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

By Martin R. Jadus, Melanie C.N. Irwin, Michael R. Irwin, Robert D. Horansky, Sant Sekhon, Karen A. Pepper, Donald B. Kohn, and H. Terry Wepsic

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\(^{n}\)-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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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. Tarnowski or Dr S. Szabo (VAMC, Long Beach, CA 90822).

CA) who purchased these animals from Harlan Sprague-Dawley (San Diego, CA).

**Cell lines.** Myeloma plasma cell lines 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 (endoxin 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 (Scherer-Plough, Kenilworth, NJ); while the L1210 cells are not infected with any retrovirus died, whereas, the infected cells continued to grow. Cells were selected on the basis of product human M-CSF using the human M-CSF Quantikine kits (R&D Systems, Minneapolis, MN). For M-CSF production, cells were grown at 10^5 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 IgG1 antibody on ice for 1 hour. Rat antinouse M-CSF (IgG1) antibody (0.1 μg/mL) was purchased from Oncogene Sciences (Manhasset, NY). The cells washed once and then incubated in a 1:10 dilution of a fluorescein isothiocyanate (FITC)-labeled rabbit antirabbit antibody (Vector Laboratories, Burlingame, CA) for an additional hour on ice. The cells were washed three times with ice cold PBS in a refrigerator 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).

**Cytostasis and cytotoxicity studies.** Macrophages used for both types of experiments were first treated with 100 μg/mL of mitomycin-C (Sigma Chemical Co) for 1 hour at 37°C to prevent macrophage-mediated division in these H-thymidine–based assays. Macrophage-mediated cytostasis experiments were performed using the procedure of Krakenbuhl and Remington 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 H-thymidine (H-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 H-TdR incorporation was determined by liquid scintillation procedures using Bio-Safe II (Research Products 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 according to the method of Melzer.** 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

**Construction of amphotrophic retroviruses to transfer the M-CSF isoforms.** The production of amphotropic retroviruses has been previously described in detail in Nolta et al. The cDNA genes encoding for functional human M-CSF both the membrane-isoform and the secreted-isoform contained in pBR325 plasmids were obtained from Dr Carl Rettenmier (Children's Hospital of Los Angeles, Los Angeles, CA) 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. These plasmids transformed DH5α bacteria and were selected in 50 μg/mL ampicillin. Aliquots of various plasmid clones were used to infect PA317 cells. The conditioned media were saved and filter-sterilized through 0.22 μm filters and were used at 25% to 33% concentrations to stimulate the growth of either murine macrophages or rat macrophages.

**Bone marrow macrophage cultures.** Bone marrow cultures were cultured in 33% M-CSF containing conditioned media for 1 week at 37°C in a humidified 5% CO2 atmosphere. Initial work with the NBXFO cells was done in RPMI-1640 media with 5% fetal bovine serum (HyClone) and then 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 washing 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 macrophages.

**Transfection of M-CSF genes into tumor cells.** Rat 9T glioma cells were infected in six-well cluster dishes (Corning, Corning, NY). One hundred thousand exponentially growing cells were incubated either in the presence or absence of the supernates of the retroviruses overnight. The cells were refed with fresh media containing 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 M-CSF production, cells were grown at 10^5 cells/mL for 3 days and then tested. For M-CSF detection, cells were tested by flow cytometric analysis as described below.
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 10⁶ target cells and freeze-thawing them three times in liquid nitrogen. Specific release is calculated using the standard equation for cytotoxicity reactions.¹²  Visual observations from multiple experiments were pooled together at each macrophage: tumor cell ratio and is then presented as the mean ± standard 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 × 10⁵ 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.²⁴  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 < .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 > .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
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 or the secreted isoform of human M-CSF (sM-CSF). 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:tumor 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>
<thead>
<tr>
<th>Effector:Target Ratio</th>
<th>Bone Marrowt</th>
<th>Spleen†</th>
<th>Thymust</th>
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<tr>
<td>10:1</td>
<td>27.5 ± 0.7</td>
<td>22.0 ± 2.8</td>
<td>2.8 ± 2.0</td>
</tr>
<tr>
<td>5:1</td>
<td>11.0 ± 4.2</td>
<td>22.5 ± 4.9</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>2.5:1</td>
<td>6.0 ± 0.0</td>
<td>14.5 ± 0.5</td>
<td>4.9 ± 0.0</td>
</tr>
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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 transfectant 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 H5-TdR labeled target cells in the presence of 30 μg/mL polymyxin B. The target cells included parental T9, one sM-CSF transfectant 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>
<thead>
<tr>
<th>Effector:Target Ratio</th>
<th>Without M-CSF</th>
<th>With M-CSF†</th>
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<tbody>
<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>
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</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.

as TNF, oncostatin-M, or interferon could be released by the cytotoxic macrophages.

This last experiment suggested that cell-to-cell contact was required for this specific cytotoxicity. We performed electron microscopy to determine whether phagocytosis could be responsible for this cytotoxicity. Figure 7A shows a typical T9 cell, while Fig 7B shows a typical rat bone marrow-derived macrophage cultured in macrophage serum-free media in the presence of M-CSF derived form H1 cells.
Macrophage-mediated killing of mM-CSF transfected clones are unaffected by catalase, superoxide dismutase, and NAME. Rat M-CSF activated macrophages grown in the presence of M-CSF for 2 weeks were cocultured with either H′-TdR-labeled parental T9, mM-CSF transfected C2 clone, or sM-CSF T9 H1 clone for 1 day in the presence of 20 μmol/L NAME, 50 U/mL catalase or 20 U/mL superoxide dismutase. Data is presented as percent specific release ± standard deviation of triplicate cultures.

FIG 6. Macrophage-mediated killing of mM-CSF transfected clones are unaffected by catalase, superoxide dismutase, and NAME.

**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 simulated 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. We created retroviral vectors to transfer the mM-CSF gene into a defined background. For personal use only.
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 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. 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. Freshly isolated adherent cells did kill mM-CSF positive T9 cells (Table...
MM-CSF INDUCES NOVEL TUMORICIDAL MACROPHAGES

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 mM-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.

Acknowledgment

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mM-CSF INDUCES NOVEL TUMORICIDAL MACROPHAGES


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

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