Regulation of Interleukin 12 p40 and p70 Production by Blood and Alveolar Phagocytes During Severe Sepsis

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SUMMARY: Paradoxically, the host response to severe sepsis may lead to immunosuppression, thereby favoring nosocomial infections. We examined the role of the two IL-12 isoforms, bioactive IL-12p70 and regulatory IL-12p40, in 16 patients with severe sepsis. We compared the capacity of purified blood and alveolar phagocytes [polymorphonuclear neutrophils (PMN) and monocytes/macrophages] to secrete each isoform. Blood monocytes had normal basal secretions. In contrast, a marked imbalance was observed after ex vivo stimulation by lipopolysaccharide plus IFN-γ, with significantly lower IL-12p70 production and higher IL-12p40 production. Conversely, stimulated IL-12p40 production by the patients' blood PMN tended to be impaired, as was their cell-surface β2 integrin and L-selectin expression, known as markers of cell activation. In the patient's bronchoalveolar lavage fluid, the production of both IL-12 isoforms after ex vivo stimulation was significantly lower with alveolar macrophages than with autologous blood monocytes and significantly higher with alveolar PMN than with autologous blood PMN. This sheds new light on the potential role of PMN in local modulation of inflammation, via secretion of the anti-inflammatory IL-12 p40 subunit. The imbalance between the bioactive and regulatory IL-12 isoforms, which is probably designed to control excessive inflammation, may also make septic patients more susceptible to nosocomial infection. (*Lab Invest 2003, 83:1353–1360*).

*T*he sepsis syndrome results from a systemic host response to infection (Bone et al, 1992). Uncontrolled release of proinflammatory mediators during sepsis, together with endothelial injury and disseminated intravascular coagulation, can result in multiple organ failure and death. Paradoxically, sepsis is also associated with a certain cellular hyporeactivity, including a reduced capacity to produce cytokines and to induce antigen-specific T-cell stimulation (Cavaillon et al, 2001). These "deficiencies" may protect the host from uncontrolled immune responses but, at the same time, may hinder appropriate responses to secondary threats such as nosocomial infections (Faist et al, 1997). This immunosuppression is dynamic and compartmentalized, depending on the cell type, the function, and the organ. In particular, we have previously reported that blood and alveolar phagocytes [polymorphonuclear neutrophils (PMN) and monocytes/macrophages] are differently regulated during acute lung insult (Chollet-Martin et al, 1996; Grenier et al, 2001; Jaffré et al, 2002). Several autocrine and para-crine immunoregulatory loops have been implicated in sepsis, with the release of numerous cytokines. Chemokines, and particularly IL-8, play a major role in phagocyte recruitment and activation during sepsis (Wagner and Roth, 1999), and we recently obtained evidence that IL-12 promotes IL-8 production by PMN participating in an amplifying loop (Ethuin et al, 2001; Gainet et al, 1998).

IL-12 is a 70-kDa heterodimeric cytokine composed of two disulfide-bound subunits designated p35 (35 kDa) and p40 (40 kDa). IL-12 is mainly produced by phagocytes (monocytes/macrophages and PMN) in response to bacterial products and intracellular parasites. IL-12 promotes immune defenses by inducing the T helper 1 phenotype and by enhancing natural killer cell cytotoxicity and IFN-γ production (Cassatella et al, 1995; Trinchieri, 1998). Both in vitro and in vivo, the p40 subunit is secreted as a monomer or homodimer, in a 10- to 50-fold excess over biologically active IL-12p70. IL-12p40 is thought to be a natural antagonist of IL-12p70, acting, at least in part, by competitive binding to the IL-12 receptor (Ling et al, 1995; Mattner et al, 1993).

During sepsis, exaggerated proinflammatory responses involving IL-12 may result in organ injury, but IL-12 also seems to be a vital component of host defenses. Indeed, IL-12 neutralization reduces survival in a murine model of sepsis (Steinhauser et al, 1999), and patients with the rare inherited IL-12 defi-

ciency are susceptible to mycobacterial infection and salmonellosis (Picard et al, 2001).

In this study we reexamined the role of IL-12 during human severe sepsis by comparing the capacity of blood and alveolar phagocytes (PMN and monocytes/macrophages) from septic patients to produce IL-12p40 and p70. We observed overproduction of regulatory IL-12p40, associated with impaired production of biologically active IL-12p70. These results point to a new mechanism of sepsis-associated immunosuppression, caused by an imbalance in the production of the two IL-12 isoforms by phagocytes.

Results

Clinical Characteristics of the Patients

Sixteen patients (6 women and 10 men) with severe sepsis or septic shock were studied. The mean age was 58 (range, 31–77) years. The main indications for intensive care unit (ICU) admission and mechanical ventilation were severe sepsis or septic shock subsequent to peritonitis ($n = 9$), postoperative pneumonia ($n = 4$), and mediastinitis, catheter-related septicemia, and pyelonephritis ($n = 1$ each). The following bacteria were isolated from the abdominal cavity: *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Candida glabrata*, and *Bacteroides fragilis*. Protected specimen brushing and/or bronchoalveolar lavage (BAL) fluid culture, performed for suspected pneumonia in all 16 patients, was positive in 4 cases, always yielding a single microorganism [*Pseudomonas aeruginosa* ($n = 2$), *Streptococcus pneumoniae*, and *Staphylococcus aureus*]. Six patients had acute respiratory distress syndrome (ARDS), as defined by the North American-European Consensus Conference (Bernard et al, 1994); in particular, the Pao_2/Fio_2 value was 128 ± 14 mmHg. The mean SAPS (simplified acute physiology score) version II score on admission was 38 ± 4.

BAL Fluid Cytology

Table 1 shows differential cell counts in BAL fluid from septic patients with and without ARDS. As expected, the percentage and absolute number of PMN were high in BAL fluid from all of the patients with ARDS. Moreover, two patients with pneumonia but without radiologic or gasometric criteria of ARDS had high absolute counts of alveolar PMN (patients 10 and 11); patients 9 and 13 had a high percentage of alveolar PMN but no evidence of pneumonia or ARDS.

IL-12p70 and p40 Levels in Plasma and BAL Fluid Supernatants

IL-12p70 was below the detection limit in the plasma of healthy volunteers and septic patients, whereas IL-12p40 levels were similar in the two groups (90 ± 13 pg/ml vs 108 ± 19 pg/ml in the patients and controls, respectively). Similarly, IL-12p70 was undetectable in BAL fluid supernatants from all of the patients, and IL-12p40 levels were low (22 ± 5 pg/ml).

Table 1. BAL Cytologic Examination

Septic patients	PMN % ($\times10^3/mm^3$)	AM % ($\times10^3/mm^3$)
With ARDS, patient no.		
1	93[a]	5[a]
2	95 (5.7)	3 (0.3)
3	85 (170)	7 (14)
4	88 (152)	8 (20)
5	87 (287)	12 (39.6)
6	81 (146)	15 (27)
Without ARDS, patient no.		
7	12 (2.4)	82 (16.4)
8	31 (0.5)	64 (1.1)
9	85 (17)	14 (2.8)
10	90 (70)	10 (7.8)
11	85 (42.5)	10 (5)
12	12 (0.6)	51 (2.5)[b]
13	92 (6.1)	5 (.3)
14	46 (5.1)	51 (5.7)
15	0	98 (14.7)
16	0	95 (2.5)

AM, alveolar macrophages.
[a] Not available.
[b] 32% eosinophils.

IL-12p70 and p40 Secretion by Isolated Blood Monocytes and PMN

As shown in Table 2, blood monocytes from the patients displayed normal basal IL-12p70 and IL-12p40 secretion. After ex vivo stimulation by lipopolysaccharide (LPS) + IFN-γ, the patients' monocytes showed different patterns of IL-12 isoform production relative to the healthy controls, with significantly lower IL-12p70 production and significantly higher IL-12p40 production in the patients. We thus calculated the ratio of IL-12p40 production to IL-12p70 production

Table 2. IL-12p40 and p70 Secretion by Blood Monocytes and PMN

	Healthy controls ($n = 10$)	Septic patients ($n = 16$)
IL-12p70 monocytes		
Basal	6 ± 1	3 ± 1
LPS + IFN-γ	198 ± 66	78 ± 22[a]
IL-12p40 monocytes		
Basal	20 ± 2	20 ± 3
LPS + IFNγ	1301 ± 419	2589 ± 387[a]
Ratio IL-12p40/IL-12p70	19 ± 4	49 ± 8[a]
IL-12p70 Neutrophils		
Basal	1 ± 0.5	1 ± 0.5
LPS + IFN-γ	2 ± 0.5	2 ± 0.9
IL-12p40 Neutrophils		
Basal	23 ± 11	17 ± 3
LPS + IFNγ	85 ± 57	43 ± 9

Results are expressed in pg/ml per 0.5×10^6 monocytes or per 10^7 PMN.
[a] Significantly different from healthy controls, $p < 0.05$.

Table 3. IL-12p40 and p70 Secretion by Alveolar Phagocytes (Macrophages or PMN)

	Macrophages from septic patients without ARDS	PMN from septic patients with ARDS
IL-12p70		
Basal	5 ± 1	2 ± 1
LPS + IFN-γ	18 ± 6	5 ± 2
IL-12p40		
Basal	19 ± 5	15 ± 5
LPS + IFN-γ	1370 ± 574	91 ± 44

Results are expressed in pg/ml per 0.5×10^6 alveolar macrophages or 10^7 PMN.

Figure 2.

Comparison of IL-12p70 and p40 production between isolated blood neutrophils and autologous alveolar polymorphonuclear neutrophils (PMN) after LPS + IFN-γ stimulation ex vivo. *p <0.05 compared with blood PMN.
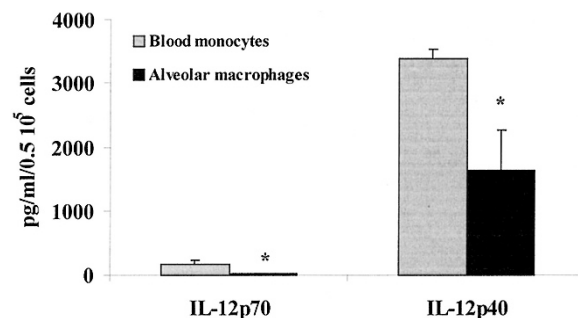
and found a pronounced imbalance in the patients compared with the controls (49 ± 8 vs 19 ± 4, $p <$ 00.1). Interestingly, the imbalance was far more marked in the 6 septic patients with ARDS (ratio 82 ± 21) than in the 10 septic patients without ARDS (ratio = 40 ± 6) ($p < 0.01$).

PMN had a lower capacity than monocytes to produce both IL-12 isoforms. Indeed, IL-12p70 production by PMN, both at rest and after ex vivo LPS + IFN-γ stimulation, was very low in the healthy controls and in the patients (Table 2). IL-12p40 isoform values were higher than IL-12p70 isoform values in both groups, both at rest and after ex vivo stimulation. There was no significant difference in IL-12p70 or IL-12p40 values between the patients and the controls, regardless of ex vivo stimulation, but IL-12p40 production by stimulated PMN tended to be lower in the patients.
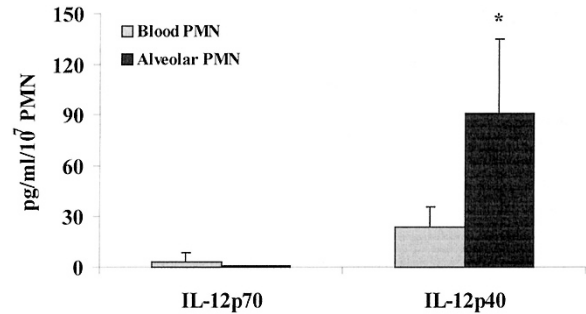
IL-12p40 and p70 Secretion by Alveolar Phagocytes (Macrophages and PMN)

The predominant cell type in each patient's BAL fluid (alveolar macrophages or PMN) was cultured and assayed for IL-12 production. IL-12p70 and IL-12p40 production was always low at rest, regardless of the cell type. LPS + IFN-γ stimulation increased the production of both IL-12 isoforms by macrophages, but only the IL-12p40 isoform by PMN (Table 3).

Interestingly, LPS + IFN-γ–stimulated production of both IL-12 isoforms by the patients' alveolar macro-

phages was significantly lower than that obtained with their autologous peripheral blood monocytes ($p < 0.05$) (Fig. 1). IL-12p40 production was higher with alveolar PMN than with autologous blood PMN (Fig. 2), both in the overall subgroup of patients with PMN-rich BAL fluid ($p < 0.05$, $n = 10$) and in the corresponding subset of patients with ARDS ($n = 6$).

Adhesion Molecule Expression by Blood PMN and Monocytes

Two phagocyte activation markers, known to be dysregulated during severe sepsis, were studied in parallel as reference parameters. As shown in Table 4, CD11b expression by resting blood PMN at 4° C was significantly higher in the septic patients than in the controls, reflecting basal activation. Ex vivo stimulation of N-formyl-methionyl-leucyl-phenylalanine (fMLP) at 37° C increased CD11b expression by blood PMN less strongly in the patients than in the controls, suggesting their deactivation. CD62-L expression at 4° C was not different between the patients and controls. fMLP stimulation at 37° C decreased CD62-L expression by blood PMN more strongly in the controls than in the patients (Table 4). Taken together, these results suggested that blood PMN were activated in the septic patients but that their capacity to be further activated ex vivo was suboptimal.

CD11b expression by whole blood monocytes was not significantly different between the patients and controls, either at rest (238 ± 81 vs 359 ± 80, respectively)

Table 4. Adhesion Molecule Expression by Whole Blood PMN

		Healthy controls ($n = 10$)	Septic patients ($n = 16$)
CD11b	4° C	94 ± 15	156 ± 15[a]
	37° C PBS	243 ± 105	440 ± 46[a]
	37° C fMLP	1395 ± 159	892 ± 80[a]
CD62-L	4° C	48 ± 8	46 ± 6
	37° C PBS	52 ± 10	44 ± 6
	37° C fMLP	5 ± 1	32 ± 20[a]

Results are expressed as the mean fluorescence index.
[a] $p < 0.05$ vs healthy controls.

Figure 1.

Comparison of IL-12p70 and p40 production between isolated blood monocytes and autologous alveolar macrophages after lipopolysaccharide (LPS) + IFN-γ stimulation ex vivo. *p <0.05 compared with blood monocytes.

or after ex vivo fMLP stimulation (1427 ± 356 vs 1472 ± 78).

Correlation Between IL-12p40 and p70 Production and Clinical Findings

Four (25%) of the 16 patients died, three of infectious complications and one of hemorrhagic shock. Neither the SAPS score nor vital outcome correlated with IL-12p70 or p40 production by blood phagocytes.

Eleven patients developed nosocomial infection during their stay in the ICU (wound abscess, pneumonia, prostatitis). Interestingly, IL-12p40 production by alveolar macrophages was significantly higher in the patients who developed pneumonia (n = 4) than in the other patients (3074 and 517 pg/ml, respectively; $p <$ 0.001). Three cases of secondary lung infection were caused by *Pseudomonas aeruginosa* and one by *Staphylococcus aureus*.

Discussion

We examined IL-12 p40 and IL-12 p70 production by the main phagocytic cells—PMN and monocytes/ macrophages—in patients with severe sepsis. Relative to healthy controls, sepsis was associated with a shift in IL-12 production by blood monocytes from the p70 isoform to the regulatory p40 isoform, resulting in a higher p40/p70 ratio. Alveolar macrophages always produced lower amounts of both IL-12 isoforms than blood monocytes. Conversely, sepsis was associated with decreased p40 production by blood PMN, whereas p40 production by the patients' alveolar PMN was within the range of values obtained with blood PMN from healthy controls. Thus, in septic patients, IL-12 production by PMN seems to be differently regulated according both to the body compartment (systemic or alveolar) and the IL-12 isoform (p70 vs p40).

Blood monocytes and PMN play a central role during sepsis, although cytokine production is differently regulated in the two cell types. We found that baseline monocyte capacity to produce both IL-12 isoforms in culture was normal in the septic patients. We chose to stimulate the cells with LPS + IFN-γ, the optimal stimulus already described by us and others (Cassatella et al, 1995; Grenier et al, 2001). After ex vivo stimulation, the monocytes' capacity to produce IL-12p70 was suboptimal, in accordance with previous reports (Ertel et al, 1997; Goebel et al, 2000; Hensler et al, 1998; Weighardt et al, 2000). Conversely, IL-12p40 production capacity was up-regulated, leading to a marked change in the IL-12p40/IL-12p70 ratio. This capacity of monocytes to produce large amounts of IL12p40 on ex vivo stimulation has already been suggested in the final phase of postoperative sepsis (Weighardt et al, 2000) and also in an animal model of *Salmonella enterica serovar Typhimurium* infection (Chang and Ou, 2002) and may participate in recovery from sepsis. Our observation that the IL-12p40/IL-12p70 imbalance was more pronounced in septic patients with ARDS than in septic patients without ARDS also suggests that this phenomenon is a regulatory response to

severe inflammation, as recently discussed by others (Abdi, 2002). Monocyte β2 integrin expression was normal in the septic patients, indicating that blood monocytes are not globally deactivated in this setting; in addition, the patients' blood monocytes were able to produce large amounts of the regulatory IL-12 isoform p40.

Profiles of IL-12 production differed between blood PMN and autologous monocytes from the septic patients. IL-12p40 and IL-12p70 were produced normally (in small amounts) by the patients' isolated blood PMN in culture, both at rest and after LPS + IFN-γ stimulation, albeit with a trend toward reduced p40 production relative to the healthy controls. These results confirm those in healthy controls (Cassatella et al, 1995) and HIV-infected patients (Gasperini et al, 1998) and point to PMN involvement in the IL-12 pathway. Our septic patients' blood PMN also showed suboptimal adhesion molecule expression in response to fMLP stimulation, with impaired up-regulation of β2 integrin CD11b/CD18 and impaired down-regulation of L-selectin CD62L; this is possibly a result of desensitization, as we have previously reported in patients with inflammatory disorders (Chollet-Martin et al, 1996; Gainet et al, 1998, 1999; Taieb et al, 2000).

Taken together, our results suggest that blood phagocytes (monocytes and PMN) have different patterns of IL-12p70 and p40 release during sepsis. Interestingly, the increased capacity of septic patients' blood monocytes to produce IL-12p40 ex vivo had no apparent influence on plasma levels of this isoform, which were normal. Moderately elevated IL-12p40 plasma levels have been described in patients with septic shock (Ertel et al, 1997; Hazelzet et al, 1997), although other authors have reported decreased levels (Wick et al, 2000). These discrepancies are probably related to the severity of sepsis and to the time of blood sampling, as recently suggested by a kinetic study performed in a baboon model of *E. coli* shock (Jansen et al, 1996).

The predominant BAL fluid cell type was alveolar macrophages in some of our patients and PMN in others. The relevant cell types were purified and cultured in each case, and the results supported a contribution of both alveolar macrophages and PMN to alveolar IL-12p70 and p40 levels. Previous studies have shown the participation of alveolar macrophages (Isler et al, 1999) and airway epithelial cells in IL-12 production (Walter et al, 2001); but, to our knowledge, this is the first evidence that human alveolar PMN produce IL-12. We also found that, contrary to autologous blood cells, alveolar cells did not respond to ex vivo LPS + IFN-γ stimulation. Alveolar PMN produced higher levels of IL-12 p40 than did their circulating counterparts, as previously reported for oncostatin M in similar patients (Grenier et al, 2001). The opposite was observed with alveolar macrophages, as previously described for IL-12 in murine models of infection (Reddy et al, 2001; Steinhauser et al, 1999) and inflammation (Huaux et al, 1999) and also in healthy subjects (Isler et al, 1999). A similar state of "deactivation" has also been reported in terms of various

cytokines: IL-6, TNF-α, and oncostatin M production during human bacterial pneumonia (Dehoux et al, 1994; Grenier et al, 2001) and hepatocyte growth factor production in pulmonary fibrosis (Crestani et al, 2002). Because there is increasing evidence that the p40 isoform may antagonize the biologic activity of IL-12 (Isler et al, 1999; Ling et al, 1995; Mattner et al, 1997), our results cast new light on the potential role of PMN in local modulation of inflammatory responses. As was the case of blood phagocytes (see above), the observed capacity of alveolar cells to produce IL-12 had minimal impact on BAL fluid supernatant IL-12 levels, because levels of both isoforms were low or undetectable. These results are in keeping with those obtained by Huaux et al (1999) in a mouse model of lung injury and are probably explained by rapid IL-12 binding to the receptors expressed by numerous alveolar cells.

Thus, depending on the cell type and the body compartment (blood vs alveoli), IL-12p40 and p70 are differently regulated in patients with severe sepsis. IL-12p40 synthesis by both blood monocytes and alveolar PMN seems to be up-regulated, possibly leading to marked dysregulation of immune defenses. This is supported by the correlation observed here between the onset of nosocomial pneumonia and high p40 production by alveolar macrophages and is in keeping with observations made in a murine model (Steinhauser et al, 1999). This important new finding adds further weight to the theory of sepsis-induced immunosuppression. The latter is based on several lines of evidence, including specific inhibition of IL-12p70 proinflammatory activity by IL-12p40 in vitro (Ling et al, 1995; Mattner et al, 1993), mouse protection from LPS-induced shock by treatment with IL-12p40 homodimer (Mattner et al, 1997), and onset of severe inflammatory colitis and high sensitivity to *Mycobacterium tuberculosis* in mice genetically deficient in IL-12p40 (Camoglio et al, 2002; Kinjo et al, 2002). IL-12p40 may thus behave as a systemic and alveolar natural inhibitor of IL-12p70, thereby regulating inflammation. Numerous studies have shown the proinflammatory effects of IL-12 p70 in vivo, through the production of IFN-γ (Giannoudis et al, 1999) or IL-8, as recently reported by our group (Ethuin et al, 2001). The second important sign of sepsis-related immunosuppression observed in this clinical study was the decreased capacity of blood monocytes and alveolar macrophages to produce IL-12 p70, as already found using markers of cell activation such as HLA class II molecule expression (Giannoudis et al, 1999; Payen et al, 2000), nuclear factor-κB translocation (Adib-Conquy et al, 2001), and proinflammatory cytokine production (Chollet-Martin et al, 1996; Ertel et al, 1995; Reddy et al, 2001).

In conclusion, these results demonstrate that blood and alveolar PMN and monocytes/macrophages show different patterns of IL-12p70 and p40 isoform release during severe sepsis, both systemically and locally in the lungs, with an overall tendency toward up-regulation of the anti-inflammatory IL-12p40 subunit and down-regulation of the proinflammatory IL-12p70

heterodimer. This imbalance between the two IL-12 isoforms seems to be designed to control excessive inflammation, but it may also render septic patients more susceptible to nosocomial infection. These findings open the way to new therapeutic immunomodulation strategies that take into account the compartmentalization of IL-12 isoform production.

Patients and Methods

Patients and Controls

Sixteen patients with clinical signs of severe sepsis or septic shock were recruited from the surgical ICU of Saint-Louis Hospital, Paris. Sepsis syndrome was defined as fever or hypothermia ($>38.3°$ C or $<35.6°$ C), tachycardia (>90 beats per minute), tachypnea (>20 breaths per minute or need for mechanical ventilation), and clinical signs of altered organ perfusion resulting in mental disorientation, oliguria, or elevated lactate levels. Septic shock was defined by a clinical diagnosis of sepsis syndrome associated with hypotension (systolic blood pressure below 90 mmHg or a 40 mmHg fall below baseline) and the need for vasopressor drugs to maintain blood pressure (Bone et al, 1992). Ten volunteers served as healthy controls. The patients (or their legal representative) and the healthy controls were informed of the purpose of the study and gave their informed consent. All procedures were conducted in accordance with the hospital's ethics committee. The patients received conventional therapy for sepsis or septic shock, including antibiotics and volume-expanding and vasoactive agents. Blood was sampled during the first 24 hours of severe sepsis or septic shock. BAL was performed in the septic patients to investigate suspected pneumonia, on the basis of lung opacities on chest radiography, a purulent endotracheal aspirate, and hypoxemia on arterial gasometry. Pneumonia was confirmed by growth of $\geq 10^3$ CFU bacteria/ml from a protected specimen. Disease severity at ICU admission was assessed by SAPS version II (Le Gall et al, 1993).

Blood PMN and Monocyte Isolation and Culture

Blood phagocytes from the healthy volunteers and patients were purified as previously described (Grenier et al, 1999). Blood was collected in sterile heparinized tubes. Leukocytes were rapidly isolated in endotoxin-free conditions by sedimentation on a separating medium containing 9% Dextran T-500 (Pharmacia, Uppsala, Sweden) and 38% Radioselectan (Schering, Lys-lez-Lannoy, France) in 0.9% saline. The leukocyte-rich suspension was then centrifuged on a Ficoll-Paque (Sigma, St. Louis, Missouri) density gradient. Monocytes were purified from the mononuclear cell ring by adherence to plastic for 1 hour. PMN from the pellet were further purified by using pan-anti-human HLA class II-coated magnetic beads (Dynabeads M-450; Dynal A.S, Oslo, Norway) for 30 minutes at 4° C with gentle rotation to deplete monocytes, B cells, and activated T cells. The cell culture medium was RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-

inactivated FCS, L-glutamine (2 mmol/ml), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Highly purified PMN (10^7/ml) and monocytes (0.5 10^6/ml) were then cultured for 48 hours and 18 hours, respectively, at 37° C with 5% CO_2, in complete medium, with or without 1 μg/ml LPS (*Escherichia coli* serotype 055:B5; Sigma) and 250 IU/ml human γ-interferon (rhIFN-γ; R&D Systems, Abingdon-Oxon, United Kingdom). Cell-free supernatants were harvested at the end of the culture periods, centrifuged at 1500 $\times g$ for 10 minutes, and stored at −80° C until cytokine assay.

For plasma preparation, blood was collected in sterile EDTA-treated tubes and centrifuged at 1200 $\times g$ for 10 minutes at 4° C to avoid cytokine synthesis or degradation in vitro. Plasma samples were stored at −80° until cytokine assay.

BAL

All of the patients underwent BAL using a standardized protocol as previously described (Chollet-Martin et al, 1996; Grenier et al, 2001). Briefly, 150 to 200 ml of saline was injected via a fiberoptic bronchoscope and immediately aspirated. The recovered fluid was filtered through gauze and centrifuged at 1500 rpm for 10 minutes at 4° C. The supernatant was stored at −80° C until cytokine assay. The BAL cell pellet was resuspended in RPMI 1640 medium, and cells were counted with a hemacytometer. Cytospin preparations (Shandon, Sewickley, Pennsylvania) were used for differential cell counts after May-Grünwald-Giemsa staining.

Alveolar Cell Isolation and Culture

BAL cell pellets were resuspended in PBS supplemented with 2% heat-inactivated FCS. When PMN were the predominant cell type in BAL, alveolar PMN were purified by 30-minute incubation at 4° C with anti-human-HLA class II-coated magnetic beads (Dynabeads M-450; Dynal) to deplete contaminating B lymphocytes, activated T lymphocytes, and macrophages. Then, 10^7 alveolar PMN/ml were cultured for 48 hours at 37° C with 5% CO_2 in complete medium, with or without LPS + IFN-γ, as described above. When PMN were not the predominant cell type, alveolar macrophages at a final density of 0.5 × 10^6/ml were purified by adherence after 1 hour of culture in culture medium and then similarly cultured. Cell-free supernatants were harvested, centrifuged at 1500 $\times g$ for 10 minutes, and stored at −80° C until cytokine assay.

PMN and Monocyte Expression of Adhesion Molecules

Phagocyte activation in vivo modifies adhesion molecule expression on the cell surface. Using flow cytometry, we thus studied the expression of the $\beta 2$ integrin CD11b/CD18 and the L-selectin CD62-L on PMN and/or monocytes, both at rest and after ex vivo stimulation with the bacterial peptide fMLP (Chollet-Martin et al, 1996; Gainet et al, 1999; Taieb et al, 2000). Heparinized whole blood was either kept on ice or incubated with fMLP (Sigma; 10^{-6} M final concen-

tration) or PBS (Pharmacia) at 37° C for 5 minutes. $\beta 2$ integrin expression was quantified using a phycoerythrin-conjugated (PE) anti-CD11b antibody (Dako, Glostrup, Denmark); L-selectin expression was measured using a FITC anti-CD62-L antibody (Immunotech, Marseille, France). An FITC-conjugated anti-CD14 antibody (Becton Dickinson) was used to identify monocytes. After 30 minutes of incubation, red blood cells were lysed with FACS lysing solution (Becton Dickinson). The remaining cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry with a Becton Dickinson FACScan (Immunocytometry Systems, San Jose, California) equipped with a 15-mW, 488-nm argon laser. The data were analyzed using LYSYS II software, and the median fluorescence intensity was used to quantify responses.

Cytokine Assays

IL-12p70 heterodimer and free IL-12p40 were assayed in plasma, BAL fluid supernatants, and alveolar and blood phagocyte culture supernatants, by using specific ELISA (Quantikine; R&D Systems, Minneapolis, Minnesota), according to the manufacturer's instructions. The detection limits were 0.5 pg/ml and 5 pg/ml, respectively, for IL-12p70 and IL-12p40.

Statistical Analysis

All results are expressed as means ± SEM. The nonparametric Mann-Whitney U test was used to determine the significance of differences between the groups. Paired comparisons were made using Wilcoxon's paired test. P values below 0.05 were considered significant. Correlations were identified with Spearman's rank correlation coefficient.

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