GM-CSF Enhances 3F8 Monoclonal Antibody-Dependent Cellular Cytotoxicity Against Human Melanoma and Neuroblastoma

By Brian H. Kushner and Nai-Kong V. Cheung

3F8 is a murine monoclonal IgG3 antibody specific for the tumor-associated antigen ganglioside GD2. Previous in vitro studies suggest that tumor regressions observed in a phase I clinical trial of 3F8 may be attributable to complement activation by 3F8 and to 3F8-dependent cellular cytotoxicity (ADCC) with lymphocytes. We now describe 3F8-mediated ADCC of GD2-positive tumor targets (melanoma and neuroblastoma) with human granulocytes and report that recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) enhanced this phenomenon. Cytotoxicity required binding of 3F8 to the low-affinity Fc receptor type III (CD16) on the granulocytes and was poor with tumor-binding monoclonal antibodies of other immunoglobulin (ie, non-IgG3) subclasses. GM-CSF (2 to 20 ng/mL) increased ADCC by 93% to 287% at limiting dilutions of 3F8 (1 μg/mL). With most GD2-positive cell lines tested, this effect translated into a tenfold or greater augmentation in 3F8 efficiency at mediating ADCC. Comparable enhancement occurred whatever GM-CSF was present in the ADCC assay or granulocytes were incubated with GM-CSF and washed before the assay. Nonoxidative mechanisms may be important for ADCC since 3F8 mediated ADCC with granulocytes from two children with chronic granulomatous disease; this cytotoxicity was also enhanced by GM-CSF. Since GM-CSF induces a neutrophilia in patients, our data suggest that this cytokine may have the potential of amplifying 3F8 antitumor activity in patients by increasing effector cell numbers and by priming granulocytes for greater cytotoxicity.

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GM-CSF. Recombinant GM-CSF (Genetics Institute, Cambridge, MA), prepared as previously described, was used at 0.0002 to 200 ng/mL, final concentration.

Cell lines. The tumor cell lines Daudi, HT144, K562, SKMel-1, SKNMC, and SKNSh were purchased from the ATCC. SKMel-31 was provided by Dr A. Houghton, Memorial Sloan-Kettering Cancer Center, New York; LAN-1 and LAN-5, were provided by Dr R. Steege, and M-14 was provided by Dr R. Irr, both of the University of California, Los Angeles; and IM-6 and NMB-7 was provided by Dr K. Liao, McMaster University, Hamilton, Ontario, Canada. All cell lines were cultured in RPMI 1640 supplemented with 1% heat-inactivated calf serum (HyClone Laboratories, Logan, UT), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL strepto-
C#3

In C-,

LI

LU

In I-.

LU

C-,

LU

80

60

40

20

0

60

50

40

30

20

10

0

3F8 CONCENTRATION (µG/ML)

- 10
- 1.0
- 0.1
- 0.01

PERCENT SPECIFIC LYSIS

EFFECTOR: TARGET RATIO

Fig 1. 3F8 dose-dependence of granulocyte ADCC against neuroblastoma NMB-7. 3F8 at 1 to 10 µg/mL mediated efficient lysis of tumor cell targets by granulocytes.

mycin (complete medium). For some experiments, RPMI with 0.5% human serum albumin (HSA) was used.

Effector cells. Granulocytes were isolated from heparinized peripheral blood (PB) by Ficoll-Paque gradients (Pharmacia) followed by 3% dextran separation and hypotonic RBC lysis. Polymorphonuclear leukocytes composed >95% of the resulting effector cell population.

Chromium release assay. Target tumor cells were labeled with sodium 51Cr chromate (Amersham, Arlington Heights, IL) at 100 Ci/10^6 cells at 37°C for one hour. After the cells were washed, loosely bound 51Cr was leaked for one hour at 37°C. After further washing, 5 x 10^4 target cells/well were admixed with effector cells, MoAbs, and/or GM-CSF in 96-well polystyrene round-bottom plates (Sarstedt, FRG) to a final volume of 250 µL/well. In some experiments, granulocytes were incubated with GM-CSF at 37°C in complete medium at 2 x 10^6/mL and washed prior to use. The plates were incubated at 37°C for four hours (except as noted) and then centrifuged at 400 g for five minutes; the released 51Cr in supernatant was counted in a γ-counter (Packard Instrument, Downers Grove, IL). Percentage of specific release was calculated using the formula 100% x (experimental cpm - background cpm)/(5% sodium dodecyl sulfate [SDS] cpm - background cpm), where cpm are counts per minute of 51Cr released. Total release was assessed by lysis with 5% SDS (Sigma), and background release was measured in the absence of granulocytes. The background was 10% to 25% for the neuroblastoma cell lines and 7% to 16% for the melanoma lines.

Lytic units. For the cell lines tested, specific lysis was a semilog function of effector:target (E:T) ratio. The linear portions of the cytotoxicity curves (usually 20% to 70% specific lysis) were used for analysis. One lytic unit was defined as the number of effector cells required to lyse 50% of 5 x 10^6 target cells. Lytic units per 10^6 effector cells were defined as follows: LU/10^6 = ke^y, where k = 5 x 10^4/5 x 10^4, y = (observed percentage of lysis - 50%)/slope of the cytotoxicity curve, and x = E:T ratio at which the observed percentage of lysis occurred.

RESULTS

3F8-mediated granulocyte ADCC. 3F8 mediated granulocyte ADCC in a dose-dependent manner (Fig 1). Maximal lysis by granulocytes was achieved with 3F8 at 10 µg/mL final concentration. Specific lysis in the absence of 3F8 was 0% to 3%. Half-maximal lysis occurred at 0.1 µg/mL. This ADCC was not inhibited by human serum (data not shown).

Only target cells expressing GD2 were sensitive to 3F8-mediated granulocyte ADCC. The neuroblastoma and melanoma cell lines shown in Table I express GD2 in high density. Efficient cell kill was achieved when these cells were exposed to granulocytes plus 3F8. Target cells that do not express GD2, such as the lymphoid line Daudi, the erythroleukemia line K562, or the neuroectodermal lines SKNMC and SKNSH, were resistant to 3F8-mediated granulocyte ADCC, confirming the specific target antigen requirement of the phenomenon.

Insignificant cytotoxicity was observed when granulocyte ADCC was studied using a panel of MoAbs that either lack the Fc region, belong to a different immunoglobulin subclass,

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>No. of Experiments</th>
<th>E:T Ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100:1</td>
<td>50:1</td>
</tr>
<tr>
<td></td>
<td>3F8</td>
<td>No 3F8</td>
</tr>
<tr>
<td>Neuroblastoma (GD2 positive)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMB-7</td>
<td>6</td>
<td>65</td>
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<td>IMR-6</td>
<td>7</td>
<td>62</td>
</tr>
<tr>
<td>LAN-1</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>LAN-5</td>
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<td>44</td>
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<tr>
<td>Melanoma (GD2 positive)</td>
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<td>SKMel-1</td>
<td>14</td>
<td>48</td>
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<td>SKMel-31</td>
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<td>42</td>
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<td>HT-144</td>
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<td>28</td>
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<tr>
<td>M-14</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>Other (GD2 negative)</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>K562</td>
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<td>6</td>
</tr>
<tr>
<td>SKNMC</td>
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<tr>
<td>SKNSH</td>
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</table>

Table 1. 3F8-Mediated Granulocyte ADCC

Results are expressed as percent specific target cell lysis. Data are arithmetic means. 3F8 = 10 µg/mL final concentration.
or recognize other target cell antigens (Table 2). Specific lysis was achieved only with the intact anti-GD2 IgG3 3F8 MoAb. Without the Fc region, the F(ab’)2 fragment of 3F8 bound to target cells but was ineffective in ADCC. 3G6, an IgM MoAb against GD2, was also ineffective at mediating granulocyte ADCC in this system. Neuroblastoma MoAbs of other subclasses—3E7 (IgG2b), 6A11 (IgM), HSAN 1.2 (IgG1a), and UJI13A (IgG1)—also failed to mediate ADCC. Finally, IgG3 MoAb that do not bind to tumor targets, such as NS.7 which recognizes SRBCs, did not mediate granulocyte ADCC against GD2-positive target cells.

### GM-CSF effects on 3F8-mediated granulocyte ADCC

Addition of GM-CSF to granulocytes and target cells in the 51Cr release assay enhanced 3F8-mediated tumor cytotoxicity. Increasing amounts of GM-CSF (0.002 to 20 ng/mL) produced graded increments of cell kill (Table 3). Maximal effects were achieved with 2 to 20 ng/mL. Suppressive effects on ADCC were noted at higher concentrations. GM-CSF alone, without granulocytes, produced no tumor cell cytotoxicity (data not shown).

At limiting concentrations of 3F8 (ie, 1 µg/mL), GM-CSF enhanced cytotoxicity of all GD2-positive targets tested by 93% to 267% (Table 4). This effect was obtained whether GM-CSF was added directly to the 51Cr release assay (Table 4) or granulocytes were incubated in complete medium at 37°C for four hours with GM-CSF and then washed before being plated with target cells in the four-hour cytotoxicity assay (data not shown). With most GD2-positive cell lines, GM-CSF activation translated into a tenfold or greater reduction in the concentration of 3F8 required for mediating granulocyte cytotoxicity (ie, granulocytes in the presence of 3F8 at 1 µg/mL plus GM-CSF achieved killing equivalent to that achieved with a tenfold higher concentration of 3F8 (10 µg/mL) and no added GM-CSF.

The time course of GM-CSF effects on granulocyte ADCC in vitro was studied by harvesting the 51Cr release assay at 0.5, 1, and 2 hours, in addition to the standard four hours. A GM-CSF effect on cytotoxicity was evident at 0.5 hours. For example, in the representative experiment shown in Fig 2, at 0.5 hours granulocytes in the presence of GM-CSF showed 0.4 lytic units of cytotoxic activity as compared with 0.2 lytic units of untreated effector cells.

### Fc receptor dependency

To define further the Fc receptor dependency of granulocyte ADCC in our system, the anti-CD16 MoAb 3G8 was added to the 51Cr release assay at time zero. 3G8 inhibited 3F8-mediated tumor cytotoxicity in a dose-dependent and highly efficient manner (Fig 3). At 1/200 (0.005 µg/mL) of the concentration of 3F8 (1.0 µg/mL), 3G8 inhibited granulocyte ADCC of three cell lines by 37% to 42%; at higher concentrations of 3G8 (0.5 to 5.0 µg/mL), this inhibition reached 90% to 100%. Addition to the 51Cr release assay of MoAbs (1.0 to 5.0 µg/mL) recognizing other antigens present on granulocytes (anti-β2-microglobulin, anti-LFA-1, and anti-Ia when LAN-1 and NMB-7 were used as targets, and anti-LFA-1 with SKMel-1 as targets) had no inhibitory effect (data not shown); this

### Table 2. Requirement of Specific Ig Subclass and Target Cell Antigen

<table>
<thead>
<tr>
<th>Antigen specificity</th>
<th>G02</th>
<th>G02</th>
<th>G02</th>
<th>NB</th>
<th>SRBC</th>
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Results are expressed as percent specific target cell lysis. Data are the means of one to four experiments. MoAb = 10 µg/mL final concentration. E:T ratio = 100:1. NB, neuroblastoma; ND, not done; None, no MoAb added; SRBC, sheep red blood cell.

### Table 3. Effect of Increasing Concentrations of GM-CSF on 3F8-Mediated Granulocyte ADCC

<table>
<thead>
<tr>
<th>GM-CSF (ng/mL)</th>
<th>LAN-1 50:1</th>
<th>25:1</th>
<th>12:1</th>
<th>NMB-7 50:1</th>
<th>25:1</th>
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<tr>
<td>20.0</td>
<td>67</td>
<td>49</td>
<td>29</td>
<td>72</td>
<td>60</td>
<td>36</td>
</tr>
</tbody>
</table>

Results of these representative studies are expressed as percent specific lysis, which was 0% to 2% in the absence of 3F8. 3F8 = 2 µg/mL final concentration.
finding suggests that the 3G8 inhibition was not a nonspecific steric effect.

Activation of granulocytes with GM-CSF (2 ng/mL) partially overcame 3G8 inhibitory effects; thus, with GM-CSF, the curves in Fig 3 were shifted to the right, changing the approximate 3G8 ID₅₀ (50% inhibition point) from 0.015 to 0.03 µg/mL.

**ADCC with chronic granulomatous disease (CGD) granulocytes.** To investigate whether reactive oxidation intermediates from granulocytes are essential in 3F8-mediated ADCC, studies were performed using granulocytes obtained from two patients with X-linked CGD. Although CGD effector cells in the presence of 3F8 demonstrated lytic activity against neuroblastoma line IMR-6 and LAN-1, the degree of lytic activity was less than the range observed with normal granulocytes. Exposure to GM-CSF activated CGD granulocytes in ADCC (Fig 4).

**DISCUSSION**

Our data show that 3F8 mediates efficient granulocyte killing of tumor cells that express the G₂₅ antigen and that GM-CSF enhances this phenomenon. Cytotoxicity was observed using concentrations of 3F8 (ie, 1 to 10 µg/mL) that are likewise effective in mediating ADCC using peripheral blood lymphocytes. These concentrations were achieved in the serum of patients who received intravenous 3F8 in our previously reported phase-I clinical trial. Thus, a MoAb of defined specificity (galactosylceramide G₂₅) that has been used clinically in human patients with demonstrated antitumor effects mediates granulocyte ADCC against a panel of tumor cell targets representative of two refractory human cancers. In contrast, several previous reports of GM-CSF effects on granulocyte ADCC against tumor cells involved rabbit antibodies or antisera with no apparent clinical utility and used one to three selected animal or human tumor cell lines as targets.

3F8 specificity for the cytotoxic process is supported by the absence of granulocyte ADCC either with IgG, MoAb against nontumor antigens or with non-IgG, MoAb directed at G₂₅ and other tumor-associated antigens. The Fc region of the 3F8 is required for granulocyte ADCC; inhibition of ADCC by the anti-CD16 3G8 MoAb suggests that the Fc region binds to the low-affinity Fc type III receptor (CD16) on granulocytes. These findings are consistent with the generally accepted view that granulocytes attach to anti-

![Fig 2](https://example.com/fig2.png)  
**Fig 2.** Time course of GM-CSF enhancement of granulocyte ADCC against neuroblastoma NMB-7. GM-CSF-enhancing effects on granulocyte ADCC were evident by 30 minutes (3F8 = 10 µg/mL final concentration).

![Fig 3](https://example.com/fig3.png)  
**Fig 3.** MoAb 3G8 effects on 3F8-mediated granulocyte ADCC. Increasing concentrations of 3G8, which binds to the low-affinity Fc receptor type III (CD16) on leukocytes, caused greater inhibition of 3F8-mediated granulocyte cytotoxicity.

![Fig 4](https://example.com/fig4.png)  
**Fig 4.** Comparison of normal v CGD granulocyte ADCC against neuroblastoma LAN-1. GM-CSF enhanced ADCC by CGD granulocytes, increasing the cytotoxicity to the range observed with normal granulocytes not exposed to GM-CSF. (Combined data from two experiments using granulocytes from a single CGD patient.)
body-coated tumor cell targets through Fc receptors. Oxidative and nonoxidative mechanisms have been implicated in granulocyte ADCC in other systems. Our experiments using granulocytes with defective oxidative metabolism obtained from patients with CGD suggest that both mechanisms may contribute to 3F8-mediated granulocyte ADCC.

Previously described in vitro and in vivo effects on GM-CSF on granulocyte proliferation and function stimulated our efforts to explore its influence on 3F8-mediated granulocyte ADCC. In addition, GM-CSF does not stimulate proliferation of melanoma or neuroblastoma cells (Usmani N, Cheung NKV, unpublished observations), unlike, for example, its effect on myeloid leukemias. Exposure of granulocytes to GM-CSF enhanced 3F8-mediated ADCC without causing a concomitant increase in spontaneous antitumor cytotoxicity activity. Maximal enhancement of granulocyte ADCC in our system was observed with GM-CSF at 0.2 to 20 ng/mL. In vitro, these concentrations of GM-CSF stimulate GM colony formation, upregulate granulocyte surface receptor expression, and prime granulocytic functional and metabolic activity. The latter actions, also detected in granulocytes harvested from animals and patients receiving GM-CSF, may directly potentiate granulocyte ADCC. Our studies documenting a rapid onset of GM-CSF enhancement of granulocyte ADCC suggest that this effect does not result merely from the previously demonstrated capacity of GM-CSF to prolong survival of granulocytes in vitro but instead favors a direct influence of GM-CSF on cytotoxic mechanisms of granulocytes and/or on the surface expression of Fc receptors or other adhesion molecules.

GM-CSF may augment the therapeutic efficacy of 3F8 in two ways: by increasing the number of circulating effector cells and, as our in vitro findings suggest, by enhancing granulocyte ADCC. Our results supplement the previously reported enhancing effects of IL-2 on 3F8-mediated ADCC by PB lymphocytes. In addition, 3F8, like other IgG, antibodies, activates complement which results in target cell lysis. In vivo, complement activation at tumor sites may elicit local inflammatory reactions that may influence the trafficking of leukocytes. Finally, previous studies have clearly demonstrated good localization of 3F8 to tumors in patients. Thus, 3F8 may have the clinical potential to mobilize and focus diverse components of the host immune system for selective ablation of tumor cells; use of cytokines such as GM-CSF and IL-2 in conjunction with 3F8 may help maximize the antineoplastic effect.

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GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma

BH Kushner and NK Cheung