (P_{cw}) <2.1 kPa (16 mmHg)). General exclusion criteria were chronic obstructive or interstitial lung disease as well as malignancy of the lung. General therapeutic approaches included parenteral nutrition, volume substitution and antibiotic drug therapy. None of the patients enrolled in this study received corticosteroids.

Healthy volunteers. We evaluated 10 healthy volunteers, comprising six males and four females (average age 24 yrs, range 23–26 yrs, four smokers, six nonsmokers). All participants in this study group were free of respiratory symptoms, were not on medication and showed normal lung functional test results, including transfer factor of the lung for carbon monoxide.

Ethics approval. This study was approved by the Medical Ethics Committee of the Justus-Liebig-University Giessen; all participants and patients or closest relatives gave written, informed consent.

Bronchoalveolar lavage

BAL was performed as a routine diagnostic procedure using flexible fiberoptic bronchoscopy as described previously [13]. Briefly, ten 20 mL aliquots of sterile saline

Table 1. – Pathogenic agents in the bronchoalveolar lavage fluid of patients with severe community-acquired pneumonia

Species	n
<i>Haemophilus influenzae</i>	3
<i>Pseudomonas aeruginosa</i>	2
<i>Streptococcus pneumoniae</i>	2
<i>Staphylococcus aureus</i>	1
<i>Serratia</i> sp.	1
<i>Klebsiella</i> sp.	1
Negative	2

Table 2. – Bronchoalveolar lavage cell characteristics of the study population

Study population	Total cells $\times 10^6$	Differential		
		AM %	PMN %	Lym %
Normal subjects (n=10)	14 \pm 4.7	90.8 \pm 4.6	0.3 \pm 0.3	8.5 \pm 4.6
Severe CAP (n=12)	25.4 \pm 17.1	45.5 \pm 24.1*	51.8 \pm 25*	2.5 \pm 1.7

Values are presented as mean \pm SD. AM: alveolar macrophages; PMN: polymorphonuclear neutrophils; Lym: lymphocytes; CAP: community-acquired pneumonia. *: $p < 0.05$, versus normal subjects.

were instilled into a subsegmental bronchus of the involved area according to chest radiographs. The fluids were aspirated by gentle suction, pooled, filtered through sterile gauze and immediately placed on ice. Centrifugation was performed at $200 \times g$ for 10 min at 4°C and the cell pellet was washed once in calcium- and magnesium-free Hank's balanced saline solution (HBSS; Gibco BRL, Eggenstein, Germany) and resuspended in minimal essential medium (MEM; Gibco BRL) until sorting of AM. The cells were counted with a haemocytometer. Viability was determined routinely by propidium iodide staining with subsequent fluorescence-activated cell sorting (FACS) analysis and was always >92% (data not shown). Pappenheim-stained (May-Grünwald and Giemsa solution; Merck, Darmstadt, Germany) cytospin preparations were prepared for morphological differentiation of BAL cells (table 2). For quantification of BAL cytokine levels, aliquots of lavage samples were stored cell-free at -86°C until use.

Flow sorting of alveolar macrophages

AM were separated by FACS as recently described in detail [11]. Briefly, a FACStar^{PLUS} flow cytometer (Becton Dickinson, San Jose, CA, USA) was employed, equipped with a 5 W argon ion laser operating at 488 nm (200 mW) and a 100 μ m ceramic nozzle that was attached to a large nozzle sort-head assembly (MacroSORT), which is especially designed for the separation of large biological particles. Standardized calibration beads (Becton Dickinson, San Jose, CA, USA) were used for optical system calibration. Prior to sorting, the sample line tubing was sterilized with 0.1% weight/volume (w/v) bleach-solution (Hedinger, Stuttgart, Germany) which was removed by backflushing the system with sterile saline. Unsorted cell suspensions and sorted AM preparations were always maintained on ice throughout the separation. The purity of sorted cells was always >96% and was analysed by: 1) post-sort analysis of sorted cells; and 2) differential cell counts of Pappenheim-stained cytocentrifuge preparations of sorted cells as well as alkaline phosphatase-stained sorted cells as recently described in detail [11]. The sheath fluids were routinely assayed for their content of endotoxin (COATEST, Chromogenix, Mölndal, Sweden) and always contained less than 10 pg·mL⁻¹ of lipopolysaccharide (LPS), the lower detection limit of the assay. These conditions have previously been shown to prevent isolation-induced pro-inflammatory cytokine expression in AM [11].

Isolation of total cellular ribonucleic acid (RNA) and reverse transcription

Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method as previously described [14]. The constituent mRNA was reverse transcribed according to the instructions of the manufacturer (StrataScript reverse transcriptase polymerase chain reaction (RT-PCR) kit; Stratagene, Heidelberg, Germany) in a final volume of 25 μ L. The synthesis of complementary deoxyribonucleic acid (DNA) was carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CA, USA) for 50 min at 37°C and enzyme inactivation was achieved by heating the reaction to 94°C for 7 min. Subsequently, the reaction mixture was diluted with ribonuclease (RNAse)-free water to 60 μ L and stored at -85°C until used.

Amplification of TNF- α , IL-6, IL-8 and β -actin complementary DNAs (cDNAs)

The polymerase chain reaction (PCR) was performed in 1 \times PCR buffer (Perkin Elmer, Norwalk, CA, USA), 1 mM of each deoxynucleoside triphosphate (dNTP) (adenine, cytosine, guanine and thymine deoxynucleotides (dATP, dCTP, dGTP and dTTP, respectively), 1 μ M of intron-spanning cytokine-specific primers (table 3; Stratagene, Heidelberg, Germany), 0.75 U AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CA, USA) and 2 μ L of first strand cDNA in a total volume of 25 μ L. PCR profiles consisted of initial denaturation at 94°C (1.5 min) followed by 25 (β -actin) or 35 cycles (TNF- α , IL-6, IL-8) of denaturation (94°C, 50 s), primer annealing (60°C, 60 s) and primer extension (72°C, 60 s) in a GeneAmp PCR System 2400. The final extension was performed at 72°C for 7 min. Aliquots of PCR-products were electrophoresed through 1.8% (w/v) Nusieve/agarose gels stained with ethidium-bromide for approximately 2 h at 75 V. Nega-tive controls were routinely performed by running PCR without cDNA template to exclude false-positive amplification products. Positive controls were performed using cDNA preparations obtained from LPS-stimulated (100 ng·mL⁻¹, 6 h) AM. To verify the specificity of PCR amplifications obtained from the above-mentioned procedure, automated DNA sequencing was carried out on the purified PCR products according to the instructions of the manufacturer (Model 373A; Applied Biosystems, Weiterstadt, Germany). By comparing the resulting cDNA sequences with the corresponding published sequences, we identified PCR products as expected seg-

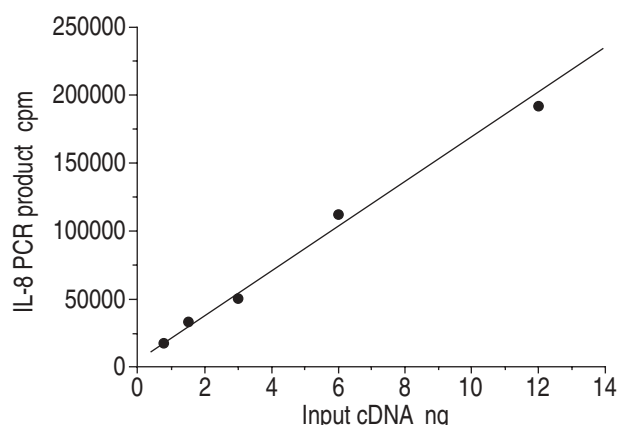


Fig. 1. – The linear relationship between the amount of input complementary deoxyribonucleic acid (cDNA) and yield of polymerase chain reaction (PCR) product. Increasing amounts of cDNA from a severe community-acquired pneumonia (PN) patient were quantified for interleukin (IL)-8 gene expression for 35 cycles of amplification. The amount of input ribonucleic acid (RNA) equivalents (abscissa) versus corresponding IL-8 signals (ordinate) are given. Results represent the mean value of three independent determinations. Similar results were obtained for specific amplification of tumour necrosis factor (TNF)- α , IL-6 and β -actin cDNA. cpm: counts per minute.

ments of spliced cytokine or β -actin mRNA species. With PCR conditions optimized for primer and magnesium concentrations and cycle numbers, amplification of cDNA samples was verified to be in the exponential phase of PCR by comparing the amount of input RNA equivalents with the yield of the respective cytokine and β -actin PCR products. Figure 1 shows a linear relationship between the increase of input cDNA concentrations and radioactively probed IL-8 PCR product, as quantified by PhosphorImager analysis (Model SF; Molecular Dynamics, Sunnyvale, CA, USA).

Quantification of amplified PCR products

The specific TNF- α , IL-6 and IL-8 PCR products were quantified as described previously [19], with some modifications. Briefly, using β -actin as housekeeping gene, varying input cDNA concentrations of different samples were adjusted with distilled water to obtain comparable cDNA contents prior to PCR amplification. Aliquots (4 μ L) of unlabelled TNF- α , IL-6, IL-8 and corresponding β -actin PCR products were denatured at 94°C for 5 min prior to blotting onto nylon membranes as previously described [20]. Membranes were baked at 80°C for 2 h, prehybridized and hybridized in 10 mL of buffer com-

Table 3. – List of primers and primer-specific sequences, as employed for reverse transcriptase polymerase chain reaction

Specificity	Oligonucleotide primer-sequences	Pos. cDNA	[Ref.]	Exon	bp
β -actin	5'-AAAGACCTGTACGCCAACACAGTGCTGTCT-3'	912–941	[15]	4	220
	5'-CGTCATACTCCTGCTTGCTGATCCACATCTG-3'	1100–1130		5	
TNF- α	5'-CGGGACGTGGAGCTGGCCGAGGAG-3'	107–130	[16]	1	355
	5'-CACCAGCTGGTTATCTCTCAGCTC-3'	437–460		4	
IL-6	5'-ATGAATCCTTCTCCACAAGCGC-3'	35–57	[17]	1/2 [†]	628
	5'-GAAGAGCCCTCAGGCTGGACTG-3'	641–662		5	
IL-8	5'-AGATGTCAGTGCATAAAGACA-3'	199–219	[18]	2	202
	5'-TGAATTCTCAGCCCTCTTCAAAA-3'	376–399		3/4 [†]	

Pos. cDNA: position complementary deoxyribonucleic acid; bp: base pairs; TNF: tumour necrosis factor; IL: interleukin. [†]: the primer itself spans intron.

posed of 5× SSC, 5× Denhardt's solution, 1% (v/v) sodium dodecyl sulphate (Sigma, Deisenhofen, Germany), 50% (v/v) de-ionized formamide (Clontech, Palo Alto, CA, USA), and heat-denatured salmon sperm DNA (100 µg·mL⁻¹; Boehringer, Mannheim, Germany) at 42°C overnight with respective probes that were labelled with α (³²P) dCTP by random hexamer priming [21]. Spin column chromatography (Boehringer) was used to remove unincorporated dNTPs as well as small probe fragments. After washing the blots twice at ambient temperature, autoradiography was performed for 90 min. The quantification of TNF- α , IL-6 and IL-8 cytokine gene expression levels was performed using a Phospho-Imager (Model SF; Molecular Dynamics, Sunnyvale, CA, USA). Results are expressed as mean ratio normalized to β -actin signals.

In vitro stimulation of flow-sorted alveolar macrophages

Flow-sorted AM obtained from the CAP patients and healthy controls were resuspended in MEM supplemented with 10% heat-inactivated foetal calf serum, 100 U·mL⁻¹ penicillin/streptomycin and 2 mM glutamine (all cell culture reagents purchased from Gibco BRL) and cultured at a density of 2×10^4 AM·mL⁻¹ in 24-well polystyrene culture plates (Nunc, Roskilde, Denmark) at 37°C/5% CO₂ in a humidified atmosphere. Incubation was performed for 16 h with either medium or medium containing 100 ng·mL⁻¹ LPS (*Salmonella abortus equi* endotoxin; Sebak, Aldenbach, Germany) or IFN- γ (300 U·mL⁻¹; Thomae, Biberach, Germany) or a combination of LPS (100 ng·mL⁻¹) plus IFN- γ (300 U·mL⁻¹). Subsequently, culture supernatants were aspirated and frozen cell-free at -85°C until cytokine quantifications.

Routine analyses of supplemented media for LPS contaminations were always found to be negative as measured by Limulus amoebocyte lysate-assay (Chromogenix).

Quantification of TNF- α , IL-6 and IL-8 protein

TNF- α , IL-6 and IL-8 protein levels from culture supernatants of flow-sorted AM and BAL fluids were measured by enzyme-linked immunosorbent assay (ELISA). Microtitre plates (Nunc, Roskilde, Denmark) were coated overnight (4°C) with polyclonal goat antibodies to human TNF- α , IL-6 (both R&D Systems, Abingdon, UK) or IL-8 (Genzyme, Rüsselsheim, Germany) followed by three washing steps with 0.05% (v/v) Tween (Sigma, Deisenhofen, Germany). Samples (50 µL) of culture supernatants or BAL fluids were dispensed into the wells and incubated for 2 h at room temperature. After washing, the plates were incubated with monoclonal mouse antibodies directed against the respective cytokine (Genzyme, Cambridge, USA) for 2 h at ambient temperature. The plates were then rinsed three times followed by incubation with a biotinylated donkey antimouse immunoglobulin (Ig) antibody (Dianova, Hamburg, Germany). After washing, avidin and biotinylated horseradish peroxidase (HRP; Dako, Glostrup, Denmark) were added to the wells for 1 h at 37°C. After a final washing step the bound enzyme was detected by incubation with 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid (6)) (ABTS) (Sigma, Deisenhofen, Germany) and hydrogen peroxide as substrate for 1

h at room temperature. Serial dilutions of the corresponding recombinant cytokines provided standard curves for each individual ELISA plate. Absorbency measurement was performed at 490 nm on an ELISA reader. The quantification of each cytokine was performed on duplicate samples with detection ranges of 3–1,000 pg·mL⁻¹ for TNF- α , IL-6 and IL-8.

Statistical analysis

Data are given as mean±SD. A standard two-sample t-test was performed to test for significant differences between group means. Correlations between parameters were analysed using Pearson's correlation coefficient. A p-value of less than 0.05 was considered significant.

Results

Bronchoalveolar lavage and flow sorting of alveolar macrophages

In BAL fluids obtained from patients with severe CAP, total cell counts were nonsignificantly elevated when compared to control subjects (table 2). As anticipated, we found significantly decreased AM proportions in parallel to increased PMN proportions in BAL samples from patients with severe CAP (both $p < 0.05$, table 2) when compared with normal volunteers. The lymphocyte proportions in BAL fluids did not differ significantly between the two groups. Differences between AM proportions contained in BAL samples of either study population did not affect cell purities.

Separation by flow sorting of AM obtained from BAL samples of either study population always yielded purities of >96%. The viability of flow-sorted AM from both study populations, as assessed by propidium iodide staining and subsequent FACS analysis, was consistently >92% (data not shown).

Alveolar macrophage ex vivo cytokine gene expression

TNF- α . Figure 2a illustrates the mean TNF- α / β -actin ratio in AM from patients with severe CAP and from healthy volunteers. AM from patients with severe CAP displayed a broader scatter of data, but had no significantly elevated mean TNF- α / β -actin ratio when compared to AM from healthy controls (CAP, 0.48 ± 0.42 versus normal 0.04 ± 0.02 , mean±SD).

IL-6. As shown in figure 2b, we found no statistically significant differences between mean IL-6/ β -actin ratios in AM from patients with CAP (0.56 ± 0.45) and normal volunteers (0.18 ± 0.12).

IL-8. Figure 2c depicts the IL-8/ β -actin ratio in AM from both study groups. AM obtained from patients with severe CAP showed a significantly upregulated IL-8 gene expression when compared with the IL-8 gene expression of AM from healthy volunteers. The mean IL-8/ β -actin ratio

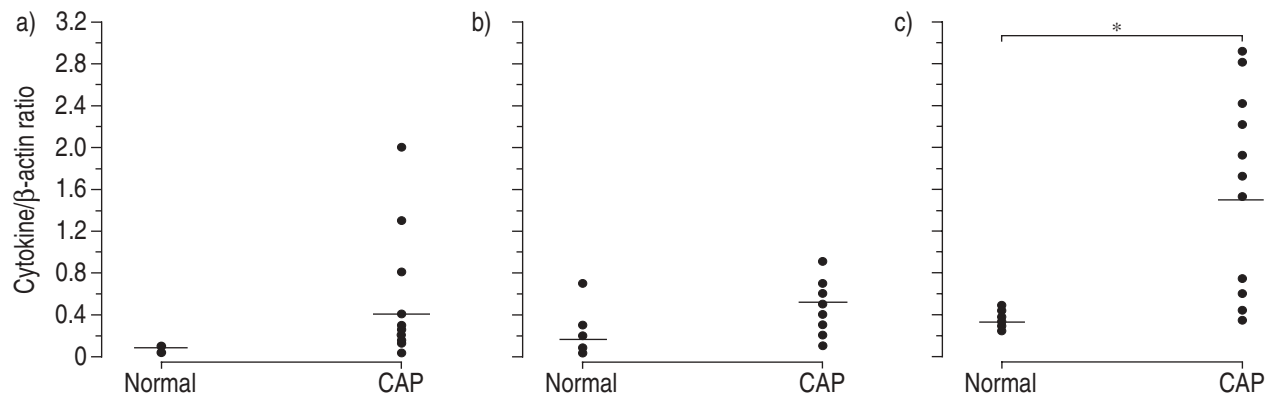


Fig. 2. – Cytokine/ β -actin ratios in alveolar macrophages (AM) from healthy volunteers ($n=10$) and patients with severe community-acquired pneumonia (CAP) ($n=12$). Gene expression of: a) tumour necrosis factor (TNF)- α ; b) interleukin (IL)-6; and c) IL-8 of flow-sorted AM was analysed using a semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. Horizontal bars indicate mean values. *: $p<0.05$, patients with CAP versus normal subjects.

in AM was 1.47 ± 0.8 for patients with CAP and 0.32 ± 0.08 for healthy volunteers. In patients with severe CAP considerable interindividual variability in AM IL-8 gene expression was observed. No statistically significant correlation was found between the IL-8/ β -actin ratio in AM from CAP patients and the cell counts of PMN per millilitre of BAL fluid obtained from corresponding patients (data not shown).

Comparative analysis of cytokine gene expression in AM and PMN from selected patients with severe CAP revealed strong signals for IL-6 and low levels of TNF- α transcripts in sorted PMN with low levels for both IL-6 and TNF- α mRNA in AM from the same patients. In both cell populations IL-8 gene expression was upregulated to the same extent (data not shown).

Alveolar macrophage in vitro cytokine secretion

TNF- α . AM from CAP patients showed a baseline TNF- α secretion that was not significantly different from that of healthy controls (fig. 3a). When stimulated with LPS ($100\text{ ng}\cdot\text{mL}^{-1}$, 16 h), we observed an evident increase of TNF- α release by AM from CAP patients when compared to baseline secretion. However, we found that AM from

CAP patients were significantly less responsive to LPS stimulation than those from healthy controls.

IL-6. As depicted in figure 3b, unstimulated AM from patients with severe CAP and from healthy volunteers were found to secrete low amounts of IL-6 protein. As pointed out for TNF- α , upon LPS stimulation we observed that in both groups AM showed an increased IL-6 release, when compared to baseline values. However, AM from CAP patients released significantly less IL-6 protein when compared to control values.

IL-8. Unstimulated AM from both study populations exhibited an elevated baseline IL-8 secretion (fig. 3c). LPS-induced IL-8 release was found to be markedly increased when compared to baseline values, but without significant differences between groups.

Influence of a concurrent LPS/IFN- γ stimulation on the AM pro-inflammatory cytokine release

In an attempt to restore the reduced capacity of AM from CAP patients to release TNF- α and IL-6 in response to LPS stimulation, we cultured AM from patients with severe CAP ($n=6$) and healthy volunteers ($n=10$) in either

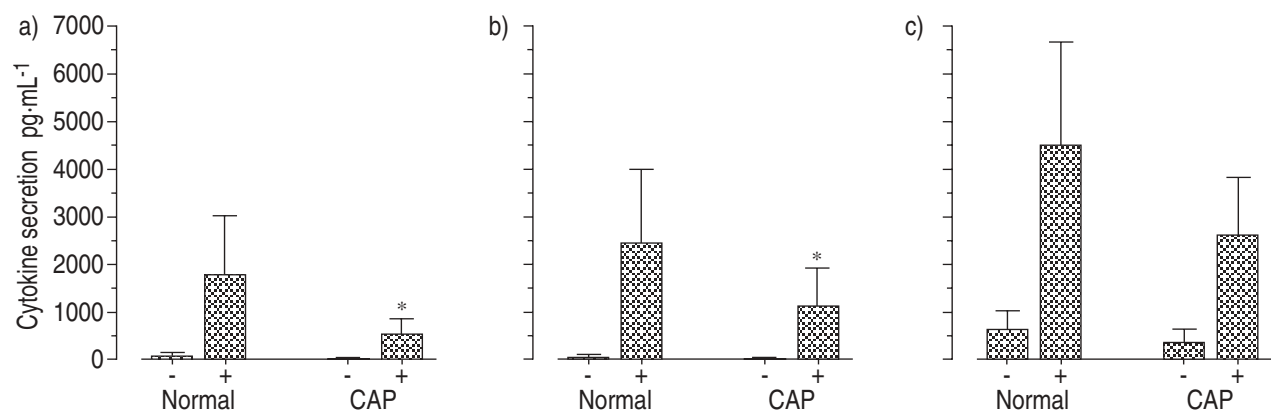


Fig. 3. – Spontaneous and lipopolysaccharide (LPS)-induced *in vitro* cytokine secretion by alveolar macrophages (AM) from healthy individuals and patients with severe community-acquired pneumonia (CAP). AM were flow-sorted and cultured at $2\times10^4\text{ cells}\cdot\text{mL}^{-1}$ with either medium (-) or LPS (+; $100\text{ ng}\cdot\text{mL}^{-1}$) for 16 h. Culture supernatants were harvested and analysed for: a) tumour necrosis factor (TNF)- α ; b) interleukin (IL)-6; and c) IL-8 protein concentrations by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean \pm SD. *: $p<0.05$, for patients with CAP versus normal subjects.

medium or medium containing LPS or IFN- γ or LPS plus IFN- γ .

TNF- α . Stimulation of AM from both study groups with IFN- γ alone did not alter baseline TNF- α secretion (fig. 4a) when compared to that of unstimulated cells. Stimulation of AM from both study groups with LPS plus IFN- γ did, however, yield a marked increase in TNF- α secretion that was particularly evident for AM from CAP patients.

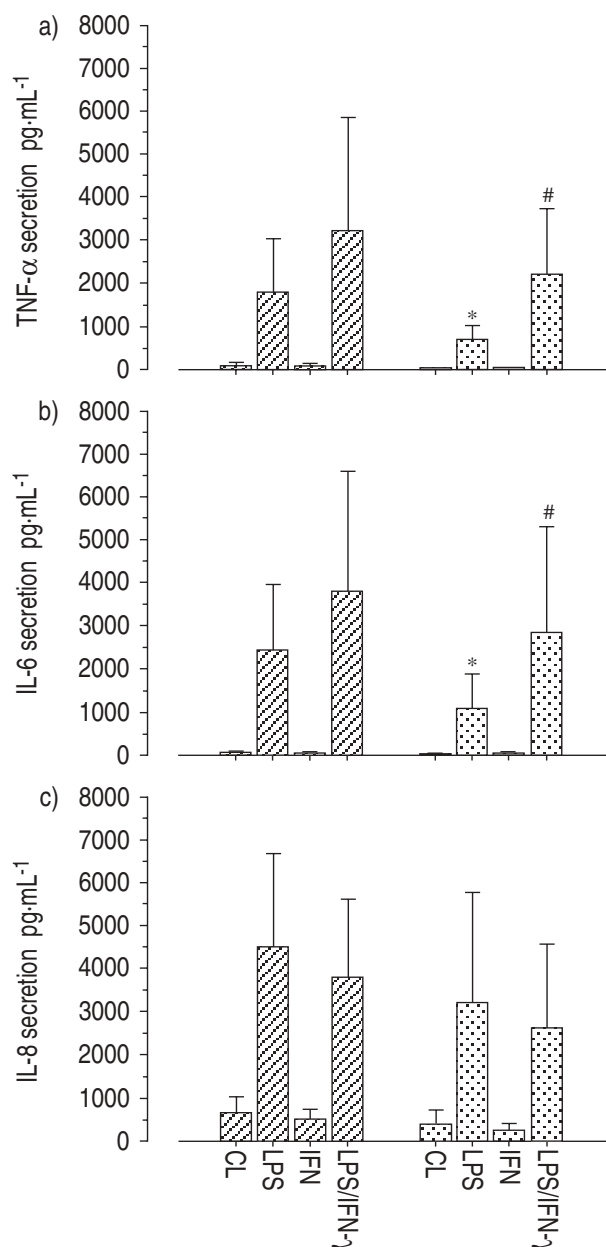


Fig. 4. — Spontaneous and lipopolysaccharide (LPS)/interferon (IFN)- γ induced *in vitro* cytokine secretion by alveolar macrophages (AM) from healthy individuals (hatched) and patients with severe community-acquired pneumonia (CAP) (dotted). Flow-sorted AM were incubated at 2×10^4 cells·mL $^{-1}$ with either medium (CL), LPS (100 ng·mL $^{-1}$), IFN- γ (300 U·mL $^{-1}$) or both LPS (100 ng·mL $^{-1}$) and IFN- γ (300 U·mL $^{-1}$) for 16 h. Culture supernatants were harvested and analysed for: a) tumour necrosis factor (TNF)- α ; b) interleukin (IL)-6 and; c) IL-8 protein contents by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean \pm SD. *: $p < 0.05$, patients with CAP versus normal subjects; #: $p < 0.05$, LPS/IFN- γ stimulation versus LPS-stimulation.

Table 4. — Pro-inflammatory cytokine concentrations in samples of bronchoalveolar lavage fluid obtained from the study population

Study population	TNF- α	IL-6	IL-8
Normal subjects (n=10)	0	4 \pm 1	0
Severe CAP (n=12)	10 \pm 7	590 \pm 460*	255 \pm 197*

CAP: community-acquired pneumonia; TNF: tumour necrosis factor; IL: interleukin. *: $p < 0.05$, versus normal subjects.

IL-6. Stimulation of AM from both healthy volunteers and patients with CAP with IFN- γ alone did not affect the IL-6 release in either study group when compared with the spontaneous IL-6 secretion of unstimulated cells (fig. 4b). However, we observed a marked elevation of IL-6 secretion by AM of both study groups following stimulation with LPS plus IFN- γ that did not differ significantly between groups. Within the CAP study group, LPS/IFN- γ stimulation yielded a significantly elevated IL-6 release when compared with the LPS-induced IL-6 secretion.

IL-8. Figure 4c illustrates that concurrent incubation of AM with LPS and IFN- γ did not further increase the IL-8 cytokine secretion levels. Stimulation of AM from both healthy volunteers and patients with severe CAP with either IFN- γ alone or in combination with LPS even resulted in a slight, although nonsignificant, reduction in IL-8 protein secretion.

BAL fluid cytokine levels

TNF- α . In lavage samples from both CAP patients and healthy volunteers we found no or only small TNF- α levels that did not differ significantly between groups (table 4).

IL-6. Markedly increased concentrations of IL-6 protein were found in BAL fluids from CAP patients, and they were significantly different from those of healthy controls (table 4; $p < 0.05$).

IL-8. We found increased IL-8 protein levels in lavage samples obtained from CAP patients that differed significantly from those values measured in BAL fluids from controls (table 4; $p < 0.05$).

Discussion

In the present study we found elevated *ex vivo* TNF- α , IL-6 and IL-8 mRNA levels in AM from patients with severe CAP compared to normal individuals. However, this elevation was found to be considerably less pronounced for TNF- α and IL-6 than for IL-8 transcript levels. Moreover, when stimulated with LPS *in vitro*, AM from CAP patients had a significantly reduced potential to secrete TNF- α and IL-6 that could, however, be restored by treatment with IFN- γ .

The elevated IL-8 transcript levels observed in flow-sorted AM from CAP patients together with a substantial *in vitro* IL-8 release as well as elevated IL-8 levels in BAL fluids from CAP patients suggest a prominent role of macrophages in the recruitment of large numbers of PMN into the alveolar compartment, since IL-8 is known to be a potent chemotactic and activating factor for neutrophils

[5, 22]. Recently, we and others demonstrated highly elevated IL-8 levels in BAL fluids from patients with severe CAP and ARDS [23–25] with IL-8 concentrations in the alveolar lining layer exceeding serum IL-8 levels by several orders of magnitude. The data of the present study support the view that in severe pneumonia macrophages substantially contribute to the compartmentalized generation of IL-8. It is noteworthy that elevated levels of IL-8 in the alveolar compartment have been associated with increased lung injury and mortality in patients with acute respiratory failure [24, 26, 27].

In the present study, AM from healthy controls were found to express near basal mRNA levels for TNF- α as well as IL-6 and IL-8, indicating that these cells were not perturbed by flow cytometry from a noninflamed microenvironment. Unexpectedly, TNF- α and IL-6 mRNA levels in AM from CAP patients were not increased to the same extent as IL-8 gene expression of these cells. Moreover, AM from CAP patients showed a significantly reduced *in vitro* potential to secrete TNF- α and IL-6 following LPS challenge. These findings may be due to: 1) accelerated degradation of the mRNA molecules with short half-lives during sorting; 2) nonspecific exhaustion of cell responses; and 3) quelling of proinflammatory responsiveness *in vivo*.

In the first instance, it is highly improbable that the sorting procedure *per se* accounts for low levels of TNF- α and IL-6 mRNA in AM from CAP patients, since high levels of IL-6 mRNA were detected in flow-sorted PMN contained in the same lavage samples. In addition, the IL-8 mRNA levels in AM from CAP patients were elevated, and there is no way by which the sorting technique could specifically affect the TNF- α and IL-6 mRNA in the macrophage population. Moreover, using *in situ* hybridization, TRAN VAN NHIEU *et al.* [6] also demonstrated nonelevated TNF- α cytokine transcripts in AM from patients with acute bacterial pneumonia.

In the second instance, a nonspecific exhaustion of cellular responses is also inconsistent with the observed elevated IL-8 mRNA levels in AM. Moreover, co-stimulation of AM with LPS and IFN- γ led to a nearly completely restored pro-inflammatory cytokine release *in vitro*.

In the final instance, AM from healthy volunteers were found to exhibit baseline cytokine gene expression, reflecting a noninflammatory microenvironment *in vivo*. When stimulated with LPS *in vitro*, these cells were heavily inducible for cytokine secretion. The unexpected finding that AM from CAP patients exhibit a markedly reduced *in vitro* LPS-inducible TNF- α and IL-6 release together with relatively low TNF- α and IL-6 mRNA expression indicates that these cells, collected from an inflammatory microenvironment, might be in a hyporesponsive state. This view is further supported by our lavage data demonstrating lack of TNF- α in BAL fluids of CAP patients, which is in accordance with our recently published work [23]. In contrast, we found elevated IL-6 concentrations in BAL fluids from CAP patients when compared to controls. However, within the alveolar compartment many cell types, such as neutrophils, fibroblasts, type II pneumocytes and AM, have been shown to secrete IL-6 in an interdependent manner [8, 10, 28]. Thus, it seems probable that cells other than AM might make a primary contribution to the increased alveolar generation of IL-6 under these conditions. In actual fact the role of IL-6 in acute

bacterial pneumonia remains controversial. Designated as alarm hormone, IL-6 is known to play a pivotal role in the induction of the systemic acute phase response. Nevertheless, IL-6 has also been reported to exhibit anti-inflammatory capacities by counteracting LPS-induced TNF- α and IL-1 β responses, both *in vitro* and *in vivo* [29].

Recently, DEHOUX *et al.* [30] demonstrated that AM from patients with CAP were hyporesponsive in terms of TNF- α and IL-6 secretion following *in vitro* LPS stimulation. Interestingly, most patients enrolled in their study had Gram-positive bacterial infections with *Streptococcus pneumoniae*, suggesting that stimuli other than LPS may also be capable of inducing a hyporesponsive state in AM. Moreover, MUNOZ *et al.* [31] have shown that desensitization to *in vitro* LPS challenge could be observed in monocytes from sepsis patients with either Gram-positive or Gram-negative infections, although it was more pronounced in patients with Gram-negative infections. The concept that AM are hyporesponsive with respect to TNF- α and IL-6 generation resembles the well-described LPS tolerance phenomenon, which is at least partly due to increased production of anti-inflammatory cytokines and is known to affect monocyte/macrophage cytokine production *in vitro* [32–34].

Because the immunomodulatory cytokine IFN- γ is known to enhance LPS-induced AM pro-inflammatory cytokine release, AM from CAP patients were challenged concurrently with endotoxin and IFN- γ . Interestingly, we found that the TNF- α and IL-6 release could be almost fully restored, leaving the IL-8 secretion unaffected. In previous reports [35, 36] it has been shown that IFN- γ is likely to restore the pro-inflammatory cytokine response by affecting the autocrine and/or paracrine production of endogenous monocyte/macrophage-derived IL-10. Thus, it may be speculated that this effect is also active in the described system.

In summary, the present work shows that highly purified alveolar macrophages from patients with severe community-acquired pneumonia exhibit a state of hyporesponsiveness with respect to tumour necrosis factor- α and interleukin-6 but not interleukin-8 production. This hyporesponsiveness could be restored to near normal levels by stimulation with interferon- γ . Whether these findings are relevant for therapeutic strategies to overcome states of impaired host defence in critically ill patients awaits further investigation.

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References

1. Fantone JC. Mechanisms of lung parenchymal injury. *Am Rev Respir Dis* 1984; 130: 484–491.
2. Nathan CF. Secretory products of macrophages. *J Clin Invest* 1987; 79: 319–326.
3. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 1990; 141: 471–501.
4. Kelley J. Cytokines of the lung. *Am Rev Respir Dis* 1990; 141: 765–788.
5. Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 1991; 17: 17–23.

6. Tran van Nhieu J, Misset B, Lebarry F, Carlet J, Bemaudin J-F. Expression of tumor necrosis factor- α gene in alveolar macrophages from patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1993; 147: 1585–1589.
7. Strieter RM, Chensue SW, Basha MA, *et al.* Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor- α , lipopolysaccharide, and interleukin-1 β . *Am J Respir Cell Mol Biol* 1990; 2: 321–326.
8. Cicco NA, Lindemann A, Content J, *et al.* Inducible production of interleukin-6 by polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Blood* 1990; 75: 2049–2052.
9. Xing Z, Kirpalani H, Torrey D, Jordana M, Gauldie J. Polymorphonuclear leukocytes as a significant source of tumor necrosis factor- α in endotoxin-challenged lung tissue. *Am J Pathol* 1993; 143: 1009–1015.
10. Elias JA, Lentz V. IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL-6 production and stabilize IL-6 messenger RNA. *J Immunol* 1990; 145: 161–166.
11. Maus U, Rosseau S, Seeger W, Lohmeyer J. Separation of human alveolar macrophages by flow cytometry. *Am J Physiol* 1997; 272 (*Lung Cell Mol Physiol* 16): L566–L571.
12. Fraser RG (ed), Paré JAP, Paré PD, Fraser RS, Genereux GP. *Diagnosis of Diseases of the Chest*. 1989. 3rd edition. Philadelphia, USA, W.B. Saunders Company.
13. Lohmeyer J, Friedrich J, Rosseau S, Pralle H, Seeger W. Multiparameter flow cytometric analysis of inflammatory cells contained in bronchoalveolar lavage fluid. *J Immunol Methods* 1994; 172: 59–62.
14. Chomczynski P, Sacchi S. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156–159.
15. Ponte P, Ng Sun-Yu NJ, Engel J, Gunning P, Cadis L. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta actin cDNA. *Nucleic Acids Res* 1984; 12: 1687–1696.
16. Wang AM, Creasey AA, Ladner MB, *et al.* Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 1985; 228: 149–154.
17. Hirano T, Yasukawa K, Harada H, *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 1986; 324: 73–76.
18. Mukaida N, Shiro M, Matsushima K. Genomic structure of the human monocyte derived neutrophil chemotactic factor IL-8. *J Immunol* 1989; 143: 1366–1371.
19. Bost TW, Riches DWH, Schumacher B, *et al.* Alveolar macrophages from patients with beryllium disease and sarcoidosis express increased levels of mRNA for tumor necrosis factor- α and interleukin-6 but not interleukin-1 β . *Am J Respir Cell Mol Biol* 1994; 10: 506–513.
20. Sorg R, Enczmann J, Sorg U, Heermeier K, Schneider EM, Wernet W. Rapid and sensitive mRNA phenotyping for interleukins (IL-1 to IL-6) and colony-stimulating factors (G-CSF, M-CSF, and GM-CSF) by reverse transcription and subsequent polymerase chain reaction. *Exp Hematol* 1991; 19: 882–887.
21. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983; 132: 6–13.
22. Leonard EJ, Yoshimura T. Neutrophil attractant/activation protein-1 (NAP-1, interleukin-8). *Am J Respir Cell Mol Biol* 1990; 2: 479–486.
23. Schütte H, Lohmeyer J, Rosseau S, *et al.* Bronchoalveolar and systemic cytokine profiles in patients with ARDS, severe CAP and cardiogenic lung oedema. *Eur Respir J* 1996; 9: 1858–1867.
24. Donnelly SC, Strieter RM, Kunkel SL, *et al.* Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 1993; 341: 643–647.
25. Villard J, Dayer-Pastore F, Hamacher J, Aubert JD, Schlegel-Haueter S, Nicod LP. GRO alpha and interleukin-8 in *Pneumocystis carinii* or bacterial pneumonia and adult respiratory distress syndrome. *Am J Respir Crit Care Med* 1995; 152: 1549–1554.
26. Miller EJ, Cohen AB, Nagao S, *et al.* Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am Rev Respir Dis* 1992; 146: 427–432.
27. Jorens PG, van Damme J, de Backer W, *et al.* Interleukin 8 (IL-8) in the bronchoalveolar lavage fluid from patients with the adult respiratory distress syndrome (ARDS) and patients at risk for ARDS. *Cytokine* 1992; 4: 592–597.
28. Crestani B, Cornillet P, Dehoux M, Rolland C, Guenounou M, Aubier M. Alveolar type II epithelial cells produce interleukin-6 *in vitro* and *in vivo*. Regulation by alveolar secretory products. *J Clin Invest* 1994; 94: 731–740.
29. Aderka D, Junming L, Vilcek J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J Immunol* 1989; 143: 3517–3523.
30. Dehoux MS, Boutten A, Ostinelli J, *et al.* Compartmentalized cytokine production within the human lung in unilateral pneumonia. *Am J Respir Crit Care Med* 1994; 150: 710–716.
31. Munoz C, Carlet J, Fitting C, Misset B, Blériot J-P, Cavaillon J-M. Dysregulation of *in vitro* cytokine production by monocytes during sepsis. *J Clin Invest* 1991; 88: 1747–1754.
32. Bogdan C, Paik J, Vodovotz Y, Nathan C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor- β and interleukin-10. *J Biol Chem* 1992; 267: 23301–23308.
33. Randow F, Syrbe U, Meisel C, *et al.* Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β . *J Exp Med* 1995; 181: 1887–1892.
34. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; 174: 1209–1220.
35. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; 147: 3815–3822.
36. Donnelly RP, Freeman SL, Hayes MP. Inhibition of IL-10 expression by IFN- γ upregulates transcription of TNF- α in human monocytes. *J Immunol* 1995; 155: 1420–1427.