The Human Ortholog of Granulocyte Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion Protein Induces Potent Ex vivo Natural Killer Cell Activation and Maturation

Claudia Penafuerte,1 Norma Bautista-Lopez,3 Boulassel Mohamed-Rachid,2 Jean-Pierre Routy,2 and Jacques Galipeau1,3

1Department of Medicine, Division of Experimental Medicine, McGill University; 2Division of Hematology, McGill University Health Centre, and 3Departments of Medicine and Oncology, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada

Abstract

Natural killer (NK) cells are appealing cellular pharmaceuticals for cancer therapy because of their innate ability to recognize and kill tumor cells. Therefore, the development of methods that can enhance the potency in their antitumor effect would be desirable. We have previously shown that a murine granulocyte macrophage colony-stimulating factor (GM-CSF)/interleukin 2 (IL-2) fusion protein displays novel antitumor properties in vivo compared with both cytokines in combination due to recruitment of NK cells. In the present work, we have found that human ortholog of the GM-CSF/IL-2 fusion protein (a.k.a. hGIFT2) induces robust NK cell activation ex vivo with significant secretion of RANTES and a 37-fold increase in IFNγ production when compared with either IL-2 or GM-CSF single cytokine treatment or their combination. Moreover, hGIFT2 upregulates the expression of NK cell activating receptors NKp44, NKp46, and DNAM-1 (CD226), as well as CD69, CD107a, and IL-2Rβγ expression. In addition, hGIFT2 promotes NK cell maturation, based on the downregulation of CD117 expression and upregulation of CD11b. This phenotype correlates with significantly greater cytotoxicity against tumor cells. At the molecular level, hGIFT2 leads to a potent activation of Janus-activated kinases (JAK) downstream of both IL-2 and GM-CSF receptors (JAK1 and JAK2, respectively) and consequently leads to a hyperphosphorylation of signal transducers and activators of transcription (STAT1), STAT3, and STAT5. In conclusion, hGIFT2 fusokine possesses unique biochemical properties distinct from IL-2 and GM-CSF, constitutes a novel and potent tool for ex vivo NK cell activation and maturation, and may be of use for cancer cell immunotherapy. [Cancer Res 2009;69(23):9020–8]

Introduction

Natural killer (NK) cells are innate effector cells that react against virus-infected or transformed cells before the onset of adaptive immunity. These cells express an array of inhibitory and activating receptors that sense the quality and quantity of self-MHC class I molecules expressed at the cell surface, which mediate the recognition of target cells and regulate the activation state of NK cells. Upon cytokine stimulation, NK cells become lymphokine-activated killer (LAK) cells that proliferate, produce cytokines [IFNγ and tumor necrosis factor (TNF) α], and upregulate effector molecules such as adhesion molecules, perforin, granzymes, Fas ligand, and TRAIL. These features endow NK cells with the ability to kill tumor cells and act as essential promoters of an adaptive immunity against tumor challenge. Indeed, several in vitro as well as in vivo studies indicate that tumor cells are recognized as NK cell targets. NK cells not only directly eliminate tumor cells, but also induce the subsequent development of tumor-specific T-cell responses to parental tumor cells. For example, the potential of NK cell–based therapy has been evoked in patients suffering of acute myeloid leukemia. In this case, adoptive transfer of allogeneic NK cells with mismatched NK inhibitory receptors and HLA class I ligands produce graft-versus-leukemia in the absence of graft-versus-host disease due to the ability of donor NK cells to attack recipient dendritic cells, which are responsible for priming donor T cells and induce graft-versus-host disease. Therefore, clinical interest in NK cells as a cancer cell pharmaceutical is rapidly growing, with >200 clinical trials examining either autologous or allogeneic NK cells as well as universal donor NK cell lines. However, there are some theoretical limitations to the use of NK cells for cancer therapy. Tumor cells expressing low levels of MHC class I may fail to trigger NK cell activation. NK cells from patients affected by advanced bulky malignancies display impaired cytolytic activity, which can be attributed to tumor-derived suppressive factors such as transforming growth factor β that exert deleterious effects of NK cell effector functions. Therefore, the infusion of primed and activated NK cells would be an approach that may enhance the effectiveness of these cells in the treatment of cancer. The use of cytokines and combinations thereof would be an obvious method to activate NK cells ex vivo. Better still, pharmacologic activation of NK cells in a manner unachievable with native cytokines may lead to desirable acquired anticancer properties. In this line of thought, we have previously shown that a murine granulocyte macrophage colony-stimulating factor (GM-CSF)/interleukin-2 (IL-2) fusion protein (a.k.a. GIF2) displays novel antitumor properties in vivo compared with both cytokines in combination regarding to tumor site recruitment of significant functional NK cell infiltration. In the present study, we show that the human ortholog of the GM-CSF/IL-2 fusion protein (hGIFT2) induces potent human NK cell activation and cytotoxicity distinct from that achieved by IL-2 or GM-CSF. The use of hGIFT2 as a means to generate oncolytic NK cells may serve an enhanced cellular platform for cancer cell therapy.
Materials and Methods

Cell lines, recombinant proteins, antibodies, and ELISA kits. 293T cells were cultured in DMEM (Wisent Technologies) supplemented with 10% fetal bovine serum (FBS; Wisent Technologies). The cell lines TF-1, CTLL-2, Daudi, K562, and U266 [American Type Culture Collection (ATCC)] were grown according to ATCC’s recommendations. Recombinant proteins (hIL-2 and hGM-CSF, R&D Systems); anti α-tubulin (Santa Cruz Biotechnology); polyclonal anti-phosphorylated Jak1, Jak2, Stat1, Stat3, Stat5, extracellular signal-regulated kinase (ERK), and IκBα, as well as their respective antibodies against full-length proteins (Cell Signaling Technology); anti-human FcR III/II, CD3, CD4, CD8, CD56, NKG2D, NKp44, NKp46, NKp30, CD107a, CD117, CD69, CD226, CD11b, CD43, KIR, IFNγ, or their isotype control antibodies mIgG1 and mIgG2a for flow cytometry (BD Biosciences); and ELISA kits for human IL-2 and GM-CSF (Invitrogen and eBioscience), and for RANTES, IFNγ, and TNFα (R&D systems). Cytotoxicity detection kit lactate dehydrogenase (LDH; Roche Applied Science).

Peripheral blood mononuclear cells and human NK cells. Blood was drawn from healthy donors, after informed consent had been obtained, into heparin- or citrate-coated CPT tubes (BD Biosciences), and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation according to the manufacturer’s recommendation. NK cells were obtained by negative purification using the untouched NK cells isolation kit (Miltenyi Biotec). Cells were treated with NK cell biotin-antibody cocktail and magnetic microbeads reagents and depleted by magnetic cell sorting system with an autoMACS column (Miltenyi Biotec) according to the manufacturer’s instructions. NK cell population (CD56+CD3−) purity assessed by flow cytometry was 96% (Supplementary Data S1). Three different donors were used for each experiment and an average of two different experiments was performed with the same donor.

Vector construct and transgene expression. The human GM-CSF cDNA (Invivogen) was modified to remove the 3′ nucleotides encoding the stop codon and subsequently cloned in frame with the cDNA encoding the human IL-2 devoid of start codon to generate the cDNA for hGIFT2 fusokine. The hGIFT2 was incorporated into a bicistronic vector (pCMV), allowing the expression of both hGIFT2 and green fluorescent protein (6). The nucleotide sequence of the hGIFT2 cDNA was confirmed by DNA sequencing at the Guelph Molecular Supercenter. Concentrated media conditioned by 293 T cells stably transfected with pCMV encoding for hGIFT2 was used to test the bioactivity of hGIFT2. The IL-2 responsive CTLL-2 or GM-CSF responsive TF-1 cell lines were plated at a density of 10^5 cells per well in a 96-well plate and treated with increasing concentration of cytokines for 48 h. Cell proliferation was assessed with a MTT assay.

Analysis of cell surface marker on leukocytes. PBMC were cultured with hGIFT2 and equimolar concentrations of cytokine controls (IL-2, GM-CSF, and IL-2 combined with GM-CSF, 2 pmol). After 7 d in culture, the cells were resuspended in PBS with 2% FBS and incubated with anti-human FcR III/II for 30 min. Subsequently, cells were labeled with a mixture of conjugated antibodies specific for cell surface markers (CD3, CD4, CD8, CD56, and their respective isotype controls) for 1 h at 4°C and analyzed by flow cytometry using a Becton Dickinson FACScan.

Flow cytometric analysis of NK cell expression markers, IFNγ expression, apoptosis, and cell proliferation. For cell surface marker staining, NK cells stimulated with hGIFT2 and equimolar concentrations...
of cytokine for 7 d were resuspended in PBS with 2% FBS, incubated with anti-human FcR III/I for 30 min, and labeled with conjugated antibodies specific for NK cell activating receptors (NKp30, NKp44, NKp46, NKp22, CD226), inhibitory receptors (CD134, KIR), maturation markers (CD11b, CD11c, CD117), NK cell activation markers (CD69, CD107a), and IL-2 receptor components (α subunit CD25, β subunit CD122, and γ subunit CD132). The expression of these cell surface markers was determined by FACS Calibur cytomter (BD) and analyzed using CellQuest software (BD). For IFN-γ intracellular staining, NK cells previously stimulated with cytokines for 2 h were treated with Brefeldin A for 4 h, were fixed, were permeabilized, and were stained with IFN-γ-conjugated antibody. For Annexin V and propidium iodide staining, NK cells cultured with cytokines (2 pmol) for 7 d were incubated with Annexin V/propidium iodide for 15 min at room temperature and analyzed by flow cytometry. For carboxyfluorescin succinimidyl ester staining, NK cells prestained with CFSE were cultured with cytokines for 4 d, and cell proliferation was assessed by flow cytometry. The data were analyzed using CellQuest software (Becton & Dickinson).

**In vitro cytotoxicity assay.** NK cells (1 × 10⁵; effector cells) previously stimulated with hGIFT2 or cytokine controls for 3 d were washed twice and cocultured with 1 × 10⁶ target cells (Daudi, K562, U266) at an effector/target cell ratio of 10:1 for 4 h. Supernatants were collected and used to measure the LDH released from the cytosol of damaged or lysed cells using a cytotoxicity detection LDH kit. As controls, we measured the spontaneous LDH released from target and effector cells. The percentage of cytotoxicity was calculated according to the formula:

\[
\text{cytotoxicity} = \left( \frac{\text{Experimental} - \text{Effector Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \right) \times 100
\]

Target Maximum: maximum LDH released from Triton X-100–treated target cells.

**Intracellular signaling.** For signaling analysis, media conditioned by stably transfected 293 T cells expressing hGIFT2, as well as media conditioned by nontransfected cells containing equimolar concentration of cytokine controls (IL-2, GM-CSF, or IL-2 combined with GM-CSF, 5 pmol each), were added to 10⁵ NK cells for 20 min before being lysed and probed by Western blot with rabbit anti-phosphorylated Jak1, Jak2, Stat1, Stat3 and Stat5, ERK, and Akt antibodies. Antibodies against total proteins were used as loading controls as well as anti-α-tubulin antibody. Three independent experiments were performed with NK cells from different donors.

**Statistical evaluation.** P values were calculated by paired Student’s t test. P < 0.05 was considered statistically significant.

**Results**

**Design and expression of hGIFT2 fusokine.** The human GMCSF/IL-2 (hGIFT2) construct was created by cloning a modified human GM-CSF cDNA missing the nucleotides encoding for the stop codon in frame with the 5’ end of the human IL-2 cDNA devoid of its start codon. The final fusokine hGIFT2 cDNA encodes for a single polypeptide chain of 302 amino acids (Fig. 1A). Denaturing immunoblotting performed on the supernatant of 293T cells stably transfected to express hGIFT2 (5 ng per million cells in 24 h) showed that the chimeric protein was secreted and has an apparent molecular weight of 45 kDa by SDS-PAGE under reducing conditions (Fig. 1B). We detected and quantified hGIFT2 by IL-2 and GM-CSF ELISA and we found that 1 pmol (45 ng) of hGIFT2 is equivalent to 97 units, whereas 1 pmol (15 ng) of recombinant IL-2 is equivalent to 221 units (Fig. 1C). Both techniques confirm that only the full-length hGIFT2 fusokine of predicted molecular weight is secreted by transfected cells.

hGIFT2 induces the proliferation of IL-2– and GM-CSF–dependent cell lines. The bioactivity of GM-CSF and IL-2 domains as part of hGIFT2 was confirmed by proliferation of GM-CSF–dependent cell line, TF-1 (Fig. 2A), and IL-2–dependent cell line, CTLL-2 (Fig. 2B). The proliferation level of each cell line was determined by MTT incorporation for the last 4 hours of incubation with cytokines. As result, hGIFT2 induces similar proliferation levels of these cell lines compared with equimolar concentration of each cytokine controls, which indicate that both GM-CSF and IL-2 as part of the fusion preserve the ability to induce an appropriate proliferative response.

**Effect of hGIFT2 on primary lymphocytes.** The effect of hGIFT2 on lymphocytes was determined by culturing PBMCs with equimolar concentrations of cytokines (2 pmol). After 7 days in culture, we observed that hGIFT2 promotes a significant increase in the number of NK cells compared with equimolar concentration of cytokine controls, whereas the number of CD8, CD4, and NKT cells did not show significant differences with each cytokine treatment (Fig. 3A). Similarly, we observed a significant increase of NK cell percentage in the hGIFT2-treated PBMC fraction from three different donors (Fig. 3B). Based on these results, we investigated whether hGIFT2 induces NK cell proliferation or enhances NK cell survival. To test these hypotheses, purified NK cells were prestained with CFSE, cultured with equimolar
concentrations of cytokines, and analyzed by flow cytometry. After 4 days in culture, IL-2 as well as IL-2 combined with GM-CSF induces NK cell proliferation (Fig. 3C). However, after 7 days in culture with cytokine controls the NK cell number decreased, although the media was changed at day 4 to avoid cell starvation. In contrast, hGIFT2 does not induce NK cell proliferation but rather protect NK cells from apoptosis (Fig. 3C and D). Based on these results and in agreement with previous reports (7, 8), we suggest that IL-2–activated NK cells undergo apoptosis in long term culture.

**hGIFT2 induces activation of NK cells.** Purified human NK cells pretreated with hGIFT2 displayed an enhanced activation profile compared with NK cells stimulated with equimolar concentration of cytokine controls, based on the expression levels of the early activation marker (CD69) and a marker of degranulation (CD107a; Fig. 4A). To evaluate the responsiveness of NK cells to hGIFT2, we analyzed the expression levels of the IL-2 trimeric receptor subunits (α, β, γ). IL-2Rα and IL-2Rγ expression levels were not modified by any cytokine treatment (data not shown), whereas IL-2Rβ expression was upregulated by hGIFT2 and downregulated by GM-CSF (Fig. 4A). Intracellular staining for IFNγ indicates that hGIFT2 induces a robust expression of IFNγ in the first 6 hours of cytokine treatment (Fig. 4B, left). By ELISA, we quantified that hGIFT2 promotes >37-fold greater IFNγ production than equimolar concentration of IL-2 after 3 days in culture (Fig. 4B, right), as well as significant secretion of RANTES (Fig. 4C, bottom). However, we did not detect significant differences regarding TNFα production (Fig. 4C, top). We also observed that GM-CSF inhibits NK cell–derived IFNγ, TNFα, and RANTES production. These results indicate that hGIFT2 displays superior properties as a tool for NK cell activation and overcomes GM-CSF–mediated immunosuppression. In addition, hGIFT2 similarly affect both CD56bright and CD56dim subsets of NK cells, which upon hGIFT2 stimulation become highly activated and granulated (Supplementary Data S2).

**hGIFT2 upregulates activating receptor expression and promotes NK cell maturation and significant greater NK cell cytotoxicity.** Spontaneous cytotoxic activity is mainly triggered by the activating receptors natural cytotoxicity receptors (NCR; NKp30, NKp44, NKp46), NK2D, and leukocyte adhesion molecule DNAX accessory molecule 1 [DNAM-1 (CD226); refs. 1, 3]. hGIFT2-stimulated NK cells upregulate NKp44, NKp46, and DNAM-1 (CD226) expression (Fig. 5A) but not NK2D (Supplementary

Figure 3. hGIFT2 increases NK survival but does not induce NK cell proliferation: PBMC or NK cells were cultured with 2 pmol of hGIFT2 or equimolar concentrations of cytokine controls (IL-2, GM-CSF, or both IL-2 and GM-CSF combined, 2 pmol each). A, PBMC cultured with cytokines for 7 d were labeled with conjugated antibodies specific for cell surface markers CD3, CD4, CD6, and CD56, and cell type number was quantified by flow cytometry. B, the percentage of NK cells from three different donors was determined in the PBMC fraction cultured with equimolar concentrations of cytokines for 7 d. Cells were labeled with conjugated antibodies specific for cell surface markers CD3 and CD56, and the percentage of NK cells was quantified by flow cytometry. C, NK cells prestaining with CFSE were incubated with cytokines for 4 d. The decrease of CFSE intensity as indicative of cell proliferation was assessed by flow cytometry. The percentage of the parent peak is indicated for each figure. D, NK cells cultured with cytokines for 7 d were staining with Annexin V and propidium iodide, and the percentage of apoptotic cells in each condition was determined by flow cytometry. These results are representative of three independent experiments performed with blood from three healthy volunteers. *, significant differences between hGIFT2 and controls in all figures (P < 0.05).
Data S3A) and NKp30 (data not shown) expression. In addition, hGIFT2 downregulates the expression of the receptor for stem cell factor c-kit (CD117) and upregulates the expression of integrin CD11b (macrophage antigen-1; Fig. 5B). hGIFT2 downregulates the expression of the inhibitory receptor CD117 and upregulates the expression of integrin CD11b (macrophage antigen-1; Fig. 5B). hGIFT2 downregulates the expression of the inhibitory receptor CD43 and does not alter the expression level of the inhibitory receptors KIR (Supplementary Data S3B and C). This phenotype indicates that hGIFT2-stimulated NK cells undergo a maturation process, which correlated with significant greater cytotoxicity than cytokine controls against both NK-resistant Daudi cells and NK-sensitive K562 and U266 tumor cells. As previously reported (9), we also observed that GM-CSF significantly suppresses NK cell cytotoxicity (Fig. 5C). After 7 days in culture with cytokines, a 30% decline of cytotoxicity was observed in hGIFT2-stimulated NK cells, and a 60% reduction of killing ability in IL-2–stimulated NK cells (Supplementary Data S4).

hGIFT2 induces hyperactivation of Jak/signal transducers and activators of transcription signaling pathway in NK cells. NK cells express components of both IL-2 and GM-CSF receptors (10). The intracellular signaling of IL-2R occurs through the β chain [JAK1/signal transducers and activators of transcription (STAT) 3] and the γ chain (JAK3/STAT5), whereas the GM-CSF receptor signaling occurs through its β chain (JAK2/STAT5). To decipher the molecular mechanism underpinning the effect of hGIFT2 on NK cells, we analyzed the activation status of JAK kinases and STAT transcription factors downstream of IL-2 and GM-CSF receptors, respectively. We observed that hGIFT2 induces robust hyperphosphorylation of JAK1 and JAK2, and this is associated with greater activation of STAT1, STAT3, and STAT5 when compared with controls. In contrast, GM-CSF completely abrogates STAT5 phosphorylation and inhibits IL-2–mediated STAT5 activation (Fig. 6A and B). We also measured the activation status of other signaling pathways downstream of IL-2 and GM-CSF receptors, such as MKK/ERK and AKT pathways (11, 12). AKT pathway was more activated by hGIFT2 than cytokine controls (Fig. 6D), which supports the observation that hGIFT2 enhances NK survival. In contrast, the activation status of NF-κB and MKK/ERK did not show significant differences in hGIFT2-stimulated NK cells compared with equimolar concentrations of cytokine controls (data not shown).

Discussion

The clinical effectiveness of GM-CSF and IL-2 combination for the treatment of cancer is controversial. A phase II clinical study of moderate-dose IL-2 and GM-CSF in patients with metastatic or recurrent cancer showed a 35% objective response rate, with a median survival of 18 months (13). In contrast, a phase III randomized controlled trial of IL-2 and GM-CSF did not demonstrate a survival benefit over conventional chemotherapy (14). The results of these studies suggest that the optimal combination of cytokines for cancer therapy may depend on the specific tumor type and patient population.

Figure 4. hGIFT2 induces NK cell activation. NK cells cultured with 2 pmol of cytokines (hGIFT2 or cytokine controls) were analyzed for the expression of NK cell activation markers and the production cytokines and chemokines. A, CD69, the early activation marker; CD107a, a marker of degranulation; and CD122, the β subunit of IL-2 receptor. B, intracellular staining for IFNγ was performed on NK cells cultured for 6 h with cytokines and treated with Brefeldin A. IFNγ expression was measured by flow cytometry using a conjugated anti-IFNγ antibody, and IFNγ production from NK cells stimulated with cytokines for 3 d was quantified by ELISA. C, NK cell–derived TNFα production was quantified by ELISA, and no significant differences were observed, whereas RANTES production was significantly increased by hGIFT2. Columns, mean (n = 3); bars, SEM. *, significant differences between hGIFT2 and controls in all figures (P < 0.05). These results are representative of three independent experiments performed with blood from three healthy volunteers.
unresectable renal carcinoma fails to manifest the synergistic therapeutic effect of these two cytokines (13). Indeed, patients receiving GM-CSF and IL-2 treatment display an impaired LAK activity compared with IL-2 alone, and specifically, GM-CSF has been shown to downregulate certain aspects of innate immune response such as NK cell cytotoxicity (14). In the present study, we have shown that hGIFT2 displays unheralded novel properties that overcome GM-CSF–mediated NK cell suppression and promotes potent NK cell activation. hGIFT2 upregulates the expression of the early activation marker CD69 and promotes greater NK cell degranulation based on acquired surface expression of LAMP-1 (CD107a). The potent immune stimulatory effects of hGIFT2 on NK cells are evidenced by a substantial upregulation of NK activating receptors NKp44 and NKp46. The human triggering receptors responsible for NK cell cytotoxicity include the NCRs and NKG2D. NCRs belong to the Ig superfamily and include NKp46 and NKp30, which are expressed by both resting and activated NK cells, and NKp44, which is expressed only by activated NK cells (15–17). Although the cellular ligands on tumor cells for NCRs are currently unknown, NCRs have been identified as crucial receptors for target cell recognition and induction of NK cell cytotoxicity toward a wide range of cancer cells. Substantial evidence indicates that there is a direct correlation between NCR surface densities and the ability of NK cells to kill different tumor target cells (18). hGIFT2 slightly upregulates the expression of DNAM-1 (CD226), which is a transmembrane glycoprotein that mediates lymphocyte

Figure 5. hGIFT2 upregulates NK cell activating receptors and promotes NK cell maturation and greater NK cell cytotoxicity than cytokine controls. NK cells cultured with 2 pmol of cytokines for 3 d were labeled with conjugated antibodies specific for (A) NK cell activating receptors (NKp44, NKp46, and DNAM-1 [CD226]). B, NK cell maturation markers (integrin, CD11b, and receptor for stem cell factor c-kit, CD117). C, NK cells previously activated with 2 pmol of cytokines for 3 d were cocultured with target cells (Daudi, K562, and U266) for 4 h, and the cytotoxicity activity of NK cells was measured by quantifying the levels of LDH released from the cytosol of damaged or lysed cells. Maximum LDH level was measured from Triton X-100–treated target cells (Pos control). Significant differences between hGIFT2 and controls in all figures are indicated. The results in all the figures are representative of three independent experiments performed with purified NK cells from three healthy donors.
adhesion and signaling. This activating receptor recognizes PVR (CD155) and Nectin-2 (CD112), two members of the lectin family that are highly expressed on carcinomas, melanomas, and some hematopoietic cell lines (19, 20). Consequently, hGIFT2-stimulated NK cells promote significant cytotoxic activity against all target tumor cells analyzed. Interestingly, hGIFT2-treated NK cells were able to lyse “NK-resistant” Daudi cells (Burkitt’s lymphoma). These tumor cells are negative for MHC class I expression, but expresses MHC class II, which correlates with the increase resistant to NK-mediated lysis (21). As expected, hGIFT2-stimulated NK cells also promote greater cell lysis of NK-sensitive K562 and U266 cell lines. K562 leukemia cells (chronic myelogenous leukemia) are negative for both MHC class I and II expression, whereas U266 cells (myeloma), although sensitive to NK-mediated cell cytotoxicity, express high levels of MHC class I (22), which supports “the missing self” hypothesis that NK cells kill certain targets because they fail to express adequate levels of MHC gene products.

NK cells stimulated with IL-2 for 7 days display a remarkable reduction of cytotoxicity activity against myeloma cells. According to previous reports that IL-2 induces NK cells apoptosis in long-term culture (7, 8), we suggest that the reduction of NK cell cytotoxicity over time is due to a decrease of IL-2–stimulated NK viability. On the other hand, hGIFT2 does not induce NK cell proliferation compared with equimolar concentration of IL-2, but rather protects NK cells from apoptosis, which result in a net increase of NK cell number over time. In addition, hGIFT2 prompts NK cells to secrete substantial amounts of IFN-γ (37-fold greater than equimolar concentrations of IL-2) as well as RANTES. The extremely high production of IFN-γ may restrain the proliferation of NK cells and at the same time prevent NK cell apoptosis, since IFN-γ has been reported to induce cell cycle arrest in several cell types (23). In LAK cells, IFN-γ in synergy with IL-2 acts as inhibitor for LAK cell proliferation but not differentiation (24). We also found that distinct from IL-2, hGIFT2 induces a NK cell maturation process characterized by the downregulation of the expression of the receptor for stem cell factor c-kit (CD117), which is expressed by NK cell precursors but is largely diminished on matured NK cells (25, 26). CD11b integrin upregulation constitute another marker of NK cell maturation (27). As molecular mechanisms underpinning hGIFT2 effect on effector cells, hGIFT2 not only upregulates IL-2Rβ (CD122) expression but also induces a hyperactivation of JAK1 and JAK2 kinases associated to β chains of both IL-2 and GM-CSF receptors, which lead to a robust phosphorylation of STAT1, STAT3, and STAT5 transcription factors. By comparing the levels of STAT5 phosphorylation in hGIFT2-stimulated NK cells stimulated for 20 min with 5 pmol of cytokines, and cell lysates were probed for (A) phosphorylated JAK1, JAK2, and their respective anti–full-length protein antibodies. B, phosphorylated STAT1, STAT3, and STAT5, as well as total STAT1, STAT3, and STAT5 antibodies. C, phosphorylated AKT and total AKT antibody. Antibodies anti–full-length proteins and α-tubulin were used as loading controls. Absorbance quantifications of immunoblots are shown in each figure. These results are representative of three independent experiments performed with NK cells from three healthy volunteers.

Figure 6. hGIFT2 induces a hyperactivation of JAK/STAT pathway downstream of GM-CSF and IL-2 receptors in NK cells. NK cells (5 × 10⁶) were stimulated for 20 min with 5 pmol of cytokines, and cell lysates were probed for (A) phosphorylated JAK1, JAK2, and their respective anti–full-length protein antibodies. B, phosphorylated STAT1, STAT3, and STAT5, as well as total STAT1, STAT3, and STAT5 antibodies. C, phosphorylated AKT and total AKT antibody. Antibodies anti–full-length proteins and α-tubulin were used as loading controls. Absorbance quantifications of immunoblots are shown in each figure. These results are representative of three independent experiments performed with NK cells from three healthy volunteers.
cells with equimolar concentration of GM-CSF, we observed that hGIFT2 overcomes GM-CSF–mediated suppressive signals on NK cells. These results suggest that hGIFT2 has higher avidity for NK cells than single cytokine control maybe due to a bivalent binding of hGIFT2 to both receptors expressed on NK cells. Interestingly, hGIFT2 fusokine promotes greater activation of phosphoinositide 3-kinase/Akt in NK cells. Activated Akt in turn phosphorylates a number of downstream target genes that prevent apoptosis and promote cell survival, which could be the mechanism used by hGIFT2 to protect NK cell from apoptosis and to enhance survival.

Several clinical trials using ex vivo IL-2–expanded and activated autologous NK cells have shown apparent limited effectiveness in the therapy of cancer, including melanoma, renal carcinoma, lung carcinoma, ovarian, and brain cancer (28). The infusion of high doses of IL-2 not only is associated with severe toxic side effects, but also sensitizes NK cells to apoptosis when they are in contact with the vascular endothelium, which may cause a decrease of NK cell migration to the tumor (29, 30). Interestingly, allogeneic NK cells characterized by a KIR/KIR-ligand incompatibility display more cytotoxicity against solid tumors and leukemia (31, 32). Remarkably, patients with acute myeloid leukemia treated with allogeneic NK cells benefit from higher rates of engraftment, survival, and reduced incidence of GvHD (33). The risk of GvHD is a consequence of the inefficient priming of allogeneic donor T cells consequent to NK cell–mediated killing of recipient antigen-presenting cells (34). However, allogeneic NK cells arising from hematopoietic stem cell transplantation require several weeks to present cytokines that may contribute to leukemia relapse (35). Therefore, we may speculate that the infusion of mature NK cells would more effectively prevent leukemic relapse, promote engraftment by eliminating patient lympho-hematopoietic cells, and reduce GvHD by lysing patient antigen-presenting cells. Therefore, biochemical NK priming that enhances their survival, effector function, and maturation would be desirable in the setting of cancer cell immunotherapy—features afforded by NK cell treatment with hGIFT2.

In summary, NK cells are the only lymphoid cells that express components of both IL-2 and GM-CSF receptors (10). Therefore, the selective effect of hGIFT2 on NK cells suggest that hGIFT2 binds to these cytokine receptors in an atypical fashion, which triggers aberrant signals downstream of both IL-2 and GM-CSF receptors, leading to the hyperphosphorylation of receptor-associated JAK/STAT proteins. Consequently, hyperactivated NK cells produce large amount of IFNγ and RANTES and overexpress NCRs involved in NK cell cytotoxicity while contemporaneously acquiring features of mature and apoptosis-resistant cells. The use of GIFT2-primed autologous NK cells may therefore be of interest in cell therapy of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 6/25/09; revised 9/30/09; accepted 10/1/09; published OnlineFirst 11/17/2009.

Grant support: Canadian Institute for Health Research operating grant MOP-15017. C. Penafuerte is a recipient of Montreal Children’s Hospital Experimental Therapeutics in Cancer Scholarship and U.S. Army Research Graduate School Scholarship. J. Galipeau is a Fonds de recherché en santé du Québec chercheur-boursier senior.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Manaf Bouchentouf, Dr. N. Eliopoulos, and Dr. Patrick Williams for technical advice and materials.

References


Correction: The Human Ortholog of Granulocyte Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion Protein Induces Potent Ex vivo Natural Killer Cell Activation and Maturation

In this article (Cancer Res 2009;69:9020–8), which was published in the December 1, 2009 issue of Cancer Research (1), the name of the third author is incorrect. The correct name is Mohamed-Rachid Boulassel.

Reference


Published OnlineFirst 06/15/2010.
©2010 American Association for Cancer Research.
doi: 10.1158/0008-5472.CAN-10-1897
The Human Ortholog of Granulocyte Macrophage Colony-Stimulating Factor and Interleukin-2 Fusion Protein Induces Potent Ex vivo Natural Killer Cell Activation and Maturation


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2322

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/11/16/0008-5472.CAN-09-2322.DC1

Cited articles
This article cites 35 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/23/9020.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/23/9020.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.