Tumor-Derived Granulocyte-Macrophage Colony-Stimulating Factor and Granulocyte Colony-Stimulating Factor Prolong the Survival of Neutrophils Infiltrating Bronchoalveolar Subtype Pulmonary Adenocarcinoma

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We evaluated the role of the tumor environment in the regulation of apoptosis of tumor-infiltrating neutrophils, the number of which correlates negatively with outcome, in patients with adenocarcinoma of the bronchioloalveolar (BAC) subtype. We examined three different parameters of apoptosis, namely morphological aspect, annexin-V expression, and DNA fragmentation. Bronchoalveolar lavage fluid (BALF) supernatants from patients with BAC significantly inhibited the 24-hour spontaneous apoptosis of normal peripheral blood neutrophils *in vitro* compared to BALF supernatants from control patients ($64 \pm 4\%$ versus $90 \pm 2\%$ measured by annexin-V flow cytometry, $P = 0.04$). The alveolar neutrophil count correlated positively with the granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations in the patient's BALF. Furthermore, neutralizing antibodies (Abs) against GM-CSF and G-CSF significantly inhibited BALF anti-apoptotic activity (15 to 40% and 34 to 63% inhibition, respectively), whereas neutralizing Abs against interleukin (IL)-8, IL-6, IL-1 β and tumor necrosis factor- α had no significant effect. In an attempt to identify the cell origin of anti-apoptotic cytokines, we tested *in vitro* the effect of BAC cells (A549 cell line and primary culture derived from a patient's BAC tumor) on the apoptosis of peripheral blood neutrophils. Cell-free supernatants from tumor

cells did not inhibit neutrophil apoptosis. In contrast, cell-free supernatants from tumor cells previously exposed to conditioned media from peripheral blood mononuclear cells and alveolar macrophages significantly inhibited spontaneous neutrophil apoptosis. This inhibition was partially lifted when conditioned media from mononuclear cells were previously treated with Abs against IL-1 β and tumor necrosis factor- α . As *in vivo*, neutralizing Abs against GM-CSF significantly inhibited the anti-apoptotic activity of cell culture supernatants, and combination with Abs against G-CSF had an additive effect. *In vivo*, GM-CSF and G-CSF were strongly expressed by tumor cells and moderately or not expressed by the normal epithelium, as assessed by immunohistochemical studies. These findings demonstrate that the tumor environment generates local conditions that prolong alveolar neutrophil survival through the production of soluble factors, thereby contributing to the persistence of the neutrophil alveolitis observed in BAC. (*Am J Pathol* 2001, 159:1423-1433)

Lung cancer is the leading cause of cancer-related death in industrialized countries.¹ Its incidence has been increasing for several decades, mainly owing to an increase in peripheral lung adenocarcinoma.² Peripheral lung adenocarcinoma consists of a heterogeneous pattern of bronchioloalveolar (BAC), papillary, acinar, and solid adenocarcinomas rather than a pure histological subtype, although small tumors are mostly of the BAC subtype.^{3,4} The long-term prognosis is uncertain, even in early TNM stages, possibly reflecting different patterns of

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tumor progression.⁵ Indeed, these tumors can either extend outward, with luminal spread and preservation of the elastotic framework, or become invasive with formation of a desmoplastic reaction.^{3,6,7} Other stroma-reaction features, such as high tumor microvessel density and tumor-infiltrating inflammatory cells, have also been evaluated as prognostic factors.^{8–13}

We have previously shown that the presence of increased numbers of tumor-infiltrating neutrophils is linked to poorer outcome in patients with adenocarcinoma of the BAC subtype¹¹ and that tumor cells drive local neutrophil recruitment and activation via C-X-C chemokine release. In the present study, we examined the role of the tumor microenvironment in promoting neutrophil survival and in the persistence of the deleterious neutrophil alveolitis observed in this subtype of adenocarcinoma. We focused our attention on the neutrophil anti-apoptotic activity of tumor-derived cytokines.

Materials and Methods

Clinical Samples

Patients with BAC

Between January, 1989, and January, 1999, BAC was diagnosed in 50 patients who were treated and followed-up in our chest department. The patients were 32 men and 18 women, with a mean age ($\pm$ SD) of 64.8 ± 11 years (range, 34 to 81 years), and comprised 34 smokers and 16 nonsmokers. The diagnosis was based on previously published criteria;^{13,14} bronchoalveolar lavage (BAL) was used as a diagnostic procedure in 41 cases.¹⁵ Briefly, 200 ml of sterile saline in four 50-ml aliquots was infused into the radiologically abnormal segment or lobe. Fluid recovered by gentle suction was pooled, filtered through sterile gauze, and used for total cell counting. Differential cell counts and tumor cell counts were performed on cytopsin preparations stained with modified Wright-Giemsa (Diff-Quick; Dade Behring, Paris, La Défense, France). Bronchoalveolar lavage fluid (BALF) containing $>5\%$ neutrophils was examined for the presence of apoptotic neutrophils (see below). For apoptosis assays and cytokine measurements, the remaining BALF was spun and the supernatant was aseptically removed and stored at -80°C .

Control Group

We used BALF supernatants obtained during a diagnostic procedure from six patients. The control patients were four men and two women aged 61 ± 7 years. Three were smokers. None had a history of neoplastic disease and all had normal results of BALF analysis (see below). Bronchoalveolar lavage was performed in the middle lobe and supernatants were stored at -80°C after centrifugation.

Measurement of 24-Hour Spontaneous Polymorphonuclear Neutrophil (PMN) Apoptosis

PMNs were isolated from peripheral blood of healthy human donors by means of density gradient centrifugation (Polymorphonuclear cell separation media; Eurobio, Les Ulis, France). PMNs were then separated from erythrocytes by hypotonic shock and washed three times in sterile saline. This method yielded $>97\%$ pure PMNs as assessed by May Grünwald Giemsa staining. Normal peripheral venous-blood PMNs at a density of $1 \times 10^6/\text{ml}$ were incubated for 24 hours in Dulbecco's modified Eagle's medium (Gibco, Cergy-Pontoise, France) with 5 mmol/L HEPES, 2 mmol/L L-glutamine, 10^5 units/L penicillin, 100 mg/L streptomycin (referred to as complete medium), and supplemented with 2% fetal bovine serum (FBS). At 24 hours the PMN preparation thus obtained was evaluated for apoptosis by using three different techniques.

Morphological Criteria for Apoptosis

One hundred μl of PMN preparation was cytopspun. Cytospin slides stained with May Grünwald Giemsa were evaluated by two independent investigators by consensus. At least 200 PMNs were graded for apoptosis by using predetermined morphological criteria. PMNs were considered apoptotic if they showed dense condensation of chromatin in the form of either a single nucleus or nuclear fragments not connected by strands.¹⁶ The results are expressed as the percentage of apoptotic PMNs.

Annexin V-Fluos Expression by Flow Cytometry

One μl of PMN preparation was washed once with ice-cold phosphate-buffered saline (PBS) and centrifuged at $200 \times g$ for 5 minutes. The cell pellet was resuspended at a density of 1×10^6 cells/100 μl in labeling buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 5 mmol/L CaCl_2 , pH 7.4) containing 20 $\mu\text{l}/\text{ml}$ of Annexin V-Fluos (Boehringer Mannheim, Meylan, France) and 20 $\mu\text{l}/\text{ml}$ of propidium iodide (50 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich, Saint Quentin Fallavier, France). Cells were incubated for 10 minutes at room temperature in the dark and analyzed within 1 hour by flow cytometry (Elite flow cytometer; Beckman Coulter, Villepinte, France) with gating set on forward scatter and side scatter to identify PMNs and to exclude cell debris.¹⁷ The results are expressed as the percentage of PMNs that met criteria for apoptosis, ie, a strong annexin V-fluorescein isothiocyanate signal and a low propidium iodide signal (early apoptotic PMNs), or a strong annexin V-fluorescein isothiocyanate signal and a strong propidium iodide signal (late apoptotic PMNs).

DNA Fragmentation Analysis

Five μl of PMN preparation was washed once with PBS, gently resuspended in 0.5 ml of lysis buffer (50

mmol/L Tris, 10 mmol/L ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate, and 250 μ g/ml proteinase K), and incubated for 24 hours at 37°C with 5% CO₂. The lysate was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and precipitated with two volumes of cold ethanol and 0.5 volume of 10 mol/L ammonium acetate at 4°C overnight. The DNA pellet was washed once with 75% ethanol, resuspended in 30 μ l of TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L ethylenediaminetetraacetic acid, pH 8.0) containing 250 μ g/ml of RNase (Sigma) and incubated at 65°C for 5 minutes. Then, 0.1 volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was added to each sample, and electrophoresis was performed in 1.2% agarose gel at 50 V for 3 hours. After staining with ethidium bromide, DNA was visualized with UV light and photographed.

Modulation of 24-Hour Spontaneous PMN Apoptosis by Tumor Cells

Effect of Tumor Cell-Conditioned Media

To determine whether tumor cells can modulate PMN apoptosis, 1×10^6 PMNs were incubated for 24 hours in 1 ml of tumor cell-conditioned medium (CM) and evaluated for apoptosis. Tumor cell-CM was obtained with: 1) the A549 cell line (American Type Culture Collection, Rockville, MD) originally established from a person with BAC¹⁸ and cultured in complete medium (see above) supplemented with 10% FBS; and 2) a primary culture (first to third passage) of tumor cells obtained from a pleural effusion specimen recovered from a patient with BAC, as previously described by Oie and colleagues.¹⁹ Briefly, pleural fluid was centrifuged and pleural cells (tumor cells, 65%; inflammatory cells, 35%) were resuspended in Dulbecco's modified Eagle's medium and separated by density gradient centrifugation (Lymphocytes separation medium, MSL1077; Eurobio, Les Ulis, France). The interface layer containing tumor cells was harvested, washed twice in Dulbecco's modified Eagle's medium, and seeded into 25-cm² flasks in complete medium with 10% FBS. The purity of the tumor cells thus obtained was verified by May Grünwald Giemsa staining. Then, cultured cells were routinely passaged every 7 to 15 days. Tumor cells, ie, A549 or primary BAC cells, were resuspended at a density of 1×10^5 /ml in complete medium with 2% FBS and cultured in 24-well plates (500 μ l/well) for 72 hours at 37°C. The medium was then harvested and confluent monolayers of tumor cells were exposed to complete medium with 2% FBS or to CM from inflammatory cells, ie, peripheral blood mononuclear cells (PBMCs), PMNs, and alveolar macrophages (AMs), prepared as described below. Cell-free supernatants were collected after 24 hours of incubation and stored at -80°C.

To obtain inflammatory cell-CM, PBMCs and PMNs were recovered from peripheral blood of healthy donors by density gradient centrifugation (Polymorphonuclear cell separation medium, Eurobio). Briefly, PMNs were

separated as above. The PBMC layer was washed three times in sterile saline solution. PBMCs comprised $65 \pm 10\%$ lymphocytes and $35 \pm 13\%$ monocytes as evaluated by flow cytometry. AMs were isolated from BALF of controls (see above). AMs accounted for >90% of BALF cells (May Grünwald Giemsa staining). Cells were separated from the remaining BALF by centrifugation. PBMCs, PMNs, and AMs were resuspended at a density of 5×10^5 /ml in complete medium with 2% FBS, then plated in 24-well plates (500 μ l/well) and cultured at 37°C in humidified 5% CO₂/95% air. After 24 hours, inflammatory cell-CM was collected and stored at -80°C.

Effect of BALF from BAC Patients

One million PMNs were incubated for 24 hours in 1 ml of BALF supernatant from patients and controls at a final concentration of 50 to 90% in complete medium with 2% FBS, and were then examined for apoptosis.

Neutralization Studies

Conditioned media and BALF supernatants were pre-incubated for 45 minutes with several goat polyclonal neutralizing antibodies (Abs) directed against human 1) granulocyte-macrophage colony-stimulating factor (GM-CSF) (1:250 dilution; R&D Systems, Abington, UK), 2) granulocyte colony-stimulating factor (G-CSF) (1:125; R&D Systems), 3) interleukin (IL)-8 (1:20; R&D Systems), 4) IL-6 (1:200; R&D Systems), 5) tumor necrosis factor- α (TNF- α) (1:250; Genzyme Diagnostics, Cergy Saint-Christophe, France), 6) IL-1 β (1:100; R&D Systems) and with irrelevant control goat polyclonal Abs (R&D systems). PMNs were then incubated for 24 hours at 37°C in Ab-treated CM or BALF supernatants and analyzed by means of flow cytometry. The results are expressed as the percentage inhibition of anti-apoptotic activity defined as follows:

$$\frac{(\% \text{ of apoptotic PMN in Ab-treated CM}) - (\% \text{ of apoptotic PMN in control Ab-treated CM})}{(\text{antiapoptotic activity of CM})} \times 100$$

in which the anti-apoptotic activity of CM was:

$$(\% \text{ of apoptotic PMN in basal conditions}) - (\% \text{ of apoptotic PMN in control Ab-treated CM})$$

Enzyme-Linked Immunosorbent Assay (ELISA) Measurements

GM-CSF, G-CSF, IL-8, IL-6, IL-1 β , and TNF- α concentrations were measured in CM and BALF supernatants by using commercially available ELISA assays (R&D Systems). The ELISA kits were used as indicated by the manufacturer and consistently detected GM-CSF, G-CSF, IL-8, IL-6, TNF- α , and IL-1 β concentrations >3, 20, 10, 0.7, 4.4, and 1 pg/ml, respectively, in linear manner.

Immunohistochemical Studies of GM-CSF and G-CSF Expression in BAC and Normal Pulmonary Tissue

We examined tumoral and distant normal pulmonary tissues from three patients with BAC. Tissue fragments were immediately frozen in liquid nitrogen and stored at -80°C . Sections $4\text{-}\mu\text{m}$ thick were fixed in acetone and reacted with appropriate dilutions of monoclonal anti-human GM-CSF (Genzyme) and G-CSF Abs (R&D Systems). Isotype-matched Abs were used as controls (MOPC 21, IgG1; Sigma, Saint Louis, MO).

Positive cells were revealed by using the Vectastain ABC-alkaline phosphatase kit (Vector, Burlingame, CA) and Fast Red substrate. To test the specificity of immunostaining, Abs were omitted or replaced by an isotype-matched control. No positive cells were identified in these conditions. The intensity of immunostaining was graded from $-$ (absent) to $+++$ (strongly positive). Complete agreement was obtained between the two independent observers.

Statistical Analysis

Spearman's ρ coefficient was used for correlation studies between quantitative variables. Comparisons were made using the Mann-Whitney nonparametric test for unpaired data and the Wilcoxon nonparametric test for paired data. Statistical analysis was performed using one-way analysis of variance for parametric data sets. Data are presented as means $\pm$ SEM. P values of <0.05 were considered to denote statistical significance.

Results

Inhibition of Spontaneous PMN Apoptosis by A549 Cells

Conditioned media from unstimulated A549 tumor cells did not inhibit constitutive PMN apoptosis at 24 hours. Numerous PMNs showed morphological characteristics of apoptotic cells, such as condensed nuclei and apoptotic bodies, which were absent at T0 in cytospin preparations (Figure 1, A and C). This phenomenon was accompanied by the emergence of annexin V labeling (Figure 1, B and D) and DNA fragmentation (Figure 2). In contrast, cell-free supernatants from A549 cells exposed to CM from PBMCs or AMs inhibited 24-hour constitutive PMN apoptosis. In these conditions, PMNs were morphologically normal (Figure 1, E and G). Flow cytometry showed the loss of annexin V labeling (Figure 1, F and H), and DNA electrophoresis showed a decrease in DNA fragmentation (Figure 2). Cell-free supernatants from A549 cell cultures exposed to PMN-CM did not inhibit 24-hour constitutive PMN apoptosis (Figure 1, I and J, and Figure 2).

To quantify this inhibition, apoptotic PMNs were counted using morphological criteria and flow cytometry (Table 1). Based on morphological criteria, the proportion

Figure 1. Effect of A549 cell line-CM on PMN apoptosis *in vitro*. PMNs were evaluated for apoptosis by morphological analysis (**left**; original magnification, $\times 400$) and annexin-V labeling (**right**) at T0 and after a 24-hour incubation in cell-free supernatant from A549 cells previously exposed to CM from PBMCs, AMs, and PMNs, as described in Materials and Methods. At T0, PMNs had a normal morphology with a polysegmented nucleus (**A**) and appear in quadrant 3 (low annexin V-fluorescein isothiocyanate signal and low propidium iodide signal) (**B**). At 24 hours, PMNs were apoptotic, with dense condensation of chromatin in the form of either a single nucleus or nuclear fragments not connected by strands (**C**, **arrows**) and appeared in quadrant 1 (**D**) (high annexin V-fluorescein isothiocyanate signal but low propidium iodide signal). Cell-free supernatant from A549 cell line cultured with PBMC-CM (**E** and **F**) or AM-CM (**G** and **H**) inhibited PMN apoptosis, whereas cell-free supernatant from A549 cell line cultured with PMN-CM (**I** and **J**) did not. This figure is representative of 10 experiments.

of apoptotic PMNs fell significantly from $40 \pm 9\%$ with A549-CM to $7 \pm 2\%$ ($P < 0.01$) and $2 \pm 1\%$ ($P < 0.01$) with cell-free supernatants from A549 cells exposed to PBMC-CM and AM-CM, respectively. Based on flow cytometry, the proportion of apoptotic PMNs fell significantly from $88 \pm 2\%$ to $27 \pm 7\%$ ($P < 0.01$) and $23 \pm 3\%$ ($P < 0.01$) in the same conditions (Table 1). As shown in

Figure 2. Effect of A549 cell line-CM on PMN apoptosis *in vitro*. Electrophoresis of DNA extracted from normal PMNs showed fragmentation, ie, the ladder-like appearance of smaller fragments characteristic of apoptosis, when PMNs were incubated for 24 hours in cell-free supernatant from A549 cells (lane 3); absent at T0 (lane 2). At T24, cell-free supernatant from A549 cells cultured in presence of medium conditioned by PBMCs (lane 4) or AMs (lane 5), but not PMNs (lane 6), inhibited PMN apoptosis. Lane 1: Molecular weight marker. This figure is representative of five experiments.

Figure 3, the observed anti-apoptotic activity was predominantly related to tumor cell release, even if PBMC or AM release also contributed to the effect (Figure 3A). Indeed, analysis of dose-response curves obtained with the different CM (Figure 3, B and C) showed that the anti-apoptotic activity derived from A549 cells stimulated by mononuclear cell-CM was respectively 10-fold and fourfold higher than that derived from PBMC-CM and AM-CM alone.

Finally, we performed neutralization experiments against IL-1 β and TNF- α to investigate their role in the ability of mononuclear cells-CM, ie, PBMCs and AMs, to induce neutrophil anti-apoptotic activity from A549-cells. As shown in Figure 4, the proportion of apoptotic PMNs increased significantly from $34 \pm 6\%$ to $66 \pm 2\%$ ($P < 0.01$) when anti-IL-1 β and anti-TNF- α Abs were previously added to PBMC-CM (Figure 4). Similarly, this pro-

Figure 3. PMN anti-apoptotic activity from A549 cells exposed to mononuclear cell-CM. The effect of cell-free supernatants on PMN apoptosis *in vitro* was measured by flow cytometry (A). As described in Materials and Methods, PMNs were incubated for 24 hours with CM from A549 cells (open bars), PBMCs, or AMs (hatched bars), and also with cell-free supernatant from A549 cells previously exposed to CM from PBMCs or AMs (filled bars). Results expressed as percentage of apoptotic PMNs are means $\pm$ SEM of six experiments. Comparisons were made using the Wilcoxon nonparametric test. Dose-response curves (B and C) performed with PBMC-CM (hatched circles), AM-CM (hatched squares), and cell-free supernatant from A549 cells previously exposed to CM from PBMCs (filled circles) or AMs (filled squares) used at different concentrations (from pure to 1/10); each point represents the mean of two experiments.

portion increased from $28 \pm 1\%$ to $51 \pm 3\%$ ($P < 0.01$) when anti-IL-1 β and anti-TNF- α Abs were added to AM-CM (Figure 4). Interestingly, the inhibition obtained with anti-IL-1 β and anti-TNF- α Abs was associated with a $>90\%$ reduction in G-CSF and GM-CSF production by A549 cells (data not shown). The fact that A549 cells did not release detectable amounts of IL-1 β or TNF- α , either constitutively or after exposure to mononuclear cell-CM (Table 2), eliminated the possibility of autocrine IL-1 β and TNF- α production by A549 cells.

Inhibition of Spontaneous PMN Apoptosis by A549 Cell-Derived Cytokines

To determine whether cytokines were responsible for the anti-apoptotic activity of cell-free supernatants from A549 cells, concentrations of cytokines known to be involved in neutrophil survival were determined in cell culture supernatants by using specific ELISAs. As shown in Table 2, unstimulated A549 tumor cells constitutively released moderate amounts of G-CSF, IL-8, and IL-6, but did not

Table 1. Measurement of PMN Apoptosis at 24 Hours

	Morphologic criteria	Annexin-V labeling
A549-CM	40 $\pm$ 9	88 $\pm$ 2
A549 + PBMC-CM	7 $\pm$ 2*	27 $\pm$ 7*
A549 + AM-CM	2 $\pm$ 1*	23 $\pm$ 3*
A549 + PMN-CM	35 $\pm$ 9	87 $\pm$ 2

PMN were isolated from whole blood and then incubated for 24 hours in cell-free supernatant from A549 cells cultured with or without CM from PBMCs, AMs, or PMNs as described in Materials and Methods. The data are the mean $\pm$ SEM percentage of cells with an apoptotic morphology or Annexin-V labeling. NS, not significant.

* $P < 0.05$ in the Mann-Whitney test, between PMNs cultured in A549-CM and A549 exposed to PBMC-CM or AM-CM. This figure is representative of 10 experiments.

Figure 4. Role of IL-1 β and TNF- α in the induction of PMN anti-apoptotic activity from A549 cells exposed to mononuclear cell-CM. The effect of A549 cell-free supernatants on PMN apoptosis *in vitro* was measured by flow cytometry. As described in Materials and Methods, PMNs were incubated for 24 hours with CM from A549 cells (open bars), cell-free supernatant from A549 cells previously exposed to CM from PBMCs, or AMs (filled bars) and IL-1 β - and TNF- α -neutralized PBMC-CM or AM-CM (hatched bars). Results (expressed as percentage of apoptotic PMNs) are means $\pm$ SEM of three experiments. Comparisons were made using the Wilcoxon test.

detectably release GM-CSF, TNF- α , or IL-1 β . PBMC-CM and AM-CM induced GM-CSF release and also enhanced G-CSF and IL-6 release. Contrary to AM-CM, PBMC-CM enhanced IL-8 release by A549 cells.

We then used neutralizing Abs to examine the relative contributions of these cytokines to the anti-apoptotic activity of A549 cells. Neutralizing Abs to G-CSF, IL-6, IL-8, IL-1 β , and TNF- α , used alone, had no significant effect on the anti-apoptotic activity of cell-free supernatants from A549 cells stimulated with PBMC-CM (data not shown). As shown in Figure 5, top, anti-GM-CSF Ab inhibited this anti-apoptotic activity by 21% when used alone and by 51% when combined with anti-G-CSF Abs. The inhibition induced by anti-GM-CSF was not enhanced by simultaneous addition of each of the other anti-cytokine Abs (data not shown). Neutralizing Abs to G-CSF, IL-6, IL-1 β , and TNF- α had no individual effect on the anti-apoptotic activity of cell-free supernatants from A549 cells stimulated with AM-CM (data not shown). Anti-GM-CSF and anti-IL-8 Abs inhibited anti-apoptotic activity by 35% and 21%, respectively, when used separately, and by 58% when combined (Figure 5, bottom). However, the IL-8

Figure 5. Role of A549 cell-derived GM-CSF and G-CSF on PMN apoptosis. Cell-free supernatants from A549 cells exposed to CM from PBMCs (top) or AMs (bottom) were preincubated with neutralizing polyclonal goat anti-human Abs against GM-CSF, G-CSF, or IL-8, used alone or in combination. PMNs were then incubated for 24 hours at 37°C in Ab-treated cell culture medium and analyzed for apoptosis by flow cytometry. Results (expressed as anti-apoptotic activity, see Materials and Methods) are means $\pm$ SEM of six experiments. Comparisons were made using the Wilcoxon test.

effect was related to AM release rather than to tumor cell release (Table 2).

Inhibition of PMN Apoptosis in Patients with BAC

The mean percentage of neutrophils in BALF was 33 $\pm$ 5% in patients with BAC and only 1.2 $\pm$ 0.2% in controls ($P = 0.03$). In the 29 BALF samples showing neutrophil alveolitis exceeding 5%, the mean proportion of apoptotic PMNs was 11 $\pm$ 2% (median, 7%; range, 0.5 to 43%). In this group, the degree of PMN alveolitis correlated negatively with the percentage of apoptotic PMNs, as shown in Figure 6A ($P = 0.024$).

Table 2. Cytokine Levels in Culture Supernatants

	GM-CSF (pg/ml)	G-CSF (pg/ml)	IL-8 (ng/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)	IL-1 β (pg/ml)
A549	ND	73 $\pm$ 8	0.5 $\pm$ 0.1	14 $\pm$ 4	ND	ND
PBMC-CM	70 $\pm$ 39	145 $\pm$ 31	27 $\pm$ 6	263 $\pm$ 89	342 $\pm$ 152	185 $\pm$ 113
A549 + PBMC-CM	227 $\pm$ 44	1777 $\pm$ 254	169 $\pm$ 22	2983 $\pm$ 1185	112 $\pm$ 42	152 $\pm$ 91
AM-CM	9 $\pm$ 3	106 $\pm$ 13	117 $\pm$ 41	451 $\pm$ 120	661 $\pm$ 384	1 $\pm$ 1
A549 + AM-CM	38 $\pm$ 18	194 $\pm$ 47	128 $\pm$ 41	1948 $\pm$ 506	227 $\pm$ 122	0.6 $\pm$ 0.6
<i>P</i> value*	0.04	0.06	0.04	0.06	0.06	0.28
<i>P</i> value†	0.01	0.10	0.86	0.01	0.10	0.10

Cytokine concentrations were measured by ELISA in CM from A549 cells, PBMCs, AMs, and A549 cells previously cultured with PBMC-CM or AM-CM. Results are means $\pm$ SEM. ND, not detectable. Wilcoxon's test was used to compare cytokine concentrations in PBMC-CM versus A549 + PBMC-CM (*) and AM-CM versus A549 + AM-CM (†). This figure is representative of 10 experiments.

Figure 6. Alteration of PMN apoptosis in patients with bronchioloalveolar carcinoma. **A:** Correlation between the percentage of apoptotic PMNs and the neutrophil count in BALF from patients with bronchioloalveolar carcinoma (BAC). Among the 41 patients with BAC, 29 had a neutrophil count $>5\%$ and could be evaluated for *in vivo* apoptosis by using morphological criteria, as described in Materials and Methods. Correlations were performed using Spearman's ρ coefficient. **B:** Effect of BALF supernatant on PMN apoptosis *in vitro*. PMNs were incubated for 24 hours with BALF supernatant from controls ($n = 6$) or patients ($n = 14$) with BAC, and evaluated for apoptosis by flow cytometry. Comparisons were made using the Mann-Whitney test.

Inhibition of PMN Apoptosis by BALF from BAC Patients

To determine whether BALF supernatants from BAC patients inhibited neutrophil apoptosis, we incubated normal peripheral blood PMNs for 24 hours in BALF supernatants from patients ($n = 14$) and controls ($n = 6$) at a final concentration of 50% in complete media. These BAL samples were selected on the basis of availability, with no knowledge of neutrophil alveolitis or cytokine

Figure 7. Role of BALF GM-CSF and G-CSF on PMN apoptosis *in vitro*. BALF from six patients (patient 1 to patient 6) with bronchioloalveolar carcinoma (BAC) was preincubated with neutralizing polyclonal goat anti-human Abs against G-CSF (open bars), GM-CSF (hatched bars), or both (filled bars). PMNs were then incubated for 24 hours at 37°C with Ab-treated BALF and analyzed for apoptosis by flow cytometry. Results (expressed as percent inhibition of anti-apoptotic activity, see Materials and Methods) are means of two different experiments.

concentrations. As shown in Figure 6B, BALF from the patients inhibited PMN apoptosis relative to BALF from controls (apoptotic PMNs: $64 \pm 4\%$ versus $90 \pm 2\%$, $P = 0.04$). This anti-apoptotic effect was concentration-dependent, as the proportion of apoptotic PMNs reached $49 \pm 5\%$ ($n = 4$), when BALF from the patients was used at a final concentration of 90%.

To evaluate whether the anti-apoptotic effects of BAC BALF depended on the cytokines previously shown to be involved in neutrophil survival *in vitro*, cytokine concentrations were determined using ELISA methods. As shown in Table 3, only G-CSF was detectable in BALF from controls, whereas all of the cytokines were detected in BALF from the patients; in addition, G-CSF concentrations were significantly higher in BALF from patients than controls. Only GM-CSF concentrations correlated with BALF anti-apoptotic activity ($P = 0.01$, $n = 14$). However, a positive correlation was also noted between the BALF G-CSF concentration and the total neutrophil count ($P = 0.01$, $n = 14$) and the neutrophil percent ($P = 0.01$, $n = 14$), as well as between the BALF GM-CSF concentration and the neutrophil percent ($P = 0.05$, $n = 14$).

To determine the contribution of the above cytokines to the anti-apoptotic activity of patient's BALF, we added neutralizing Abs to the six BALFs with the highest anti-apoptotic activity (30 to 60% inhibition). Neutralizing Abs to IL-8, IL-6, IL-1 β , and TNF- α had no significant effect. Combinations of anti-cytokine Abs did not further enhance PMN apoptosis (data not shown). By contrast,

Table 3. Cytokine Levels in BAL Fluid Supernatants from BAC Patients and Controls

	GM-CSF (pg/ml)	G-CSF (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)	IL-1 β (pg/ml)
Patients ($n = 14$)	2.5 [0–353]	88 [0–566]	219 [0–2612]	55 [0–375]	2 [0–21]	1.4 [0–86]
Controls ($n = 6$)	ND	9 [0–22]	ND	ND	ND	ND
<i>P</i> value	0.01	0.01	<0.01	<0.01	0.02	0.02

Cytokine concentrations were measured by ELISA in bronchoalveolar lavage fluid supernatants from patients with bronchioloalveolar carcinoma and from healthy controls. Results are medians and ranges. ND, not detectable. *P* value calculated by the Mann-Whitney test.

Figure 8. PMN anti-apoptotic activity of primary cultured tumor cells from a patient with BAC (BAC cells), exposed to mononuclear cell-CM. The effect of cell-free supernatants on PMN apoptosis *in vitro* was measured by flow cytometry. As described in Materials and Methods, PMNs were incubated for 24 hours with CM from BAC cells (open bars), PBMCs, or AMs (hatched bars), and also with cell-free supernatant from BAC cells previously exposed to CM from PBMCs or AMs (filled bars). Results (expressed as percentage of apoptotic PMNs) are means $\pm$ SEM of three experiments. Comparisons were made using the Wilcoxon *n* test.

anti-GM-CSF and anti-G-CSF Abs inhibited 15 to 40% and 34 to 63% of the BALF anti-apoptotic activity, respectively, with an additive effect of the two Abs used in combination (Figure 7). Moreover, the observed levels of G-CSF and GM-CSF in these BALF (65 to 566 pg/ml and 10 to 353 pg/ml, respectively, Figure 7) were in the range of concentrations of the corresponding recombinant cytokines (50 to 2000 pg/ml G-CSF and 5 to 200 pg/ml GM-CSF) preventing 24-hour neutrophil apoptosis *in vitro* (data not shown).

Inhibition of PMN Apoptosis by Primary Culture Supernatants of Tumor Cells from a BAC Patient

To confirm observations with the A549 cell line, we used tumor cells directly derived from the lung tumor of a patient with BAC (BAC cells). As shown in Figures 8 and 9, results with BAC cells were similar to those obtained with A549 cells (Figures 3 and 4). First, the proportion of apoptotic PMNs fell significantly from $82 \pm 3\%$ with BAC-CM to $20 \pm 6\%$ ($P < 0.01$) and $18 \pm 6\%$ ($P < 0.01$) with cell-free supernatants from BAC cells exposed to PBMC-CM and AM-CM, respectively. Second, neutralizing Abs against GM-CSF and G-CSF significantly inhibited the anti-apoptotic activity of cell-free supernatants from BAC cells exposed to PBMC-CM or AM-CM, with an additive effect of the two Abs used in combination, whereas neutralizing Abs against IL-8 had a significant effect only on the cell-free supernatant of BAC cells exposed to AM-CM (Figure 9).

Cellular Origin of GM-CSF and G-CSF in the Tumor Environment

To determine the cellular origin of GM-CSF and G-CSF in the tumor environment *in vivo*, we performed an immu-

Figure 9. Role of G-CSF and GM-CSF produced by primary cultured tumor cells from a patient with BAC (BAC cells) in PMN apoptosis. Effect of neutralizing antibodies on apoptosis *in vitro*. Cell-free supernatants from BAC cells exposed to CM from PBMCs (top) or AMs (bottom) were preincubated with neutralizing polyclonal goat anti-human Abs against GM-CSF, G-CSF, or IL-8, used alone or in combination. PMNs were then incubated for 24 hours at 37°C in Ab-treated cell culture and analyzed for apoptosis by flow cytometry. Results (expressed as anti-apoptotic activity, see Materials and Methods) are means $\pm$ SEM of three experiments. Comparisons were made using the Wilcoxon test.

nohistochemical study of both tumoral and adjacent normal lung tissue (Figure 10). The results obtained with three different specimens were similar. Tumor cells strongly expressed GM-CSF (+++), whereas interstitial mononuclear cells (++), AMs (++), and resident cells (++) expressed it less intensely. G-CSF was also strongly expressed by tumor cells and less intensely by interstitial mononuclear cells (++) and AMs (++) but not by resident cells. The expression of the two cytokines was homogenous and cytoplasmic. In normal lung tissue distant from the tumor, GM-CSF was also expressed by normal epithelial cells (++) and interstitial mononuclear cells (+), whereas G-CSF was only expressed by bronchiolar cells (+).

Discussion

BALF supernatants from patients with BAC protected peripheral blood neutrophils from spontaneous apoptosis in a concentration-dependent manner. No such effect was observed with BALF supernatants from controls. Significant anti-apoptotic activity was observed with all but one of the patients' BALF supernatants. This anti-apoptotic effect might have been underestimated, as the epithelial lining fluid recovered by BAL is usually 100-fold diluted.²⁰ Several soluble factors with neutrophil anti-apoptotic activity, such as cytokines, complement factor

Figure 10. GM-CSF and G-CSF expression in bronchioloalveolar carcinoma *in vivo*. Histological analysis and immunohistochemical evaluation of cytokine expression in tumor and adjacent normal tissue obtained by surgical excision of the right upper lobe of a patient with bronchioloalveolar carcinoma (BAC). **A:** H&S-stained paraffin section showing the typical aspect of a BAC tumor; note the presence of neutrophils at the luminal pole. **B:** Negative control using nonimmune mouse IgG on tumor tissue. **C–H:** Immunostaining using monoclonal Abs and the Vectastain ABC-alkaline phosphatase technique. **C:** Immunodetection of GM-CSF in tumor tissue showing that tumor cells are strongly positive (+++). **D:** Immunodetection of G-CSF in tumor tissue showing that tumor cells are also strongly positive for this cytokine (+++). **E and F:** Immunodetection of GM-CSF in normal tissue distant from the tumor, showing that this factor is moderately expressed in normal lung and mostly produced by bronchiolar epithelium (++). **G and H:** Immunodetection of G-CSF in normal tissue distant from the tumor; only normal bronchiolar cells express the cytokine (+). Original magnifications: $\times 200$ (**A**, **C**, **G**, and **H**); $\times 125$ (**B** and **D** to **F**). al, Alveolar lumen; bl, bronchiolar lumen.

5a, or eicosanoids^{16,21,22} were probably contained in BALF supernatants from the patients but not the controls. We focused on epithelium-derived cytokines, and especially those known to have neutrophil anti-apoptotic activity *in vitro*, namely GM-CSF, G-CSF, IL-1 β , IL-8, IL-6, and TNF- α .^{21,23–26} All these cytokines were present in patients' BALF, whereas only G-CSF (at a significantly lower concentration) was found in controls. Importantly, G-CSF and GM-CSF concentrations in patients correlated with the intensity of neutrophil alveolitis, and GM-CSF concentrations also correlated with neutrophil anti-apoptotic activity. Both GM-CSF and G-CSF seem to be involved in the anti-apoptotic effect, because Abs against the two cytokines significantly and independently inhibited BALF supernatant anti-apoptotic activity. Our use of peripheral blood neutrophils instead of alveolar neutro-

phils could limit the *in vivo* relevance of these findings. Indeed, endothelial transmigration by neutrophils could modulate their sensitivity to apoptosis, through adhesion molecule engagement.²⁷ We thus counted apoptotic alveolar neutrophils in BALF cytospin preparations from patients with BAC. Interestingly, we found a negative correlation between the degree of alveolar neutrophil apoptosis and that of neutrophil alveolitis, suggesting that an alteration of programmed neutrophil death might contribute to the persistence of neutrophil alveolitis *in vivo*.

In an attempt to mimic the tumor environment and identify the respective roles of tumor cells and inflammatory cells in the prolongation of neutrophil survival, we tested the effect of A549 cell-derived cytokines *in vitro* on the 24-hour spontaneous apoptosis of peripheral blood neutrophils after culturing A549 cells under basal condi-

tions and with media conditioned by several types of inflammatory cells. Although A549 cells constitutively released several of the anti-apoptotic cytokines tested (G-CSF, IL-8, and IL-6), A549 cell CM obtained under basal conditions did not prevent neutrophil apoptosis. By contrast, CM of A549 cells exposed to inflammatory cells (PBMcs and AMs) inhibited 24-hour spontaneous neutrophil apoptosis by 68% and 78%, respectively. This phenomenon was also observed when primary culture of BAC cells directly derived from lung tumors was used instead of the long-term established A549 cell line. Media directly conditioned by PBMcs and AMs also had anti-apoptotic activity, but less than tumor cells.

As in *in vivo* experiments, only tumor-derived GM-CSF and G-CSF were clearly involved in the *in vitro* prolongation of neutrophil survival. IL-8 had an additive effect on GM-CSF in the AM model but was released by AMs and not by tumor cells. Moreover, the similar pattern of results obtained with A549 cells and primary cultured BAC cells directly derived from lung tumor, as well as with patients' BALF supernatants, strongly supports the *in vivo* relevance of our *in vitro* findings. Unlike Daffern and colleagues,²⁸ we found that IL-6 did not contribute to the anti-apoptotic activity of tumor epithelial cells. However, these latter authors used nasal-derived primary epithelial cells previously stimulated by high concentrations of recombinant TNF- α .²⁸ In the same way, IL-1 β and TNF- α did not play a role in neutrophil survival in our study, but these cytokines were not produced by the A549 cell line whatever the stimulus used. By contrast, we showed that PBMcs and AMs derived IL-1 β and TNF- α contribute to the release of neutrophil anti-apoptotic factors (GM-CSF and G-CSF) by tumor epithelial cells, as previously suggested by other studies.^{28–30} Furthermore, TNF- α up-regulation of GM-CSF receptor expression on PMNs might also indirectly contribute to this phenomenon.³¹ Finally, the fact that anti-GM-CSF and anti-G-CSF Abs did not completely abrogate the anti-apoptotic effect of tumor cell CM suggests that cytokines or soluble factors not studied here could partly explain our findings.^{21,28}

To examine the respective roles of tumor cells and tumor-infiltrating inflammatory cells in the production of anti-apoptotic factors *in vivo*, we then evaluated G-CSF and GM-CSF protein expression in specimens of tumorous and distant normal pulmonary tissue from patients with BAC. We observed strong expression of both cytokines by tumor cells. GM-CSF, but not G-CSF, was strongly expressed by the tumor-associated stroma reaction thickening the tumor alveolar wall. In distant normal lung tissue, GM-CSF was detected in epithelial cells as well as in monocytes/macrophages, fibroblasts, and endothelial cells, whereas G-CSF was only detected in bronchial cells. However, immunostaining of these two cytokines was less intense in distant normal lung than in tumoral areas. The accumulation of GM-CSF and G-CSF in tumoral areas, demonstrated by immunochemical studies and BALF cytokine measurements, suggest a possible up-regulation of GM-CSF and G-CSF production by the tumor microenvironment. Our *in vitro* results support the hypothesis that mononuclear cells such as AMs and PBMcs, which have close contacts with tumor cells

in vivo,^{11,13} might contribute to this cell's previously reported paracrine feedback loop between tumoral and inflammatory.^{32–35} It is also reasonable to suppose that mononuclear cell-derived TNF- α and IL-1 β influence GM-CSF and G-CSF production by tumoral epithelia *in vivo*.

Although the mechanisms by which neutrophils influence the prognosis of BAC is unclear, it has been postulated that the persistence of neutrophil alveolitis would result in persistent release of proinflammatory mediators such as cytokines, proteases, and reactive oxygen and nitrogen species that can damage DNA and activate oncogenes.^{36,37} We have also previously postulated that neutrophils might be involved in luminal tumor spread by promoting tumor cell shedding.¹¹ In addition, GM-CSF itself could be involved in tumor progression through its proliferative effect on type II alveolar epithelial cells, possible precursors of BAC.^{38,39} Lastly, *in vivo* alterations of phagocytic clearance of apoptotic neutrophils also results in a lack of release of anti-inflammatory mediators such as transforming growth factor- β ,^{40,41} which functions as a classic tumor suppressor.⁴²

In conclusion, we demonstrate that the BAC tumor microenvironment generates local conditions that prolong alveolar neutrophil survival through the production of soluble factors, and thereby contributes to the persistence of the deleterious neutrophil alveolitis observed in this setting. We also provide additional evidence of possible cooperation between tumor and inflammatory cells in tumor progression, possibly warranting clinical trials of local anti-inflammatory adjuvant therapy.

References

1. Davis DL, Hoel D, Fox J, Lopez A: International trends in cancer mortality in France, West Germany, Italy, Japan, England and Wales, and the USA. *Lancet* 1990, 336:474–481
2. Auerbach O, Garfinkel L: The changing pattern of lung carcinoma. *Cancer* 1991, 68:1973–1977
3. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, Shimasato Y: Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995, 75:2844–2852
4. Roggli VL, Vollmer RT, Greenberg SD, McGavran MH, Spjut HJ, Yesner R: Lung cancer heterogeneity: a blinded and randomized study of 100 consecutive cases. *Hum Pathol* 1985, 16:569–579
5. Postoperative T1 N0 non-small cell lung cancer: squamous versus nonsquamous recurrences. The Lung Cancer Study Group. *J Thorac Cardiovasc Surg* 1987, 94:349–354
6. Eto T, Suzuki H, Honda A, Nagashima Y: The changes of the stromal elastotic framework in the growth of peripheral lung adenocarcinomas. *Cancer* 1996, 77:646–656
7. Shimosato Y, Suzuki A, Hashimoto T, Nishiwaki Y, Kodama T, Yoneyama T, Kameya T: Prognostic implications of fibrotic focus (scar) in small peripheral lung cancers. *Am J Surg Pathol* 1980, 4:365–373
8. Fontanini G, Lucchi M, Vignati S, Mussi A, Ciardiello F, De Laurentiis M, De Placido S, Basolo F, Angeletti CA, Bevilacqua G: Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J Natl Cancer Inst* 1997, 89:881–886
9. Takanami I, Takeuchi K, Kodaira S: Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology* 1999, 57:138–142
10. Lee TK, Horner RD, Silverman JF, Chen YH, Jenny C, Scarantino CW: Morphometric and morphologic evaluations in stage III non-small cell lung cancers. Prognostic significance of quantitative assessment of infiltrating lymphoid cells. *Cancer* 1989, 63:309–316
11. Bellocq A, Antoine M, Flahault A, Philippe C, Crestani B, Bernaudin

- JF, Mayaud C, Milleron B, Baud L, Cadranet J: Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome. *Am J Pathol* 1998, 152:83-92
12. Takise A, Kodama T, Shimosato Y, Watanabe S, Suemasu K: Histopathologic prognostic factors in adenocarcinomas of the peripheral lung less than 2 cm in diameter. *Cancer* 1988, 61:2083-2088
13. Tosi P, Sforza V, Santopietro R, Lio R, Gotti G, Paladini P, Cevenini G, Barbini P: Bronchiolo-alveolar carcinoma: an analysis of survival predictors. *Eur J Cancer* 1992, 28:1365A-1370A
14. Barsky SH, Cameron R, Osann KE, Tomita D, Holmes EC: Rising incidence of bronchioloalveolar lung carcinoma and its unique clinicopathologic features. *Cancer* 1994, 73:1163-1170
15. Springmeyer SC, Hackman R, Carlson JJ, McClellan JE: Bronchiolo-alveolar cell carcinoma diagnosed by bronchoalveolar lavage. *Chest* 1983, 83:278-279
16. Lee A, Whyte MK, Haslett C: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 1993, 54:283-288
17. Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingersperger CP, Roos D: Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood* 1995, 85:532-540
18. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 1976, 17:62-70
19. Oie HK, Russell EK, Carney DN, Gazdar AF: Cell culture methods for the establishment of the NCI series of lung cancer cell lines. *J Cell Biochem* 1996, 24:S24-S31
20. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, Weiner-Kronish J, Sticherling M, Christophers E, Matthay MA: Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am Rev Respir Dis* 1992, 146:427-232
21. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 1992, 80:2012-2020
22. Hebert MJ, Takano T, Holthofer H, Brady HR: Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxigenase-derived eicosanoids. *J Immunol* 1996, 157:3105-3115
23. Brach MA, deVos S, Gruss HJ, Herrmann F: Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* 1992, 80:2920-2924
24. Kettritz R, Gaido ML, Haller H, Luft FC, Jennette CJ, Falk RJ: Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney Int* 1998, 53:84-91
25. Murray J, Barbara JA, Dunkley SA, Lopez AF, Van Ostade X, Condliffe AM, Dransfield I, Haslett C, Chilvers, ER: Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* 1997, 90:2772-2783
26. Biffl WL, Moore EE, Moore FA, Barnett Jr CC: Interleukin-6 suppression of neutrophil apoptosis is neutrophil concentration dependent. *J Leukoc Biol* 1995, 58:582-584
27. Watson RW, Rotstein OD, Nathens AB, Parodo J, Marshall JC: Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. *J Immunol* 1997, 158:945-953
28. Daffern PJ, Jagels MA, Hugli TE: Multiple epithelial cell-derived factors enhance neutrophil survival. Regulation by glucocorticoids and tumor necrosis factor-alpha. *Am J Respir Cell Mol Biol* 1999, 21:259-267
29. Cromwell O, Hamid Q, Corrigan CJ, Barkans J, Meng Q, Collins PD, Kay AB: Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. *Immunology* 1992, 77:330-337
30. Koyama S, Sato E, Masubuchi T, Takamizawa A, Kubo K, Nagai S, Izumi T: Alveolar type II-like cells release G-CSF as neutrophil chemotactic activity. *Am J Physiol* 1998, 275:L687-L693
31. Brailly H, Pebusque MJ, Tabilio A, Mannoni P: TNF alpha acts in synergy with GM-CSF to induce proliferation of acute myeloid leukemia cells by up-regulating the GM-CSF receptor and GM-CSF gene expression. *Leukemia* 1993, 7:1557-1563
32. Negus RP, Stamp GW, Relf MG, Burke F, Malik ST, Bernasconi S, Allavena P, Sozzani S, Mantovani A, Balkwill FR: The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *J Clin Invest* 1995, 95:2391-2396
33. Colasante A, Castrilli G, Aiello FB, Brunetti M, Musiani P: Role of cytokines in distribution and differentiation of dendritic cell/Langerhans' cell lineage in human primary carcinomas of the lung. *Hum Pathol* 1995, 26:866-872
34. Mantovani A: Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. *Lab Invest* 1994, 71:5-16
35. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JPD, Toews GB, Westwick J, Strieter RM: Interleukin-8 gene expression by a pulmonary epithelial cell line: a model for cytokine networks in the lung. *J Clin Invest* 1990, 86:1945-1953
36. Weitzberg AB, Weitzman SA, Destremes M, Latt SA, Stossel TP: Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells. *N Engl J Med* 1983, 308:26-30
37. Jackson JH, Vollenweider M, Hill J, Rodriguez H, Schwabacher AW, Mitra G, Kuo CY: Stimulated human leukocytes cause activating mutations in the K-ras protooncogene. *Oncogene* 1997, 14:2803-2808
38. Huffman Reed JA, Rice WR, Zsengeller ZK, Wert SE, Dranoff G, Whitsett JA: GM-CSF enhances lung growth and causes alveolar type II epithelial cell hyperplasia in transgenic mice. *Am J Physiol* 1997, 273:L715-L725
39. Kitamura H, Kameda Y, Ito T, Hayashi H: Atypical adenomatous hyperplasia of the lung. Implications for the pathogenesis of peripheral lung adenocarcinoma. *Am J Clin Pathol* 1999, 111:610-622
40. Haslett C: Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999, 160: S5-S11
41. Savill J, Fadok V, Henson P, Haslett C: Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* 1993, 14:131-136
42. Tang B, Bottinger EP, Jakowlew SB, Bagnall KM, Mariano J, Anver MR, Letterio JJ, Wakefield LM: Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998, 4:802-807