Phenotypic characterization of alveolar monocyte recruitment in acute respiratory distress syndrome

SIMONE ROSSEAU, PETER HAMMERL, ULRICH MAUS, HANS-DIETER WALMRATH, HARTWIG SCHÜTTE, FRIEDRICH GRIMMINGER, WERNER SEEEGER, AND JÜRGEN LOHMEYER

Department of Internal Medicine, Justus-Liebig-University, 35385 Giessen, Germany

Received 17 August 1999; accepted in final form 2 February 2000

Rosseau, Simone, Peter Hammerl, Ulrich Maus, Hans-Dieter Walmrath, Hartwig Schütte, Friedrich Grimminger, Werner Seeger, and Jürgen Lohmeyer. Phenotypic characterization of alveolar monocyte recruitment in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 279: L25–L35, 2000.—In 49 acute respiratory distress syndrome (ARDS) patients, the phenotype of alveolar macrophages (AMs) was analyzed by flow cytometry. Bronchoalveolar lavage (BAL) was performed within 24 h after intubation and on days 3–5, 9–12, and 18–21 of mechanical ventilation. The 27E10(high)/CD11b(high)/CD71(low)/25F9(low)/HLA DR(low)/RM3/1(low) AM population in the first BAL indicated extensive monocyte influx into the alveolar compartment. There was no evidence of increased local AM proliferation as assessed by nuclear Ki67 staining. Sequential BAL revealed two distinct patient groups. In one, a decrease in 27E10 and CD11b and an increase in CD71, 25F9, HLA DR, and RM3/1 suggested a reduction in monocyte influx and maturation of recruited cells into AMs, whereas the second group displayed sustained monocyte recruitment. In the first BAL from all patients, monocyte chemoattractant protein (MCP)-1 was increased, and AMs displayed elevated MCP-1 gene expression. In sequential BALs, a decrease in MCP-1 coincided with the disappearance of monocyte-like AMs, whereas persistent upregulation of MCP-1 paralleled ongoing monocyte influx. A highly significant correlation between BAL fluid MCP-1 concentration, the predominance of monocyte-like AMs, and the severity of respiratory failure was noted.

The recruitment of leukocyte populations is one of the fundamental mechanisms involved in inflammatory processes. Neutrophil influx into the alveolar space has long been known in acute respiratory distress syndrome (ARDS) and severe pneumonia (10, 14, 31), but excessive expansion of the alveolar mononuclear phagocyte population in ARDS has also been reported (35). It has recently been suggested that local proliferation causes expansion of the alveolar macrophage (AM) population in inflammatory lung disease (5), but there is growing evidence that enlargement of the AM pool in sarcoidosis (18), hypersensitivity pneumonitis (20), idiopathic lung fibrosis (ILF) (22), and human immunodeficiency virus (HIV)-related pulmonary disease (42) is attributed to the recruitment of peripheral blood monocytes into the lung. These AM precursors were identified by their immature monocyte-like immunophenotype, and augmented release of proinflammatory mediators was ascribed to these cells (3, 22, 43).

Monocyte chemoattractant protein (MCP)-1 belongs to the supergene family of C-C chemokines located on chromosome 17 and has specificity for the recruitment of mononuclear leukocytes (24). Elevated MCP-1 levels in bronchoalveolar lavage (BAL) fluids have been demonstrated in patients with sarcoidosis, ILF (9), hypersensitivity pneumonitis (38), and ARDS (16). In addition to its role as a chemoattractant, MCP-1 may participate in the pathogenesis of monocyte-mediated lung injury by activating mononuclear phagocytes through the generation of receptor-mediated calcium influx (33), increasing the release of reactive oxygen species (41), and modulating the adhesion properties of these cells (40). MCP-1 expression in monocytes is induced by transendothelial migration (39) and interaction with extracellular matrix proteins (27). Thus one might speculate that extravasated monocytes themselves trigger sustained monocyte recruitment and promote ongoing inflammation.

In the present study, we employed fluorescence-activated cell-sorting (FACS) analysis for detailed immunophenotyping of the alveolar space mononuclear phagocyte population in acute lung injury. Sequential BAL was performed in patients with ARDS induced by sepsis, pancreatitis, or pneumonia. We analyzed the expression of monocyte differentiation, maturation, and activation markers; BAL fluid MCP-1 levels; and AM MCP-1 gene expression and inquired about a correlation between alveolar monocyte recruitment and MCP-1 levels and lung injury in these patients.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Study population. Patients included in the present study were recruited from the intensive care unit of the Department of Internal Medicine, Justus-Liebig-University (Gießen, Germany). Healthy volunteers and mechanically ventilated patients suffering from cardiogenic lung edema (CLE) served as control groups. The study was approved by the local ethics committee, and written informed consent was obtained from all participants or the patient’s closest relatives.

All patients were mechanically ventilated; the inspired O₂ fraction (FIO₂) and respirator settings, including positive end-expiratory pressure, were chosen according to the requirements of pulmonary insufficiency. General therapeutic approaches included nutrition, volume substitution, and antibiotics. Vasoactive or inotropic drugs were administered according to right heart catheterization. In all patients, the first BAL was performed within 24 h after intubation. The second BAL was performed on days 3–5, the third on days 9–12, and the fourth on days 18–21, when possible, after the onset of mechanical ventilation. General exclusion criteria for entry into this study were cancer, interstitial lung disease, and HIV infection.

BAL was performed in 16 patients with ARDS after sepsis or pancreatitis and in 33 patients with pneumonia-induced ARDS (ARDS/PNEUMONIA; Table 1). The general criteria for the diagnosis of ARDS were roentgenographically diffuse and bilateral alveolar infiltrates, pulmonary capillary wedge pressure < 16 mmHg, and the absence of acute or chronic left heart failure. First and second BALs were performed in 49 patients, 39 patients underwent a third BAL, and only 18 patients underwent a fourth BAL due to prior extubation or death.

Eight mechanically ventilated CLE patients underwent bronchoscopy and BAL within 24 h after intubation. Sequential BAL was not performed because all CLE patients were extubated 72 h after the onset of respirator therapy.

Fifteen healthy volunteers underwent a single bronchoscopy and BAL. The participants had no history of cardiac or pulmonary disease, were free of respiratory symptoms, did not take any medication, and displayed normal lung function when tested.

BAL. Bronchoscopy and BAL were performed by means of a fiber-optic bronchoscope, with 10 aliquots of 20 ml of warm sterile saline being infused into one segment and removed by gentle suction (35–60% recovery in ARDS patients, 65–85% recovery in healthy volunteers, and 78–113% recovery in patients with CLE). Lavage fluid was filtered through sterile gauze, collected on ice, and immediately centrifuged at 200 g at 4°C for 10 min to sediment BAL fluid cells. The cells were counted with a hemocytometer, viability was assessed by trypan blue exclusion, and differential cell counting was performed in Pappenheim-stained cyt centrifuge preparations. Supernatant aliquots were frozen in liquid nitrogen and stored at −80°C for subsequent cytokine measurements.

Immunofluorescence staining of BAL fluid cells. Immunofluorescence labeling of BAL fluid cells was performed as previously described (25). Briefly, 2 × 10⁵ cells were distributed to each well of a flexible-bottom microtitrator plates (Falcon, Heidelberg, Germany) and washed with PBS (Sigma, Munich, Germany) containing 0.1% BSA (Sigma) and 0.02% sodium azide (NaN₃; Merck, Darmstadt, Germany). Before the addition of monoclonal antibodies, 20 μl of a human immunoglobulin preparation (Behring, Marburg, Germany) were added to block AM Fcγ receptors. Saturating amounts of antibodies directed against CD14 (My4, Coulter Immunology, Hialeah, FL), CD71 (OKT9, Ortho, Raritan, NJ), HLA DR (L243, Becton Dickinson, San Jose, CA), 25F9 (45), 27E10 (3), RM3/1 (Dianova, Hamburg, Germany); 46, CD11b (M522; 26), MHC I (positive control, W6/ 32.HL) previously provided by A. Ziegler, Humboldt University, Berlin, Germany), 1, or isotype controls (negative control; Dianova) were added and incubated for 30 min at 4°C. The plates were washed twice with PBS and incubated with biotin-labeled F(ab)₂ fragments of sheep anti-mouse immunoglobulin antibodies (Biozol, Munich, Germany) for 30 min on ice. In a further 30-min incubation step, biotinylated secondary antibodies were developed with streptavidin-coupled phycoerythrine-cyanine 5-tandem conjugate (TriColor, Medac, Hamburg, Germany). This technique circumvents the problem of autofluorescence imposed by the AMs. After two final washes, the cells were suspended in PBS and kept on ice until flow cytometric analysis.

Quantification of proliferating AMs. The monoclonal antibody Ki67 (Dianova) was used for the quantification of proliferating AMs in ARDS patients and healthy control subjects. Immunocytochemical staining was performed on cytocentrifuge prepara tions of BAL fluid cells with an immunnoalkaline phosphatase technique [alkaline phosphatase-anti-alkaline phosphatase (APAAP)] as previously described (12). Briefly, cyto spins were incubated with the Ki67 antibody followed by rabbit anti-mouse Ig (DAKO, Glostrup, Denmark), APAAP complexes, and the alkaline phosphatase substrate (Dianova). Counterstain was performed with Gill’s hematoxylin (Sigma). Finally, the percentage of positive cells was estimated from 200 AMs.

Flow cytometric phenotyping of AMs. Flow cytometry was performed with the use of a FACStarPLUS flow cytometer from Becton Dickinson equipped with a 5-W argon-ion laser operating at 488 nm and 200 mW. AMs were gated by dual light scatter and autofluorescence characteristics. The data on forward- and right-angle light scatter, green autofluorescence, and TriColor fluorescence intensity were recorded for 10⁴ cells on the FACStarPLUS data-handling system and further analyzed with PC-LYSYS research software (Becton Dickinson). Specific TriColor fluorescence distribution of the AMs is expressed as the fluorescence intensity index (fluorescence intensity index of MAb = (MFI of MAb − MFI of isotype)/MFI of W6/32.HL − MFI of isotype), where MFI is the mean fluorescence intensity corrected for the negative control and signal-to-noise ratio values and MAb is the monoclonal antibody.

Flow sorting of AMs. AMs from healthy control subjects and from the first BAL of ARDS patients were separated by flow sorting with the FACStarPLUS flow cytometer described in Flow cytometric phenotyping of AMs. A 100-μm ceramic nozzle was attached to a large-nozzle sort-head assembly.
Ampli

GTGGAGTGAGTGTTCAAGTC-3

1 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1 μM intron-spanning specific primers (β-actin, 5'-AAAAGAACCTTGACCTGCAACAGTGCGTCT-3' and 5'-CCTTACCTCCTGCGTCTAGATCCACACTCTG-3', and MCP-1, 5'-TGAAGCTCGCACTCTCGCCT-3'; Stratagene), 0.75 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 2 μl of first-strand cDNA in a total volume of 25 μl. PCR profiles consisted of initial denaturation at 94°C (1.5 min) followed by 25 (β-actin) or 35 (MCP-1) cycles of denaturation (94°C for 50 s), primer annealing (60°C for 60 s), and primer extension (72°C for 7 min). Aliquots of PCR products were electrophoresed through 1.8% (wt/vol) NuSieve-agarose gels stained with ethidium bromide for ~2 h at 75V. Negative controls were routinely performed by running PCR without a cDNA template to exclude false-positive amplification products. Positive controls were performed with cDNA preparations obtained from lipopolysaccharide (LPS)-stimulated AMs. To verify the specificity of PCR amplifications obtained from the above-mentioned procedure, automated DNA sequencing was carried out on the purified cDNA samples according to the instructions of the manufacturer (model 373 A, Applied Biosystems, Darmstadt, Germany). By comparing the resulting DNA sequences with the corresponding published sequences, we identified PCR products as expected segments of spliced MCP-1 or β-actin mRNA species. With PCR conditions optimized for primer and magnesium concentrations and cycle number, 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 MCP-1 and β-actin PCR products. The specific MCP-1 product was quantified as previously described (6) with some modifications. Briefly, with β-actin as a housekeeping gene, input cDNA concentrations of different samples were adjusted with distilled water to obtain comparable cDNA contents before PCR amplification. Aliquots of unlabelled MCP-1 and corresponding β-actin PCR products were denatured at 94°C for 5 min before being blotted onto nylon membranes (32). Membranes were baked at 80°C for 2 h, prehybridized, and hybridized in 10 ml of buffer composed of 5× saline-sodium citrate, 5× Denhardt’s solution, 1× sodium dodecyl sulfate (Sigma), 50% deionized formamide (Clontech, Palo Alto, CA), and heat-denatured salmon sperm DNA (Boehringer Mannheim) at 42°C overnight with respective probes that were labeled with [α-32P]dCTP by random hexamer priming (15). Spin column chromatography (Boehringer Mannheim) was used to remove unincorporated deoxynucleotide triphosphates as well as small probe fragments. After two washes, quantification of MCP-1 gene expression levels was performed with the use of a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA). The results are expressed as mean ratios normalized to β-actin signals.

**BAL fluid MCP-1 levels.** MCP-1 protein in BAL samples was measured with the ELISA sandwich Maxisorp microtiter plates (Nunc, Wiesbaden, Germany) that were labeled with [111In]InCl3 (10 mCi/ml; Amersham, Braunschweig, Germany) tropolon (Fluka Chemical, Hauppauge, NY) as previously described (13). Human pulmonary artery endothelial cells (5×10⁴, passages 4 and 5; Clonetics, San Diego, CA) were seeded onto 100,000 pore size Transwell inserts (diameter 6.5 mm; Costar, Cambridge, MA) and grown to confluence in 4–5 days. BAL supernatants from healthy control subjects and ARDS patients were added to the lower compartments in the absence and presence of a neutralizing mouse anti-human MCP-1 antibody (R&D Systems), and 1×10⁶ radiolabeled monocytes were added to the filter inserts of Transwell chambers. After 120 min, the monocytes from the lower compartment were collected and counted in a gamma counter (Packard Canberra, Frankfurt, Germany), and the number of monocytes that migrated through the endothelial barrier is expressed as a percentage of counts in the lower compartment in relation to the counts initially added to the upper compartment.

**Statistical analysis.** Differences among the study groups were analyzed with the Kruskal-Wallis test followed by Dunn’s multiple contrast hypothesis or the Wilcoxon-Mann-Whitney test considering Bonferroni’s correction. For correlation of AM maturity and immaturity markers with BAL fluid MCP-1 concentration and oxygenation index [arterial Po2 (PaO2)/FiO2], the data were log transformed to achieve
normal distribution as assessed by the Kolmogorov-Smirnov test. P values < 0.05 were considered to represent a significant difference. Statistical procedures were performed with the SPSS for MS Windows analysis system.

RESULTS

AM phenotype and MCP-1 levels in healthy control subjects, patients with CLE, and in first BAL of ARDS patients. In the first BAL after the onset of mechanical ventilation, a significant increase in total cell count and a massive neutrophil influx were noted in all patients with ARDS (Table 2). Compared with that in the healthy control subjects, the percentage of AMs was decreased in ARDS patients, but absolute cell counts revealed a significant expansion of the AM population (Table 2). AM and neutrophil counts in patients with ARDS after sepsis or pancreatitis were not significantly different from the cell counts in ARDS/PNEUMONIA patients. In CLE patients, absolute cell counts were not different from those in healthy control subjects, but neutrophil granulocytes were significantly increased (Table 2).

The immunophenotype of AMs from healthy control subjects, patients with CLE, and the first BAL from ARDS patients is presented in Fig. 1. In ARDS patients, expression of CD14 and CD11b on AMs was markedly increased and of CD71, HLA DR, and the marker of mature tissue macrophages, 25F9, was significantly decreased. This AM phenotype resembled the expression pattern of peripheral blood monocytes, suggesting monocyte recruitment into the alveolar compartment in ARDS. In addition, 27E10, recognizing inflammatory acute-phase monocytes/macrophages (3), was highly elevated, whereas expression of RM3/1, a monocyte/macrophage marker associated with the downregulatory phase of the inflammatory process (46), was hardly expressed. AMs presenting this "immature" phenotype (27E10 high, CD11b high, CD71 low, HLA DR low, 25F9 low, and RM3/1 low paralleled by CD14 low), a monocyte/macrophage marker associated with the downregulatory phase of the inflammatory process (46), was hardly expressed. AMs presenting this "immature" phenotype (27E10 high, CD11b high, CD71 low, HLA DR low, 25F9 low, and RM3/1 low paralleled by CD14 low), again suggesting monocyte origin of this AM subpopulation (Fig. 2). The AM phenotype in patients with ARDS after sepsis or pancreatitis did not significantly differ from the AM phenotype in patients with ARDS caused by pneumonia (Fig. 1), and the AM phenotype in CLE patients closely resembled the AM phenotype in healthy control subjects.

Nuclear staining of Ki67 antigen for quantification of AM proliferation, which might contribute to the expansion of the AM population in ARDS, revealed only 0.5–1.5% positive AMs both in healthy control subjects and in the first BAL from ARDS patients (not significant).

### Table 2. Cell counts in first BAL

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>BAL Fluid Cells, (\times 10^5/\text{ml})</th>
<th>Neutrophils, (\times 10^5/\text{ml})</th>
<th>AMs, (\times 10^5/\text{ml})</th>
<th>Lymphocytes, (\times 10^5/\text{ml})</th>
<th>Eosinophils, (\times 10^5/\text{ml})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>7.5 ± 1.8</td>
<td>0.01 ± 0.002</td>
<td>6.6 ± 1.4</td>
<td>0.8 ± 0.1</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>CLE</td>
<td>8</td>
<td>9.1 ± 3.4</td>
<td>0.2 ± 0.04*</td>
<td>8.2 ± 2.3</td>
<td>0.6 ± 0.05</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>ARDS</td>
<td>16</td>
<td>24.9 ± 4.6*</td>
<td>9.7 ± 2.6*</td>
<td>14.3 ± 3.1*</td>
<td>0.9 ± 0.1</td>
<td>0.03 ± 0.006</td>
</tr>
<tr>
<td>ARDS/PNEUMONIA</td>
<td>33</td>
<td>34.3 ± 3.2*</td>
<td>14.3 ± 2.4*</td>
<td>17.2 ± 1.7*</td>
<td>2.5 ± 0.2*</td>
<td>0.3 ± 0.12*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. BAL, bronchoalveolar lavage; AM, alveolar macrophage. *P < 0.01 compared with control group.
Compared with those in healthy control subjects, MCP-1 levels were elevated by more than one order of magnitude in the first BAL fluid from ARDS patients, with no significant difference between ARDS induced by sepsis or pancreatitis and ARDS with pneumonia as the underlying disease (Fig. 3). Correction of BAL fluid MCP-1 data according to the urea method was performed routinely to obtain concentrations in the assumed volume of alveolar lining fluid. This resulted in a fold (mean; range 4- to 27-fold) higher cytokine concentrations. However, statistical analysis of these urea-corrected data did not reveal any previously unobserved differences (data not shown). Monocyte-specific chemotactic activity in the first BAL fluid from ARDS patients was significantly upregulated compared with that in healthy control subjects (Fig. 3), with no significant difference between ARDS and ARDS/PNEUMONIA patients. Immunophenotyping of AMs in the course of sequential BALs revealed two distinct patient groups. The first subgroup (subgroup A; Figs. 4 and 5; a representative FACS analysis is presented in Fig. 6) displayed pronounced downregu-

Flow-sorted AMs in the first BAL from ARDS patients showed strong upregulation of MCP-1 gene expression compared with that in healthy control subjects. The MCP-1-to-β-actin ratio differed significantly from the control value, without a statistical difference between ARDS and ARDS/PNEUMONIA patients (Fig. 3).

**AM phenotype and MCP-1 levels in sequential BALs from ARDS patients.** AM absolute cell counts increased, whereas neutrophil cell counts slightly decreased in sequential BAL from ARDS patients (Table 3), with no significant difference between ARDS and ARDS/PNEUMONIA patients. Immunophenotyping of AMs in the course of sequential BALs revealed two distinct patient groups. The first subgroup (subgroup A; Figs. 4 and 5; a representative FACS analysis is presented in Fig. 6) displayed pronounced downregu-

**Fig. 2.** Flow cytometric identification of 2 different macrophage populations in the first BAL fluid from a patient with ARDS. BAL was performed 4 h after onset of mechanical ventilation. Macrophage subpopulations [monocytes (MONO) and AMs] were gated by autofluorescence characteristics and CD14 intensity (dot plot analysis; top) and were further analyzed for forward-scatter properties (histogram; bottom). Small CD14high immature AMs exhibiting lower autofluorescence intensity (MONO) and large, highly autofluorescent CD14low mature AMs were discriminated. PMN, neutrophil granulocytes; LY, lymphocytes.

**Fig. 3.** Top: monocyte chemoattractant protein (MCP)-1 levels in BAL supernatant from healthy volunteers (n = 15) and in 1st BAL fluid from ARDS patients (n = 16) and ARDS/PNEUMONIA patients (n = 33). Data are means ± SE. *P < 0.001 compared with healthy volunteers. Middle: monocyte-specific chemotactic activity in BAL fluid from healthy volunteers (n = 15) and in 1st BAL fluid from ARDS (n = 16) and ARDS/PNEUMONIA (n = 33) patients. Monocytes (1 × 10⁶) transmigrated monolayers of human pulmonary artery endothelial cells in the presence of BAL supernatant or BAL supernatant plus saturating amounts of a neutralizing antibody against human MCP-1. Data are means ± SE. *P < 0.01 compared with healthy volunteers. Bottom: MCP-1-to-β-actin ratio in AMs from healthy control subjects and 1st BAL fluid from patients with ARDS (n = 12) and ARDS/PNEUMONIA (n = 27). MCP-1 gene expression of flow-sorted AMs was analyzed with semiquantitative RT-PCR. Data are means ± SE. *P < 0.001 compared with healthy volunteers.
3 h after intubation, 2nd BAL was performed on points (BAL number). First BAL was performed within ARDS after sepsis or pancreatitis at 4 different time
Fig. 4. Immunophenotyping of AMs from patients with markedly between the subgroups, with a sustained
sequential BALs from ARDS patients (not significant
AM proliferation revealed 0.3–1.1% positive AMs in
sustained in these subgroups. CD14 was persistently downregu-
lation of 27E10 and CD11b and significant upregula-
tion of CD71, HLA DR, and 25F9, i.e., a transition of
the predominance of immature to mature phenotype.
In addition, expression of RM3/1, the monocyte/macro-
phage marker associated with the downregulatory
phase of the inflammatory process, was markedly
increased. A biphasic course was noted for CD14. It was
significantly decreased on AMs obtained from the sec-
ond BAL compared with those from the first BAL,
healthy control subjects, and CLE patients. In the
following lavages, CD14 was again upregulated but did
not surpass the expression level of AM from healthy
control subjects or CLE patients. The second subgroup
(subgroup B; Figs. 4 and 5) exhibited prolonged pre-
dominance of the immature phenotype in the course of
sequential BALs, indicated by a continuously elevated
expression of 27E10 and CD11b on AMs and the ab-

Fig. 4. Immunophenotyping of AMs from patients with ARDS after sepsis or pancreatitis at 4 different time
table points (BAL number). First BAL was performed within 24 h after intubation, 2nd BAL was performed on
days 3–5, 3rd BAL on days 9–12, and 4th BAL on days 18–21
after onset of mechanical ventilation. Two different
subgroups of ARDS patients were identified: those with
transient (solid lines) or persistent (dotted lines) changes in the AM immunophenotype profile. Values of the
3rd and 4th BALs are missing in the case of prior extubation or death. All single values (expressed as
fluorescence intensity index) of the time course of mono-
cyte markers (CD14 and CD11b), macrophage differen-
tiation antigens (25F9, HLA DR, and CD71), and in-
flammatory acute (27E10)- and late (RM3/1)-phase markers are given.

Correlation with the clinical data. In ARDS patients,
the expression of AM immaturity markers correlated
inversely with lung function given as PaO2/FIO2 (shown
for 27E10 in Fig. 8; CD11b: r = −0.62; P < 0.01; n = 155)
samples); in the third BAL, r = 0.53 (P < 0.05; n = 39 samples); and in the fourth BAL,
r = 0.55 (P < 0.05; n = 18 samples).

Nuclear staining of Ki67 antigen for quantification of
AM proliferation revealed 0.3–1.1% positive AMs in
sequential BALs from ARDS patients (not significant
compared with that in healthy control subjects).

MCP-1 levels in sequential BALs did, however, differ
markedly between the subgroups, with a sustained
versus transient AM “immaturity” profile. Patients
were ascribed to the two subgroups depending on the
course of CD11b, 27E10, CD71, HLA DR, RM3/1, and
25F9 expression by AMs as displayed in Figs. 4 and 5.
Patients displaying progressive macrophage matura-
tion exhibited a steady decrease in MCP-1 levels on
repetitive BAL (Fig. 7, subgroups A), whereas patients
with ongoing predominance of immaturity markers
demonstrated sustained or even increased BAL fluid
MCP-1 levels (Fig. 7, subgroups B). When analyzed for
all ARDS patients and lavages (n = 155), BAL fluid
MCP-1 levels were significantly correlated to the AM
surface expression of 27E10 (Fig. 8). This correlation
was also found at individual days of sequential BAL. In
the first BAL, MCP-1 and 27E10 correlated, with r =
0.56 (P < 0.01; n = 49 samples); in the second BAL, r =
0.61 (P < 0.01; n = 49 samples); in the third BAL, r =
0.53 (P < 0.05; n = 39 samples); and in the fourth BAL,
r = 0.55 (P < 0.05; n = 18 samples).

Table 3. Cell counts in sequential BALs

<table>
<thead>
<tr>
<th>n</th>
<th>BAL Fluid Cells, ×10⁴/ml</th>
<th>Neutrophils, ×10⁴/ml</th>
<th>AMs, ×10⁴/ml</th>
<th>Lymphocytes, ×10⁴/ml</th>
<th>Eosinophils, ×10⁴/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd BAL</td>
<td>16</td>
<td>30.2 ± 4.4</td>
<td>13.1 ± 1.7</td>
<td>15 ± 2.1</td>
<td>2.07 ± 0.6</td>
</tr>
<tr>
<td>3rd BAL</td>
<td>14</td>
<td>30.3 ± 3.6</td>
<td>10.2 ± 0.9</td>
<td>18.7 ± 1.3*</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>4th BAL</td>
<td>7</td>
<td>30.1 ± 2.9</td>
<td>8.2 ± 1.2</td>
<td>20.8 ± 2.4*</td>
<td>1 ± 0.07</td>
</tr>
<tr>
<td>ARDS/PNEUMONIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd BAL</td>
<td>33</td>
<td>32.8 ± 5.2</td>
<td>15.1 ± 2.5</td>
<td>14.6 ± 2.3</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>3rd BAL</td>
<td>25</td>
<td>33.2 ± 3.1</td>
<td>11.3 ± 1.6</td>
<td>17.1 ± 1.4</td>
<td>4.2 ± 0.8*</td>
</tr>
<tr>
<td>4th BAL</td>
<td>11</td>
<td>35.7 ± 5.2</td>
<td>8.9 ± 1.3*</td>
<td>20.8 ± 2.1*</td>
<td>5.5 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. *P < 0.01 compared with 1st BAL (Table 2).
RM3/1: \( r = 0.59, P < 0.01 \), HLA DR: \( r = 0.64, P < 0.01; n = 155 \) samples). When individual days of sequential BAL were analyzed, the correlation of the immaturity marker 27E10 with \( \text{Pa}_2/\text{Fi}_2 \) was \( r = 0.52 \) (\( P < 0.05; n = 49 \) samples) in the first BAL, \( r = 0.6 \) (\( P < 0.05; n = 49 \) samples) in the second BAL, \( r = 0.55 \) (\( P < 0.05; n = 39 \) samples) in the third BAL, and \( r = 0.54 \) (\( P < 0.05; n = 18 \) samples) in the fourth BAL. The correlation of \( \text{Pa}_2/\text{Fi}_2 \) with the maturity marker CD71 was \( r = 0.53 \) (\( P < 0.05; n = 49 \) samples) in the first BAL, \( r = 0.62 \) (\( P < 0.05; n = 49 \) samples) in the second BAL, \( r = 0.49 \) (\( P < 0.05; n = 39 \) samples) in the third BAL, and \( r = 0.53 \) (\( P < 0.05; n = 18 \) samples) in the fourth BAL. The overall survival in ARDS patients was 63.3\% (31 of 49 patients). In the subgroup with a progressive transition to mature AMs and a decrease in MCP-1 levels in sequential BAL, 28 of 37 patients (75.7\%) survived. The survival rate was only 25\% (3 of 12) in patients with a sustained predominance of immaturity markers on AMs and elevated MCP-1 levels.

**DISCUSSION**

The present study shows that in addition to the well-known phenomenon of neutrophil recruitment, there is an early profound expansion of the sessile pool of AMs, with an impressive shift from a mature to an immature cell type in both ARDS after sepsis or pancreatitis and ARDS caused by severe pneumonia. Immunophenotyping of the alveolar mononuclear phagocytes, together with the demonstration of a lack of increased local proliferation, strongly suggests that a rapid influx of monocytes from the vascular compartment represents the predominant underlying event. In addition, evidence is presented that upregulation and secretion of MCP-1 in the alveolar compartment may be centrally linked to this process. Interestingly, a
state of sustained monocyte influx was found to be significantly correlated with the severity of respiratory failure.

As anticipated for sessile mature macrophages, the alveolar mononuclear phagocyte population of healthy control subjects was characterized by a 27E10low/CD11bhigh/CD71high/HLA DRhigh/25F9high/RM3/1high phenotype profile (4, 18, 20, 30, 45). This contrasted sharply with the profile of 27E10high/CD11bhigh/CD71low/HLA DRlow/25F9low/RM3/1low, which was consistently noted in the early lavage fluids of ARDS patients and represents a monocyte-like immunophenotype. In parallel with the observation of an overall increase in BAL fluid total AM numbers, these findings suggest that the rapid expansion of the AM population was predominantly caused by an influx of monocytes. This view is further supported by the analysis of forward scatter properties, demonstrating smaller average cell size in the initial lavage fluids from ARDS patients and by the lower autofluorescence characteristics of these cells. The monocyte-like population of AMs in ARDS patients revealed exceptional upregulation of the calprotectin complex 27E10, a heterodimer of the calcium-binding proteins MRP8/MRP14, which are only present on inflammatory acute-phase macrophages (3) and which have previously been shown to be upregulated on AMs in pneumonia (36). No evidence for increased local proliferation of AMs in ARDS patients was obtained as judged by nuclear Ki67 staining. Such local AM replication has been suggested as an alternative mechanism that may increase AM numbers in the alveolar space (5).

Because all patients with ARDS were lavaged under conditions of mechanical ventilation in contrast to healthy control subjects, one might argue that this therapeutic intervention rather than the underlying lung inflammatory disease might be responsible for the alveolar monocyte influx. Because the AM phenotype of mechanically ventilated patients suffering from CLE closely resembled the AM phenotype of healthy control subjects, respirator therapy as well as lung edema formation per se may not be responsible for the marked alveolar monocyte recruitment in ARDS patients.

Fig. 7. MCP-1 protein levels in sequential BAL fluids from ARDS (top) and ARDS/PNEUMONIA (bottom) patients. Two different subgroups of ARDS and ARDS/PNEUMONIA patients were identified: those with transient (ARDS-A and ARDS/PNEUMONIA-A) or persistent (ARDS-B and ARDS/PNEUMONIA-B) changes in the AM immunophenotype profile as described in RESULTS. ARDS-A: n = 11 subjects for 1st and 2nd BALs, 9 subjects for 3rd BAL, and 6 subjects for 4th BAL; ARDS-B: n = 5 subjects for 1st, 2nd, and 3rd BALs and 1 subject for 4th BAL; ARDS/PNEUMONIA-A: n = 26 subjects for 1st and 2nd BALs, 18 subjects for 3rd BAL, and 9 subjects for 4th BAL; ARDS/PNEUMONIA-B: n = 7 subjects for 1st, 2nd, and 3rd BALs and 2 subjects for 4th BAL. Data are means ± SE. *P < 0.01 compared with values of the respective subgroup A.

Fig. 8. Correlation of BAL fluid MCP-1 protein levels with the expression of 27E10 on AMs (top) and correlation of oxygenation index (arterial PO2/inspired O2 fraction) with 27E10 expression on AMs (bottom) analyzed for all ARDS patients and all lavages (n = 155). Values were log transformed to achieve normal distribution as assessed by the Kolmogorov-Smirnov test.
Performance of sequential BAL over 2–3 wk after the onset of respirator therapy showed the appearance of two distinct groups of ARDS patients. The larger subgroup was characterized by a progressive disappearance of the monocyte-like alveolar mononuclear phagocyte population concomitant with an increasing predominance of the mature AM phenotype. In addition, enhanced expression of RM3/1, a marker present on inflammatory late-phase monocytes/macrophages (46), was noted. These findings clearly suggest a decrease in monocyte influx into the alveolar space in these patients. In contrast, the other subgroup displayed evidence for sustained monocyte recruitment, with persistent predominance of the immature AM phenotype profile. Interestingly, these immunophenotype features were highly significantly correlated with the severity of disease, and a tendency toward higher mortality with presumed ongoing influx of monocytes was found in the subgroup.

In contrast to the other immunophenotype markers, the course of CD14 expression on AMs in sequential BAL was more complex. Clearly elevated in the initial BAL, CD14 expression sharply dropped and subsequently increased again toward normal values in the patients with a predominance of AM maturation, whereas the CD14 levels remained low in the second to fourth BAL fluids from patients with presumed ongoing monocyte influx. The expression of the LPS/LPS-binding protein receptor on myeloid cells seems to be subject to a complex regulatory mechanism because monocyte maturation either increased or decreased CD14 expression depending on the microenvironment of the cell (19, 44). Such complex regulation was also suggested to underlie the observation of both enhanced and depressed AM CD14 expression in sarcoidosis (18, 20, 37). Without being able to elucidate the regulation of CD14 in the present study in more detail, the fact that the control values of CD14 expression, with a progressive appearance of mature AMs, were noted in later BAL samples from ARDS patients further supports the notion of a normalization of the alveolar mononuclear phagocyte population in these subgroups.

Elevated MCP-1 levels in BAL fluid have been previously demonstrated in patients with sarcoidosis (9), ILF (9), and ARDS (16). The present study offers strong evidence of a major role for MCP-1 in alveolar monocyte recruitment under the currently investigated conditions of ARDS. First, in parallel with the appearance of immature monocyte-like AMs in the alveolar compartment, the initial BAL fluid MCP-1 levels were increased in all patients with acute inflammatory lung injury, and the BAL fluid of ARDS patients displayed increased monocyte chemotactic activity. This monocyte-specific chemotactic activity was significantly reduced by a neutralizing anti-MCP-1 antibody. BAL fluid MCP-1 concentrations were also measured in patients requiring mechanical ventilation due to CLE, and MCP-1 levels in these patients were not different from those in healthy control subjects, suggesting that lung inflammation rather than respirator therapy or lung edema formation caused the alveolar space MCP-1 response. Second, the patient subgroup presenting a decrease in monocyte-like AMs in sequential BAL exhibited a marked decline in MCP-1 to near control values, whereas the subgroup displaying evidence for sustained monocyte influx showed continuously elevated or even further increased BAL fluid MCP-1 levels. Third, when analyzed for all ARDS patients independent of the affiliation to the different subgroups, there was a highly significant correlation between a monocyte-like immunophenotype of the AM population and BAL fluid MCP-1 concentrations. Fourth, concomitant with elevated MCP-1 protein levels in the initial BAL, flow-sorted AMs from ARDS patients showed extensively upregulated MCP-1 gene expression. These data, obtained under clinical conditions, are well in line with investigations in experimental lung injury, suggesting that the induction of MCP-1 in AMs may be a major contributor to the recruitment of peripheral blood monocytes into the alveolar compartment (7, 8, 21). MCP-1 has previously been shown to be sufficient for the transmigration of monocytes across the alveolocapillary barrier in a transgenic mouse model (17), which, of course, does not deny a substantial contribution of other monocyte-specific chemokines (23). Peripheral blood monocytes do not express MCP-1 message constitutively, but this is strongly induced by transendothelial migration (39) and interaction with extracellular matrix proteins (27). Taken altogether, one might thus speculate that extravasated monocytes themselves, in a positive feedback loop, trigger further alveolar monocyte influx by MCP-1 synthesis. Monocytes and macrophages may not, however, be the only cells responsible for the regulation of the alveolar space MCP-1 response in acute lung injury. Epithelial cells have been shown to secrete MCP-1 constitutively (2, 23) and in response to the macrophage-derived early-response cytokines TNF-α and interleukin-1β (34), and they showed a polar secretion of this chemokine into the apical compartment for initiating or maintaining a chemotactic gradient (29).

In conclusion, evidence is presented that the early expansion of the alveolar mononuclear phagocyte population observed in patients with ARDS is mainly due to a rapid influx of monocytes from the vascular compartment. Upregulation of MCP-1 synthesis and its secretion into the alveolar space are suggested to be centrally involved in this process. These events were found to be significantly correlated with the severity of respiratory failure. Further studies are encouraged to elucidate the mechanisms responsible for MCP-1 regulation and the on- and off-switching of the monocyte recruitment response in more detail.

We thank M. Lohmeyer, R. Maus, and G. Wahler for excellent technical assistance and Dr. R. L. Snipes for proofreading the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft, Klinische Forschergruppe “Respiratorische Insuffizienz” (Justus-Liebig-University, Giessen, Germany).
REFERENCES


33. Standiford TJ, Kunkel SL, Phan SH, Rollins BJ, and Strie- ter RM. Alveolar macrophage-derived cytokines induce mono-


