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David H. Munn · Nai-Kong V. Cheung

Antibody-independent phagocytosis of tumor cells by human monocyte-derived macrophages cultured in recombinant macrophage colony-stimulating factor

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Abstract Human monocytes exposed in vitro to recombinant macrophage-colony-stimulating factor (rhMCSF) differentiate into monocyte-derived macrophages (MDM), which mediate efficient antibody-dependent cytotoxicity (ADCC) against tumor cells. We and others have shown that this form of ADCC is unusual in that phagocytosis, rather than extracellular lysis, appears to play the major role in target cell killing. In this study, we asked whether the phagocytic form of cytotoxicity seen with ADCC could occur in the absence of an opsonizing antibody. We now report that, whereas cell lines derived from solid tumors are often resistant to antibody-independent cytotoxicity, malignant cells of lymphoid origin appear particularly susceptible to such antibody-independent killing. We found that all of nine lymphocytic leukemia and lymphoma cell lines tested in a total of 35 experiments, plus all four samples of fresh leukemic blasts, were consistently susceptible to antibody-independent MDM cytotoxicity. Antibody-independent cytotoxicity against these cells was efficient (40%–63% killing) at effector:target (E:T) ratios as low as 2:1. Like ADCC, antibody-independent cytotoxicity involved phagocytosis of target cells, as demonstrated by ingestion of fluorescently labeled targets and analysis by flow cytometry. At the time of phagocytosis, the majority of target cells retained membrane integrity, as indicated by the direct transfer of intracellular [^{51}Cr]chromate from radiolabeled targets to phagocytosing MDM, without release of the label into the medium. However, in contrast to ADCC, we found that the degree of antibody-independent cytotoxicity was not a function of the E:T ratio. Instead, a constant proportion of the available target cells were killed regardless of the E:T ratio, suggesting that target cell recognition, rather than effector cell potency, might be the limiting factor in determining cytotoxicity. In additional experiments, we have also identified a second tumor cell type, nueroblastoma, as being susceptible to antibody-independent phagocytosis (all of five cell lines tested, cytotoxicity 40%–93%, E:T = 3:1). Our data thus indicate that the cytotoxicity induced by rhMCSF is not confined to antibody-mediated killing, and that phagocytosis can play a significant role in target cell destruction even in the absence of opsonizing antibody.

Key words Macrophage · Cytotoxicity · Phagocytosis Tumor cells · Macrophage-colony-stimulating factor

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D. H. Munn (✉)
Section of Pediatric Hematology-Oncology,
Medical College of Georgia, Augusta, GA 30912, USA

D. H. Munn
Institute for Molecular Medicine and Genetics,
Medical College of Georgia, Augusta, GA 30912, USA

N.-K. V. Cheung
Department of Pediatrics,
Memorial Sloan-Kettering Cancer Center,
New York, NY 10021, USA

Introduction

Studies from our laboratory and others have shown that human monocytes cultured in recombinant human macrophage-colony-stimulating factor (rhMCSF) mediate efficient cytotoxicity against tumor cells that have been opsonized by a suitable monoclonal antibody [1–3]. This form of antibody-dependent cytotoxicity (ADCC) is acquired during differentiation, with fresh monocytes initially showing only weak ADCC, but acquiring increasingly effective cytotoxicity as they mature into monocyte-derived

macrophages (MDM) under the influence of rhMCSF [2, 4].

One unusual feature of rhMCSF-induced ADCC is the fact that direct phagocytosis of the target cells appears to play a major role in target cell destruction [5–7], in contrast to the more familiar mechanism of extracellular lysis [8, 9]. This observation is potentially of interest not only because it describes an efficient mechanism of cytotoxicity, but also because antigens from phagocytosed cells may be able to enter the macrophage MHC class I antigen-presentation pathway, leading to induction of cytotoxic T lymphocyte (CTL) responses [10–13]. Thus, rhMCSF-activated macrophages have the theoretical potential to serve both as direct cytotoxic effector cells and, subsequently, as antigen-presenting cells for CTL.

However, as a form of cytotoxicity, ADCC is limited by the requirement for a suitable antibody. Although many studies have shown that macrophages can mediate antitumor cytotoxicity in the absence of antibody, this type of killing typically involves extracellular lysis rather than phagocytosis, and often requires "priming" with cytokines such as interferon γ, followed by "triggering" with additional agents such as lipopolysaccharide or phorbol ester (see the reviews in [8, 9, 14]).

In this study, we asked whether rhMCSF-induced MDM could mediate the phagocytic form of antitumor cytotoxicity without the need for opsonizing antibody, and without requiring additional priming or triggering signals. After screening a variety of tumor cell lines, we found that lymphoid malignancies appeared reproducibly sensitive to recognition and destruction by rhMCSF-derived MDM. We now describe and characterize the rhMCSF-induced antibody-independent macrophage cytotoxicity against lymphoid-derived cell lines and fresh leukemic blasts.

Materials and methods

Target cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, Md.: Leukemia/lymphoma lines ARH, CESS, SKW, HSB-2, and MOLT-4, and melanoma line SK-MEL-1. The KR-2 cell line was established from a patient with acute lymphoblastoid leukemia at Case Western Reserve University, Cleveland, Ohio. RSH (lymphoblastoid leukemia) was obtained from Dr. Richard O'Reilly, Memorial Sloan-Kettering Cancer Center, New York, NY. HPB107 and 8402 (lymphoblastoid leukemias) were obtained from Dr. Ronald Levy, Stanford University, Stanford, Calif. LS180 (colon carcinoma) was obtained from Dr. Deborah Young, Genetics Institute, Cambridge, Mass. A-431 (epidermoid carcinoma) was obtained from Drs. Hideo Masui and John Mendelsohn, Memorial Sloan-Kettering. Neuroblastoma line LA-N-1 was the gift of Dr. Robert Seeger, University of California, Los Angeles, Calif.; LA1-6s, LA1-15n and LA1-21n were the gift of Dr. June Biedler, Memorial Sloan-Kettering; NMB-7 was the gift of Dr. Shuen-Kuei Liao, McMaster University, Hamilton, Ontario, Canada.

Mycoplasma testing

Cell lines were either obtained as *Mycoplasma*-free cultures from ATCC, or were tested for *Mycoplasma* by culture in the Memorial Sloan-Kettering microbiology laboratory. Constitutive *Mycoplasma* carriage was detected in four lines (LA-N-1, 8402, KR-2, and HPB107). Analysis of our data with or without the *Mycoplasma*-positive lines produced identical conclusions, so we report the results for all cell lines.

Preparation of fresh leukemic blasts

Heparinized whole blood was obtained from patients with acute lymphocytic leukemia prior to the institution of therapy, in accordance with Institutional Review Board guidelines governing informed consent. Mononuclear cells from these samples were more than 95% leukemic blasts by Wright's stain morphology, and were used as target cells for cytotoxicity assays.

Isolation and culture of peripheral blood monocytes

Our monocyte culture technique has been described in detail previously [1]. Briefly, peripheral blood leukocytes were obtained from volunteer donors by phlebotomy or apheresis. Monocytes were centrifuged sequentially over Ficoll-Hypaque and isotonic Percoll gradients, then either used immediately or cryopreserved in 10% dimethylsulfoxide freezing medium. Cryopreserved monocytes were functionally indistinguishable in our assays from fresh monocytes; reported results were observed with both fresh and frozen cells. Before use, monocytes were further purified by adherence to serum-coated 96-well tissue-culture plates followed by vigorous washing (typically purity using this technique was above 95% monocytes by morphology and CD14 expression). Purified monocytes were then cultured in RPMI-1640 medium with 10% bovine calf serum and 200 ng/ml rhMCSF (gift of Genetics Institute, Cambridge, Mass.) for 7–12 days to permit functional differentiation into MDM. All media and additives contained less than 0.03 endotoxin U/ml by *Limulus* amebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.) in the concentrations used.

Despite these purification steps and the period of in vitro culture, it remains possible that some cytotoxic lymphocytes might have carried over into our MDM preparations. However, such contaminating lymphocytes would not mediate the phagocytic form of cytotoxicity that we describe, and should have been readily apparent on chromium-release assays (which they were not). Therefore, we do not believe that contaminating lymphocytes are likely to account for our results.

Phagocytosis assay

We have adapted existing assays for macrophage phagocytosis [15, 16] to permit sensitive quantification of phagocytosis of live human tumor cells. Details of our flow-cytometry-based assay have been reported previously [4–6]. Briefly, target cells (either cell lines or fresh leukemic blasts) were labeled with the green-fluorescent PKH2 dye (Zynaxis Cell Science, Malvern, Pa.), then added to cultures of MDM and incubated for 18 h at 37 °C. The cocultures were harvested with EDTA, MDM counterstained with phycoerythrin-conjugated anti-macrophage antibodies (CD14 plus CD11b, Becton Dickinson, Mountain View, Calif.), and phagocytosis of dye-labeled tumor cells was measured by two-color flow cytometry, as described in Results. Wide gates for forward and 90° light scatter were used to ensure that the extremely large, granular macrophage population was fully included. Quadrants for analysis were established on the

basis of the fluorescence of unphagocytosed target cells in cocultures. Only macrophages that had acquired a green fluorescence intensity equal to that of an intact target cell were considered positive for phagocytosis (this restriction probably failed to include macrophages that phagocytosed a target cell and subsequently lost or degraded a portion of the tracking dye, but it provided a conservative estimate of cytotoxicity and avoided ambiguous levels of intermediate fluorescence).

Control target cells, including the fresh leukemic cells, were typically more than 90% viable by trypan blue dye exclusion following the PKH2 labeling and 18 h incubation. Each data point represents eight replicate cultures, which were pooled and analyzed together. The measured E:T ratio and the absolute number of target cells undergoing phagocytosis were calculated from the flow cytometry data as described in [5].

We have previously documented that this assay measures true phagocytosis of target cells, rather than the mere formation of conjugates, as demonstrated by electron microscopy, fluorescence microscopy, and transfer of cytoplasmic radiolabeling compounds [5]. In this study, we monitored each flow-cytometric assay with fluorescence microscopy to ensure that phagocytosis rather than conjugate formation had occurred. In addition, we demonstrated direct transfer of radionuclide from labeled targets to macrophages, as described below.

Lymphocyte controls

As negative controls to demonstrate the specificity of macrophage cytotoxicity we tested normal human lymphocytes (the non-malignant counterpart of our leukemia/lymphoma cell lines). Allogeneic lymphocytes were used in these studies in order to reflect the allogeneic nature of the tumor cell studies. Unlike the tumor cell lines, which labeled uniformly and intensely with PKH2 dye, resting lymphocytes labeled heterogeneously. To guard against false-negative results, we therefore modified the cytotoxicity assay to provide more stringent quantitative information on recovery of viable lymphocytes following co-culture with macrophages. Lymphocytes were labeled with PKH2 as described above then incubated for 18 h with or without macrophages. Cultures were harvested with EDTA, macrophages were counterstained as described, and any dead cells were marked by incubation with 1 μg/ml propidium iodide for 15 min. Viable lymphocytes (propidium-iodine-negative, counterstain-negative) were then enumerated by flow cytometry, and absolute lymphocyte recovery was calculated on the basis of hemacytometer counts of harvested cultures. Cytotoxicity was defined as the ratio of surviving lymphocytes in macrophage cocultures to lymphocytes cultured alone. As discussed in Table 1, lymphocyte recovery was quantitatively identical with or without macrophages, indicating that neither the labeling process nor the allogeneic nature of the system produced artifactual cytotoxicity.

Chromium-release assay

Target cells were labeled with sodium [^{51}Cr]chromate (Amersham, Arlington Heights, Ill.) as described [17]. Labeled targets were added at various concentrations to wells containing cultured MDM, to yield approximate E:T ratios of 8, 4, 2, and 1:1, then incubated for 18 h at 37 °C. Following incubation, an aliquot of supernatant was removed for gamma counting to determine extracellular lysis. The macrophage monolayers were then washed to remove unphagocytosed target cells and solubilized with 1% sodium dodecyl sulfate to recover intracellular ^{51}Cr (a measure of phagocytosis). Specific lysis and target cell phagocytosis were calculated as described [5, 17].

We have previously shown that incorporation of heavy-metal labeling compounds such as chromium into MDM monolayers under these conditions is due to direct phagocytosis of target cells [5]. However, to check that soluble chromium was not being released during extracellular lysis and then nonspecifically taken up by MDM, we performed the following validation experiments. Target cells (LA-N-1) were labeled with chromium and lysed by freezing/thawing and the resultant chromium-containing supernatant was incubated with MDM for 18 h. Under these conditions we observed no detectable uptake of soluble chromium by MDM (three experiments).

Results

Antibody-independent phagocytosis of lymphoid leukemia cell lines

We used our previously described flow-cytometric assay to measure tumor cell phagocytosis. An example of the raw data generated by this assay is shown in Fig. 1. Cytotoxicity was calculated as the ratio of phagocytosed to unphagocytosed target cells (quadrant 2 to quadrant 4, 66% in the example shown). The E:T ratio was calculated as the ratio of total MDM (quadrant 1 + quadrant 2) to total target cells (quadrant 2 + quadrant 4). Monocytes were seeded at a density anticipated to yield an E:T ratio of approximately 4:1 when target cells were added; variations in MDM and target cell proliferation during the assay produced actual measured E:T ratios between 1:1 and 6:1 (mean 3:1). In our system, cytotoxicity was not significantly affected by fluctuations in the E:T ratio over this range (see below), so no attempt was made to normalize results to a standard E:T ratio.

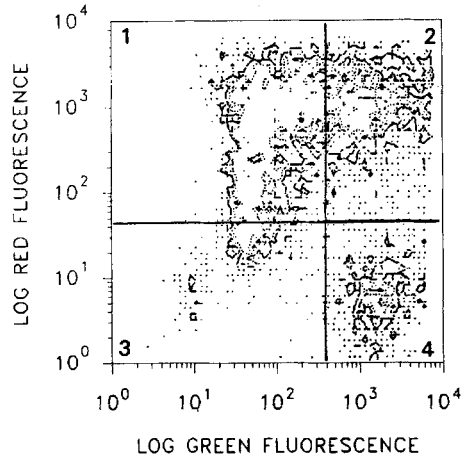

Fig. 1 Antibody-independent phagocytosis of leukemic cells by MDM. Monocyte-derived macrophages (MDM) were cultured in rhMCSF for 7 days. Target cells (ARH in this example) were labeled with flourescent tracking dye and incubated with MDM for 18 h. The cocultures were then harvested and MDM counter-stained with phycoerythrin-conjugated anti-macrophage antibodies. Quadrant 1 contains a population of MDM alone, quadrant 4 contains unphagocytosed target cells, and quadrant 2 contains double-positive cells representing MDM that had ingested a dye-labeled target cell

Table 1 Phagocytosis of lymphoid leukemia/lymphoma cell lines by MDM. Human tumor cell lines were used as targets in a phagocytosis assay with cultured monocyte-derived macrophages (MDM), as described in Fig. 1. The average E:T ratio was 3:1. Results are means ± SD. For the control, the phagocytosis assay was modified as described in Materials and methods to guard against false negative results when normal lymphocytes were used

Cell line	Type	Phagocytosis (%)	*n*
HSB-2	Leukemia/lymphoma	50 ± 29	8
CESS	Leukemia/lymphoma	55 ± 17	5
SKW 6.4	Leukemia/lymphoma	63 ± 19	5
RSH	Leukemia/lymphoma	55 ± 24	4
ARH	Leukemia/lymphoma	60 ± 22	4
HPB107	Leukemia/lymphoma	57 ± 25	3
Molt-4	Leukemia/lymphoma	40 ± 13	2
8402	Leukemia/lymphoma	63 ± 10	2
KR-2	Leukemia/lymphoma	49 ± 23	2
SK-MEL-1	Melanoma	12 ± 4	7
LS-180	Colon carcinoma	21 ± 9	3
A-431	Epidermoid carcinoma	58 ± 16	4
Control	Normal lymphocytes	2 ± 2	4

Table 1 shows the sensitivity to antibody-independent cytotoxicity for 9 different leukemia/ lymphoma cell lines (we did not attempt to distinguish between leukemia and lymphoma lines; all were lymphoid-derived and grew as suspension cultures). In a total of 35 experiments, each of these lines was found to be reproducibly susceptible to MDM-mediated killing. To confirm that these results are not merely an artifact of our assay system, we have included in the table data from 3 representative solid-tumor cell lines (of 14 analyzed) showing various degrees of sensitivity to antibody-independent phagocytosis. Normal (non-transformed) allogeneic lymphocytes were also analyzed as negative controls, and these displayed no susceptibility to macrophage-mediated cytotoxicity.

Cytotoxicity against fresh leukemic blasts

We next asked whether susceptibility to antibody-independent cytotoxicity was a property only of cultured cell lines, or whether fresh leukemic blasts could also be phagocytosed by MDM. In these experiments, we employed the same allogeneic system used with the cell lines above, since the donors of leukemic blasts were myelosuppressed by their disease and autologous monocytes were not available. We found that, like lymphoid cell lines, fresh leukemic blasts were consistently susceptible to killing by rhMCSF-derived MDM (Table 2).

Absence of chromium release during cytotoxicity

Our flow-cytometry assay was designed to detect phagocytosis rather than extracellular lysis. To determine

Table 2 Cytotoxicity by allogeneic MDM against fresh leukemic blasts. Fresh blasts from patients with leukemia were used as target cells in a cytotoxicity assay with allogeneic MDM, as described in Fig. 1. The E:T ratio was 2:1. *ALL* acute lymphocytic leukemia

Patient	Diagnosis	Phagocytosis (%)
1	ALL, relapsed	52
2	ALL, newly diagnosed	38
3	ALL, relapsed	41
4	ALL, relapsed	28

the contribution of extracellular lysis to cytotoxicity in our system, we employed an 18-h ^{51}Cr-release assay. We have previously shown that radionuclide-release assays may fail to detect MDM cytotoxicity because of sequestration of the heavy-metal radiolabeling compounds following phagocytosis [1, 5]. This ingested radionuclide can be recovered, however, by detergent lysis of the MDM monolayer following removal of residual target cells, and thus provides an estimate of phagocytic activity [18]. Figure 2 shows data from a representative assay of this type, illustrating that there was little extracellular release of chromium during incubation with MDM, whereas significant phagocytosis of the radionuclide occurred (comparable to the degree of phagocytosis detected by flow cytometry, cf. Fig. 1). The lack of chromium release during target cell killing suggests that phagocytosis occurred while cells were alive, prior to the loss of membrane integrity.

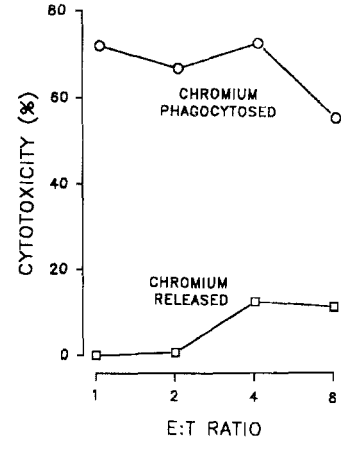

Fig. 2 Extracellular lysis is not a major mechanism of cytotoxicity induced by recombinant human macrophage-colony-stimulating factor (rhMCSF). MDM were cultured for 7 days in rhMCSF. Chromium-labeled leukemia cell lines (ARH in this example) were used as targets in an 18-h radionuclide-release assay. Chromium release due to extracellular lysis was first measured in the supernatant (□). Residual target cells were then removed by washing, and chromium from phagocytosed targets recovered by detergent lysis of the macrophage monolayer (○). Duplicate determinations; SD < 5% for all points. Representative of 9 experiments with 6 cell lines

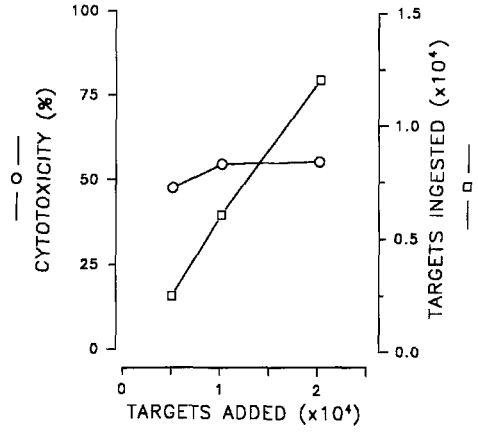

Fig. 3 The degree of phagocytosis is not a function E:T ratio. MDM were cultured for 7 days in rhMCSF. Fresh leukemic blasts were added in increasing numbers to replicate cultures of MDM and cytotoxicity measured by flow cytometry (○). The same data are re-plotted as the absolute number of target cells ingested (□). Note that a similar proportion of the available target cells were phagocytosed regardless of the target cell number or E:T ratio

Influence of E:T ratio

The data from experiments such as that shown in Fig. 2 suggested that there was little relationship between the degree of phagocytosis and the E:T ratio. To investigate this unexpected observation, we added increasing numbers of target cells to a fixed number of MDM, and measured phagocytosis by flow cytometry. As shown in Fig. 3, as more target cells were added to the cocultures the absolute number of targets killed increased, but the proportion of target cells undergoing phagocytosis remained essentially constant. This was a consistent observation with both chromium-release ($n=10$) and flow-cytometry ($n=4$) assays, and occurred with both fresh leukemic blasts and tumor cell lines.

Phagocytosis of neuroblastoma cell lines

In the course of the current study, we also screened a variety of solid-tumor cell lines for sensitivity to antibody-independent MDM cytotoxicity. Results with solid tumors were variable, and the degree of cytotoxicity was in general less than that observed with lymphoid malignancies. However, one group of cell lines, those derived from neuroblastoma tumors, showed consistent sensitivity to MDM cytotoxicity (Table 3). Thus, while the efficiency of antibody-independent cytotoxicity was related to the cell lineage, it was not restricted solely to lymphoid cell lines.

Table 3 Phagocytosis of neuroblastoma cell lines by MDM. Human neuroblastoma cell lines were tested as described in Table 1. The average E:T ratio was 3:1. Results are means ± SD

Cell line	Phagocytosis (%)	*n*
LA-N-1	58 ± 19	4
LA1-6s	54 ± 15	4
LA1-15n	40 ± 13	3
NMB-7	93 ± 1	2
LA1-21n	61 ± 3	2

Discussion

In this study we show that human MDM cultured in rhMCSF can display significant tumor cell phagocytosis even in the absence of an opsonizing antibody. Phagocytosis as a mechanism of macrophage antitumor cytotoxicity has not been widely studied, despite the fact that it has been shown to occur in both antibody-dependent [19–24] and antibody-independent [18, 25] systems, with human and animal models. Moreover, the general ability of macrophages to ingest nucleated cells, whether malignant or nonmalignant, is by no means a novel observation. Macrophages are known to phagocytose a variety of cell types as the cells become senescent or undergo apoptosis [26]. In the specific case of tumor cells, however, the limiting factor governing phagocytosis appears to be recognition of the target cells as suitable candidates for attack.

The rhMCSF-derived MDM used in this report are highly phagocytic, with over 75% of MDM being capable of ingesting antibody-opsonized target cells [6]. Given this potential efficiency, the rate-limiting step in target cell killing without opsonizing antibody would appear to be the initial recognition step. Support for this hypothesis comes from three observations: (a) large numbers of effector cells appeared no more efficient than smaller numbers at mediating antibody- independent killing, suggesting that some intrinsic property of the target cell governs its recognition; (b) some cell lines such as SK-MEL-1 are highly resistant to killing in the absence of antibody, but are quite sensitive when opsonized by antibody [6]; and (c) tumors derived from certain tissues (lymphoid malignancies and neuroblastoma in this report) appear to be recognized more consistently than those of other histological types. Sensitivity to macrophage cytotoxicity did not appear related to adherent or nonadherent properties of the targets, since the adherent neuroblastoma and A-431 lines were sensitive, and the non-adherent SK-MEL-1 cells and fresh lymphocytes were resistant. Taken together, these findings suggest that phagocytosis of target cells depends significantly upon intrinsic features of the target cells themselves which govern the initial recognition step by macrophages.

The allogeneic nature of our assay system did not appear to influence macrophage cytotoxicity, as demonstrated by the fact that allogeneic lymphocytes were

not killed (Table 1). This finding was not unexpected, since macrophages do not possess the T cell receptors required to distinguish "self" from non-self, and thus would be expected to mediate MHC-unrestricted cytotoxicity. Natural killer cells, which also mediate MHC-unrestricted killing, have traditionally been assayed against allogeneic targets with good success [27].

Previous studies have shown that a brief (1 day) exposure to rhMCSF can enhance the antitumor cytotoxicity of fresh monocytes [28]. Our present work extends these observations by describing a second, developmentally acquired form of rhMCSF-induced cytotoxicity, which emerges as fresh monocytes differentiate into MDM. In our system, rhMCSF is an obligate survival factor, and monocytes cultured for 7 days without MCSF do not live. Thus, it is difficult to distinguish between "maturation" and "activation" in describing MCSF-induced cytotoxicity. However, we have recently reported that interferon γ is able to inhibit the development of MCSF-induced antibody- dependent cytotoxicity without affecting macrophage survival [4], suggesting that survival in culture is not by itself sufficient to promote cytotoxicity.

One potentially interesting aspect of tumor cell phagocytosis is the recently discovered ability of phagocytic macrophages to act as antigen-presenting cells for CTL. A variety of accessory-cell types are known to process and present antigens derived from other cells (i.e., proteins taken up by phagocytosis or pinocytosis) [29]. However, such exogenously derived antigens are normally presented in the context of MHC class II molecules and elicit only a humoral immune response, not the CTL response required to kill tumor cells. CTL are typically elicited only against a target cell's own endogenous antigens, presented in the context of the cell's class I MHC molecules. Tumor cells often fail to present endogenous antigens in a way that can provoke an effective CTL response [30]. However, it has recently been recognized that exogenous antigens can elicit an efficient CTL response if they are first phagocytosed by macrophages, then presented in the context of the macrophage's class I MHC molecules [10–13]. This unusual form of class-I-mediated antigen presentation appears to be dramatically more efficient if the antigen has been phagocytosed in a particulate form (as occurs in our system) rather than a soluble form [11, 12]. Presentation of tumor-associated antigens by macrophages in this fashion makes it possible to circumvent the tendency of tumor cells to evade CTL immunity.

We do not yet know whether rhMCSF-induced macrophages display tumor-associated antigens on their surface following target cell phagocytosis, nor whether such antigen presentation is able to elicit a CTL response. However, in view of the possibility that these macrophages may be able to act both as direct cytotoxic effector cells and as antigen-presenting cells for CTL, we believe that they warrant further study.

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