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Leukemic Cell Lysis by Activated Human Macrophages: Significance of Membrane-associated Tumor Necrosis Factor

Yukiharu Nakabo,[1,4] Nari Harakawa,[1] Kohkichi Yamamoto,[1] Minoru Okuma,[1] Kazuko Uno[2] and Masataka Sasada[1,3]

[1]*The First Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaramachi, Sakyo-ku, Kyoto 606,* [2]*Institut Pasteur de Kyoto, Tanaka-Monzencho, Sakyo-ku, Kyoto 606 and* [3]*College of Medical Technology, Kyoto University, 53 Shogoin-Kawaramachi, Sakyo-ku, Kyoto 606*

In this study, we analyzed the mechanism(s) of leukemic cell lysis by human macrophages. Peripheral blood monocyte-derived macrophages were activated with recombinant interferon-γ and lipopolysaccharide and their lytic activity against two leukemic cell lines (K562 and HL-60 cells) was assessed by an ^{111}In releasing assay. Activated macrophages lysed these leukemic cells, and the lytic activity against leukemic cells was almost completely inhibited by anti-tumor necrosis factor (TNF) antibody. The macrophage-lysate prepared from activated macrophages also exhibited significant lytic activity against leukemic cells; this lytic activity was inhibited by anti-TNF antibody. The leukemic cells that we used for the cytotoxicity assays were resistant to recombinant TNF. The culture supernatant of activated macrophages did not show any lytic activity. These findings suggest that cell-associated TNF plays a role in macrophage-mediated cytotoxicity against leukemic cells.

Key words: Macrophage — Macrophage activation — TNF — Leukemic cell — Cytotoxicity

Macrophages, which play an important role in host defense against microbes,[1-4] also have antitumor activity when activated. Many agents, such as bacterial lipopolysaccharide (LPS),[5] interferon-γ (IFN-γ),[6-8] granulocyte-macrophage colony-stimulating factor,[9,10] macrophage colony-stimulating factor,[11,12] and interleukin-2 (IL-2)[13,14] have been shown to activate these cells.

In general, activated macrophages preferentially destroy tumor cells but not normal cells.[1,15-18] The macrophages seem to discriminate between tumor cells and normal cells when they come into contact. Therefore cell-to-cell contact is considered to be an important event in macrophage-mediated tumor cytotoxicity. The binding between macrophages and tumor cells is known to involve integrins and lectin-like receptors.[19-21]

Several substances have been reported to be responsible for the lysis of tumor cells, including oxygen metabolites,[22] neutral protease,[23] complement C3a,[24] arginase,[25] tumor necrosis factor (TNF),[26-28] IL-1,[29] nitrogen metabolites,[30-33] and so on. TNF was first described as a cytotoxic factor for certain tumor cells.[34] TNF-sensitive tumor cells are usually sensitive to TNF secreted by macrophages. However, the membrane-associated TNF has also been shown to be involved in the lysis of some TNF-sensitive tumor cells.[28]

We have recently reported that activated human macrophages destroyed leukemic cells.[35] But little is known about the mechanism(s) by which leukemic cells are lysed by human macrophages. Accordingly, in this study, we investigated the machanism(s) by using macrophage lysate in order to examine whether the cell-associated TNF participates in the leukemic cell killing.

[4] To whom correspondence should be addressed.

MATERIALS AND METHODS

Reagents Human recombinant IFN-γ (rIFN-γ)(2×10^7 U/mg) which was kindly provided by Shionogi & Co., Ltd. (Osaka) and LPS (*E. coli* 026:B6) from Difco (Detroit, MI) were used to activate the macrophages. Anti-TNF antibody (2.6×10^5 neutralizing units/ml) was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama). Human recombinant TNF (rTNF) (3×10^6 JRU/mg) was kindly provided by Dainippon Pharmaceutical Co., Ltd. (Osaka). All materials that we used contained less than 20 pg/ml LPS, the concentration of which was measured by use of the Limulus test.

Preparation of macrophages Human peripheral blood monocyte-derived macrophages were prepared as previously described with modifications.[35] Briefly, mononuclear cells were isolated from heparinized peripheral blood on Percoll gradients. To enhance the purity of the monocytes, the cells were loaded into a Beckman elutriation centrifuge (J6-MC, JE-5.0, standard chamber, Beckman, Tokyo). The monocyte-rich fraction (purity more than 95%, determined by Giemsa staining) was obtained at a speed of 3000 rpm and at a flow rate of 36–40 ml/min. The cells were suspended in RPMI 1640 (GIBCO, Grand Island, NY) containing 5% human heat-inactivated

AB serum (HIABS), 50 U/ml penicillin, and 50 μg/ml streptomycin (complete medium) at a concentration of 1.5×10^6 monocytes/ml. The monocytes (1.5×10^5/well) were plated on 96-well flat-bottomed microplates (Falcon 3072, Becton Dickinson, New Jersey). For the preparation of the macrophage lysate, 3.0×10^6 monocytes were plated on 35 mm culture dishes (Falcon 3001). After incubation for 2 h in a 5% CO_2 incubator, plates or dishes were washed twice vigorously to eliminate nonadherent cells and were incubated in a CO_2 incubator after addition of complete medium. On day 5 of culture, more than 98% of the adherent cells were macrophages, as estimated by staining of non-specific esterase and surface marker (CD 14, Becton Dickinson). The number of cells on the microplates on day 5 was around 1×10^5/well. After macrophage monolayers were washed with complete medium, rIFN-γ (50 U/ml) and/or LPS (10 ng/ml) were added. A cytotoxicity assay was performed after 48 h of incubation.

Target cells Two leukemic cell lines, K562 (erythroleukemia cell line)[36] and HL-60 (acute myelocytic leukemia cell line)[37, 38] were used as target cells of the cytotoxicity assay of macrophages. These cells were maintained in complete medium and passaged three times weekly. Cultures were checked periodically for mycoplasma, and confirmed to be free from contamination. In some experiments, lymphocytes obtained by counterflow centrifugal elutriation were also used as target cells.

Cytotoxicity assay Cytotoxic activity of macrophages towards target cells was assayed as previously described.[35] Briefly, on day 7 of culture of macrophages, ^{111}In-labeled target cells (5×10^3) were added to each well containing macrophages with or without treatment. The total reaction volume was 0.2 ml. After 48 h of incubation in a CO_2 incubator, the microplates were centrifuged for 5 min at 250g and 0.05 ml of supernatant was taken for counting of radioactivity. The percent specific lysis was calculated as follows:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\%,$$

where the maximum release was that obtained from target cells exposed to 1% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, MO), and spontaneous release was that obtained from target cells cultured in macrophage-free medium. All assays were performed in triplicate. The spontaneous release was always less than 15% of the maximum release.

Preparation of macrophage lysate Macrophages cultured for 7 days were washed with phosphate-buffered saline (PBS) containing 5% HIABS (PBS with HIABS), then collected by scraping with a rubber policeman. The cells were suspended in PBS with HIABS at a concentration of 2×10^7/ml and sonicated for 15 s three times at a power setting of 40 W (Ultrasonic) at 4°C. The supernatant was obtained after centrifugation for 10 min at 150g, and used as macrophage lysate in this study. Its protein concentration was around 2.0 mg/ml by Lowry's method when the number of cells was 2×10^7/ml in the lysate preparation. In the assays of cytotoxic activity of the macrophage lysate, round-bottomed microplates (Falcon 3077) instead of flat-bottomed microplates were used.

Nitrite measurement The concentration of nitrite in the culture supernatant and macrophage lysate was determined by a microplate assay method as described by Ding *et al.*[39]

RESULTS

Cytolysis of leukemic cells by macrophages Untreated macrophages did not show any lytic activity against leukemic cells. Macrophages treated with rIFN-γ or LPS exhibited lytic activity against leukemic cells of K562 and HL-60 cell lines (Fig. 1). Macrophages treated with both rIFN-γ and LPS showed higher lytic activity against both

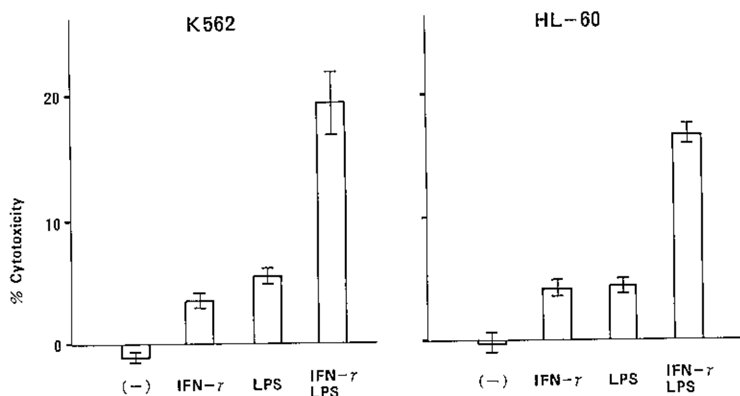

Fig. 1. Leukemic cell lysis by human macrophages. Human macrophages were treated with IFN-γ and/or LPS and their cytolytic activity towards K562 or HL-60 cells was assessed after an additional 48 h of incubation at the macrophage-to-target cell ratio of 20:1. The values represent the mean±SEM of five separate experiments.

of the leukemic cell lines (Fig. 1). The culture supernatant of macrophages activated with rIFN-γ and LPS did not exhibit any lytic activity against either leukemic cell line (data not shown).

Role of TNF in the macrophage-mediated cytotoxicity against leukemic cells To investigate the mechanism(s) of leukemic cell lysis by the macrophages, the effect of anti-TNF antibody on the lytic activity of the macrophages was examined. Anti-TNF antibody almost completely inhibited the macrophage cytolytic activity against K562 and HL-60 cells (Fig. 2), while mouse IgG_1 as a control had no effect on macrophage cytolytic activity. The inhibitory effect of anti-TNF antibody was concentration-dependent (Fig. 3). Therefore, TNF was considered to play a role in the leukemic cell lysis by the macrophages. We examined whether TNF itself had lytic activity on the leukemic cells. However, rTNF could not destroy K562 and HL-60 cells at all even at a concentration of 10^4 U/ml (data not shown). Here, U937 (histiocytic lymphoma cell line) cells, which are regarded as TNF-sensitive cells, were lysed by TNF (10^4 U/ml) by 25.6±3.2%, and this lytic activity was blocked by anti-TNF antibody (2.6×10^4 neutralizing units/ml) (n=3)

Cytolysis of leukemic cells by macrophage lysate The macrophage lysate was examined for lytic activity against leukemic cells. As shown in Fig. 4, the lysate from untreated macrophages did not lyse any leukemic cells. However, lysate from activated macrophages had significant lytic activity. The lytic activity increased with the amount of lysate used in the assay as shown in Fig. 5. Maximum activity was obtained at the concentration of 1 mg/ml. A time course study demonstrated that the

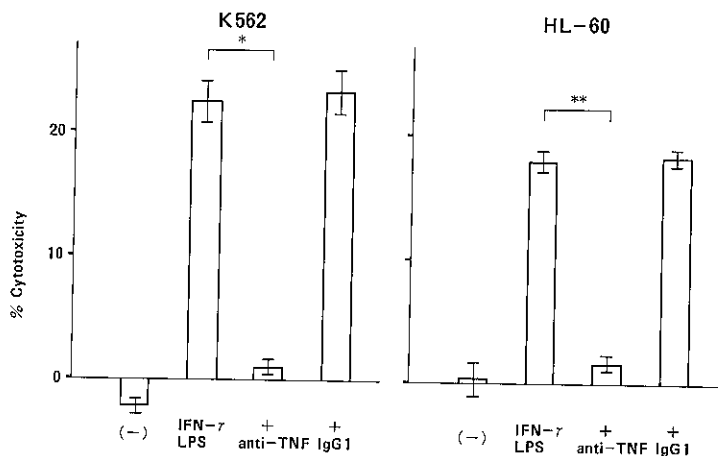

Fig. 2. Effect of anti-TNF antibody on macrophage cytotoxicity towards leukemic cells. Human macrophages were treated with IFN-γ and LPS. Anti-TNF antibody (2.6×10^3 neutralizing units/ml) or mouse IgG_1 (control) was added to the macrophages 20 min before leukemic cells were added. The values represent the mean±SEM of three separate experiments. * $P<0.025$. ** $P<0.01$.

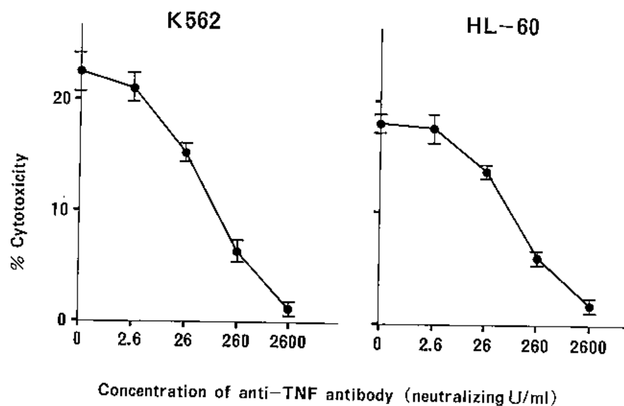

Fig. 3. Concentration dependence of anti-TNF antibody action. The inhibitory effect of anti-TNF antibody was examined at various concentrations as in Fig. 2. The values represent the mean±SEM of three separate experiments.

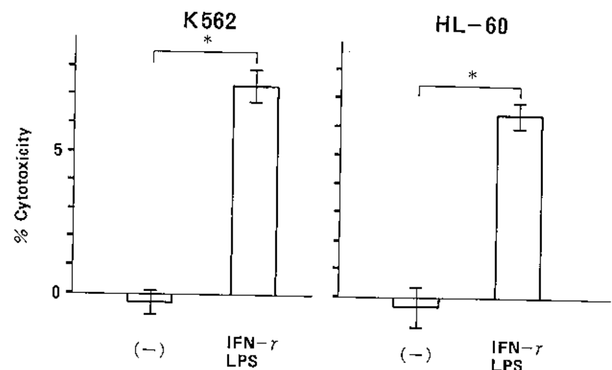

Fig. 4. Cytolysis of leukemic cells by macrophage lysate. Macrophage lysate was prepared from untreated or treated macrophages as described in "Materials and Methods." Cytotoxic activity towards K562 or HL-60 cells was assessed after 48 h of incubation. The values represent the mean±SEM of four separate experiments. * $P<0.01$.

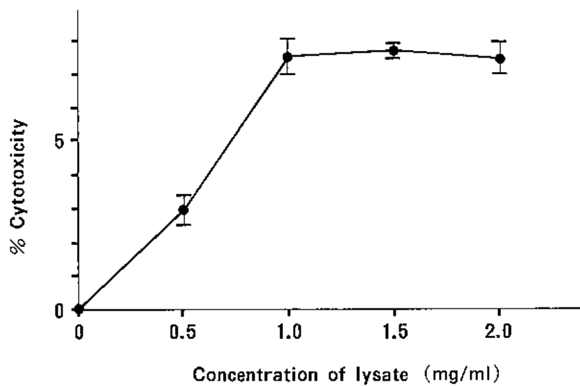

Fig. 5. Cytotoxic activity of lysate prepared from activated macrophages. Leukemic cells (K562) were incubated with the lysate at the indicated concentrations. The values represent the mean±SEM of three separate experiments.

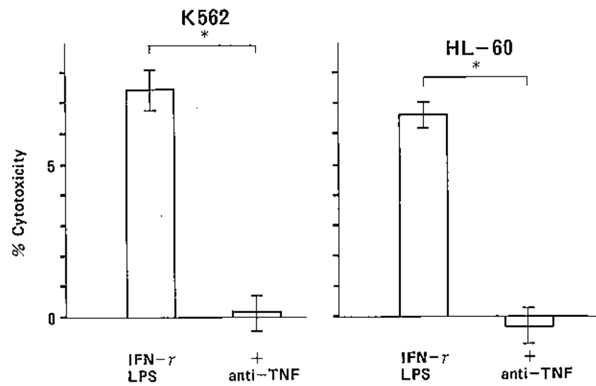

Fig. 7. Effect of anti-TNF antibody on the cytotoxicity of lysate towards leukemic cells. Macrophage lysate prepared from IFN-γ plus LPS-treated macrophages exhibited cytotoxic action against leukemic cells, K562 and HL-60 cells. Anti-TNF antibody (2.6×10^3 neutralizing units/ml) inhibited this activity almost completely. * $P<0.025$ (n=3).

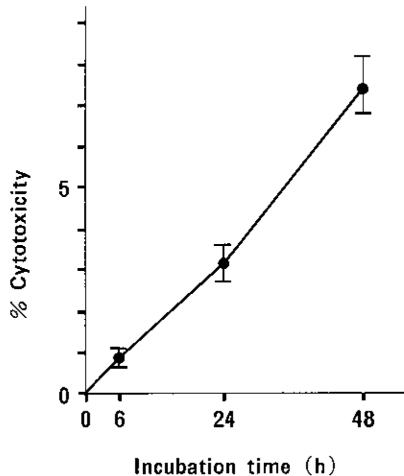

Fig. 6. Time course of leukemic cell lysis by macrophage lysate. Leukemic cells (K562) were incubated with the lysate for the indicated period. The values represent the mean± SEM of two separate experiments.

macrophage lysate had significant lytic activity against K562 cells after 48 h of incubation (Fig. 6). A similar result was obtained using HL-60 cells as target cells (data not shown). The inhibitory effect of anti-TNF antibody was examined in the reaction with macrophage lysate. Anti-TNF antibody abrogated the cytotoxic activity against K562 and HL-60 cells almost completely (Fig. 7).

Normal lymphocytes as target cells To examine whether or not the lytic activity of macrophages was specific for leukemic cells, normal lymphocytes were used as target cells. Activated macrophages did not show any lytic activity against normal lymphocytes (Table I). Similarly,

Table I. Cytotoxic Activity of Human Macrophages towards Normal Lymphocytes

Target cells		Treatment of macrophages[a]	
		(−)	rIFN-γ + LPS
		% Cytotoxicity[b]	
Normal	allogeneic lymph	−4.8±1.8	−7.1±1.6
	autologous lymph	−6.0±1.0	−8.2±1.5
K562 cells		−1.5±0.6	20.3±1.3

a) Macrophages were untreated or treated with rIFN-γ plus LPS.
b) The cytotoxic activity of the macrophages towards target cells was assayed after cocultivation for 48 h at the macrophage-to-target cell ratio of 20:1. Results are expressed as the mean±SEM of three separate experiments.

Table II. Cytotoxicity of Macrophage Lysate to Normal Lymphocytes

Target cells		Treatment of macrophages[a]	
		(−)	rIFN-γ + LPS
		% Cytotoxicity[b]	
Normal	allogeneic lymph	−1.6±1.4	−2.3±1.8
	autologous lymph	−1.1±1.6	−1.8±0.9
K562 cells		−0.3±0.4	7.5±1.1

a) Macrophage lysate was prepared from macrophages untreated or treated with IFN-γ plus LPS.
b) The cytotoxic activity against target cells was assessed after 48 h of incubation. The values show the mean±SEM for three experiments.

Table III. Production of Nitrite by Human and Murine Macrophages[a)]

Source of macrophages	Preparation	Treatment of macrophages	
		(−)	rIFN-γ + LPS
		(nmol/10^6 cells)	
Human	Supernatant	2.5±0.4	2.8±0.3
	Lysate	1.5±0.4	1.7±0.5
Murine	Supernatant	2.1±0.3	40.2±1.3

a) Supernatant and lysate were prepared from the macrophages cultured in the same conditions as for the cytotoxicity assay, and the content of nitrite was determined. Murine macrophages were obtained after injection of LPS (50 μg) into the peritoneum, and these were untreated or treated with rIFN-γ and LPS for 48 h. The values show the mean±SEM of three experiments.

the lysate from activated macrophages did not show any lytic activity against normal lymphocytes (Table II).

Measurement of nitrite We could hardly detect nitrite in culture supernatant or lysate of human macrophages whereas murine macrophages produced a significant amount of nitrite (Table III).

DISCUSSION

Activated macrophages are believed to destroy tumor cells effectively. However, the mechanism of tumor cell lysis by macrophages has not been fully elucidated. In the present study, we analyzed the mechanism(s) of leukemic cell lysis by activated human macrophages. We treated human macrophages with IFN-γ and LPS, since the combined treatment with these agents is considered to be optimal for activation of macrophages.[40)] We prepared the macrophage lysate to pursue the effector molecule(s) involved in leukemic cell lysis. The macrophage lysate had significant lytic activity against leukemic cells, although the extent of lytic activity was lower than that of intact macrophages (Figs. 1 and 4). The method used to evaluate the lytic activity, an ^{111}In-releasing assay,[41)] was sufficiently sensitive to quantitate the low activity of human macrophages. Leukemic cell lysis by intact human macrophages was almost completely inhibited by anti-TNF antibody (Fig. 2). However, the leukemic cells that we used in this study were so-called TNF-resistant cells since TNF added exogenously did not exhibit any cytolytic activity against them. The culture supernatant of the activated macrophages did not exhibit any lytic activity against leukemic cells. A similar observation was reported by Klostergaard *et al.*, who showed that the cytotoxicity of activated macrophages against TNF-resistant cells (EMT-6) was partially inhibited by anti-TNF antibody,[42)] although the experimental conditions were different from ours, since they used murine macrophages and the macrophages were activated with BCG. The lytic activity of the macrophage lysate was also inhibited by anti-TNF antibody. These findings suggest that cell-associated TNF is involved in human macrophage-mediated cytotoxicity towards leukemic cells. Membrane-associated TNF was shown to be present on the surface of human monocyte-derived macrophages and alveolar macrophages[43)] when these cells were activated. The membrane-associated TNF of murine macrophages has been reported to be responsible for the lysis of some TNF-sensitive tumor cells.[28)] We further demonstrated that the membrane-associated TNF of human macrophages was also involved in lysis of TNF-resistant cells. It is, however, unclear why only membrane-associated, but not secreted TNF of human macrophages had lytic activity against leukemic cells. Almost all cells, including K562 and HL-60 cells,[44)] are considered to have TNF receptors on their surface. However, the mechanism of signal transduction of the TNF receptor remains unclear.

Activated macrophages exerted significant lytic activity against leukemic cells but not normal lymphocytes. Many investigators have also reported that macrophages destroy tumor cells but not normal cells. In this study, the macrophage lysate also exhibited significant cytolytic activity against leukemic cells but not normal lymphocytes. Two explanations are possible. First, substances produced by macrophages selectively react with leukemic cells. Second, these substances react with both leukemic cells and normal lymphocytes, but are lethal only to leukemic cells. While TNF has cytotoxic activity towards TNF-sensitive cells, it has diverse (but not cytotoxic) activities towards normal cells. Effector molecule(s) of activated human macrophages in this study might have a function similar to TNF.

TNF was involved in the cytotoxicity of activated human macrophages towards leukemic cells. However, other factor(s) must also be involved since these leukemic cells were resistant of TNF. Therefore, we examined the involvement of nitric oxide (NO) by determining nitrite, since it was recently shown to play an important role in tumor cell lysis in the murine system.[30-33)] As to the production of NO in human macrophages, there are conflicting reports. Denis and others detected nitrite in culture supernatant of human macrophages[45, 46)] whereas James *et al.* and others did not.[47, 48)] We quantitated nitrite in culture suernatant or lysate of activated macrophages, and it was less than one-tenth of that in the culture supernatnat of activated mouse macrophages (Table III). Furthermore, addition of N^G-monomethyl-L-arginine (an inhibitor of NO production) had no effect on the cytolysis of leukemic cells (data not shown). Thus

L-arginine-dependent reactive nitrogen intermediates might not be involved in leukemic cell lysis by human macrophages.

The mechanism of macrophage-mediated cytotoxicity towards tumor cells has been shown to differ markedly depending on the source of macrophages, the method of activation, and the kind of target cells. The present study has partially elucidated the mechanisms of leukemic cell lysis by human macrophages. Further studies will provide important information on human macrophages and may also lead to applications in clinical therapy.

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REFERENCES

1) Adams, D. O. and Hamilton, T. A. The cell biology of macrophage activation. *Annu. Rev. Immunol.*, **2**, 283–318 (1984).
2) Klebanoff, S. J. Oxygen metabolism and the toxic properties of phagocytes. *Ann. Intern. Med.*, **93**, 480–489 (1980).
3) Sasada, M. and Johnston, R. B., Jr. Macrophage microbicidal activity: correlation between phagocytosis-associated oxidative metabolism and the killing of *Candida* by macrophages. *J. Exp. Med.*, **152**, 85–98 (1980).
4) Sasada, M., Kubo, A., Nishimura, T., Kakita, T., Moriguchi, T., Yamamoto, K. and Uchino, H. Candidacidal activity of monocyte-derived human macrophages: relationship between *Candida* killing and oxygen radical generation by human macrophages. *J. Leukocyte Biol.*, **41**, 289–294 (1987).
5) Chen, A. R., McKinnon, K. P. and Koren, H. S. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. *J. Immunol.*, **135**, 3978–3987 (1985).
6) Fischer, D. G., Golightly, M. G. and Koren, H. S. Potentiation of the cytolytic activity of peripheral blood monocytes by lymphokines and interferon. *J. Immunol.*, **130**, 1220–1225 (1983).
7) Le, J., Prensky, W., Yip, Y. K., Chang, Z., Hoffman, T., Stevenson, H. C., Balazs, I., Sadlick, J. R. and Vilcek, J. Activation of human monocyte cytotoxicity by natural and recombinant immune interferon. *J. Immunol.*, **131**, 2821–2826 (1983).
8) Sadlik, J. R., Hoyer, M., Leyko, M. A., Horvat, R., Parmely, M., Whitacre, C., Zwilling, B. and Rinehart, J. J. Lymphocyte supernatant-induced human monocyte tumoricidal activity: dependence on the presence of γ-interferon. *Cancer Res.*, **45**, 1940–1945 (1985).
9) Grabstein, K. H., Urdal, D. L., Tushinski, R. J., Mochizuki, D. Y., Price, V. L., Cantrell, M. A., Gillis, S. and Conlon, P. J. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science*, **232**, 506–508 (1986).
10) Heidenreich, S., Gong, J.-H., Schmidt, A., Nain, M. and Gemsa, D. Macrophage activation by granulocyte/macrophage colony-stimulating factor: priming for enhanced release of tumor necrosis factor-α and prostaglandin E_2. *J. Immunol.*, **143**, 1198–1205 (1989).
11) Sampson-Jahannes, A. and Carlino, J. A. Enhancement of human monocyte tumoricidal activity by recombinant M-CSF. *J. Immunol.*, **141**, 3680–3686 (1988).
12) Suzu, S., Yokota, H., Yamada, M., Yanai, N., Saito, M., Kawashima, T., Saito, M., Takaku, F. and Motoyoshi, K. Enhancing effect of monocytic colony-stimulating factor on monocyte tumoricidal activity. *Cancer Res.*, **49**, 5913–5917 (1989).
13) Malkovsky, M., Loveland, B., North, M., Asherson, G. L., Gao, L., Ward, P. and Fiers, W. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. *Nature*, **325**, 262–265 (1987).
14) Philip, R. Cytolysis of tumor necrosis factor (TNF)-resistant tumor targets: differential cytotoxicity of monocytes activated by the interferons, IL-2, and TNF. *J. Immunol.*, **140**, 1345–1349 (1988).
15) Hibbs, J. B., Jr., Lambert, L. H., Jr. and Remington, J. S. Control of carcinogenesis: a possible role of the activated macrophage. *Science*, **177**, 998–1000 (1972).
16) Hibbs, J. B., Jr. Macrophage nonimmunologic recognition: target cell factors related to contact inhibition. *Science*, **180**, 868–870 (1973).
17) Fidler, I. J. and Kleinerman, E. S. Lymphokine-activated human blood monocytes destroy tumor cells but not normal cells under cocultivation conditions. *J. Clin. Oncol.*, **2**, 937–943 (1984).
18) Fidler, I. J. and Schroit, A. J. Recognition and destruction of neoplastic cells by activated macrophages: discrimination of altered self. *Biochim. Biophys. Acta*, **948**, 151–173 (1988).
19) Strassman, G., Springer, T. A., Somers, S. D. and Adams, D. O. Mechanisms of tumor cell capture by activated macrophages: evidence for involvement of lymphocyte function-associated (LFA)-1 antigen. *J. Immunol.*, **136**, 4328–4333 (1986).
20) Oda, S., Sato, M., Toyoshima, S. and Osawa, T. Binding of activated macrophages to tumor cells through a macrophage lectin and its role in macrophage tumoricidal activity. *J. Biochim.*, **105**, 1040–1043 (1989).
21) Bernasconi, S., Peri, G., Sironi, M. and Mantovani, A. Involvement of leukocyte (β2) integrins (CD18/CD11) in

human monocyte tumoricidal activity. *Int. J. Cancer,* **49,** 267–273 (1991).
22) Nathan, C. F., Silverstein, S. C., Brukner, L. H. and Cohn, Z. A. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.,* **149,** 100–113 (1979).
23) Adams, D. O., Kao, K.-J., Farb, R. and Pizzo, S. V. Effector mechanisms of cytolytically activated macrophages. II. Secretion of a cytolytic factor by activated macrophages and its relationship to secreted neutral proteases. *J. Immunol.,* **124,** 293–300 (1980).
24) Ferluga, J., Schorlemmer, H. U., Baptista, L. C. and Allison, A. C. Production of the complement cleavage product, C3a, by activated macrophages and its tumorolytic effects. *Clin. Exp. Immunol.,* **31,** 512–517 (1978).
25) Currie, G. A. Activated macrophages kill tumour cells by releasing arginase. *Nature,* **273,** 758–759 (1978).
26) Urban, J. L., Shepard, H. M., Rothstein, J. L., Sugarman, B. J. and Schreiber, H. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc. Natl. Acad. Sci. USA,* **83,** 5233–5237 (1986).
27) Feinman, R., Henriksen-DeStefano, D., Tsujimoto, M. and Vilcek, J. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. *J. Immunol.,* **138,** 635–640 (1987).
28) Decker, T., Lohmann-Matthes, M.-L. and Gifford, G. E. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J. Immunol.,* **138,** 957–962 (1987).
29) Onozaki, K., Matsushima, K., Aggarwal, B. B. and Oppenheim, J. J. Human interleukin 1 is a cytocidal factor for several tumor cell lines. *J. Immunol.,* **135,** 3962–3968 (1985).
30) Hibbs, J. B., Jr., Taintor, R. R. and Vavrin, Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science,* **235,** 473–476 (1987).
31) Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z. and Rachlin, E. M. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.,* **157,** 87–94 (1988).
32) Stuehr, D. J. and Nathan, C. F. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.,* **169,** 1543–1555 (1989).
33) Keller, R., Geiges, M. and Keist, R. L-Arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res.,* **50,** 1421–1425 (1990).
34) Carswell, E. A., Old, L. J., Kassel, R. L., Greeen, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA,* **72,** 3666–3670 (1975).
35) Kakita, T., Sasada, M., Moriguchi, T., Nishimura, T., Yamamoto, K. and Uchino, H. Lysis of human leukemic cells by monocyte-derived macrophages activated with interferon-γ and interleukin-2. *Jpn. J. Cancer Res.,* **80,** 59–64 (1989).
36) Lozzio, C. B. and Lozzio, B. B. Human chronic myelogeneous leukemic cell-line with positive Philadelphia chromosome. *Blood,* **45,** 321–334 (1975).
37) Collins, S. J., Gallo, R. C. and Gallagher, R. E. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature,* **270,** 347–349 (1977).
38) Dalton, W. T., Jr., Ahearn, M. J., McCredie, K. B., Freireich, E. J., Stass, S. A. and Trujillo, J. M. HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood,* **71,** 242–247 (1988).
39) Ding, A. H., Nathan, C. F. and Stuehr, D. J. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol.,* **141,** 2407–2412 (1988).
40) Adams, D. O. and Hamilton, T. A. Molecular transductional mechanisms by which IFNγ and other signals regulate macrophage development. *Immunol. Rev.,* **97,** 5–27 (1987).
41) Wiltrout, R. H., Taramelli, D. and Holden, H. T. Measurement of macrophage-mediated cytotoxicity against adherent and non-adherent target cells by release of ^{111}indium-oxine. *J. Immunol. Methods,* **43,** 319–331 (1981).
42) Klostergaard, J., Stoltje, P. A. and Kull, F. C., Jr. Tumoricidal effector mechanisms of murine BCG-activated macrophages: role of TNF in conjugation-dependent and conjugation-independent pathways. *J. Leukocyte Biol.,* **48,** 220–228 (1990).
43) Nii, A., Sone, S., Orino, E. and Ogura, T. Induction of a 26-kDa membrane-form tumor necrosis factor (TNF)-α in human alveolar macrophages. *J. Leukocyte Biol.,* **53,** 29–36 (1993).
44) Scheurich, P., Ücer, U., Krönke, M. and Pfizenmaier, K. Quantification and characterization of high-affinity membrane receptors for tumor necrosis factor on human leukemic cell lines. *Int. J. Cancer,* **38,** 127–133 (1986).
45) Denis, M. Tumor necrosis factor and granulocyte-macrophage colony-stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium*: killing effector mechanism depends on the generation of reactive nitrogen intermediates. *J. Leukocyte Biol.,* **49,** 380–387 (1991).
46) Martin, J. H. J. and Edwards, S. W. Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. *J. Immunol.,* **150,** 3478–3486 (1993).
47) James, S. L., Cook, K. W. and Lazdins, J. K. Activation of human monocyte-derived macrophages to kill schistosomula of *Schistosoma mansoni in vitro. J. Immunol.,* **145,** 2686–2690 (1990).
48) Padgett, E. L. and Pruett, S. B. Evaluation of nitrite production by human monocyte-derived macrophages. *Biochem. Biophys. Res. Commun.,* **186,** 775–781 (1992).