Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion cDNA for Cancer Gene Immunotherapy

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Abstract
Genetic engineering of tumor cells to express both granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2 can induce synergistic immune antitumor effects. Paradoxically, the combination has also been reported to down-regulate certain immune functions, highlighting the unpredictability of dual cytokine use. We hypothesized that a GM-CSF and IL-2 fusion transgene (GIFT) could circumvent such limitations yet preserve synergistic features. We designed a fusion cDNA of murine GM-CSF and IL-2. Protein structure computer modeling of GIFT protein predicted for intact ligand binding domains for both cytokines. B16 mouse melanoma cells were gene modified to express GIFT (B16GIFT), and these cells were unable to form tumors in C57bl/6 mice. Irradiated B16GIFT whole-cell tumor vaccine could also induce absolute protective immunity against challenge by live B16 cells. In mice with established melanoma, B16GIFT therapeutic cellular vaccine significantly improved tumor-free survival when compared with B16 expressing both IL-2 and GM-CSF. We show that GIFT induced a significantly greater tumor site recruitment of macrophages than combined GM-CSF and IL-2 and that macrophage recruitment arises from novel chemotactic feature of GIFT. In contrast to suppression by GM-CSF of natural killer (NK) cell recruitment despite coexpression of IL-2, GIFT leads to significant functional NK cell infiltration as confirmed in NK-defective beige mice. In conclusion, we demonstrated that a fusion between GM-CSF and IL-2 can invoke greater antitumor effect than both cytokines in combination, and novel immunobiological properties can arise from such chimeric constructs.

Introduction
The delivery of cytokines, or their encoding cDNA sequences, has been broadly explored to increase tumor cell immunogenicity. Interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are among the most potent cytokines able to induce tumor-specific systemic immunity, both in experimental models and clinical trials (1, 2). By comparing the antitumor effect of different cytokines in the B16 mouse melanoma model, Dranoff et al. (3) reported that GM-CSF was the most effective in generating systemic immunity protecting mice against a distant tumor, whereas IL-2 was the most effective at inducing locoregional tumor rejection. Given the complementing nature of their actions, several groups have since demonstrated powerful antitumor synergy between GM-CSF and IL-2 (4, 5). However, other studies reported that the combination of GM-CSF and IL-2 could induce inhibitory signals down-regulating the functions of certain immune effectors (6, 7). These conflicting results highlight the importance—and the difficulty—of optimizing the activity between two agents with different pharmacologic properties. Alternatively, bifunctional proteins generated from the fusion of two distinct cytokines have been shown to recapitulate synergistic effects while eliminating the need for dual delivery (8). Moreover, a fusion protein may possess unheralded biopharmaceutical properties, which may trigger novel beneficial responses. We here report the first engineering of a GM-CSF and IL-2 fusion transgene (GIFT). We provide evidence that this GM-CSF and IL-2 fusion displays novel antitumor properties greater than those of combined GM-CSF and IL-2 for cancer immunotherapy.

Materials and Methods

Animals and Cell Lines. The C57bl/6-derived B16F0 (B16) mouse melanoma cells were generously given by M. A. Alaoui-Jamali (Lady Davis Institute, Montreal, Quebec, Canada) and maintained in Dulbecco’s modified Eagle’s medium (Wisent Technologies, Rocklin, CA), 10% fetal bovine serum (Wisent Technologies), and 50 U/mL Pen/Strep (Wisent Technologies). CTL-L2 and JAWSII cells were purchased from American Type Culture Collection (Manassas, VA) and maintained as per American Type Culture Collection recommendations. C57bl/6 wild-type female mice were obtained from Charles River (Laprairie Co., Quebec, Canada). Immundeficient CD8−/−, CD4−/−, and beige mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were used for experimentation at 4 to 8 weeks of age.

Vector Construct and Virtual Protein Modeling. Mouse IL-2 and GM-CSF cDNAs were obtained from the National Gene Vector Laboratories (The University of Michigan, Ann Arbor, MI), excised by restriction digest and inserted into bicistronic retroviral plasmids allowing coexpression of green fluorescent protein (GFP) (9). The nucleotide sequence of the fusion product of GM-CSF and IL-2 cDNAs was confirmed by DNA sequencing at the Guelph Molecular Supercenter (University of Guelph, Ontario, Canada). Based on the templates 2gfn, 1m47, and 4h1b from P3I-BLAST (10), searches with a three-dimensional model of the GIFT gene product was built using Modeler 6.2 (11). Here, 50 structure models were generated, and the one with lowest objective function was selected for analysis using the Procheck3.5 software (12).

Transgene Expression. The retroviral plasmids were introduced into GP+AM12 packaging cells (American Type Culture Collection) and supernatant used to gene modify B16 cells. Single B16 clones were isolated by colony sorting and further expanded. Supernatant from clonal populations was tested by enzyme-linked immunosorbent assay for cytokine expression (BioSource, San Diego, CA) or immunoblotted using antiserum IL-2 or antiserum GM-CSF antibodies (BD Biosciences, San Jose, CA).

Cytokine-Dependent Proliferation Assays. CTL-L2 or JAWSII cells were plated at 104 cells/well of a 96-well plate with increasing concentrations of cytokines from gene-modified B16 cells. The cells were incubated for 48 hours, and 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution were incorporated for the last 4 hours of incubation. The reaction was stopped by adding 200 µL of dimethyl sulfoxide and absorbance read at 570 nm.

Murine B16 Tumor Implantation and Therapeutic Modeling. One million cytokine-secreting B16 cells were injected subcutaneously (n = 14 per group) in C57bl/6 mice, and tumor growth was monitored over time. For prophylactic B16 vaccinations, one million irradiated (50°Cy) cytokine-secreted...
ing B16 cells were injected subcutaneously and challenged 14 days later on the contralateral flank with 5 × 10^6 wild-type B16 cells. For therapeutic B16 vaccination experiments, 2 × 10^6 wild-type B16 cells were injected subcutaneously into wild-type, CD8^+/-, CD4^+/-, or beige mice and treated at days 1 and 7 with peritumoral injection of 10^6 irradiated (50Gy) B16-GIFT, B16-GMCSF, B16-IL-2, or 10^6 B16-GMCSF plus 10^6 B16-IL-2 cells (n = 10 per group). This experiment was repeated (n = 10 per group) in wild-type mice, and the results were combined for statistical analysis. All implanted B16 clones produced similar and comparable molar quantities of the cytokine(s) analyzed (0.7 ± 0.2 pmol per 10^6 cells per 24 hours).

**Immune Effector Infiltration Analysis.** One million cytokine-secreting B16 cells (in 50 μL of PBS) were mixed to 500 μL of Matrigel (BD Biosciences) at 4°C and injected subcutaneously in C57bl/6 mice (n = 4 per group). After 2 days, implants were surgically removed and incubated for 90 minutes with a solution of 1.6 mg/mL collagenase type IV (Sigma-Aldrich, Oakville, Ontario, Canada) and 200 μg/mL Dnasel (Sigma-Aldrich) in RPMI 10% fetal bovine serum with or without 6 nmol/L of GIFT, GM-CSF, IL-2, or 6 nmol/L of GM-CSF and 6 nmol/L of IL-2 obtained from the supernatant of cytokine-secreting B16 cells. After 1 hour at 4°C with antimouse PE-Mac3 and biotin-Ly6G6C, PE-NK1.1, or proper isotypic controls, followed by streptavidin–allophycocyanin for 15 minutes. Labeled cells were subsequently analyzed by flow cytometry with a Becton-Dickinson FACScan.

**Macrophage Migration Assay.** Murine peritoneal macrophages were isolated from C57bl/6 mice by lavage of the abdominal cavity with RPMI and were consistently >65% Mac-1 positive by flow cytometric analysis. Immediately after isolation, 10^6 cells per well were plated in the top chamber of a 0.15% gelatin-coated 50-μm Transwell plate. The lower chambers were filled in duplicates with 600 μL of RPMI 10% fetal bovine serum with or without 6 nmol/L of GIFT, GM-CSF, IL-2, or 6 nmol/L of GM-CSF and 6 nmol/L of IL-2 obtained from the supernatant of cytokine-secreting B16 cells. After 5 hours at 37°C, the top chamber was removed, thoroughly washed, removed from the cells on the top filter with a cotton swab, fixed in methanol, and stained with violet blue dye. The cells on the bottom filter of 10 high power fields from cells on the top filter with a cotton swab, fixed in methanol, and stained with violet blue dye. The cells on the bottom filter of 10 high power fields (×400) were counted for each well, and the results are depicted on a histogram. The experiment was performed twice will similar results.

**Results and Discussion**

Part of the synergy between GM-CSF and IL-2 comes from the fact that GM-CSF can promote proliferation and differentiation of antigen-presenting cells, which may initiate a tumor-specific immune response that can be subsequently amplified by IL-2 (1). For tumor-infiltrating lymphocytes, addition of GM-CSF to IL-2 has been reported to result in faster proliferation and enhanced tumor cytotoxicity (13). In addition, GM-CSF and IL-2 have been shown to enhance monocyte activation and cytotoxicity against melanoma cells in vitro (14, 15) and to prolong polymorphonuclear neutrophil survival (16, 17). However, GM-CSF and IL-2 used in combination can sometimes induce paradoxical effects. Skog et al. (6) reported that combined GM-CSF and IL-2 therapy could induce inhibitory signals in colorectal carcinoma patients, down-regulating the functions of monocytes, natural killer (NK) cells, and B cells compared with therapy with GM-CSF alone. Lee et al. (7) reported that although GM-CSF and IL-2 expression was synergistic at inhibiting primary mouse colon adenocarcinoma growth, it abrogated the protective effect against wild-type tumor challenge compared with single cytokine expression.

The difficulty in predicting the outcome of GM-CSF and IL-2 combined therapy may come from their distinct pharmacokinetic and biological properties. The half-life of IL-2 in the circulation is extremely short (approximately 10 minutes), whereas the half-life of GM-CSF can extend from 50 to 85 minutes (18). Furthermore, GM-CSF is a potent initiator of an adaptive immune response, whereas IL-2 promotes innate antitumor activity. Soliciting these two functional immune pathways contemporaneously may lead to unalloyed antagonism. Indeed, GM-CSF has been shown to down-regulate certain aspects of the innate immune response such as NK cytotoxicity (19), and these may in part explain the observations of others (6, 7) especially if tumor production of GM-CSF and IL-2 varies in space and time. We hypothesize that a single bifunctional fusion protein, predicted amino acid sequence of the fusion transgene.

![Fig. 1. Cloning of a bifunctional mouse GIFT. A, predicted amino acid sequence of the fusion transgene. B, computer model of GIFT gene product built by comparative modeling. The region of the fusion that corresponds to the signal peptide (orange ribbon) and glutamic tract (blue ribbon) of mouse IL-2 precursor was predicted to form α-helix. There are 98.9% of residues in the most favored and allowed regions of the Ramachandran plot, which indicates that stereochemical quality of the model is excellent. Residue G114 (green) connects GM-CSF (purple ribbon) to IL-2 (orange, cyan, pink, and blue ribbons). The COOH-terminal α-helix is shown in blue. The side chains of solvent-accessible residues that are important for mouse IL-2 to interact with IL-2Rα are in red sticks, whereas the four residues that are important for interaction with other subunits of IL-2Rα are in black sticks. C, immunoblotting of conditioned supernatant from GIFT gene-modified B16 cells with antimouse IL-2 and antimonouse GM-CSF monoclonal antibodies (Lanes 1 and 5, recombinant mouse IL-2; Lanes 2 and 4, recombinant mouse GM-CSF; Lanes 3 and 6, supernatant from B16-GIFT). CTL-T-2 (D) and IAWSII (E) cell proliferation assays as determined by MTT incorporation after 48 hours of incubation with increasing concentrations of cytokines from conditioned supernatant of gene-modified B16 cells (CTL-T-2, P > 0.05 between B16-GIFT and B16-IL-2; IAWSII, P > 0.05 between B16-GIFT and B16-GMCSF). Mean of triplicates are shown ± SE of one representative experiment of three.](image-url)
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through constant equimolar availability of both subunits, could limit paradoxical effects. Granted, such a fusion protein would be bereft of a true physiologic role and may trigger novel responses. In this study, we report the engineering of a GM-CSF and IL-2 fusion transgene. We provide evidence that a fusion between two cytokines can invoke greater antitumor effect than both cytokines in combination.

The cDNAs for mouse GM-CSF and mouse IL-2 were cloned in frame after a 33-bp deletion at the 3' end of the GM-CSF cDNA. We used the IL-2 signal peptide sequence as an intercytokine bridge. The resulting fusion transgene, named GIFT, was confirmed by sequencing analysis. Fig. 1A illustrates the predicted amino acid sequence of the gene product encoded by GIFT. Computer-based analysis of the GIF gene product predicted that the signal peptide (Fig. 1B, orange ribbon) and glutamic tract (Fig. 1B, pink ribbon) of the mouse IL-2 precursor would form α-helix structure linking the mature GM-CSF to the mature IL-2 (Fig. 1B), allowing proper folding of both subunits and availability of crucial receptor binding residues.

Bicistronic retrovectors allowing coexpression of the GFP reporter were then generated for GIFT, GM-CSF, and IL-2 and used to gene-modify B16 mouse melanoma cells. Immunoblotting of cultured cell supernatant confirmed that GIFT gene product was secreted and consisted of a protein of expected molecular weight (Fig. 1C). Clonal populations of cytokine-secreting B16 cells were then isolated by fluorescence-activated cell sorting, and their conditioned media were tested by enzyme-linked immunosorbent assay for cytokine secretion. For comparison purposes, we selected clonal populations secreting comparable molar quantities of GIFT, GM-CSF, or IL-2 (0.7 ± 0.2 pmol per 10^6 cells per 24 hours).

To test the bifunctionality of GIFT, cytokine conditioned supernatant was tested at different concentrations for its ability to stimulate proliferation of IL-2-dependent JAWSII cells (Fig. 1D) and GM-CSF–dependent JAWSII cells (Fig. 1E). As demonstrated by three distinct MTT incorporation experiments, GIFT was able to stimulate CTLL-2 cells at a similar level to IL-2 (P > 0.05 by t test) and to stimulate JAWSII cells at a similar level to GM-CSF (P > 0.05 by t test). Our results thus confirmed the in vitro bifunctionality of GIFT gene product.

To assess the GIFT in vivo antitumor effect, we first proceeded with a set of experiments in which 10^6 live cytokine-secreting B16 cells were injected subcutaneously into cohorts of immunocompetent syngeneic C57bl/6 mice (n = 14). Consistent with previous studies (3), we observed that IL-2 expression but not GM-CSF expression by live B16 cells could prevent tumor growth (respectively 78 and 0% of mice rejected the implant). In comparison, all mice that received injections of GIFT-expressing B16 cells rejected the tumor implant (P < 0.05 by t test with IL-2; Fig. 2A). Importantly, the observed absence of tumor growth was not the result of clone-specific cell proliferation rates, as determined by MTT incorporation assays in vitro (P > 0.05; data not shown). Neither was it due to an idiosyncratic property of this clone, because polyclonal B16-GIFT tumors were also rejected (data not shown).

We then tested whether GIFT-engineered B16 cells could induce protective immunity against a wild-type challenge of B16 cells—in essence a prophylactic tumor cell vaccine. C57bl/6 mice received subcutaneous injections into the right flank with 10^6 irradiated (50 Gy) cytokine-secreting B16 cells and challenged 14 days later with 5 × 10^4 wild-type B16 cells into the contralateral flank. Consistent
with previous studies (3), we observed that GM-CSF expression but not IL-2 expression could induce systemic protective immunity when given as an irradiated cellular tumor vaccine (respectively 80 and 0% of mice rejected the challenge). In comparison, all mice vaccinated with irradiated B16-GIFT rejected the subsequent challenge (P > 0.05 with GM-CSF; Fig. 2B). Taken together, our results demonstrate that in addition to its potent locoregional effect against live tumor cells, GIFT is able to induce systemic antitumor immunity, protecting mice against a distant injection of wild-type B16 cells, thereby combining the innate immune effects of IL-2 and the adaptive immune effects of GM-CSF, without any observable mutual interference.

We also compared the antitumor action of GIFT to a combination of IL-2 and GM-CSF as assessed in a therapeutic cancer cell vaccine strategy. First, 2 × 10^6 B16 cells were injected subcutaneously into C57Bl/6 mice. Then on days 1 and 7, the same mice with pre-established live B16 tumors received peritumoral injections of 10^6 irradiated B16-GIFT cells or a mixture of 10^6 B16-GMCSF and 10^6 B16-IL2 cells (Fig. 3A). At equimolar cytokine secretion rates, the treatment with a GIFT-expressing cellular vaccine was significantly greater than a vaccine expressing both IL-2 and GM-CSF (P = 0.0407 by log rank), IL-2 alone (P = 0.035 by log rank), or GM-CSF alone (P = 0.0003 by log rank). Treatment of CD4^+^- tumor-bearing mice with GIFT was indistinguishable from treatment of wild-type tumor-bearing mice, indicative of a T helper–independent immune response (Fig. 3B). In contradistinction, CD8^-^ mice treated with GIFT failed to develop antitumor immune response (P < 0.05 by log rank compared with wild-type). NK cells were also implicated, because treatment of NK-defective beige tumor-bearing mice was significantly reduced but not completely abolished, compared with treatment of wild-type mice (P < 0.05 by log rank compared with wild-type).

Our observation that GIFT tumor cell vaccines were more effective than a combination of both GM-CSF and IL-2 at equimolar concentration suggested that GIFT may possess supplementary and novel immunopharmacologic properties when compared with the combination of GM-CSF and IL-2. We hypothesized that immune cells expressing both the GM-CSF and the low-affinity IL-2 receptors could mediate such distinct properties in response to GIFT. Macrophages and neutrophils are known to express both the GM-CSF and the low affinity IL-2 receptors and have been reported to play a role in the antitumor effect induced by GM-CSF and IL-2. We thus compared the level of macrophage and neutrophil infiltration of early cytokine-secreting B16 tumors. As shown in Fig. 4, GIFT induced a significantly more robust infiltration of macrophages than GM-CSF, IL-2, or even a combination of both GM-CSF and IL-2 (P < 0.05 by t test). On the other hand, the number of neutrophils was significantly greater in response to GIFT compared with IL-2 or GM-CSF alone (P < 0.05 by t test), but similar to the number of neutrophils in response to combined GM-CSF and IL-2 (P > 0.05 by t test). To determine whether the enhanced macrophage infiltration was the result of a direct chemoattractive effect of GIFT, migration assays were performed with mouse peritoneal macrophages. As shown in Fig. 4C, GIFT was able to induce migration of significantly more macrophages than equimolar concentration of combined GM-CSF and IL-2 (P < 0.05 by t test).

An intriguing observation was the significant suppression of NK infiltration by GM-CSF when compared with controls (P < 0.05 between GM-CSF and GFP by t test). The effect was not rescued by coexpression of IL-2 (P > 0.05 between GM-CSF and GM-CSF + IL-2 by t test). However, GIFT retained the ability to recruit NK cells as did IL-2 alone (Fig. 4D). Recombinant human GM-CSF has been shown to suppress NK cell formation in vitro (20) and NK cytotoxicity in vitro (19). This may explain in part the inability of GM-CSF alone to reject live tumor cells as we and others (3) have observed. The dominant-negative effect of GM-CSF on NK cells may also help explain in part the apparent inferiority of GM-CSF and IL-2 combination to GIFT as part of a therapeutic vaccine.

In conclusion, we have demonstrated that the nucleotide sequence encoding for the fusion of GM-CSF and IL-2 cDNA can be used as a therapeutic transgene for gene therapy of cancer, recapitulating the potent antitumor effects of both GM-CSF and IL-2. Furthermore, this fusion gene product appears to have immunopharmacologic properties distinct of GM-CSF and IL-2 used alone or in combination. This is the first report that a fusion between two cytokines can invoke greater antitumor effect than both cytokines in combination and suggest that chimeric fusion cytokine transgenes may serve as novel genetic biotherapeutics for cancer immunotherapy.

References
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