Biologically Active Intercellular Adhesion Molecule-1 Is Shed as Dimers by a Regulated Mechanism in the Inflamed Pleural Space

Mario Melis, Elisabetta Pace, Liboria Siena, Mario Spatafora, Annalisa Tipa, Mirella Profita, Anna Bonanno, Antonio M. Vignola, Giovanni Bonsignore, Christopher H. Mody, and Mark Gjomarkaj

Institute of Respiratory Physiopathology, Italian National Research Council; Institute of General Medicine and Pneumology, University of Palermo, Palermo, Italy; and Department of Microbiology and Infectious Diseases and Department of Internal Medicine, University of Calgary, Calgary, Alberta, Canada

Intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule that plays a crucial role in cell–cell interactions involved in the recruitment of cells and immune responses. Under some circumstances, ICAM-1 is found as a soluble protein that has the potential to influence the nature of immunoinflammatory responses. By examining cells and fluid from the pleural compartment of patients with cancer, tuberculosis, and congestive heart failure, the cellular source, conformation, control, and biological activity of soluble ICAM-1 (sICAM-1) were investigated. The results suggest that dimeric sICAM-1 was released locally in the pleural compartment of tuberculous and malignant effusions. sICAM-1 was shed from preexpressed surface ICAM-1 rather than produced *de novo*, and both CD45-positive leukocytes and cytokeratin-positive epithelial and mesothelial cells expressed ICAM-1, suggesting multiple cellular sources for sICAM-1. The expression of sICAM-1 was regulated because pleural macrophages caused release of sICAM-1 via a tumor necrosis factor-α-dependent mechanism. The functional significance of sICAM-1 was demonstrated by showing that pleural sICAM-1 interfered with conjugate formation between LAK cells and K562 cells, suggesting that pleural sICAM-1 plays an immunosuppressive role by inhibiting adhesion of cytotoxic lymphocytes and tumor cells. Thus, sICAM-1 is shed from the surface of cells in a regulated manner and has the potential to influence the immune response in the pleural space.

Keywords: intercellular adhesion molecule-1; lung cancer; mononuclear phagocyte; pleural effusion; tuberculosis

Despite the recognition of a soluble form of intercellular adhesion molecule-1 (sICAM-1), the cellular source, protein conformation, factors controlling its release, and physiologic importance of sICAM-1 in disease states remain unclear.

ICAM-1 is a member of the immunoglobulin supergene family that is widely distributed among hematopoietic and nonhematopoietic cells (1). Although both monomeric and dimeric forms of ICAM-1 are present on the cell membrane, the dimeric form is the predominant one (2, 3) and is able to bind leukocyte function associated-1 (LFA-1) molecules with greater affinity compared with monomers (3). Because of the ability of surface-expressed homodimers of ICAM-1 to act as a ligand for LFA-1 and MAC-1, it plays a crucial role in intercellular interactions involved in the development of inflammatory responses (2). Accordingly, ICAM-1 is actively expressed on the surface of leukocytes, and the expression of ICAM-1 is upregulated by inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ (4). However, the upregulation of surface ICAM-1 expression by inflammatory (and neoplastic) cells can be associated with the release of sICAM-1 in the extracellular milieu (2, 5).

Elevated concentrations of sICAM-1 have been detected in serum and various extracellular fluids in a variety of inflammatory and neoplastic diseases (6, 7) and it has been demonstrated to play an important role in the regulation of some immune response mechanisms. In this regard we have previously shown that sICAM-1 that is produced by a lung cancer cell line inhibits cell adhesion and cytotoxicity between LAK cells and a lung cancer cell line (8).

The pleural space is frequently involved in inflammatory and neoplastic diseases that may compromise both pleural vascular and mesothelium permeability and lead to the accumulation of fluid enriched in cells and proteins into the pleural space, that is, the development of an exudative pleural effusion (9, 10). As a closed compartment that is involved in a variety of different human diseases, the pleural space has many advantages that facilitate the study of immune responses. Different diseases that result in pleural effusions present an ideal opportunity to study different models of the inflammatory mechanisms that are used within the pleural compartment. Although pleural effusions are common, the mechanisms involved in the pleural immune response are not well elucidated; in particular, little is known about the role that is played by adhesion molecules in promoting the intercellular interactions that may contribute or inhibit the development of pleural inflammation. It is not clear which cells produce sICAM-1 in the pleural compartment. Nor has it been clearly established whether sICAM-1 is produced *de novo* and immediately released or initially expressed on the surface of pleural cells and then shed from their surface. It is also not known whether this event is regulated and whether sICAM-1, which is produced *in vivo*, could play a role in the regulation of inflammatory and antitumor responses within the pleural space.

The aims of this study were (*1*) to evaluate whether sICAM-1 is produced locally within the inflamed pleural space, (*2*) to identify the mechanism of release and cell source(s) and protein conformation of pleural sICAM-1, and (*3*) to investigate the possible role(s) of sICAM-1 within the inflamed pleural space. Moreover, because pleural macrophages (PleMs) have been demonstrated to play a role in the regulation of inflammatory and immune reactions within the pleural space (11, 12), we evaluated the role played by PleMs in the modulation of

(*Received in original form July 4, 2002; accepted in final form January 15, 2003*)

Supported by the Italian National Research Council and by the Alberta Lung Association. C.H.M. is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

Correspondence and requests for reprints should be addressed to Mark Gjomarkaj, M.D., Istituto di Fisiopatologia Respiratoria, Consiglio Nazionale delle Ricerche, Via Ugo La Malfa, 153-90146 Palermo, Italy. E-mail: marco@ifr.pa.cnr.it

This article has an online supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Am J Respir Crit Care Med Vol 167. pp 1131–1138, 2003
Originally Published in Press as DOI: 10.1164/rccm.200207-654OC on February 5, 2003
Internet address: www.atsjournals.org

ICAM-1 expression and sICAM-1 release by pleural effusion cells.

METHODS

Pleural Fluid and Blood Sample Collection

Pleural fluid was collected by therapeutic thoracentesis from hospitalized patients with congestive heart failure (CHF) (n = 9 [6 males and 3 females]; age range, 56 to 84 years), tuberculosis (TB) (n = 8 [6 males and 2 females]; age range, 26 to 71 years), and cancer (n = 15 [10 males and 5 females]; age range, 39 to 81 years). All subjects gave informed written consent and the study was approved by the institutional review board for human studies. Standard clinical, laboratory, and radiological investigations established the diagnosis for each patient as previously described (12). In detail, CHF effusions were defined as transudates associated with an enlarged heart, distended neck veins, and cardiac gallop that improved with therapy for the congestive heart failure; tuberculous effusions were defined as exudates with positive culture for *Mycobacterium tuberculosis* or with positive smear for acid-fast bacilli of pleural fluids; malignant effusions were defined as exudates associated with a pathologic diagnosis of cancer from cytologic examination of pleural fluids and from pathologic examination of lung tissues. The effusions were classified as transudates or exudates by meeting at least one of the criteria described by Light and coworkers (13). The fluids were drawn into polypropylene tubes containing heparin (10–20 IU/ml). Red blood cells were differentiated from nucleated pleural cells by Turk's stain. The total cell counts and cell viability (more than 95%) were performed by trypan blue dye exclusion. Differential cell counts were performed by using the following monoclonal antibodies (mAbs): anti-CD68, specific for mononuclear phagocytes (Dako, Glostrup, Denmark); anti-cytokeratin (MNF116), specific for epithelial and mesothelial cells (Dako); anti-human neutrophil elastase (clone NP57; Dako), specific for neutrophils; anti-human eosinophil cationic protein (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden), specific for eosinophils; anti-CD3, specific for T lymphocytes (Dako); anti-CD20, specific for B lymphocytes (Dako); anti-CD16 (clone DJ130c), specific for natural killer (NK) cells (Dako); and isotype-matched immunoglobulins (Dako) as negative controls. The identification of cancer cells was performed by the Department of Pathology of our hospital (Ospedale V. Cervello, Palermo, Italy) according to standard diagnostic procedures, using a panel of mAbs, including anti-human calretinin (Dako), as recommended by the current consensus (14, 15).

The pleural fluids were centrifuged at 400 × *g* for 10 min. Cell-free fluids were immediately frozen at –70°C until they were analyzed for subsequent experiments. Autologous serum specimens were obtained from blood samples collected from patients on the same day as thoracentesis and from healthy control subjects (n = 11 [5 males and 6 females]; age range, 25 to 59 years) after informed written consent had been obtained.

Detection of sICAM-1 in Pleural Fluid and Serum

Pleural cell-free fluids and sera were brought to room temperature. sICAM-1 concentration was measured with a commercially available ELISA kit (British Biotechnology, Abingdon, UK) in accordance with the manufacturer's instructions. The sensitivity of the assay was less than 0.35 ng/ml. The sICAM-1 concentration (ng/ml) was also expressed as a ratio to albumin concentration, which was used as a marker of vascular permeability (16). Albumin (mg/ml) was measured by a nephelometric assay, using specific mAbs (Beckman assay protein system; Beckman, Milan, Italy) (17).

Immunoprecipitation and Western Blotting of sICAM-1 in Pleural Fluid

Pleural cell–free fluids were incubated overnight with anti-CD54 mAb (anti–ICAM-1) (British Biotechnology). Protein G–Sepharose was then added for 90 minutes and centrifuged at 10,000 × *g*. The pellets containing the antigen–antibody complexes were recovered, and supernatants were used in subsequent sICAM-1 depletion experiments (*see* below). The expression of ICAM-1 was evaluated by Western blot as described previously (18). Fifty-microgram samples of total protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 4–12% gradient gels (Novex, San Diego, CA) with or without 10 mM dithio-L-threitol (DTT) and were blotted onto nitrocellulose membranes. These were blocked with phosphate-buffered saline (PBS) containing 3% BSA–0.1% Tween 20 and then probed with a mouse antibody specific to human ICAM-1 (British Biotechnology) at 4°C overnight. After serial washes with PBS containing 0.1% Tween 20, membranes were incubated with peroxidase-conjugated anti-mouse antibody (Dakopatts, Älvsjö, Sweden). The bands were visualized with an enhanced chemiluminescence system (New England Nuclear, Boston, MA) followed by autoradiography.

Depletion of sICAM from Pleural Fluids

Depletion of sICAM from pleural fluids was performed by immunoprecipitation as previously described (*see above*). The sICAM-1-depleted fluids contained negligible concentrations of sICAM-1 as assessed by ELISA.

Phenotypic Characterization of ICAM-1-positive Cells

The expression of ICAM-1 on the surface of pleural cells was determined by direct double-label immunofluorescence using a FACStar Plus (Becton Dickinson, Mountain View, CA) analyzer. The following mAbs were used in this study: phycoerythrin (RPE)-conjugated mouse anti-human ICAM-1 (anti-CD54) (LB2-PE; Becton Dickinson), fluorescein isothiocyanate (FITC)-conjugated anti-human leukocyte common antigen specific for human leukocytes (CD45), and FITC-conjugated anti-human cytokeratin (MNF116) specific for mesothelial and epithelial cells (all from Dakopatts). Before labeling with anti-cytokeratin antibodies the cells were permeabilized with 0.1% Triton X-100 in PBS and washed in PBS (19). Negative and positive controls were performed with an FITC-conjugated mouse IgG1 and with dual-color mouse IgG1–FITC and mouse IgG1–RPE mAbs, respectively (both from Dakopatts). Data are expressed as the percentage of positive cells.

Isolation of Macrophages from Pleural Effusions

Macrophages were isolated from pleural effusions obtained from patients with CHF, using previously described methodologies developed in our laboratory with minor modifications (Ficoll-Hypaque gradient followed by discontinuous Percoll gradient, omitting the overnight plastic adherence step) (11, 20). The isolated cell population was composed of more than 90% macrophages, as confirmed by nonspecific esterase staining and expression of CD68 (macrophage EBM 11; Dako), which is specific for mononuclear phagocytes. PleMs were resuspended in complete medium (CM) (RPMI 1640 plus 1% fetal calf serum, 25 mM HEPES, 2 mM glutamine, penicillin [100 ng/ml], and streptomycin [100 ng/ml]) (GIBCO, Paisley, UK) at 10^6 cells/ml and incubated (4 hours, 37°C, 5% CO_2) in the absence or in the presence of lipopolysaccharide (LPS; 1 μg/ml) from *Escherichia coli* (serotype 0111:B4; Sigma, St. Louis, MO) to stimulate TNF-α production (11). After incubation, tubes were centrifuged (600 × *g*, 10 minutes) and culture supernatants were collected and stored at –70°C until used in subsequent experiments examining modulation of ICAM-1 expression and sICAM-1 release.

Modulation of ICAM-1 Expression and sICAM-1 Release and Shedding

To examine the release of surface ICAM-1, pleural cells were first incubated with EZ-Link sulfo-NHS-biotin (Pierce, Rockford, IL), as previously described (21), for biotinylation of cell surface proteins.

To evaluate the ability of PleMs to modulate ICAM-1 expression and sICAM-1 release and shedding, both unbiotinylated and biotinylated pleural cells were cultured in CM (24 hours, 37°C, 5% CO_2) in the presence or absence of PleM culture supernatants. LPS from *E. coli* (Sigma) (1 μg/ml), recombinant TNF-α (1,000 IU/ml), or sufficient anti-TNF-α polyclonal antibody to neutralize 1,000 IU of TNF-α as indicated by the manufacturer (polyclonal rabbit anti-human TNF-α; Genzyme Diagnostics, Cambridge, MA), were used as controls. The optimal concentrations of LPS and TNF-α were chosen on the basis of preliminary dose–response experiments (data not shown) (11). After incubation, tubes were centrifuged (600 × *g*, 10 minutes) and supernatants of unbiotinylated cells were collected and stored at –70°C until used for the evaluation of sICAM-1 concentrations by the same methodology used for pleural fluids. Supernatants of biotinylated cells were

TABLE 1. CHARACTERISTICS OF PLEURAL EFFUSIONS

	TB	Cancer	CHF
Albumin, g/dl	2.48 ± 0.3	2.15 ± 0.4	1.4 ± 0.6
sICAM-1, ng/ml	705 ± 317	591 ± 350	123 ± 62
Total, cells/μl	2,391 ± 333	2,974 ± 1,054	758 ± 299
Differential cell counts, %			
Lymphocytes	88.1 ± 5.2	56.5 ± 22	55.6 ± 9.4
Neutrophils	2.3 ± 3	14.9 ± 9.3	0.3 ± 0.8
Macrophages	4.4 ± 3.6	10.3 ± 9	27.7 ± 4.8
Eosinophils	1.7 ± 2.7	2.7 ± 3.9	0
Mesothelial cells	1.7 ± 1.7	9.2 ± 6.6	16.3 ± 9
Tumor cells	0	6.4 ± 6.4	0

Definition of abbreviations: CHF = congestive heart failure; sICAM-1 = soluble intercellular adhesion molecule-1; TB = tuberculosis.

Values represent means ± SD.

immunoprecipitated with the anti-CD54 mAb and the methodology described above. To detect only biotinylated ICAM-1, Western blot analyses of biotinylated immunoprecipitates were performed with streptavidin conjugated with peroxidase and a peroxidase-conjugated anti-mouse antibody (Dako).

Pleural cells were recovered from the bottom of the tubes and tested for the expression of surface ICAM-1 by cytofluorimetric analysis, as described above. Data from these experiments are expressed as mean fluorescence intensity.

Conjugate Formation Assay

To evaluate the role of pleural sICAM-1 in cell–cell adhesion, we performed a conjugate formation assay according to previously described methodology (22). Briefly, LAK cells, which were generated from healthy adult peripheral blood (after obtaining informed written consent from the donor) as previously described (8) and the erythromyeloid cell line K562 were used as effector and target cells, respectively. LAK and K562 cells were stained with sulfofluorescein diacetate (green fluorescence) and with hydroethidine (red fluorescence), respectively, and were then coincubated at a ratio of 4:1 for 15 minutes at 37°C. Conjugates were detected by the simultaneous expression of particles expressing red and green fluorescence. To determine the effect of sICAM-1 in pleural fluids, untreated exudative pleural fluid was compared with pleural fluid that had been depleted of sICAM-1. As a control, this was compared with K562 cells that had been preincubated for 1 hour with an anti-CD54 mAb (20 mg/ml) or with LAK cells that had been preincubated with an anti-CD11a (anti-LFA-1) mAb (10 mg/ml) or with recombinant sICAM-1 (50 ng/ml). A total of 10^4 particles was analyzed and the results were expressed as a percentage of variation of conjugate formation in comparison with baseline condition (medium alone).

Statistical Analysis

Data are expressed as means ± SD. The Spearman test was used for assessment of correlations. The Mann–Whitney U test was used for comparison between patient groups. The Wilcoxon test was used to compare levels of sICAM-1 in pleural effusions and in autologous sera. For all tests, $p < 0.05$ was accepted as the threshold of statistical significance.

RESULTS

Expression of sICAM-1 in Pleural Effusions

Table 1 shows the main characteristics of pleural effusions; as expected, effusions recovered from patients with TB (n = 8) and cancer (n = 15) had higher total cell numbers as well as sICAM-1 and albumin concentrations when compared with effusions from patients with CHF (n = 9).

To determine whether sICAM-1 was produced locally or accessed the compartment by extravasation from the blood into the pleural space, the concentrations of sICAM-1 and albumin were compared in pleural fluids and in sera. Albumin is of lower molecular weight and was selected as a marker of vascular/mesothelial permeability. Therefore, if the sICAM-1/albumin ratio were the same in pleural fluid and sera, it would suggest a vascular and a mesothelial leak, whereas a ratio that was higher in pleural fluid than in sera would suggest local production of sICAM-1. sICAM-1/albumin ratios were significantly higher in pleural fluids than in autologous serum in patients with TB (Figure 1A) and cancer (Figure 1B); in marked contrast, no significant difference was detected between sICAM-1/albumin ratios in pleural fluid and autologous serum in patients with CHF (Figure 1C). The serum sICAM-1 concentrations, evaluated in normal subjects, were 5.5 ± 1.5 ng/mg of albumin and were significantly ($p < 0.05$) lower than the serum sICAM-1 concentrations in the other three study groups. These data suggest that sICAM-1 is produced locally in tuberculosis and cancer, but that it is due to leak from the blood in patients with CHF.

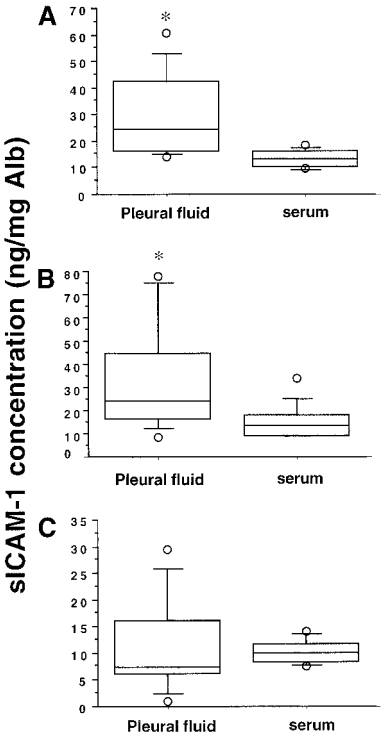

Figure 1. Comparison of soluble intercellular adhesion molecule-1 (sICAM-1)/albumin ratios between pleural effusion and autologous serum in patients with tuberculosis (TB) (*A*), cancer (*B*), and congestive heart failure (CHF) (*C*). *$p < 0.05$.

Characterization of sICAM-1 in Pleural Effusions

To evaluate whether sICAM-1 was present in pleural fluids in a monomeric or in a dimeric form, we performed Western blot analysis to characterize its molecular weight. Because the treatment with reducing agents (DTT) used in the Western blot analysis may affect the stability of dimeric sICAM-1 molecules, we tested the presence of sICAM-1 in pleural fluids under both nonreducing (in the absence of DTT) and reducing (in the presence of DTT) conditions. Under nonreducing conditions, native pleural sICAM-1 showed only a strong dimeric band at about 190 kD, and no other significant bands were detected at different molecular weights. Under reducing conditions, no band was detected at 190 kD whereas two bands were detected at about 60 and 95 kD, respectively, corresponding to different monomeric and/or fragmented dimeric sICAM-1 forms (Figure 2). These results demonstrate that sICAM-1 is present, in inflammatory pleural effusions, in a dimeric form of about 190 kD and that the other forms detected under reducing conditions are due to technical artifacts.

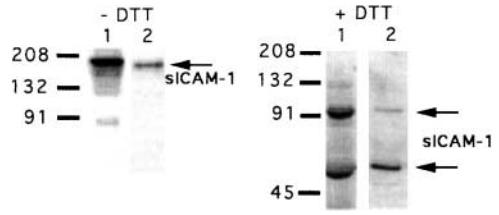

Figure 2. Western blot analysis of pleural sICAM-1. Pleural fluids were immunoprecipitated with the anti-CD54 monoclonal antibody (mAb) and Western blot analyses of immunoprecipitates were performed under both nonreducing (−dithio-L-threitol [DTT]) and reducing (+DTT) conditions (*see* METHODS). *Lanes 1* and *2* show two representative experiments.

Correlation between sICAM-1 Dimers and Membrane Surface ICAM-1 in Pleural Effusions

sICAM-1 dimers could be released into the pleural fluid by two mechanisms. They could be expressed on the surface of cells and shed from cells into the fluid, or they could be synthesized and released *de novo* from cells that did not express ICAM-1 on their surface. To examine the possibility that ICAM-1 was shed from cells that express it on their cell surface, we first determined whether sICAM-1 levels correlated with the expression of surface ICAM-1. Cells from all three patient groups expressed ICAM-1 (Table 2). Although there was no significant difference in the percentage of cells expressing ICAM-1 among the three study groups, the number of cells per volume of pleural fluid expressing ICAM-1 was significantly higher in patients with cancer and patients with TB than in patients with CHF (Table 2). Further, a positive correlation was observed between the number of cells expressing ICAM-1 on their surface and the sICAM-1/albumin ratio in pleural fluid (Figure 3). This suggested that the increased levels of sICAM-1 dimers in pleural fluids of patients from these two study groups were due to cells expressing and releasing ICAM-1.

Cell Type Expressing ICAM-1 in Pleural Effusions

To evaluate the cell type that expressed, and was therefore capable of releasing, sICAM-1 dimers, cells recovered from pleural effusions were labeled with anti-ICAM-1 and with anti-cytokeratin mAb specific for mesothelial and epithelial cells or anti-CD45 for leukocytes. In patients with TB the ICAM-1 molecule was present almost exclusively on the surface of CD45-positive cells (Figure 4A), suggesting that lymphocytes, the overwhelmingly predominant cell population in TB effusions (Table 1), were responsible for the presence of sICAM-1 dimers within the pleural space in patients with TB. In contrast, in patients with cancer, cells that were CD45 positive expressed a low level of surface ICAM-1 (Figure 4B), whereas cells that were cytokeratin positive expressed high levels of surface ICAM-1 (Figure 4B), suggesting that a variety of cells of both bone marrow and

TABLE 2. EXPRESSION OF MEMBRANE INTERCELLULAR ADHESION MOLECULE-1 BY CELLS RECOVERED FROM PLEURAL EFFUSIONS

	TB	Cancer	CHF
CD54 (anti–ICAM-1), %	36.1 ± 4.4	30.4 ± 11.4	33.6 ± 16.6
ICAM-1–positive cells/μl	866 ± 182*	902 ± 472*	235 ± 112

For definition of abbreviations *see* Table 1.
Values represent means ± SD.
*$p < 0.05$ versus CHF.

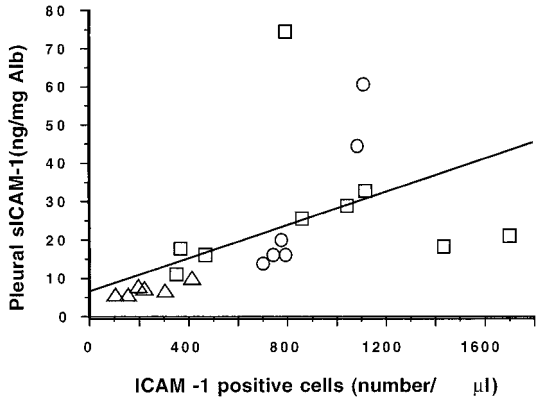

Figure 3. Correlation between the sICAM-1/albumin ratio in pleural effusions and the total number of ICAM-1–positive cells per milliliter. The sICAM-1/albumin ratio and the number of ICAM-1–positive cells were determined in pleural fluids recovered from patients with TB (*circles*; n = 6), cancer (*squares*; n = 9), and CHF (*triangles*; n = 6) (*see* METHODS for details). $\rho = 0.86$; $p < 0.0002$.

mesenchymal origin could contribute to the release of sICAM-1 dimers within the pleural space in patients with cancer.

Mechanism of sICAM-1 Dimer Production: Role of Pleural Macrophages

We next determined whether the sICAM-1 dimers present in the fluids were shed from the surface of ICAM-1-positive cells

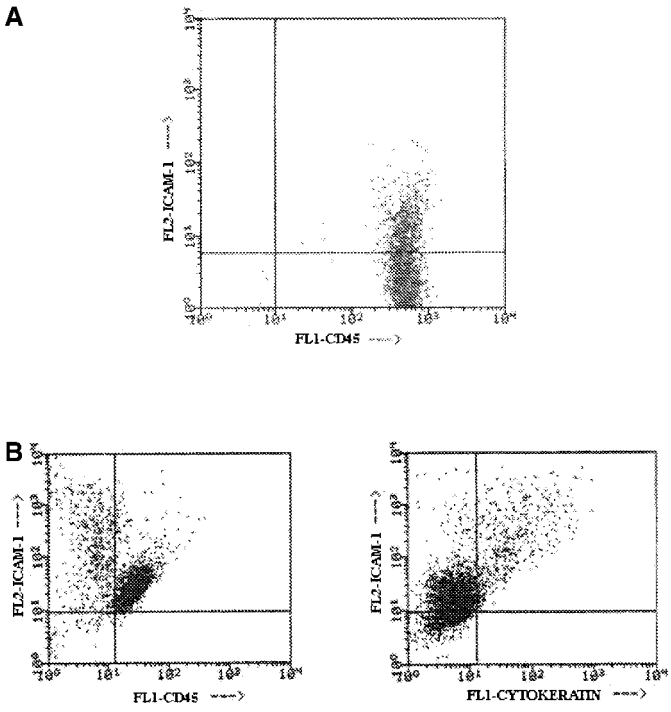

Figure 4. Representative cytofluorimetric analysis of pleural effusion cells from patients with TB (*A*) and lung cancer (*B*). Cells were stained with RPE-conjugated anti-ICAM-1 mAb (*y axis*) and with fluorescein isothiocyante (FITC)-conjugated anti-CD45 mAb (*x axis of* [*A*] and of *left dot plot* of [*B*]) or with FITC-conjugated anti-cytokeratin mAb (*x axis, right dot plot* of [*B*]). On all dot plots, double-positive cells are visible on the upper right side.

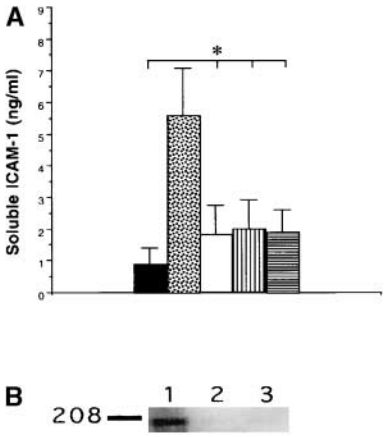

Figure 5. Modulation of sICAM-1 release (*A*) and sICAM-1–shedding (*B*) by pleural effusion cells. (*A*) Pleural effusion cells were cultured (*see* METHODS for details) in the absence (*solid bar*; baseline) or in the presence of the following: conditioned supernatants from LPS-stimulated pleural macrophages (PleMs) (*stippled bar*); conditioned supernatants from LPS-stimulated PleMs plus anti–tumor necrosis factor (TNF)-α polyclonal antibody (*open bar*); recombinant TNF-α (1,000 IU/ml) (*vertical hatched bar*); and LPS (1 μg/ml) (*horizontal hatched bar*). Data are expressed as nanograms of sICAM-1 per milliliter and represent means ± SD of five experiments. Macrophage culture supernatants versus baseline, $p < 0.05$; macrophage culture supernatants versus macrophage culture supernatants plus anti-TNF-α mAb, $p < 0.05$; macrophage culture supernatants versus TNF-α alone, $p < 0.05$; macrophage culture supernatants versus LPS alone, $p < 0.05$; TNF-α versus LPS, p = NS. (*B*) Surface proteins of pleural cells were biotinylated and then incubated in the presence of PleM supernatants or in the presence of TNF-α and LPS alone. Culture supernatants were immunoprecipitated with the anti-CD54 mAb and Western blot analyses of immunoprecipitates were performed under nonreducing conditions (*see* METHODS). *Lane 1* represents culture supernatants from cells incubated in the presence of PleM-conditioned medium. *Lanes 2* and *3* represent culture supernatants from cells incubated in the presence of TNF-α and LPS alone, respectively.

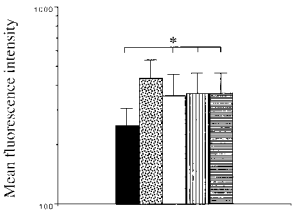

Figure 6. Modulation of surface ICAM-1 expression on pleural effusion cells. Pleural effusion cells were cultured (*see* METHODS for details) in the absence (*closed bar*) or in the presence of the following: conditioned supernatants from LPS-stimulated PleMs (*stippled bar*); conditioned supernatants from LPS-stimulated PleMs plus anti-TNF-α polyclonal antibody (*open bar*); recombinant TNF-α (1,000 IU/ml) (*vertical hatched bar*); and LPS (1 μg/ml) (*horizontal hatched bar*). Data are expressed as mean fluorescence intensity and represent means ± SD of five experiments. Macrophage culture supernatants versus baseline, $p < 0.05$; macrophage culture supernatants versus macrophage culture supernatants plus anti-TNF-α polyclonal antibody, $p < 0.05$; macrophage culture supernatants versus TNF-α alone, $p < 0.05$; macrophage culture supernatants versus LPS alone, $p < 0.05$; TNF-α versus LPS, p = NS.

and whether this mechanism was regulated. For this purpose, pleural macrophages were used as a source of soluble factors that might alter surface ICAM-1 expression and sICAM-1 dimer production by pleural effusion cells.

Only negligible concentrations of sICAM-1 were detected in conditioned medium from PleMs (data not shown). However, incubation of pleural cells with PleM-conditioned medium dramatically increased the release of sICAM-1 by pleural effusion cells (Figure 5A). Anti-TNF-α mAb significantly reduced the release of sICAM-1, suggesting that the presence of TNF-α in PleM-conditioned medium was required for the release of ICAM-1 by pleural effusion cells. However, neither recombinant TNF-α nor LPS alone was also able to significantly upregulate sICAM-1 release by pleural cells (Figure 5A), suggesting that both TNF-α and LPS may act indirectly via the release of other soluble factors by PleMs.

To determine whether the sICAM-1 detected in pleural culture supernatants was shed from the surface of pleural cells, the cell surface proteins were biotinylated and culture supernatants were analyzed for biotinylated ICAM-1. We reasoned that if sICAM-1 was released from the cell surface, it would be biotinylated, whereas if it were released from within the cell or by *de novo* synthesis, it would not be biotinylated. Immunoblot analysis, performed under nonreducing conditions, for biotinylated proteins that had been immunoprecipitated with anti-ICAM-1 demonstrated the presence of a biotinylated band at about 190 kD, corresponding to the native sICAM-1 dimers, only after incubation with PleM-conditioned medium (Figure 5B). In addition, conditioned medium from LPS-stimulated PleMs caused a statistically significant increase in surface ICAM-1 expression on pleural effusion cells when compared with cells cultured in unconditioned medium (Figure 6). Moreover, anti-TNF-α mAb significantly reduced the percentage of cells expressing ICAM-1, suggesting that the presence of TNF-α in PleM culture supernatants was responsible, at least in part, for the expression of ICAM-1 by pleural effusion cells. However, incubation with either recombinant TNF-α or LPS alone was also able to modestly, but significantly ($p < 0.05$), upregulate ICAM-1 expression by pleural cells when compared with baseline. This upregulation was significantly lower ($p < 0.05$) than that generated by PleM culture supernatants (Figure 6). Thus, pleural macrophages were able to induce the shedding of dimeric sICAM-1 as a 190-kD protein from the surface of pleural cells and to upregulate the expression of ICAM-1 on the surface of pleural cells. Moreover, these results suggest that the control of ICAM-1 shedding is more complex than the control of ICAM-1 surface expression and is not merely mediated by TNF and LPS alone.

Role of Pleural sICAM-1 in Adhesion of Cytotoxic Lymphocytes and Tumor Cells

To determine whether the sICAM-1 might be active and play a role in the immune response, experiments were performed to determine whether sICAM-1 inhibited conjugate formation between LAK effector cells, which were generated from healthy adults, and K562 target cells. K562 cells were used because these cells express high levels of surface ICAM-1 molecules, which are required for cellular cytotoxicity (23). We compared the ability of untreated pleural effusion with the ability of sICAM-1-depleted pleural effusion to affect the formation of conjugates. When untreated pleural fluid was added to the LAK effector cells and K562 target cells there was a slight augmentation in conjugate formation, possibly because of immunostimulation by the pleural fluid. On depletion of sICAM-1 there was a significant increase in conjugate formation when compared with the addition of untreated pleural fluids (Figure 7A), indicating that sICAM-1 present in pleural fluids plays a role in the inhibition of cell–cell adhesion. As expected, the addition of recombinant sICAM-1, anti–ICAM-1 or anti–LFA-1 mAbs significantly inhibited the formation of conjugates when compared with baseline (Figure 7B).

DISCUSSION

The current studies have utilized cells and fluid from the pleural space to demonstrate that (*1*) sICAM-1 is produced locally by cells within an inflammatory compartment (the pleural space); (*2*) sICAM-1 is present in pleural fluid as a dimer and it is

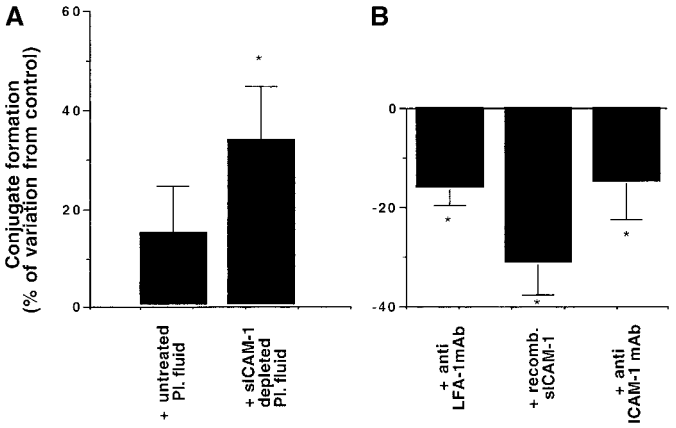

Figure 7. Role of pleural sICAM-1 in cell–cell adhesion. LAK cells and K562 cells were used as effector and target cells, respectively, in conjugate formation assays using exudative pleural fluids, or fluid that had been depleted of sICAM-1, recovered from patients with TB (n = 2) and cancer (n = 4) (*see* METHODS for details) (*A*). Cultures in the presence of anti-LFA-1 mAb, recombinant sICAM-1, and anti ICAM-1 mAb were included as positive controls (*B*). Data are expressed as the percentage of variation with respect to negative control (cultures in complete medium). Comparison with baseline, *$p < 0.05$.

shed from the surface of cells that express surface ICAM-1; (*3*) shedding of sICAM-1 dimers occurs in a regulated fashion via the products of stimulated macrophages; and (*4*) sICAM-1 is capable of influencing immune responses.

We first demonstrated that pleural effusions recovered from patients with TB and cancer have significantly higher concentrations of sICAM-1 than effusions recovered from patients with CHF. These findings confirm and extend the previous observations by Hoffmann and coworkers (24), who demonstrated a similar increase in exudative pleural effusions of heterogeneous etiologies, compared with transudative effusions.

The pleural compartment is separated from the blood circulation by two different limiting membranes, represented by the vascular endothelium and the pleural mesothelium, respectively, both contributing to limit the passage of cells and of soluble proteins. To clarify whether increased sICAM-1 concentration in pleural effusions was due to local production of this molecule or simply due to increased extravasation of serum sICAM-1 into the pleural space, we normalized sICAM-1 to albumin concentrations both in pleural effusions and in sera. Albumin is considered a good marker of capillary permeability in the pleura, because there is no evidence that albumin is produced or stored in pleural mesothelium. Further, the pleural microvascular endothelium is semipermeable (9); albumin originates only from serum and reaches different organ compartments via passive diffusion (25); the concentrations of total protein and albumin in pleural fluid are lower than in serum (26); and albumin and sICAM-1 have similar molecular weights. Therefore, sICAM-1 concentrations were normalized to albumin to gain insights into local sICAM-1 production. The ratio of sICAM-1 to albumin was elevated in TB and cancer, suggesting that sICAM-1 is produced locally in the pleural space during inflammatory processes, whereas no local production of sICAM-1 could be demonstrated in the absence of active inflammation, that is, in transudative effusions from patients with CHF. Interestingly, the serum sICAM-1 concentrations in patients with CHF were higher than in normal subjects. In this regard, it has been demonstrated that the concentrations of TNF-α in plasma are higher in patients with CHF than in normal subjects (27). Because TNF-α is one of the major stimuli for production of sICAM-1 (5), it is possible that this cytokine is involved in the described increase of serum sICAM-1 concentrations.

To characterize the molecular weight of sICAM-1 present within the pleural fluids we performed immunoblot analysis under both nonreducing and reducing conditions because it was demonstrated that under reducing conditions, the ICAM-1 dimeric molecule dissociates, yielding monomers or fragments that might not natively be present (28). Under nonreducing conditions the immunoblot identified a band of about 190 kD, with no other significant bands identified at lower molecular weights. Under reducing conditions two bands were identified at about 60 and 95 kD, corresponding to different monomeric and/or fragmented dimeric sICAM-1 forms. In this regard it has been demonstrated that the dimeric extracellular forms of sICAM-1 may be represented by U-shaped and by ringlike dimers. Moreover, two U-shaped dimers may compose a W-shaped tetramer. All these forms of polymeric sICAM-1 have molecular weights ranging from 112 and 200 kD (28). Accordingly, a reducing agent may break a dimeric native pleural sICAM-1 into monomers (MW 60 kD) or into a partially fragmented dimer (MW 95 kD). These findings demonstrated that sICAM-1 was present in pleural fluid in a native dimeric form and that the presence of smaller sICAM-1 molecules is due to technical artifacts related to the use of reducing agents (DTT).

Soluble dimers of ICAM-1 may have important implications for immune responses. Membrane ICAM-1 is expressed predominantly as a dimer rather than as monomers (2, 3). Dimers have increased affinity for their ligand as demonstrated by an engineered form of soluble ICAM-1 dimer that has increased affinity for rhinovirus (one of the ligands for ICAM-1) compared with monomers of ICAM-1 (29), and reduced LFA-1-dependent transendothelial chemotaxis of peripheral blood mononuclear cells (30). ICAM-1 has two dimerization sites. The first is Val-51 in strand E of domain 1 (31). This appears to be critical for dimerization because this position is occupied by valine or isoleucine in all ICAM-1 molecules, whereas in ICAM-2, which does not dimerize, the amino acid at this position is hydrophobic (3, 31). The second dimerization site is within the transmembrane domain (2, 3). Although recombinant ICAM-1 is usually present in solution as monomers, we show that in a biological fluid it is a dimer. There are a number of possible mechanisms for this dimerization. The first is that Val-51 may be sufficient for dimerization, which is contrary to prior opinion (31). The second is that ICAM-1 dimers in pleural fluid may contain a portion of the transmembrane domain that contributes to dimerization. Finally, a ligand for ICAM-1 may contribute to the dimerization. The events that lead to this process will require further study.

Because the dimeric form is the most biologically active form of sICAM-1, it prompted us to study the mechanisms of release and activity of this molecule within the pleural space. It has been demonstrated that cells that are capable of expressing membrane ICAM-1 may upregulate the expression of this molecule after stimulation, and may also increase the subsequent release of sICAM-1 (3). To test the hypothesis that sICAM-1 might be due to shedding of ICAM-1 from the cell surface, we first sought a correlation between the expression of ICAM-1 on cell membranes and sICAM-1 levels. We found that the sICAM-1/albumin ratio in pleural fluid correlated with the number of pleural effusion cells expressing surface ICAM-1. Further, by labeling the surface molecules of the pleural cells with biotin before the incubation with PleM culture supernatants, we were able to show that an approximately 190-kD band was immunoprecipitated with an anti-ICAM-1 antibody. This suggests that the sICAM-1

is cleaved from the surface of pleural cells and shed as an intact surface extracellular dimeric molecule.

To verify whether structural or immunocompetent pleural cells expressed surface ICAM-1, we labeled cells with an anti-ICAM-1 mAb together with an anti-leukocyte (CD45) or an anti-cytokeratin mAb. In TB effusions, surface ICAM-1 was expressed, almost exclusively, on CD45-positive cells. Because it has been demonstrated that activated T lymphocytes express ICAM-1 (6) and T lymphocytes represent the majority of cells present in tuberculous pleurisy (32), it suggests that a compartmentalized immune response occurs at the site of disease and that T lymphocytes may actively contribute to the release of sICAM-1. Moreover, it has been demonstrated that in pulmonary sarcoidosis, another granulomatous disorder characterized by the activation of T lymphocytes, sICAM-1 concentrations correlate with the percentage of lymphocytes in bronchoalveolar lavage (33).

With regard to cancer effusions, we demonstrated that surface ICAM-1 is present on CD45-positive as well as cytokeratin-positive cells. Lymphocytes and macrophages represent the main cell population expressing surface ICAM-1 among CD45-positive leukocytes (6). With regard to cytokeratin-positive cells, we characterized our cytokeratin-positive cell population with an anti-cytokeratin mAb specific for cytokeratin MNF16 present on the surface of both mesothelial and cancer cells. In cancer effusions, all the cytokeratin-positive cells were also positive for the ICAM-1 molecule. Because every cancer effusion was specifically characterized in terms of the percentage of cancer cells by using a panel of mAbs including calretinin (*see* METHODS) and because both cancer (8) and mesothelial cells (34) express ICAM-1 on their surface, it is conceivable that both mesothelial and cancer cells constitute the ICAM-1-positive/cytokeratin-positive cell population present in cancer effusions. We have previously demonstrated that lung cancer cell lines express membrane ICAM-1 and release sICAM-1 after incubation with cytokines, including TNF-α and IFN-γ (8); moreover, TNF-α has been shown to be present in pleural effusions of patients with lung cancer (35, 36). Taken together, these findings suggest that different cell types present in TB and cancer pleural effusions express membrane ICAM-1 and that, during inflammation, these cells may contribute to the release of sICAM-1 within the pleural space. It is possible that mesothelial cells lining the visceral and parietal pleura may contribute to the release of sICAM-1 after inflammatory insults. Further studies on pleural biopsies are necessary to clarify this phenomenon.

The data presented here suggest that PleMs play a crucial role in regulating ICAM-1 signaling. In the presence of TNF-α and LPS, cells were able to express ICAM-1, but did not shed sICAM-1. This would facilitate ICAM-1/LFA-1 interactions. By contrast, these same stimuli cause PleMs to produce factors, including TNF-α (11), that caused shedding of sICAM-1, which would potentially downregulate ICAM-1/LFA-1 interactions. This evidence suggests that sICAM-1 shedding is a more complex phenomenon than membrane ICAM-1 expression. Other factors, in addition to TNF-α and LPS, are necessary to induce sICAM-1 shedding, and TNF-α and LPS might indirectly promote sICAM-1 shedding by interacting with other soluble mediators present in PleM culture supernatants and by inducing the release of other soluble mediators. Further studies are necessary to better clarify these mechanisms.

Although ICAM-1 is considered to play an important role in the immunologic host response against tumor cells and in the metastatic process (1), the role of sICAM-1 in cell-mediated host defense, within the pleural space, was not previously defined. In this regard, it has been suggested that the release of sICAM-1 might be one of the mechanisms by which cancer cells escape physical interactions with LFA-1-positive cytotoxic cells (5, 8). Consistent with this hypothesis, it has been demonstrated that, in patients with lung cancer, serum levels of sICAM-1 are associated with advanced tumor stages and metastatic potential (7, 37). Our finding that the *in vitro* adhesion between cancer and LAK cells was reduced by the addition of pleural fluid containing sICAM-1 compared with fluid that had been depleted of sICAM-1 suggests that, within the pleural space, sICAM-1 downregulates the interaction between cytotoxic T cells and tumor cells. To our knowledge, this is the first report demonstrating a functional role of pleural sICAM-1 as suppressive factor in host–cancer interactions. Further, it has been demonstrated that dimerization of sICAM-1 is required for optimal binding to its receptor (28). Accordingly, our data demonstrate that dimeric sICAM-1, the form of sICAM-1 present in pleural fluids, is capable of inhibiting intercellular adhesion.

In conclusion, our study suggests that in the pleural space, during inflammation, the compartmentalized production of dimeric sICAM-1 is related to the expression of membrane ICAM-1 by heterogeneous inflammatory, structural, and neoplastic cells. sICAM-1, within the pleural space, may play a suppressive role in the ICAM-1-dependent interaction between cytotoxic lymphocytes and cancer cells. PleMs play a crucial role in the regulation of this phenomenon by releasing soluble factors capable of upregulating ICAM-1 expression and sICAM-1 release by other pleural effusion cells.

References

1. Springer TA. Adhesion receptors of the immune system. *Nature* 1990;346: 425–434.
2. Miller J, Knorr R, Ferrone M, Houdei R, Carron CP, Dustin ML. Intercellular adhesion molecule-1 dimerization and its consequences for adhesion mediated by lymphocytes function associated-1. *J Exp Med* 1995; 182:1231–1241.
3. Reilly PL Jr, Woska JR, Jeanfavre DD, McNally E, Rothlein R, Bormann BJ. The native structure of intecellular adhesion molecule-1 (ICAM-1) is a dimer: correlation with binding to LFA-1. *J Immunol* 1995;155:529–532.
4. Rothlein RM, Chajkowski M, O'Neill MM, Marlin SD, Mainolfi E, Merluzzi VJ. Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by inflammatory cytokines. *J Immunol* 1988; 141:1665–1670.
5. Becker JC, Dummer R, Hartmann AA, Burg G, Schmidt RE. Shedding of ICAM-1 from human melanoma cell lines induced by IFN-γ and tumor necrosis factor-α: functional consequences on cell mediated cytotoxicity. *J Immunol* 1991;147:4398–4401.
6. van de Stolpe A, van der Saag PT. Intercellular adhesion molecule-1. *J Mol Med* 1996;74:13–33.
7. Grothey A, Heistermann P, Philippou S, Voigtmann R. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1, CD54) in patients with non-small-cell lung cancer: correlation with histological expression of ICAM-1 and tumour stage. *Br J Cancer* 1998;77:801–807.
8. Melis M, Spatafora M, Melodia A, Pace E, Gjomarkaj M, Merendino AM, Bonsignore G. ICAM-1 expression by lung cancer cell lines: effects of upregulation by cytokines on the interaction with LAK cells. *Eur Respir J* 1996;9:1831–1838.
9. Sahn SA. The pleura: state of the art. *Am Rev Respir Dis* 1988;138:184–234.
10. Kroegel C, Antony VB. Immunobiology of pleural inflammation: potential implication for pathogenesis, diagnosis and therapy. *Eur Respir J* 1997;10:2411–2418.
11. Gjomarkaj M, Pace E, Melis M, Spatafora M, Toews GB. Mononuclear cells in exudative malignant pleural effusions: characterization of pleural phagocytic cells. *Chest* 1994;106:1042–1049.
12. Pace E, Gjomarkaj M, Melis M, Profita M, Spatafora M, Vignola AM, Bonsignore G, Mody CH. Interleukin-8 induces lymphocyte chemotaxis into the pleural space: role of pleural macrophages. *Am J Respir Crit Care Med* 1999;159:1592–1599.
13. Light RW, McGregor MI, Luchsinger PC, Ball WC. Pleural effusions: the diagnostic separation of transudates and exudates. *Ann Intern Med* 1972;77:507–513.
14. Queiroz C, Barral-Netto M, Bacchi CE. Characterizing subpopulations

of neoplastic cells in serous effusions: the role of immunocytochemistry. *Acta Cytol* 2001;45:18–22.
15. Comin CE, Novelli L, Boddi V, Paglierani M, Dini S. Calretinina, thrombomodulin, CEA, and CD15: a useful combination of immunohistochemical markers to differentiate pleural epithelial mesothelioma from pulmonary adenocarcinoma. *Hum Pathol* 2001;32:529–536.
16. Rankin JA, Naegel GP, Schrader CE, Matthay RA, Reynolds HY. Airspace immunoglobulin production and levels in bronchoalveolar lavage fluid of normal subjects and patients with sarcoidosis. *Am Rev Respir Dis* 1983;127:442–448.
17. Ritchie R, Alper J, Person GN, Larson C. Automated quantitation of proteins in serum and other biologic fluids. *Am J Clin Pathol* 1973; 59:151–159.
18. Thorne RF, Marshall JF, Shafren DR, Gibsons PG, Hart IR, Burns GF. The integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ physically and functionally associate with CD36 in human melanoma cells. *J Biol Chem* 2000;275:35264–35275.
19. Li CY, Lazcano VO, Pierre RV, Yam LT. Immunocytochemical identification of cells in serous effusions: technical considerations. *Am J Clin Pathol* 1987;88:696–706.
20. Gjomarkaj M, Pace E, Melis M, Spatafora M, Profita M, Vignola AM, Bonsignore G, Toews GB. Phenotypic and functional characterization of normal rat pleural macrophages in comparison with autologous peritoneal and alveolar macrophages. *Am J Respir Cell Mol Biol* 1999; 20:135–142.
21. Altin JG, Pagler EB. A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. *Anal Biochem* 1995;224:382–389.
22. Becker JC, Termeer C, Schmidt RE, Brocker EB. Soluble intercellular adhesion molecule 1 inhibits MHC-restricted specific T cell/tumor interaction. *J Immunol* 1993;151:7224–7232.
23. Parikh HH, Li WC, Ramanathan M. Evaluation of an alternative to the Kolmogorov–Smirnov test for flow cytometric histogram comparisons. *J Immunol Methods* 1999;229:97–105.
24. Hoffmann JC, Kruger H, Luhrs J, Hamm H. Detection of soluble adhesion molecules in pleural effusions. *Chest* 1996;110:107–113.
25. Broaddus VC, Staub NC. Pleural liquid and protein in health and disease. *Semin Respir Med* 1987;9:7–12.
26. Pistolesi M, Miniati M, Giuntini C. Pleural liquid and solute exchange. *Am Rev Respir Dis* 1989;140:815–847.
27. Milani RV, Mehra MR, Endres S, Eigler A, Cooper ES, Jr Lavie CJ, Ventura HO. The clinical relevance of circulating tumor necrosis factor-α in acute decompensated chronic heart failure without cachexia. *Chest* 1996;110:992–995.
28. Jun CD, Shimaoka M, Carman MCV, Takagi J, Springer T. Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion. *Proc Natl Acad Sci USA* 2001;98:6830–6835.
29. Casasnovas JM, Springer TA. Kinetics and thermodynamics of virus binding to receptor: studies with rhinovirus, intercellular adhesion molecule-1 (ICAM-1), and surface plasmon resonance. *J Biol Chem* 1995;270:13216–13224.
30. Weber C, Springer TA. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to $\alpha_{IIb}\beta_3$ and stimulated by platelet-activating factor. *J Clin Invest* 1997;100:2085–2093.
31. Casasnovas JM, Stehle T, Liu JH, Wang JH, Springer TA. A dimeric crystal structure for the N-terminal two domains of intercellular adhesion molecule-1. *Proc Natl Acad Sci USA* 1998;95:4134–4139.
32. Rossi GA, Balbi B, Manca F. Tuberculous pleural effusions: evidence for selective presence of PPD-specific T-lymphocytes at site of inflammation in the early phase of the infection. *Am Rev Respir Dis* 1987;136:575–579.
33. Ishii Y, Kitamura S. Elevated levels of soluble ICAM-1 in serum and BAL fluid in patients with active sarcoidosis. *Chest* 1995;107:1636–1640.
34. Nasreen N, Mohammed KA, Hardwick J, Van Horn RD, Sanders KL, Doerschuk CM, Antony VB. Polar production of interleukin-8 by mesothelial cells promotes the transmesothelial migration of neutrophils: role of intercellular adhesion molecule-1. *J Infect Dis* 2001;183: 1638–1645.
35. Xirouchaki N, Tzanakis N, Bouros D, Kyriakou D, Karkavitsas N, Alexandrakis M, Siafakas NM. Diagnostic value of interleukin-6, and tumor necrosis factor in pleural effusions. *Chest* 2002;121:815–820.
36. Ishii Y, Uchiyama Y, Hasegawa S, Kinoshita T, Mitsui K, Kojima H, Fujita T. Detection of tumour necrosis factor/cachectin in pleural effusion of patients with lung cancer. *Clin Exp Immunol* 1990;80:350–353.
37. Sprenger A, Schardt C, Rotsch M, Zehrer M, Wolf M, Havemann K, Heymanns J. Soluble intercellular adhesion molecule-1 in patients with lung cancer and benign lung diseases. *J Cancer Res Clin Oncol* 1997;123:632–638.