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Production of Tumor Necrosis Factor-α by Alveolar Macrophages of Lung Cancer Patients

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The abilities of human alveolar macrophages (AM) obtained from healthy donors and patients with lung cancer to produce tumor necrosis factor (TNF) were compared with those of their blood monocytes after activation with lipopolysaccharide (LPS). TNF activity was assayed by measuring cytotoxicity against actinomycin D-treated L929 cells and TNF was determined quantitatively by sandwich enzyme-linked immunosorbent assay (ELISA) with polyclonal and monoclonal antibodies against TNF-*α*. Unstimulated AM from healthy donors released variable amounts of TNF spontaneously, whereas blood monocytes did not. When treated with LPS for 24 h, AM and monocytes produced TNF dose-dependently, but TNF production by AM was significantly more than that by blood monocytes. This TNF activity was inhibited completely by monoclonal anti-TNF-*α* antibody. Macrophages generated by *in vitro* maturation of monocytes induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) produced more TNF than freshly isolated monocytes. No difference was found in the abilities of AM from healthy donors and patients with lung cancer to produce TNF after activation stimuli. These observations suggest that human AM may be important in *in vivo* antitumor defense of the lung through TNF-*α* production.

Key words: Macrophage — Tumor necrosis factor-*α* — Lung cancer — Monocyte

Studies in murine systems have shown that activated monocyte-macrophages are important in defense against primary and/or metastatic neoplasias.[1,2] Human blood monocytes and alveolar macrophages (AM)[2] are also rendered tumoricidal by various activating stimuli, such as lipopolysaccharide (LPS),[3] muramyl dipeptide (MDP),[4] macrophage activating factor (MAF),[5] and interferons (IFN).[6] The mechanism of monocyte- or macrophage-mediated tumor cell killing is still controversial, but is known to be mediated by several effector molecules such as oxygen intermediate,[7] arginase,[8] neutral protease,[9] interleukin-1 (IL-1),[10] and tumor necrosis factor-*α* (TNF-*α*).[11]

TNF-*α*, first found in the sera of mice treated with *Bacillus Calmette-Guerin* and endotoxin,[12] induces hemorrhagic necrosis of transplanted tumors *in vivo* and is cytotoxic and/or cytostatic to several cancer cell lines *in vitro.*[13] In addition, TNF-*α* is now recognized to have a wide range of biological activities, including antiviral activity, stimulation of fibroblast growth, augmentation of neutrophil function, stimulation of class I antigen expression, and induction of colony-stimulating factors (CSF).[14]

AM, which differentiate from blood monocytes,[15] are the main mononuclear phagocytes in the lung for defense against foreign particles reaching the alveolar space.[16] The lung is a common site of primary or metastatic cancer, so studies on the effects of biological response modifiers (BRM) on the anti-tumor functions of AM seem important. Indeed, some BRM were reported to be effective in preventing lung metastases by activation of AM in animal systems.[1,2] Little is known, however, about their effects in humans. In this study, we found by biological and quantitative assays that in response to appropriate stimuli, human AM of healthy donors have a higher capacity than their monocytes to produce TNF-*α*. We also observed that human AM of patients with lung cancer can be activated to produce as much TNF-*α* as that produced by AM from healthy donors.

MATERIALS AND METHODS

Patients The subjects examined were 19 healthy donors of 21 to 28 years old (mean age: 24±2 years), and 16 patients with primary lung cancer of 37 to 78 years old (mean age: 59±10 years) in Tokushima University Hospital who had not received any conventional anticancer therapy. The histories and physical examinations of the healthy donors (12 nonsmokers and 7 smokers) gave no evidence of any lung disease. All but two of the patients with lung cancer were smokers. Of the cancers, 5 were diagnosed histologically as squamous cell carcinomas, 8 as adenocarcinomas, 2 as small cell carcinomas and 1 as a large cell carcinoma. The stage of lung cancer was determined according to the tumor-node-metastasis classifi-

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[2] Abbreviations used: AM, alveolar macrophages; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

cation system (Union Internationale Contre le Cancer, 1987); stage I, 6; stage II, 3; stage IIIa, 3; stage IIIb, 1; stage IV, 3.

Cell culture L-929 cells, transformed murine fibroblasts, were maintained on plastic flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (designated as CPRMI 1640), at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagents FBS was purchased from M.A. Bioproducts (Walkerville, MD). Natural TNF-α (specific activity, 4.0 $\times 10^5$ JRU/mg of protein) and monoclonal anti-TNF-α antibody (IgM) against natural TNF-α (neutralizing activity, 2.2×10^5 U/ml) were gifts from Hayashibara Institute (Okayama). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; specific activity 6.7×10^6 units/mg glycoprotein) was kindly supplied by the Genetics Institute (Cambridge, MA). None of these materials contained endotoxin, as judged by *Limulus* amebocyte assay (sensitivity limit, 0.1 ng/ml) (Seikagaku Kogyo Co., Tokyo). LPS (*E. coli* 055: B5) was obtained from Difco Laboratories, Detroit, MI.

Preparation of human AM AM were harvested by bronchoalveolar lavage as described in detail elsewhere.[3, 17] Briefly, 0.5 mg of atropine sulfate was administered sc. Then, the oral cavity and upper airway were anesthetized with lidocaine spray (10 ml of 4% lidocaine suspension), and the tip of an Olympus fiberoptic bronchoscope (Model BF-1T; Olympus Co., Tokyo) was inserted po and wedged into the non-tumor-bearing segment of the right or left lobe. The lungs were washed with 50 ml of sterile 0.9% NaCl solution at 37°C, and the fluid was gently sucked out with a 50-ml syringe. This process was repeated 3 times. A total of 150 ml of 0.9% NaCl solution was instilled, of which about 65% was recovered. The lavaged cells were passed through sterile gauze and washed twice with PBS. Finally the cells were suspended in RPMI 1640 supplemented with 5% FBS. More than 90% of the cells were viable, as determined by the trypan blue dye exclusion test, and differential counts showed that more than 89% of these cells were AM, the other cells being either lymphocytes or neutrophils, which were eliminated during subsequent washings. There was no significant difference in the compositions of cells from normal volunteers and patients with lung cancer. The AM were plated into the wells of Microtest III plates (Falcon Plastic Co.) at a concentration of 1×10^5 AM per well, unless otherwise indicated. Non-adherent cells were removed by washing with medium after 60 min. At that time, more than 99% of the adherent cells were mononuclear, as judged by morphologic examination and tests of esterase activity.

Preparation of human blood monocytes Blood monocytes were prepared from peripheral venous blood taken into a heparinized syringe at the time of bronchoalveolar lavage from normal volunteers and patients with lung cancer. Mononuclear cells were separated from the peripheral blood by discontinuous gradient centrifugation in lymphocyte separation medium (Litton Bionetics, Kensington, MD). Then monocyte-rich cells were isolated from mononuclear cells by discontinuous gradient centrifugation in 46% Percoll solution at 1800 rpm (600*g*) for 30 min. The monocyte-rich cell suspension was washed twice with medium. More than 80% of the cells were monocytes, as judged by their morphology and their staining for nonspecific esterase. Samples of 10^5 blood monocytes were plated into each well of Microtest III plates and incubated for 60 min at 37°C. Then the monolayers were washed with medium to remove non-adherent cells. At this point, the purity of the monocytes was >99% as judged from their morphology and non-specific esterase staining.

***In vitro* activation of monocytes and AM** Samples of 1×10^5 monocytes or AM were incubated for 24 h at 37°C in medium with or without 1 μg/ml of LPS. Then, the cell-free supernatants were harvested, filtered through a 0.45-μm Millipore membrane, and stored at -20°C until use.

Assay of TNF activity The activity of TNF was measured by dye uptake assay as described previously.[18] Briefly, 3×10^4 L929 cells were grown in wells of a 96-well Microtest III plate in the presence of 1 μg/ml of actinomycin D and test samples. After 18 h, the plates were washed to remove the test samples, and cell lysis was detected by staining the plate with a 0.5% solution of crystal violet in methanol/water (1:4, v/v). The end point on the microtiter plate was determined with an Automatic Titertek Multiscan autoreader set for absorption at 540 nm. Preliminary experiments showed that under the conditions described here, there was a linear correlation between the degree of dye uptake by target cells and the number of adherent cells (data not shown). Percent cytotoxicity was calculated by means of the following formula:

$$\%\ \text{Cytotoxicity} = \frac{C-T}{C} \times 100$$

where C is the absorption of the control, and T is that of the treated sample. Lysis of cells incubated in medium alone was set at 0% and that of cells incubated with 3 *M* guanidine hydrochloride solution as 100%. One unit of TNF activity was defined as that causing 50% cytotoxicity. Units were calculated as the reciprocals of the supernatant dilutions causing 50% lysis of the L929 cell layer.

Enzyme-linked immunosorbent assay for TNF Immunoassay of TNF-α (sensitivity limit, 5.5 pg/ml) was performed in essentially the same way as that of inter-

leukin-1 reported elsewhere.[19] Briefly, microtitration plates were coated with anti-TNF-α monoclonal antibody (10 μg/ml) in 100 μl/well of phosphate-buffered saline, pH 7.4 (PBS). After overnight incubation at 4°C, the wells were blocked with 1% skimmed milk in PBS solution for at least 1 h at room temperature and washed three times with PBS containing 0.05% Tween-20 (Tween-PBS). All subsequent washings were performed with this buffer. Then 200 μl of culture supernatant (sample) in PBS containing 0.1% bovine serum albumin (BSA) was introduced into the wells and the plates were incubated at 37°C for 2 h. They were then washed three times, and 100 μl of rabbit anti-TNF-α antibody (diluted to 1/1000 with PBS containing 0.1% BSA) was introduced into each well. The plates were then incubated for 2 h at 37°C, washed, treated with 100 μl/well of peroxidase-labeled goat anti-rabbit IgG (diluted to 1/1000 with PBS containing 0.1% BSA) and incubated at room temparature for 2 h. Finally, 100 μg/well of enzyme substrate (1 mg/ml *o*-phenylenediamine in 0.1 *M* sodium citrate buffer, pH 5.0) was added and the plates were incubated at room temperature for 5 min. The reaction was stopped by adding 100 μl/well of H_2SO_4 and the absorbance at 492 nm was determined with a Titertek Multiskan.

Statistical analysis The statistical significance of differences between test groups was analyzed by the use of Student's *t* test (two-tailed).

RESULTS

Spontaneous release of TNF by AM We examined whether blood monocytes or AM from healthy donors could produce TNF spontaneously, without activation. For this, samples of 1×10^5 monocytes or AM from 19 healthy donors (12 non-smokers and 7 smokers) were incubated for 24 h in medium alone, and the cell-free supernatants were harvested. Then the appropriately diluted culture supernatants were added to 3×10^4 L929 cells/well with 1 μg/ml of actinomycin D, and 18 h later, the reactions were terminated. As shown in Fig. 1, blood monocytes did not release any factor that was cytotoxic to L929 cells, whereas low levels of cytotoxic activity were detected in the supernatants of cultured AM. These levels varied, but were all below that of 1 U/ml of TNF-α. These results indicate slight spontaneous release of TNF by AM.

Production of TNF by activated monocytes and AM For comparison of the abilities of blood monocytes and AM to produce TNF, samples of 1×10^5 of these two types of cells from healthy donors were incubated with various amounts of LPS for 24 h, and then the culture supernatants were harvested and tested for TNF activity. TNF production by AM was significantly more than that by blood monocytes at concentrations ranging from 0.01 to 10 μg/ml of LPS (data not shown). Next, we examined whether the cytotoxic activity in the supernatants obtained from cultures of blood monocytes or AM from healthy donors after LPS stimulation was really that of TNF. For this, 1×10^5 blood monocytes or AM from healthy donors were incubated with optimal concentration of LPS (1 μg/ml) for 24 h, and the supernatants were harvested and diluted as indicated in Table I. L-929 cells treated with actinomycin D were susceptible to the cytotoxicity of TNF, but not that of 1000 U/ml of IFN-α or 100 U/ml of IL-1 (α or β). Under these experimental conditions, TNF production by human AM was much more than that by blood monocytes at all dilutions tested. In a parallel experiment, the supernatants obtained from cultures of activated monocytes or AM were treated for 14 h with sufficient monoclonal anti-TNF-α antibody to neutralize 200 U/ml of TNF-α, and then their TNF activity against actinomycin D-treated L-929 cells was tested (Table I). The results showed that the cytotoxic activity of the supernatants was completely neutralized by anti-TNF-α antibody, suggesting that the cytotoxic effect on L-929 cells was due to TNF-α.

We also measured TNF-α quantitatively by sandwich enzyme immunoassay. For this, samples of 1×10^5 monocytes or AM from five healthy donors were incubated in medium with or without LPS (1 μg/ml) for 24 h, and the supernatants were harvested for quantitative measurement of TNF-α. As shown in Table II, spontaneous

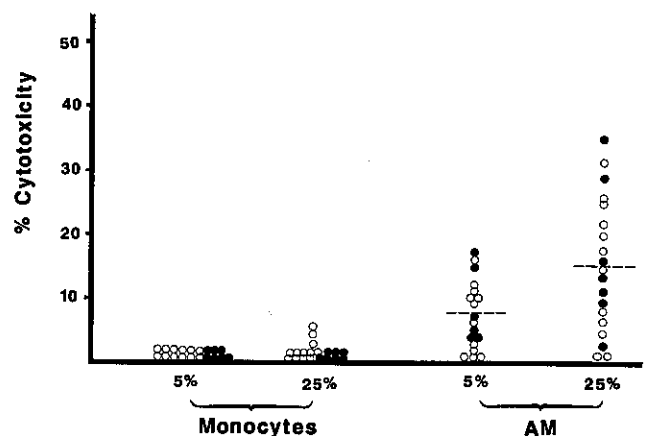

Fig. 1. Spontaneous release of TNF by AM from healthy donors. Samples of 10^5 blood monocytes or AM from 19 healthy donors (○, nonsmokers: ●, smokers) were incubated in medium for 24 h. Then the supernatants were harvested and incubated at concentrations of 25% and 5% with 3×10^4 L-929 cells with 1 μg/ml of actinomycin D for 18 h. The percentage cytotoxicity was calculated as described in "Materials and Methods."

Table I. Effects of Treatment with Monoclonal Anti-TNF-α Antibody on TNF Activity of Supernatants of Monocytes and AM

Supernatant or cytokine		% Cytotoxicity against L929 cells[a)]	
		Medium	Anti-TNF-α antibody
Set 1: Unstimulated monocytes or AM			
Monocyte supernatant	×8	3±1[b)]	0
	×16	0	0
AM supernatant	×8	28±4	0
	×16	11±3	0
Set 2: LPS-stimulated monocytes or AM			
Monocyte supernatant	×8	77±2	0
	×16	56±3	0
	×32	36±3	0
AM supernatant	×8	91±2	4±2
	×16	86±1	1±2
	×32	81±1	0
Set 3: Cytokine			
TNF (U/ml)	10	92±4	0
	1	74±2	0
	0.1	10±4	0
IL-1α (U/ml)	100	0	0
IL-1β (U/ml)	100	0	0
IFN-α (U/ml)	1000	0	0

a) Supernatants obtained from culture of 10^5 monocytes or AM treated with or without LPS (1 μg/ml) or various cytokines were incubated for 30 min with or without monoclonal anti-TNF antibody capable of neutralizing 200 U/ml of TNF activity before assay of cytotoxic activity against actinomycin D-treated L-929 cells.
b) Mean±SD for triplicate cultures. Data are representative of two separate experiments.

Table II. Abilities of Blood Monocytes and AM to Produce TNF-α after Stimulation with LPS

Cell	TNF-α (pg/ml)[a)]	
	Medium	LPS
Monocytes	36±21[b)]	1311±72
AM	799±372	1614±108[c)]

a) Samples of 10^5 monocytes or AM of healthy donors were incubated for 24 h in medium with or without 1 μg/ml of LPS, and then TNF-α in the supernatants was measured by sandwich ELISA as described in "Materials and Methods."
b) Data are means±SD's of values for cells from five healthy donors.
c) Significantly different from the value for LPS-activated monocytes.

Table III. Effect of Pretreatment with GM-CSF on Production of TNF by Blood Monocytes

Experiment No.	TNF activity in supernatant (U/ml)[a)]				
	Fresh monocytes		Monocyte treatment	5-Day monocytes	
	Medium	LPS		Medium	LPS
1	<1	8	Medium	<1	3
			GM-CSF	<1	29
2	<1	7	Medium	<1	6
			GM-CSF	<1	50
3	<1	25	Medium	<1	22
			GM-CSF	<1	75
4	<1	6	Medium	<1	4
			GM-CSF	<1	20
5	<1	6	Medium	<1	3
			GM-CSF	<1	17

a) Samples of 10^5 freshly isolated monocytes or blood monocytes that had been cultured in suspension for 5 days with GM-CSF (100 U/ml), were incubated for 24 h in medium with or without 1 μg/ml of LPS. Then, TNF activity in the culture supernatants was assayed as described in "Materials and Methods."

TNF-α production by fresh AM was more than that by blood monocytes (799±372 (SD) pg/ml vs. 36±21 pg/ml). Moreover, after activation, AM produced significantly more TNF-α than blood monocytes.

Augmented production of TNF-α by monocyte-derived macrophages To determine whether the difference between the TNF-α productions by blood monocytes and AM was related to cell maturity, we examined the production of TNF-α by macrophages generated from blood monocytes *in vitro*. For this, monocytes were cultured for 5 days in medium with 100 U/ml of recombinant human GM-CSF, which is known to induce cell maturation of monocytes *in vitro*.[20)] After incubation for 5 days, more than 70% of the fresh monocytes were recovered as viable macrophage-like cells. Morphologically the GM-CSF-treated monocytes had a typical macrophage-like appearance, showing enlargement, spreading, and decrease in the nuclear/cytoplasmic ratio. These cells (1×10^5/well) were washed thoroughly with medium, and incubated in medium with or without 1 μg/ml of LPS for 24 h. Results on their production of TNF assayed as described above are shown in Table III. In the absence of LPS, no TNF-α activity was detectable in the supernatants of cultures of fresh monocytes or monocytes that had been incubated in medium with or without GM-CSF. But, after stimulation with LPS, GM-CSF-treated monocytes showed significantly more ability than fresh monocytes to produce TNF-α, though the productions by both types of cells varied.

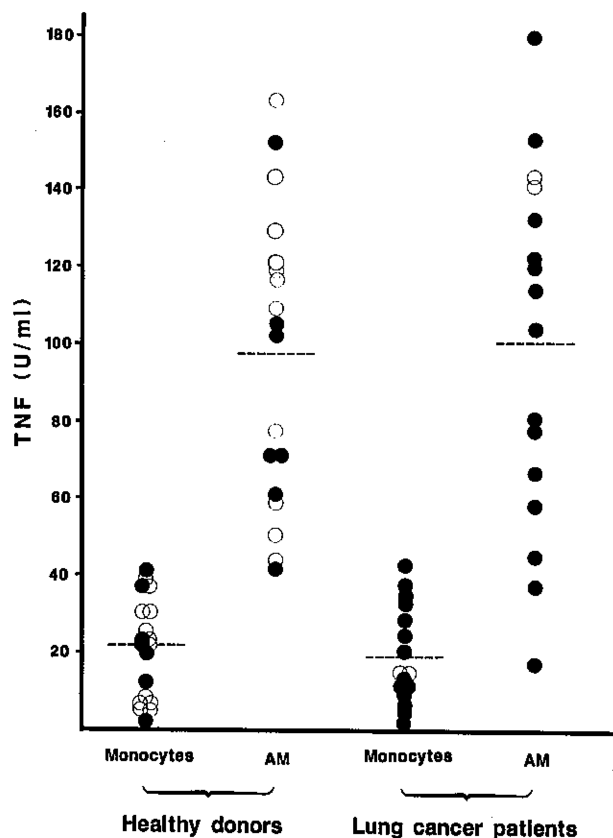

Fig. 2. TNF productions by blood monocytes and AM from healthy donors and patients with lung cancer (○, nonsmokers; ●, smokers) in response to LPS. Samples of 10^5 blood monocytes or AM from 19 healthy donors or 16 lung cancer patients were incubated with 1 μg/ml of LPS for 24 h. Then the supernatants were harvested and their TNF contents were assayed as described in "Materials and Methods." Bars show means for each group.

Production of TNF-α by blood monocytes and AM from lung cancer patients Finally, the abilities of blood monocytes and AM obtained from 19 healthy donors (12 non-smokers and 7 smokers) and 16 patients with lung cancer (2 non-smokers and 14 smokers) to produce TNF-α after incubation with 1 μg/ml of LPS were compared as follows. Samples of 1×10^5 monocytes or AM were incubated for 24 h in medium with or without LPS (1 μg/ml), and then the supernatants were harvested, diluted serially and assayed for TNF-α activity. AM of patients with lung cancer also produced low levels of TNF-α spontaneously, but their monocytes did not (data not shown). As shown in Fig. 2, after stimulation with LPS, the AM from both groups produced significantly more TNF-α than the blood monocytes ($P<0.01$). In the group of healthy donors, there was no significant difference between the production of TNF-α by monocytes or AM of non-smokers and smokers (monocytes: 20±12 (SD) U/ml vs. 22±12 U/ml, AM: 102±36 U/ml vs. 86±33 U/ml). Moreover there was no significant difference between the productions of TNF-α by blood monocytes or AM of healthy donors and patients with lung cancer (monocytes: 21±12 (SD) U/ml vs. 19±6 U/ml, AM: 96±36 U/ml vs. 99±46 U/ml).

DISCUSSION

In the present study using both bioassay and ELISA we found that TNF-α was produced spontaneously by human AM, but not by blood monocytes, and that in response to activation stimuli, AM of healthy donors produced significantly more TNF-α than their blood monocytes. Moreover, we found no difference in the abilities of blood monocytes or AM of healthy donors and patients with lung cancer to produce TNF-α.

TNF-α is a potent cytotoxic and/or cytostatic factor responsible for macrophage-mediated tumor cell killing *in vitro* and *in vivo*.[11, 13] Human AM are spontaneously cytostatic and/or cytolytic to allogeneic or autologous tumors.[21–23] We found previously that human AM spontaneously produced antitumor monokine(s) that killed tumorigenic cells, including TNF-α- and IL-1-sensitive A375 melanoma cells, but not non-tumorigenic cells.[17] Consistent with a report by others,[24] we recently found that fresh human AM of healthy donors do not produce detectable IL-1 without stimulation.[25] These observations, together with the present finding that human AM produced TNF-α spontaneously, suggest that the antitumor monokine responsible for spontaneous cytotoxic activity of AM may be TNF-α. This possibility was supported by the finding that the spontaneous cytotoxic activity against L-929 cells was completely inhibited by addition of monoclonal anti-TNF-α antibody (Table I). Moreover, quantitative measurement of TNF-α showed that AM had more capacity than blood monocytes to produce TNF-α (Table II). These observations also confirm and extend our previous finding[26] that freshly isolated blood monocytes do not show spontaneous cytotoxicity or produce TNF-α. One possible explanation for this difference between AM and monocytes could be that AM in the lower respiratory tract are continuously exposed to inhaled foreign particles, and so some of them may be "stimulated and/or activated" to produce TNF-α.

Recently, activated AM from healthy humans were shown by bioassay using TNF-sensitive target cells (i.e., L-929 cells) to have a greater capacity than blood monocytes of the same donors to produce TNF-α.[27, 28] These observations were confirmed and extended by the present measurements of TNF-α by both bioassay and ELISA,

showing that AM of healthy donors produced significantly more TNF-α than their blood monocytes, irrespective of their activation.

Monocytes circulating in the blood are precursors of tissue macrophages such as AM and macrophages of the pleural cavity.[15] Thus, the increased production of TNF-α by activated human AM could be due to cell maturation. Indeed, we recently found that macrophages from the pleural cavity of patients with lung cancer without malignant pleural effusion produced more TNF-α than their blood monocytes.[29] This was also confirmed by the present findings that production of TNF-α by macrophages induced by maturation of monocytes with GM-CSF *in vitro* was greater than that by fresh monocytes. Thus, consistent with the findings of others,[27,30,31] these findings indicate that the production and secretion of TNF-α by the human mononuclear phagocyte system can be regulated by its state of differentiation and/or maturation.

In several studies,[21,32,33] but not all,[23] no difference was found between the AM-mediated cytotoxicities of lung cancer patients and healthy donors. Little is known, however, about the ability of AM of patients with lung cancer to produce TNF-α. In the present study we found that AM from patients with lung cancer produce as much TNF-α as AM from healthy donors. Although TNF-α is not the only factor responsible for AM-mediated tumor cytotoxicity, these findings suggest that AM may be important as effector cells in *in situ* antitumor defense in the lung either directly or indirectly through production of TNF-α.

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