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Concise Review: Engineering the Fusion of Cytokines for the Modulation of Immune Cellular Responses in Cancer and Autoimmune Disorders

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ABSTRACT

As our understanding of the basic precepts of immunobiology continue to advance at a rapid pace, translating such discoveries into meaningful therapies for patients has proved challenging. This is especially apparent in the use of cytokine-based immunotherapies for cancer. Unanticipated and serious side effects, as well as low objective response rates seen in clinical trials, have dealt setbacks to the field. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and common γ-chain (γ-c) interleukins are cytokines that have been used as stand-alone immunotherapies with moderate success. Our group has found that the fusion of GM-CSF to members of γ-c interleukins results in the generation of novel proteins with unique signaling properties and unheralded biological effects. These fusion proteins, termed GIFT (GM-CSF interleukin fusion transgenes) fusokines, are the result of combining GM-CSF and a γ-c interleukin into a single, bifunctional polypeptide. In our experience, GIFT fusokines often confer immune cells with a gain of function that cannot be explained by the mere sum of their constituent moieties. They act as bispecific ligands, coupling activated GM-CSF and interleukin receptors together to drive unique downstream signaling events. The synergy that arises from these fusions has shown great promise in its ability to modulate the immune response and overcome maladaptive biological processes that underlie diseases such as cancer and autoimmune conditions. In this review, we discuss the ways in which the GIFT fusokines are able to alter the immune response, particularly in disease states, with a special emphasis on how these novel molecules may be translated into effective therapies in the clinical setting. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:66–73*

INTRODUCTION

Cytokines hold an important place within the field of immunology; soluble substances secreted from white blood cells that could alter the behavior of other cells were predicted in the early 20th century but were not confirmed until 1957 with the discovery of interferon and, later on, with the identification of a certain T cell-derived "lymphocyte-activating factor," which was ultimately demonstrated to be interleukin-2 (IL-2) in 1976 [1–3]. Since then, the field of cytokine biology has expanded rapidly with the advancement of molecular cloning techniques and, more recently, with genome-wide screens. Cytokines control everything from the maintenance of homeostasis to activation, proliferation, and even the programmed cell death of immune cells [4]. This made cytokines a natural target for manipulation and clinical translation. With all the important functions ascribed to cytokines and in particular the interleukins, it was hoped that they would behave as a sort panacea for all the ailments of the immune system. However, despite the large numbers of clinical trials established over the past several decades, IL-2 remains the only common γ-chain (γ-c) interleukin approved by the Food and Drug Administration (FDA) for clinical use, others having been abandoned for reasons such as low therapeutic efficacy or harmful side effects.

In an attempt to improve upon the efficacy of endogenous cytokines, our group and others have explored the fusion of cytokines, or fusokines, as an experimental way to augment the effector functions of the immune response [5, 6]. We have discovered that fusokines can pharmacologically impel the clustering of unrelated but activated cytokine receptors together, transducing unique and supraphysiological signals that ultimately confer novel biological effects in responsive cellular subsets [7]. Whereas monomeric cytokine therapy may elicit only physiological immune responses owing to the natural coevolution of regulatory mechanisms to limit cytokine-mediated effects, fusokines are not bound by the same regulatory constraints [5]. Fusokines also allow for two different bioactive ligands to act in the same time and space, an important synergy that cannot be

guaranteed even in combinatorial cytokine treatment. Moreover, fusokines direct their immunomodulating effects specifically to cellular subsets that express receptors for both moieties of the fusion. In this way, fusokines may be rationally designed to target only the cells from which we wish to elicit an effect. This important consideration has obvious implications for the therapeutic index of fusokines in the clinical setting, a problem that has posed a significant roadblock for the widespread use of endogenous cytokines, which have significant off-target effects [8–10]. With this in mind, we turn our attention to the ways in which GIFT (granulocyte-macrophage colony-stimulating factor [GM-CSF] interleukin fusion transgenes) fusokines are able to modulate the immune response, particularly in cancer and autoimmune conditions.

GIFTs From an Arranged Marriage of Cytokines

GM-CSF is a FDA-approved recombinant molecule predominately used in bone marrow transplant settings to aid patients in the reconstitution of their granulocytic and monocytic hematopoietic compartments [11]. More experimentally, it has been repeatedly shown to be an effective cytokine at inducing antitumor responses in cancer vaccine models [12, 13]. This has been attributed to its ability to drive the maturation of dendritic cells (DCs), which present tumor-associated antigens to T cells, thereby triggering an adaptive immune response against the tumor. With a relatively safe toxicity profile, GM-CSF became the parental cytokine from which the GIFTs were derived [14].

The γ-c family of cytokines consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The unifying characteristic of all these cytokine members is that they use the γ-c chain (also known as CD132) as a part of their receptor complexes. These cytokine receptor complexes also include an α-chain, which confers ligand specificity to each individual cytokine. IL-2 and IL-15 additionally use a shared β chain (CD122) as a part of their receptor complexes.

The γ-c cytokines were a logical target for fusion to GM-CSF because of their well-studied and potent proinflammatory effects. It is worth noting that interspecies differences in cytokine signaling exist to varying degrees, depending on the cytokine in question [15]. However, the major functions of individual cytokines are generally well-conserved in mammalian species (e.g., mice, nonhuman primates, and humans), from which all the GIFT preclinical data discussed in this review have been derived. These cytokines have pleiotropic functions that are critical for both the homeostasis and effector functions of the immune cellular repertoire [16, 17]. Although these cytokines use shared receptor components for ligand binding and signal transduction, the repertoire of receptors expressed are tightly regulated and vary among different immune cell types, ensuring fine-tuned specificity in response to cytokine stimulation.

A GIFT for Every Occasion

GIFT-2

IL-2 was the first candidate to be fused to GM-CSF because of its potent effects on T-cell proliferation and activation. As one of the few cytokines approved by the FDA for the treatment of metastatic disease, IL-2 (aldesleukin) is also known to be one of the most effective cytokines at promoting the locoregional rejection of live tumor cells [18, 19]. GM-CSF, on the other hand, has proven to be superior at inducing long-term antitumor immunity in irradiated tumor vaccine studies [20]. The hope was to create a fusokine capable of recruiting the complementary arms of the anticancer response in both the lymphoid and myeloid components of the immune system. Priming of tumor antigen presentation and maturation of dendritic cells enhanced by GM-CSF can spur an adaptive antitumor response that may be subsequently amplified by IL-2.

GIFT-2 was created by cloning the cDNA of IL-2 in frame directly 3′ to GM-CSF [21]. Given the primary amino acid sequence of GIFT-2, computer-based molecular modeling predicted that both moieties of the protein would be able to properly fold without major structural changes to the domains responsible for receptor binding. This was confirmed with bioactivity assays assessing the proliferative capacity of CTLL-2 and JAWS-II cells when cultured in the presence of GIFT-2, two cell lines that are dependent on IL-2 and GM-CSF, respectively. With in vitro confirmation that both moieties of the fusion protein were bioactive, we next moved in vivo to ascertain the therapeutic effects of GIFT-2 in a mouse model of melanoma. GIFT-2 was just as effective as IL-2 at inducing locoregional tumor rejection and outperformed GM-CSF in an irradiated tumor vaccine model in which mice that received irradiated B16 melanoma cells secreting GIFT-2 (B16-GIFT-2) were protected from subsequent tumor challenge. Furthermore, in a therapeutic cancer vaccine model, more mice with pre-established B16 tumors were able to progress to a tumor-free state when given irradiated B16-GIFT-2 cells compared with mice receiving irradiated B16 cells that secreted GM-CSF and IL-2.

One interesting observation made in the GIFT-2 studies was that B16 tumors secreting GIFT-2 recruited significantly higher numbers of NK cells into the tumor microenvironment compared with B16 cells secreting GM-CSF. Although GM-CSF has been observed to impair NK cell function, which may explain why GM-CSF cannot durably induce rejection of live tumor cells, the addition of B16 cells secreting IL-2 to these B16-GMCSF tumors could not rescue NK cell infiltration into the tumor [22]. This observation illustrates an important point when considering combinatorial cytokine therapy; use of individual cytokines together may elicit conflicting biological responses [23]. Such antagonistic responses may be overcome with the use of a single, fused molecule, as is the case with GIFT-2. This observation also showcases how fusokines may act in a synergistic manner to confer a gain of function above and beyond simply administering two individual cytokines in the same time and space. Indeed, in follow-up studies using the human ortholog of GIFT-2 (hGIFT-2), Penafuerte et al. [22] were able to show that compared with treatment with GM-CSF and IL-2, hGIFT-2 could differentially activate human NK cells by increasing their expression of surface markers associated with enhanced cytotoxicity, allowing them to more effectively lyse target cells.

GIFT-15

IL-15 was next to be fused to GM-CSF because of its similarity to IL-2. IL-15 uses a receptor complex very similar to that of IL-2, with the exception of an IL-15 receptor α-chain (IL-15Rα-chain), which specifically allows for IL-15 to associate with the shared IL-2/15Rβ-chain and a γ-c [24]. Like IL-2, IL-15 has been shown to stimulate T-cell proliferation and NK cell activation. However, IL-15 is

distinct in that it can prevent activation-induced cell death of T cells and stimulate the proliferation of memory phenotype $CD8^+$ T cells, both processes that are actually inhibited in the presence of IL-2 [25, 26]. It was thought that GIFT-15 would behave similarly to GIFT-2, acting as a proinflammatory molecule. It was therefore an unexpected surprise when GIFT-15 behaved as a potent immunosuppressive agent. Rafei et al. [27] first noticed this intriguing phenomenon when B16 cells transduced to express GIFT-15 grew at a significantly faster rate compared with wild-type B16 cells when implanted into mice. Moreover, human U87 glioblastoma cells expressing GIFT-15 could be successfully xenografted into immunocompetent mice without subsequent rejection, pointing to this fusokine's profoundly immunosuppressive properties.

GIFT-15's surprises did not end there. When unfractionated splenocytes from mice were treated with this fusokine, a population of naïve MHC-II+ B cells was enriched. Although B cells express both GM-CSF and IL-15 receptors, they were not predicted to be the most responsive subset of immune cells to GIFT-15, especially given IL-15's well-characterized effects on NK and T cells [28, 29]. Moreover, GIFT-15 converted these naïve B cells into a regulatory phenotype, characterized by high surface expression of CD1d and the secretion of IL-10 [30]. IL-10-secreting regulatory B cells (Bregs) have become a topic of intense interest in the field of clinical immunology, owing to their ability to negatively modulate the immune response in autoimmune conditions [31]. Their presence and dysregulation have been noted in conditions ranging from rheumatoid arthritis to systemic lupus erythematous in both human patients and experimental mouse models. Akin to their T-cell counterparts, Bregs are able to suppress overt inflammatory processes by secretory factors, such as IL-10 and transforming growth factor-β, which inhibit IFN-γ production by T_H1 cells, as well as the in vitro differentiation of T_H17 cells [32, 33]. Although naturally occurring Bregs exist in a variety of phenotypes in humans, they occur in very small numbers and are difficult to propagate ex vivo. GIFT-15 provided an obvious remedy to this problem by converting large numbers of naïve B cells into Bregs. With this insight, Rafei et al. [27] found that Bregs derived from GIFT-15 treatment (GIFT-15 Bregs) could durably induce remission of disease when adoptively transferred into mice with experimental autoimmune encephalomyelitis (EAE), a murine model of the autoimmune demyelinating human disease, multiple sclerosis.

The transfer of two million GIFT-15 Bregs ameliorated severe neurological symptoms, such as hind-limb paralysis, in mice with EAE. These findings also correlated with histopathological data showing significantly lower numbers of proinflammatory cell infiltrates in the central nervous system of GIFT-15 Breg-treated mice. Interestingly, GIFT-15 Bregs derived from IL-$10^{-/-}$ mice were unable to suppress neuroinflammation, suggesting that the secretion of IL-10 was necessary for their immunosuppressive effects. However, although IL-10 is necessary for suppression, it is not sufficient as GIFT-15 Bregs derived from MHC-$II^{-/-}$ mice were also incapable of dampening neuroinflammation. This latter observation suggests that interaction with CD4 T cells plays a critical role in the therapeutic efficacy of GIFT-15 Bregs in EAE. In addition to their ability to stop the progression of autoimmune reactions, GIFT-15 Bregs may also be effective in suppressing exuberant allogeneic responses. This has exciting clinical implications in the realm of solid organ or *allo*-bone marrow transplant in which such allogeneic responses are detrimental.

GIFT-15's narrative serves to remind us of two very important points in the development of the fusokine platform. First, although it is possible to rationally design fusion proteins with a desired biological phenomenon in mind, the resulting fusokine may have novel, unpredictable, and unanticipated effects. Second, although fusokines may be used as a protein biologic for direct administration to patients with autoimmune disease or cancer, their utility as agents to augment immune cells ex vivo may prove just as valuable. Indeed, the Bregs generated by GIFT-15 ex vivo becomes the therapeutic product that may be used to treat patients, as opposed to GIFT-15 itself. The dichotomy between creating a pharmaceutical molecule versus a cellular product for clinical use may seem like an arbitrary distinction but has meaningful implications related to regulatory approval.

GIFT-21

IL-21 is the most recently identified member of the γ-c family of cytokines and predominately acts to promote the function of mature effector cells in the immune system [34]. IL-21 differentiates $CD4^+$ T cells down the T_H17 pathway, activates NK cells, and stimulates $CD8^+$ T cells to mount antitumor responses [35–37]. We hypothesized that fusing GM-CSF and IL-21 (GIFT-21) would lead to synergistic anticancer effects because of each cytokine's respective role in mediating inflammation. The GIFT-21 fusokine had unanticipated hypermorphic effects on the monocyte lineage of cells, inducing their maturation into a distinct DC population with tumoricidal properties [38]. GIFT-21-induced DCs (GIFT-21 DCs) display enhanced antigen presentation properties and secrete substantially more proinflammatory cytokines that ultimately drive a T_H1-polarized response [39]. When adoptively transferred into B16 melanoma- or D2F2 breast cancer-bearing mice, GIFT-21 DCs were able to inhibit tumor growth even without prior antigen priming. Analysis of tumor explants revealed that GIFT-21 DCs could readily migrate into the tumor microenvironment within 24 hours post-transfer to sample antigen, whereas conventional DCs (monocytes matured into DCs with GM-CSF and IL-4) were absent. DCs have been the subject of many immunotherapeutic studies in clinical oncology, because they act as gatekeepers to an effective adaptive immune response [40]. Unfortunately, DCs are relatively rare, difficult to isolate, and even more challenging to propagate ex vivo to numbers that result in meaningful clinical outcomes when administered to patients. Furthermore, many clinical trials focus on priming DCs by exposing them to a single (or a few preselected) antigen(s) before transfusion, limiting the repertoire of antigenic determinants presented and the efficacy of the ensuing adaptive immune response [41]. GIFT-21 provides an attractive alternative to DC-based immunotherapy by converting readily available and abundant monocytes into hyperactivated DCs, capable of inducing effective antitumor responses even without prior exposure to tumor antigens.

GIFT-4

GIFT-4, borne of the linkage of GM-CSF and IL-4, triggers an antitumor response that is B cell-dependent [42]. Mice with preestablished B16 or melan-a $GNAQ^{Q209L}$ melanoma tumors treated with GIFT-4 displayed significant inhibition of tumor growth compared with control mice receiving GM-CSF and IL-4 treatments. B16 tumors engineered to express GIFT-4 (B16-GIFT-4) were

significantly attenuated when implanted into wild-type C57BL/6J mice, but this effect was lost when the same cells were implanted into B cell-deficient μMT mice. The latter result provides evidence that GIFT-4's ability to suppress melanoma growth is dependent on B cells. Deng et al. [42] were further able to show that the human ortholog of GIFT-4 could stimulate the proliferation and activation of B cells derived from the peripheral blood of healthy human subjects. These GIFT-4 treated B cells (GIFT-4 B cells) expressed substantially higher costimulatory and antigen-presentation markers, in addition to secreting significantly higher concentrations of IL-2 and IL-6, among other proinflammatory cytokines. GIFT-4 B cells robustly promoted T-cell proliferation in in vitro cocultures and primed them to become antitumor cytotoxic effectors by inducing T-cell production of granzyme B, granulysin, and IFN-γ. These GIFT-4 B cell-primed T cells specifically lysed A375 human melanoma cells in vitro and in vivo when adoptively transferred into NOD *scid* γ mice lacking T, B, and NK cells. Interestingly, GIFT-4 has very little direct effect on purified T cells but will only license T cells to become antitumor effectors in the presence of GIFT-4-activated B-cell mediators. GIFT-4 opens the exciting possibility of developing B cells as effectors for cancer immunotherapy.

GIFT-7

The fusion of IL-7 to GM-CSF resulted in GIFT-7, a fusokine that preferentially affects the T-cell compartment. This was not surprising, given that lymphopoeisis and many other facets of T-cell development are critically dependent upon the function of IL-7 [43]. Indeed, IL-7$^{-/-}$ mice are severely lymphopenic because of an arrest of T-cell development at the pro- to pre-T-cell transition in the thymus [44]. Further, IL-7 is required for the homeostatic proliferation and survival of naïve T cells [45]. GIFT-7 acted as a potent mitogen for IL-7 receptor high T-cell precursors derived from the thymus. The earliest of these T-cell precursors, the double-negative (DN; CD4$^-$, CD8$^-$) thymocytes, were the most responsive, with further subset analysis revealing that a CD44 intermediate-expressing population of DN thymocytes expanded by more than fourfold over GM-CSF- and IL-7-treated thymocytes. In vivo administration of GIFT-7 to young mice resulted in transient hyperplasia of the thymic cortex, an important site for T-cell selection and maturation. This effect was even more pronounced in aged mice (10–15 months old), in which GIFT-7 treatment led to hypercellularity of thymic cortical tissue and enhanced output of T cells into the periphery. The potential clinical significance of this observation was not lost on Hsieh and colleagues, who found that aged mice pretreated with GIFT-7 and subsequently challenged with murine cytomegalovirus (MCMV) were superior at inducing anti-MCMV specific T-cell responses compared with mice pretreated with GM-CSF and IL-7 (Hsieh J, Bosinger S, Wu J et al., manuscript submitted for publication). Thymic atrophy and involution occurs as a natural process of ageing in mice, as well as humans. As we age, thymic output decreases, reducing the number of T cells in the peripheral circulation. This is one of the reasons we become more susceptible to infections as we age [46]. The ability of GIFT-7 to reverse the effects of thymic atrophy makes it an attractive molecule for clinical translation, particularly in conditions under which immune senescence and exhaustion are contributors. Chronic viral infections, cancer, and age-associated immune deficiencies are just some of the conditions that could immensely benefit from a molecule that enhances both the number and the repertoire of circulating T cells.

GIFT-9

IL-9 is perhaps the least studied of the γ-c cytokines and the last of this family to be fused into a GIFT fusion protein. Best known for its affect on mast cell growth and function, IL-9 has also been ascribed a protective role against parasitic infections and implicated as a critical mediator of allergic inflammation [47–49]. Akin to IL-9, GIFT-9's predominant biological effect was seen on mast cells. GIFT-9 behaved as a hyperagonist of the IL-9 receptor (IL-9R) and was better able to induce the growth of bone-marrow mast cells compared with equimolar concentrations of GM-CSF and IL-9 [7].

GIFTs Can Send Mixed Signals

In order to understand how GIFT fusokines deploy their unprecedented biological effects, we must look at the way in which they transduce their signal within immune cells. The ability of GIFTs to bring together receptors belonging to different superfamilies results in radically altered signaling events downstream that are distinct from the signals transduced by monomeric cytokines (Fig. 1). GM-CSF typically triggers signaling by inducing the heterodimerization of cell surface GM-CSF receptor (GMCSFR) α-chains (αc) and β-chains (βc). The dimerization results in conformational changes that result in the phosphorylation and activation of GMCSFR βc-associated JAK2. In turn, JAK2 will phosphorylate the cytoplasmic tails of GMCSFR α- and β-chains, resulting in the recruitment and activation of STAT5 in a SH-2-dependent manner. γ-c interleukins trigger a similar signaling cascade, generating a heterodimer (or trimer, in the case of IL-2 and IL-15) of the γ-c and the interleukin receptor (IL-R) αc. The resulting complex activates γ-c-associated JAK3 and IL-Rα-chain-associated JAK1. Depending on the IL bound, the STAT molecules that become phosphorylated will vary, because different IL-Rα chains preferentially associate with different STAT molecules [50]. The phosphorylation of STAT molecules allows them to homodimerize and translocate into the nucleus, where they alter transcription [51]. Of note, none of the γ-c interleukins use JAK2 as part of their signal transduction machinery.

Although natural cooperativity between unrelated cytokine receptors have been observed, we endeavored to engineer a family of proteins that bridge unrelated receptors together [52, 53]. Coimmunoprecipitation experiments using GIFT-4 and GIFT-9 have shown that GM-CSFR βc pulls down significant amounts of IL-4 and IL-9 receptor α-chains, respectively. This suggests that in the presence of GIFT fusokines, GM-CSF and interleukin receptors are able to interact with one another. Direct evidence of receptor clustering was provided by immunofluorescence staining coupled with confocal microscopy. These studies revealed that antibodies directed toward GMCSFR βc and the γ-c on MC/9 mast cells were colocalized when treated with GIFT-9 but remained separated across the cell surface when treated with GM-CSF and IL-9, alone or in combination. Deng et al. [42] were readily able to replicate these data, showing extensive colocalization of IL-4Rα and the GMCSFR βc with GIFT-4 treatment of primary B cells.

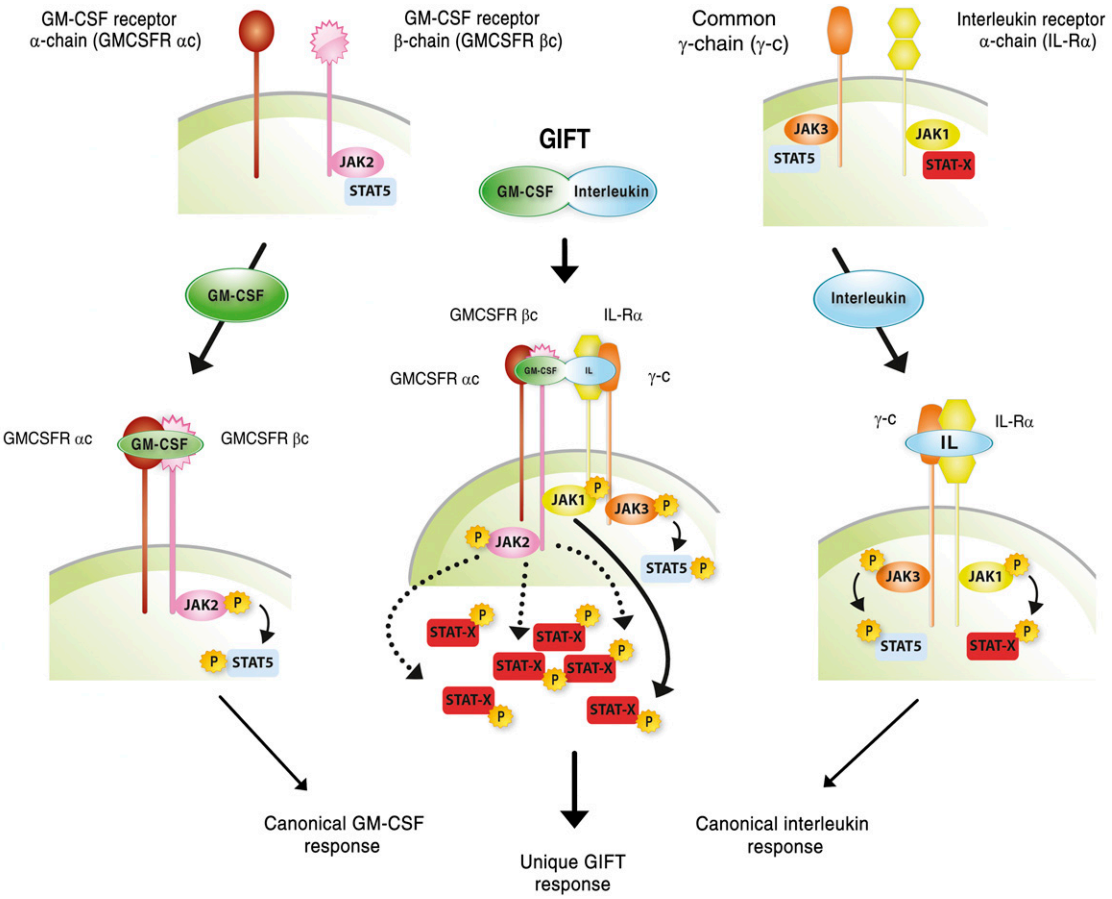

Figure 1. GIFT-induced receptor clustering. GIFTs are able to bring together activated GM-CSF and interleukin receptors belonging to the common γ-c family. GM-CSF ligand binding to the GM-CSF receptor triggers the dimerization of α- and β-chains, resulting in the activation of β-chain-associated JAK2/STAT5. γ-c cytokines initiate a similar signaling cascade by bringing together the γ-c and a cytokine-specific IL-Rα. JAK3 associates exclusively with the γ-c in lymphomyeloid cells and activates STAT5 upon IL binding. JAK1 associated with the IL-Rα will activate different STATs (STAT-X), depending on the IL bound. GIFTs trigger the coclustering of all four activated receptor components, resulting in transphosphorylation of IL-Rα-associated STAT-X substrates by JAK2 (dotted arrows). Changes in the balance of STAT phosphorylation events induce a unique GIFT-mediated response that is distinct from canonical GM-CSF- and IL-mediated responses. Abbreviations: γ-c, γ-chain; GM-CSF, granulocyte/macrophage colony-stimulating factor; GMCSFR αc, GM-CSF receptor α-chain; GMCSFR βc, GM-CSF receptor β-chain; IL, interleukin; IL-Rα, IL receptor α-chain.

Heterologous receptor clustering has significant physiological consequences for immune cells. In addition to bringing GM-CSF and interleukin receptors together, the kinases and molecules of signal transduction that are uniquely associated with these receptors are also colocalized as a result of GIFT binding. In a set of mechanistic experiments, Li et al. [7] showed that GIFT-9 leads to hyperactivation of IL-9R-associated STAT1 through a JAK2-dependent mechanism. This finding is significant because JAK2 is typically associated with GMCSFR βc and does not physiologically interact with γ-c interleukin receptor complexes. The ability of GIFT-9 to hyperactivate STAT1 is a result of its capacity to recruit and activate GMCSFR βc-associated JAK2 into a complex that also includes IL-9R-associated STAT1. In other words, JAK2 is able phosphorylate STAT1 because GIFT-9 acted as a chaperone clustering the IL-9R and its associated signaling molecules to GMCSFR βc-associated JAK2. Specific blockade of JAK2 with a small molecule inhibitor abrogated GIFT-9's ability to hyperactivate STAT1, demonstrating that this effect was JAK2-specific. In essence, an environment is created where JAK1, JAK2, and JAK3 are assembled in a manner not otherwise physiologically permissible, hence resulting in a gain of function (Fig. 1).

GIFT-9 provided a proof of concept that receptor clustering is responsible for the altered signaling that is observed across the GIFT family of fusokines (Table 1). By bringing together different combinations of activated JAKs and STATs that do not physiologically interact with one another, GIFT fusokines launch novel downstream signaling events that cannot be achieved by any other method described to date. The phenotypic outcome and eventual effector functions of GIFT-responsive cell types rely on the balance of STATs that become activated [54]. Mechanistically, GIFTs alter cellular signaling to confer a STAT-driven mitogenic response in lymphomyeloid cells, inducing their proliferation. Cellular physiology is also altered as a result of GIFT treatment, including but not limited to changes in cell surface marker expression, cytokine and chemokine secretion (i.e., secretome), and susceptibility to apoptosis. GIFTs confer a unique transcriptional program to lymphomyeloid cells because they are able to cause nonphysiological activation of various combinations of STAT molecules not seen in nature. For this reason, we prefer to think of GIFTs as a novel

Table 1. Summary of GIFT-mediated biological effects and potential clinical applications

Fusokine	Responder cell subsets	Biological effect and phenotype	Potential clinical application	Signal transduction	References
GIFT-2	Macrophages, NK cells (H/M)	Expansion and hyperactivation of NK cells	Cancer immunotherapy, viral infections	Hyper pSTAT-1/3/5	19, 20
GIFT-4	Naïve B cells (H/M)	Expansion and conversion of naïve B cells to B-effector cells; GIFT-4 B cells may also license naïve T cells to become antitumor CTLs	Cancer immunotherapy	Hyper pSTAT-1/3/5/6	40
GIFT-7	Thymocytes, peripheral T cells (H/M/NHP)	Expansion of peripheral $CD4^+$ T cells and double-negative thymocytes	Age-associated thymic insufficiency, chronic viral infections, cancer immunotherapy	N/A	Hsieh et al.[a]
GIFT-9	Mast cells (M)	Expansion of bone marrow-derived mast cells		Hyper pSTAT-1	6
GIFT-15	Naïve B cells (H/M/NHP)	Conversion of naïve B cells into immunosuppressive regulatory B cells	Autoimmune disease (e.g., SLE, RA, MS), chronic inflammatory conditions, solid organ transplant, *allo*-BMT	Hyper pSTAT-3 Hypo pSTAT-5	25, 28
GIFT-21	Monocytes, DCs (H/M)	Conversion and maturation of monocytes into hypermorphic DCs	Cancer immunotherapy	Hyper pSTAT-3	36, 37

The responder cell subsets, the species from which these cells were derived, and the known cellular signaling events activated by each member of the GIFT family are listed. Hyper/hypophosphorylation effects (hyper/hypo pSTAT) are based on the strength of GIFT-induced phosphorylation of STAT substrates relative to phosphorylation by granulocyte/macrophage colony-stimulating factor and/or the derivative monomeric common γ-chain cytokine. The clinical applications outlined for each GIFT are based on in vitro effects observed and in vivo data derived from experimental animal models.

[a]Hsieh J, Bosinger S, Wu J et al., manuscript submitted for publication.

Abbreviations: allo-BMT, allogeneic bone marrow transplant; CTLs, cytotoxic T-lymphocytes; DCs, dendritic cells; H, human; M, mouse; MS, multiple sclerosis; NHP, nonhuman primate; N/A, not available; SLE, systemic lupus erythematous; RA, rheumatoid arthritis.

cytokine class and not simply the combination of two existing cytokines.

Although JAK2's promiscuous transphosphorylation of different STAT substrates provides a unifying mechanism explaining the gain of function observed, it might not be the only mechanism by which GIFTs act. The conformational changes induced by GIFT binding to receptor complexes may allow for hyperactivation and increased recruitment of JAKs themselves [22]. Although this was not seen with GIFT-9, the hyperactivation of JAK1 and JAK3 was seen with GIFT-2 treatment in CTLL-2 T cells. Indeed, the stoichiometry of GIFT ligand to receptor complexes and their associated signal adaptor molecules remains to be worked out in detail. GIFT-15 provides an example of how fusokines may engage with their cognate receptors in unique ways, because this is only GIFT molecule that acts as a partial agonist. Although GM-CSF and IL-15 are both activators of STAT5 signaling, GIFT-15 stimulation activates STAT3 without concurrent activation of STAT5, driving a transcriptional program that coaxes naïve B cells to take on a regulatory phenotype. This would suggest that GIFT-15 engages its receptors in a way that alters the ability of adaptor molecules to transduce an intracellular signal [27]. We also cannot exclude the possibility that receptor clustering alters the way in which negative regulators of cytokine signaling function. Protein phosphatases such as the SH-2 domain-containing protein-tyrosine phosphatase-1 (SHP-1), suppressor of cytokine signaling family members (e.g., SOCS1 and SOCS3), CD45, and protein inhibitors of activated STATs (PIAS), which negatively regulate the JAK/STAT pathway, could inadvertently be affected by receptor clustering, a hypothesis that bears further testing [55–58].

Conclusion

With GM-CSF fusions to all of the known γ-c cytokines completed, we have summarized the major findings of our GIFT fusokine research program in this article. The GIFT family of fusokines demonstrates that the marriage of two bioactive leukins results in the formation of a novel fusion protein endowed with the ability to significantly alter lymphomyeloid cell physiology. This is due to the GIFTs' ability to cluster activated GM-CSF and interleukin receptors together, an unnatural interaction that results in the transduction of a unique signal, ultimately conferring responsive cells with unheralded phenotypes and effector functions, functions that may be exploited for therapeutic use in a clinical setting.

Our study of GIFTs has yielded novel insights into cytokine biology and cellular signal transduction. More importantly, they have established the fusion of cytokines as a viable biopharmaceutical platform to elicit a gain of function from specific cellular subsets based on their receptor expression patterns. In addition to clinical trials currently under way using γ-c cytokines (supplemental online Table 1) [59], it is our hope that the GIFT family of fusokines will open the door to hypothesis-driven cytokine coupling strategies and meaningfully add to the growing armament of immunotherapeutic biologics.

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Author Contributions

S.N. and J.G.: manuscript writing.

Disclosure of Potential Conflicts of Interest

J.G. is an inventor on U.S. provisional patents of GIFT molecules. The other author indicated no potential conflicts of interest.

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