Macrophages Can Recognize and Kill Tumor Cells Bearing the Membrane Isoform of Macrophage Colony-Stimulating Factor

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NBXFO hybridoma cells produced both the membrane and secreted isoforms of macrophage colony-stimulating factor (M-CSF). Murine bone marrow cells stimulated by the secreted form of M-CSF (sM-CSF) became Mac1+, Mac2+, Mac3+, and F4/80+ macrophages that inhibited the growth of NBXFO cells, but not L1210 or P815 tumor cells. In cytotoxicity studies, M-CSF activated macrophages and freshly isolated macrophages killed NBXFO cells in the presence of polymyxin B, eliminating the possibility that contaminating lipopolysaccharide (LPS) was responsible for the delivery of the cytotoxic signal. Retroviral-mediated transfection of T9 glioma cells with the gene for the membrane isoform of M-CSF (mM-CSF), but not for the secreted isoform of M-CSF, transferred the ability of macrophages to kill these transfected T9 cells in a mM-CSF dose-dependent manner. Macrophage-mediated killing of the mM-CSF transfected clone was blocked by using a 100-fold excess of recombinant M-CSF. Catalase, superoxide dismutase, and the nitric oxide inhibitor, N\(^{o}\)-nitro-arginine methyl ester (NAME), did not effect macrophage cytotoxicity against the mM-CSF transfected T9 clones. T9 parental cells when cultured in the presence of an equal number of the mM-CSF transfected cells were not killed, indicating specific target cell cytotoxicity by the macrophages. Electron microscopy showed that macrophages were capable of phagocytosing mM-CSF bearing T9 tumor cells and NBXFO hybridoma cells; this suggested a possible mechanism of this cytotoxicity. This study indicates that mM-CSF provides the necessary binding and triggering molecules through which macrophages can initiate direct tumor cell cytotoxicity.

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ACROPHAGES PLAY a complex role in tumor biology; their presence within a tumor can either correlate with tumor destruction or tumor growth.\(^{1,4}\) Macrophages become cytotoxic for tumor cells in a two-step process.\(^{2,6}\) Cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)),\(^{7}\) granulocyte-macrophage colony-stimulating factor (GM-CSF),\(^{8}\) tumor necrosis factor (TNF),\(^{9}\) and macrophage colony stimulating factor (M-CSF) for extended periods of time. Transfection experiments with the mM-CSF gene by Stein et al\(^{10}\) showed that this isoform was a functional molecule because paraformaldehyde-fixed cells stimulated macrophage colony formation when coincubated with bone marrow stem cells. The true physiological significance of this isoform is not currently known. Alternatively, if the mRNA is processed into either the 4 kb or 2.3 kb mRNA, the protein has retained the proteolytic sensitive site that will be cut within the secretory vesicle. This cut M-CSF protein will be released from the cell when the secretory vesicle fuses with the membrane. This secreted form of M-CSF (sM-CSF) then stimulates cells in either autocrine, paracrine, or endocrine manners. Many different cells including several tumor types are known to produce M-CSF\(^{17}\) and this cytokine may be responsible for the presence of macrophages within the tumor.

In preliminary studies, we discovered that NBXFO hybridoma cells (16 \(\times\) 10\(^6\) cells injected intraperitoneally (IP) per syngeneic mouse) did not develop tumors, even if the mice were first immunesuppressed with high-dose cyclophosphamide (300 mg/kg), which induced potent macrophage suppressor cells.\(^{22,23}\) We previously identified that NBXFO cells produced both the secreted and membrane forms of M-CSF.\(^{24}\) Bone marrow cells stimulated with the secreted form of M-CSF (sM-CSF) became macrophages that directly killed the NBXFO cells without the use of exogenous LPS. We tested the hypothesis that the membrane isoform of M-CSF (mM-CSF) present on tumor cells could provide a recognition molecule for macrophages to induce direct tumor cytotoxicity. By using retroviral gene transfer technology, we provide evidence that macrophages kill tumor cells expressing the membrane isoform of M-CSF.

MATERIALS AND METHODS

Animals. Male DBA/2J mice (4 to 6 weeks old) were purchased from Jackson Labs (Bar Harbor, ME). Mice housed in our facility for 6 months have tested negative for various viruses and mycoplasma on routine screening. Sprague-Dawley rats were obtained from either Dr A. Tarnawski or Dr S. Szabo (VAMC, Long Beach, California, Irvine and the Long Beach Research foundation. Anirnrds. Male DBA/2J mice (4 to 6 months have tested negative for various viruses and mycoplasma on routine screening. Sprague-Dawley rats were obtained from either Dr A. Tarnawski or Dr S. Szabo (VAMC, Long Beach, California, Irvine and the Long Beach Research foundation. Anirnrds.
CA) who purchased these animals from Harlan Sprague-Dawley
(San Diego, CA).

Cell lines. Myeloplasma free cells as determined using the Gen- 
Probe assay (Fisher Scientific, Tustin, CA) were grown either in 
RPMI-1640 media supplemented with 5% fetal bovine serum 
(Hyclone, Logan, UT) or in a macrophage serum free media (endo-
toxin levels were undetectable; GIBCO, Grand Island, NY) for 2 to 
4 days as a monolayer until confluence, when they were passaged 
1:6. The conditioned media were saved and filter-sterilized through 
0.22 μm filters. The NBXFO cells were obtained from Dr Beverly 
Barton (Schering-Plough, Kenilworth, NJ); while the L1210 cells 
were obtained from Dr Lewis Slater (Department of Pathology, Uni-
versity of California, Irvine, CA). T9 glioma cells were obtained 
from M. Graf and Dr J. Hiserodt (Departments of Molecular Biology 
and Pathology, University of California, Irvine, CA). The P815, 
PA317, and WEHI-3 cells were purchased from the American Type 
Culture Collection (ATCC, Rockville, MD).

The hybridomas producing monoclonal antibodies against murine 
Mac 1a (TIB 128), Mac 2 (TIB 166), Mac 3 (TIB 168), and F4/80 
(HB 198) were purchased from the American Type Culture Collec-
tion. The supernates derived from NBXFO and M-CSF transfected 
clones were filtered through 0.22 μm filters and used at 25% to 
33% concentrations to stimulate the growth of either murine mac-
rophages or rat macrophages.

Bone marrow macrophage cultures. Bone marrow cells were 
cultured in 33% M-CSF containing conditioned media for 1 week 
at 37°C in a humidified 5% CO₂ atmosphere. Initial with the 
NBXFO cells was done in RPMI-1640 media with 5% fetal bovine 
serum (Hyclone) and using NBXFO supernate as the source of M-
CSF. The work using rat macrophages was done with macrophage 
serum-free media (GIBCO) using M-CSF transfected supernate as 
the source of M-CSF. After 1 week, the media was replaced with 
fresh 33% conditioned media. All culture materials were disposable 
plastics and free of endotoxin. Macrophages were removed by wash-
ing off the tissue culture media, and then incubating the cells in 
clinical grade irrigation saline (Kendal McGaw Inc, Irvine, CA) for 
30 minutes to 1 hour at 4°C. The cells were scraped using a cell 
scraper. This procedure results in >95% viability of the macro-
phages.

Construction of amphotrophic retroviruses to transfer the M-CSF 
isofoms. The production of amphotropic retroviruses has been pre-
viously described in detail in Nolta et al.25,26 The cDNA genes 
encoding for functional human M-CSF both the membrane-isoform 
and the secreted-isoform contained in pBR325 plasmids were ob-
tained from Dr Carl Rettenmier (Children’s Hospital of Los Angeles, 
Los Angeles, CA)27 after Materials Transfer Agreements were signed 
with Chiron Corporation (Emoryville, CA). The M-CSF genes were 
excised using Xho I and then ligated into the Xho I site of the pLXSN 
shuttle vector.27 These plasmids transformed DH5α bacterial and 
were selected in 50 μg/mL ampicillin. Aliquots of various plasmid 
clonal isolates were then digested with various restriction enzymes 
to insure that the M-CSF genes were oriented in the sense position. 
Plasmids containing the proper gene orientation were then used to 
transfect GEP cells via DOTAP (Boehringer-Manhtem, Indiana-
apolis, IN) to produce ecotropic retroviruses.28 After 2 days, the supernates 
from these retroviruses were used to infect PA317 cells.29 Cells 
were selected in 1 mg/mL G418 (Geneticin, GIBCO) for 1 week. 
 Afterwards, the PA317 cells that were resistant to G418 were cloned. 
Clones of PA317 producing high titers of retrovirus (10² to 10³ 
fected infectious units/mL) were selected and tested for functional retroviral 
activity.

Transfection of M-CSF genes into tumor cells. Rat T9 glioma 
cells were infected in six-well cluster dishes (Corning, Corning, 
NY). One hundred thousand exponentially growing cells were in-
culated either in the presence or absence of the supernates of the 
retroviruses overnight. The cells were refed with fresh media con-
taining 1 mg/mL G418. After 2 weeks of G418 selection, cells that 
were not infected with any retrovirus died, whereas, the infected 
cells continued to grow. Cells were selected based on production 
of human M-CSF using the human M-CSF Quantikine kits (R&D 
Systems, Minneapolis, MN). For sM-CSF production, cells were 
grown at 10⁵ cells/mL for 3 days and then tested. For M-CSF 
detection, cells were tested by flow cytometric analysis as described 
below.

Antibodies and flow cytometry. Cells to be phenotyped were 
first incubated in phosphate-buffered saline (PBS) for 10 minutes, 
followed by a 5 to 10-minute incubation at 37°C with 1 mg/mL 
collagenase (Sigma Chemical, St Louis, MO). After cells detached 
from the plastic, they were centrifuged and resuspended in PBS and 
counted. One-half million cells in 50 μL were first incubated with 
25 μL of normal rabbit serum for 5 minutes on ice to saturate all 
membrane bound Fc receptors followed by an incubation with 2.5 
μL of the anti-M-CSF antibody or 2.5 μL of an isotypic IgGl antibi-
ody on ice for 1 hour. Rat antinouse M-CSF (IgGl) antibody (0.1 
μg/mL) was purchased from Oncogene Sciences (Manhasset, NY).24 
The cells were washed once and then incubated in a 1:10 dilution 
of a fluorescein isothiocyanate (FITC)-labeled rabbit antirat antibody 
(Vector Laboratories, Burlingame, CA) for an additional hour on 
ice. The cells were washed three times with ice cold PBS in a 
refrigerated centrifuge. Ten thousand cells were analyzed on the 
EPICS Profile. Data was collected and then analyzed on the Multi-
2D program (Phoenix Flow Systems, San Diego, CA).

Hybridoma cells producing monoclonal antibodies against the mu-
rine Mac-1, Mac-2, Mac-3, and F4/80 determinants were purchased 
from ATCC. These cells were grown in vitro, and then the antibodies 
were isolated using the affinity purification reagents available from 
Sigma Chemical. Monoclonal antibodies against rat macrophage de-
terminant ED1 was purchased from Harlan Bioproducts for Science 
(Indianapolis, IN).

Cytostasis and cytotoxicity studies. Macrophages used for both 
types of experiments were first treated with 100 μg/mL of mitomy-
cin-C (Sigma Chemical Co) for 1 hour at 37°C to prevent macro-
phage-mediated division in these H-thymidine-based assays. Mac-
rophage-mediated cytostasis experiments were performed using 
the procedure of Krahenbuhl and Remington29 in 96 flat well plates. 
Here macrophages were incubated at various ratios with the tumor 
cells starting at 2:1 and finishing at 0.25:1. Fifty thousand tumor 
cells were plated with the macrophages in a final volume of 200 μL 
of macrophage serum-free media. On the next day, 8 hours before 
harvesting the cells, the individual wells were pulsed with 1 μCi of 
3H-thymidine (3H-TdR; New England Nuclear, NET-027A, 74 GBq/ 
mmol) in a volume of 25 μL. Immediately before the cultures were 
harvested, cultures were viewed under an inverted microscope to 
confirm whether tumor cells were present or absent under the various 
experimental conditions. Cells were then aspirated through a 
glass wool fiber filter with a multiple sample harvester (PHD Harvester, 
Cambridge, MA), and total 3H-TdR incorporation was determined 
by liquid scintillation procedures using Bio-Safe II (Research Pro-
ducts Int, Mount Prospect, IL). Data are expressed as the mean counts 
per minute (CPM) ± standard deviation (SD) per triplicate culture. 
Visual observations from each experiment confirmed the cytostasis 
results.

Macrophage mediated cytotoxicity studies were performed ac-
ording to the method of Meltzer.30 Target tumor cells were labeled 
with 4 μCi of 3H-TdR overnight in the media. The next morning, 
tissue culture media was replaced with fresh media and allowed to 
incubate a further 1 to 3 hours to reduce spontaneous release by the 
tumor cells. Ten thousand target cells were incubated in 200 μL
of macrophage serum-free media overnight with graded doses of macrophages ranging from 10:1 to 0.75:1 at 37°C in a humidified 5% CO2 incubator. Immediately before the supernates were harvested, cultures were viewed under an inverted microscope to confirm whether tumor cells were present or absent under the various experimental conditions. Afterwards, 100 μL of supernate was removed and placed into 2 mL of scintillation fluid. Spontaneous release after 24 hours was about 10% of maximum release. Maximum release is calculated by taking $10^4$ target cells and freeze-thawing them three times in liquid nitrogen. Specific release is calculated using the standard equation for cytotoxicity reactions. Visual observations from each experiment confirmed the cytotoxicity results. Cytotoxicity data from multiple experiments were pooled together at each macrophage: tumor cell ratio and is then presented as the mean ± standard deviation of the error of the means. Cytotoxicity is not considered relevant if values are ≥10% specific release.

Data from the cytostasis and cytotoxicity assays were analyzed using Student’s $t$ tests on the Sigma Plot Version 5.0 (Jandel Scientific, San Rafael, CA) computer program. Values were considered significantly different at the $P < 0.05$ levels.

Clinical grade Cetus M-CSF (activity: $6.94 \times 10^7$ units/mg; endotoxin content <0.08 EU/mL) was kindly provided by Chiron Corporation (Emoryville, CA).

*Electron microscopic studies.* Cells were gently scraped from monolayer cultures and then centrifuged (1,000g) in a 15-mL centrifuge tube for 10 minutes. The cells were then prepared the same way as described in detail in Jadus et al. The grids were examined with a Joel Electron microscope (Peabody, MA).

**RESULTS**

*M-CSF–activated bone marrow-derived macrophages inhibit the growth of NBXFO cells.* NBXFO cells expressed the membrane isoform of M-CSF (mM-CSF) in Fig 1; the supernate from these cells supported the growth of murine bone marrow macrophages. These M-CSF stimulated cells are >90% positive for Mac1, Mac2, Mac3, and F4/80 antigens, consistent with a macrophage phenotype. When these macrophages were cocultured with various tumor cells in a cytostasis experiment, the NBXFO cells were significantly ($P < 0.05$) inhibited in their growth at 2:1 to 0.5:1 macrophage: tumor ratios (Fig 2). These same macrophages failed to inhibit the growth of L1210 and P815 tumor cells ($P > 0.05$) when assayed concurrently. Similar results were found in a repeated experiment. These studies suggested that macrophages specifically inhibited the growth of NBXFO cells.

*Bone marrow-derived macrophages kill NBXFO cells, but not NIH 3T3-transfected cells with mM-CSF.* Figure 3 demonstrated that the bone marrow derived macrophages killed NBXFO cells in cytotoxicity assays. We used 30 μg/mL polymyxin-B to bind any endotoxin that could have contaminated the media or the cells. This data was pooled together at each effector:target ratio from eight independent assays. M-CSF–activated macrophages from two separate experiments did not kill NIH 3T3 cells transfected with the human mM-CSF gene. By flow cytometric analyses, these mM-CSF–transfected 3T3 cells were >90% for mM-CSF. This indicates that only tumor cells with the mM-CSF phenotype are killed by these macrophages.

*Freshly isolated adherent cells also kill NBXFO cells.* We took adherent cells obtained from murine bone marrow and spleen and assayed them to determine if freshly isolated macrophages could kill NBXFO cells. Table 1 shows that these adherent cells lysed the NBXFO cells after 24 hours. Thus, freshly isolated macrophages without prior in vitro
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exposure to M-CSF are capable of killing these target cells. Thymocytes did not kill the NBXFO cells, eliminating the trivial possibility that physical overcrowding was responsible for the death of the NBXFO cells.

Transfected tumor cells displaying mM-CSF are killed by M-CSF activated macrophages. The previous studies suggested that macrophages killed NBXFO cells by recognizing the mM-CSF found on NBXFO cells. Because NBXFO cells are hybridoma cells and may have other molecules that could provide macrophages with other ligands for binding, we performed more definitive experiments using T9 glioma cells expressing mM-CSF.

Rat T9 glioma cells were infected with retroviruses constructed to transfect the genes for either the membrane isoform of three randomly chosen T9 mM-CSF transfectant clones all expressed mM-CSF, but to variable amounts. The C2 clone was the most fluorescent, while the F6 clone was the least fluorescent.

Rat bone marrow derived macrophages (>90% ED1+) were tested for their ability to kill these transfected T9 cells in Fig 5. The macrophages killed the mM-CSF infected clones in a mM-CSF concentration-dependent manner. The C2 clone was killed the best, followed by the F2 clone. The F6 clone was not killed any better than were H1 or the T9 parental cells. Thus, there is a required threshold amount of mM-CSF present on the target cell before the macrophages will kill that target cell. We selected the C2 and H1 clones for all further work. The macrophages have reproducibly killed only the C2 cells and not H1 or the parental cells.

We modified the M-CSF enzyme-linked immunosorbent assay (ELISA) assay to quantitate the amount of mM-CSF present on C2 cells (Jadus et al, manuscript submitted) We found that 10,000 C2 cells express 1,002 pg of mM-CSF, while an equivalent number of H1 clones or parental T9 cells were negative for mM-CSF. We used a 100-fold excess of M-CSF (100,000 pg) to completely block macrophage cytotoxicity against the C2 clones as shown in Table 2. This experiment has been successfully reproduced one more time.

The mechanism of cytotoxicity displayed by the macrophages against the mM-CSF transfectant C2 clone does not involve a soluble factor, but may include phagocytosis. Tumoridical macrophages can kill through the production of short-lived soluble factors such as superoxide radicals, hydrogen peroxide, and nitric oxide. We tested whether inhibitors of these cytotoxins could prevent macrophage-mediated killing of the C2 clones. Figure 6 shows that 50 U/mL of catalase, 20 U/mL superoxide dismutase, and 20 μmol/L NAME failed to prevent macrophage-mediated killing of the C2 clones (upper panel). All experimental results at each macrophage:target cell ratio were not significantly different (P > .05) from the untreated control cells. None of these reagents affected macrophage cytotoxicity of the parental T9 cells or H1 clones (lower two panels).

To eliminate the possibility that other unknown soluble cytotoxins are responsible for this macrophage-mediated cytotoxicity against the mM-CSF clones, we performed mixing experiments. Here labeled T9 or H1 clones were mixed with an equal number of unlabeled mM-CSF C2 cells in the presence of the bone marrow-derived macrophages. In Table 3, the macrophages did kill the mM-CSF C2 clone, but the macrophages did not kill the labeled parental T9 or H1 clone, either alone or in the presence of the unlabeled C2 clone. This study eliminates that any soluble cytotoxic factor such

Table 1. Freshly Isolated Adherent Splenocytes and Bone Marrow Cells Can Lyse NBXFO Cells

| Effector:Target Ratio | Bone Marrow | Spleen | Thymus
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<tr>
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<tr>
<td>10:1</td>
<td>27.5 ± 0.7</td>
<td>23.0 ± 2.8</td>
<td>2.8 ± 2.0</td>
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<tr>
<td>5:1</td>
<td>11.0 ± 4.2</td>
<td>22.5 ± 4.9</td>
<td>4.9 ± 1.0</td>
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<tr>
<td>2.5:1</td>
<td>5.0 ± 0.0</td>
<td>14.5 ± 0.5</td>
<td>4.9 ± 0.0</td>
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* Macrophage mediated cytotoxicity was measured after a 24-hour incubation in the presence of 20 μg/mL polymyxin B.

† Freshly isolated mouse adherent spleen and bone marrow cells were isolated.

‡ Freshly isolated mouse thymocytes were used.
Fig 4. mM-CSF flow cytometric profile of cloned transfected T9 glioma cells. Various cloned transfected T9 glioma cells (H1: sM-CSF; C2, F2, F6: mM-CSF) were incubated with either an IgG1 isotypic or an anti-M-CSF antibody. The surface fluorescence of 10,000 cells were collected. The isotypic controls were subtracted from the anti-M-CSF fluorescence values and are labeled percent positive in upper right corner of each graph.

Fig 5. Macrophages kill cloned mM-CSF transfected T9 glioma cells. Rat bone marrow-derived cells were cultured in M-CSF containing media for the first week. The cells were refed with fresh media containing M-CSF for another week. These M-CSF activated macrophages were cultured with H3-TdR labeled target cells in the presence of 30 µg/mL polymyxin B. The target cells included parental T9, one sM-CSF transfected clone: H1; three randomly picked mM-CSF transfected clones: C2, F2, F6. After 1 day, the supernates were harvested. Data is presented as percent specific release ± standard deviation of triplicate cultures.

Table 2. A 100-fold Excess of Recombinant M-CSF Will Prevent Bone Marrow Macrophages From Killing the mM-CSF Transfectant C2 Clone

<table>
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<th>Effector:Target Ratio</th>
<th>Without M-CSF</th>
<th>With M-CSF†</th>
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<tr>
<td>20:1</td>
<td>25 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>10:1</td>
<td>23 ± 4</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>5:1</td>
<td>18 ± 9</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>2.5:1</td>
<td>8 ± 1</td>
<td>6 ± 0</td>
</tr>
</tbody>
</table>

* Macrophage mediated cytotoxicity was measured after a 24-hour incubation in the presence of 20 µg/mL polymyxin B.
† Ten thousand C2 cells possess 1,002 pg mM-CSF; recombinant M-CSF (100,000 pg) was added to achieve a 100-fold excess.
for 2 weeks. The macrophage contained numerous granules. Additionally, these cells possessed several lipid droplets. These fat droplets provided us with a fortunate internal marker for the macrophages so we can positively identify the macrophage when we mixed these cells with the T9 tumor cells. Figure 7C shows a macrophage that was incubated with the mM-CSF transfectant cells after 24 hours. Here a macrophage has ingested several mM-CSF transfected T9 tumor cells (labeled T). The macrophage is identified because the macrophage is the bigger cell, and the lipid droplets are present. These lipid droplets have coalesced into bigger ones. The numerous granules found in the normal macrophage have disappeared in these phagocytic macrophages. Presumably these granules fused with the mM-CSF bearing tumor cells when they formed the phagolysosome.

To determine whether phagocytosis could be responsible for the cytotoxicity observed against NBXFO cells. We also performed electron microscopy with the murine macrophages and NBXFO cells. Again, we found macrophages that ingested NBXFO cells, one such cell is shown in Fig 8. These murine macrophages were previously cultured in complete RPMI-1640 media along with the NBXFO supernate and did not display any fat droplets as seen in the rat macrophages when cultured in the macrophage serum-free media. This study illustrates that the presence of fat droplets within the macrophages are not necessary for macrophage cytotoxicity.

**DISCUSSION**

In tumor biology, macrophages may be considered a ‘‘double edged sword’’. Macrophages frequently associate within breast and ovarian tumors in response to M-CSF produced by these tumors. Macrophages may induce tumor growth by releasing stimulatory or angiogenic factors or by acting as immunosuppressor cells. Other studies have concluded that macrophages were beneficial for the host.

In several cytokine transfection models, macrophages were one effector cell when the tumor cells were expressing interleukin-2 (IL-2), IL-4, IL-6, IL-7, IFN-γ, and TNF. For macrophages to become tumoricidal in vitro, they must be stimulated in two ways. First, cytokines prime the macrophages, while secondary signals allow the macrophage to kill the tumor cell. It is tempting to speculate that this ‘‘double edged sword’’ effect could be explained by the two-signal model. When macrophages only receive the priming signal, these macrophages promote tumor growth and metastases. Whereas, when both signals are received the macrophages mediated tumor regression. One possible mechanism to tip the balance toward a favorable prognostic response is to devise a molecule that delivers to the macrophage both cytotoxic delivery signals simultaneously. In studies presented here, macrophage cytotoxicity against tumor cells may be accomplished by a single molecule, namely the membrane isoform of macrophage colony stimulating factor.

In this report, we found that M-CSF-activated macrophages inhibited the growth of mM-CSF expressing NBXFO cells (Fig 2) and killed NBXFO cells in an endotoxin-free environment (Fig 3). NBXFO cells are hybridoma cells and may possess other cell surface molecules that may induce immune responsiveness as shown by Guo et al.6 We created retroviral vectors to transfer the mM-CSF gene into a defined

| Table 3. Macrophages Will Not Kill T9 or the sM-CSF Clone, H1, in the Presence of the mM-CSF Clone, C2 |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| % Specific Release ± SD*       | Addition of Unlabeled Target |
| Macrophage:Target Ratio       | None            | T9              | H1              | C2              |
| Labeled T9 target             |                  |                 |                 |                 |
| 10:1                          | 12 ± 2           | 9 ± 2           | 10 ± 6          | 11 ± 1          |
| 5:1                           | 10 ± 0           | 9 ± 3           | 4 ± 2           | 8 ± 2           |
| 2.5:1                         | 6 ± 3            | 4 ± 3           | 1 ± 4           | 5 ± 2           |
| Labeled H1 target             |                  |                 |                 |                 |
| 10:1                          | 12 ± 2           | 9 ± 3           | 10 ± 5          | 13 ± 6          |
| 5:1                           | 13 ± 3           | 8 ± 2           | 8 ± 2           | 7 ± 1           |
| 2.5:1                         | 8 ± 3            | 7 ± 2           | 4 ± 3           | 6 ± 1           |
| Labeled C2 target             |                  |                 |                 |                 |
| 10:1                          | 34 ± 3           |                 |                 |                 |
| 5:1                           | 25 ± 2           |                 |                 |                 |
| 2.5:1                         | 22 ± 2           |                 |                 |                 |

* Macrophage mediated cytotoxicity was measured after a 24-hour incubation in the presence of 20 μg/mL polymyxin B.

† Labeled target cells were cultured in the presence of an equal number of unlabeled target cells throughout the course of this experiment.
tumor cell to prove our hypothesis that macrophages can kill mM-CSF bearing tumor cells.

Rat T9 glioma clones transfected with the mM-CSF retrovirus, did express mM-CSF (Fig 4) and were killed by M-CSF activated macrophages in a dose-dependent juxtacrine manner (Fig 5). Parental T9 cells and sM-CSF transfected H1 clones were not killed by these macrophages. This macrophage-mediated cytotoxicity against the mM-CSF transfectant was prevented by using a 100-fold excess of recombinant M-CSF (Table 2). NIH 3T3 fibroblasts expressing mM-CSF were not killed by macrophages (Fig 3). Membrane M-CSF by itself is insufficient to allow macrophages to kill nontransformed cells bearing this molecule. Therefore, another tumor specific molecule allows the macrophages to distinguish the tumor cell from a normal cell. This finding is consistent with previous work\(^{57}\) showing that macrophages only kill transformed tumor cells and not rapidly growing fibroblasts.

Most M-CSF producing cells only make the secreted form of M-CSF. When sM-CSF transfected myeloma cells grew as a tumor, macrophages were found within the tumor bed.\(^{58}\) This work showed that M-CSF acted as a strong chemoattractant for macrophages, but probably did not induce any direct tumoricidal activity, perhaps by not allowing the macrophage to physically contact the tumor cell. When we injected NBXFO cells IP into mice (up to 16 million cells/syngeneic mouse), no tumors ever developed, even in mice first treated with high-dose cyclophosphamide, which induced potent macrophage suppressor cells.\(^{22,23}\) Freshly isolated adherent cells did kill mM-CSF positive T9 cells (Table 3).
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1. Thus, killing of mM-CSF tumor cells is not just restricted to M-CSF activated macrophages. If this is true in vivo, the injection of the mM-CSF retrovirus directly into a tumor with a high macrophage content may induce the endogenous macrophages to kill those infected tumor cells and perhaps reduce the tumor burden. Another therapeutic approach may involve allowing the M-CSF transfected tumor cell to be killed by the macrophages in vivo and then allowing these macrophages to act as antigen presenting cells and then stimulating systemic immune responses. The exact physiological role of mM-CSF is unknown, but we believe this unique molecule may represent a novel way of targeting tumor cells to macrophages to stimulate an immune response.

In summary, we have found that M-CSF activated macrophages inhibited the growth and killed NBXFO hybridoma cells that express the membrane isoform of macrophage colony-stimulating factor. This cytotoxic activity was mediated by both M-CSF–activated bone marrow-derived macrophages and freshly isolated macrophages. When T9 glioma cells were transfected with retroviruses containing the gene for mM-CSF, macrophages killed those T9 glioma cells that only expressed the mM-CSF. This killing was inhibited by using a 100-fold excess of recombinant M-CSF. One putative mechanism of macrophage mediated killing may include direct phagocytosis of the mM-CSF tumor cells, because no evidence that a soluble cytotoxic mediator was found.

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Macrophages can recognize and kill tumor cells bearing the membrane isoform of macrophage colony-stimulating factor

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