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Novel TGF-β Antagonist Inhibits Tumor Growth and Angiogenesis by Inducing IL-2 Receptor-Driven STAT1 Activation

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Carcinoma derived TGF-β acts as a potent pro-oncogenic factor and suppresses antitumor immunity. To antagonize TGF-β mediated effects in tandem with a proinflammatory immune stimulus, we generated a chimeric protein borne of the fusion of IL-2 and the soluble extracellular domain of TGF-βRII (FIST). FIST acts as a decoy receptor trapping active TGF-β in solution and interacts with IL-2-responsive lymphoid cells, inducing a distinctive hyperactivation of STAT1 downstream of IL-2R, which in turn promotes SMAD7 overexpression. Consequently, FIST-stimulated lymphoid cells are resistant to TGF-β-mediated suppression and produce significant amounts of proinflammatory cytokines. STAT1 hyperactivation further induces significant secretion of angiostatic CXCL10. Moreover, melanoma cells expressing FIST fail to form tumors in CD8−/−, CD4−/−, B cell-deficient (μMT), and beige mice, but not in NOD-SCID and Rag2/γc knockout mice, consistent with the pivotal role of FIST-responsive, cancer-killing NK cells in vivo. In summary, FIST constitutes a novel strategy of treating cancer that targets both the host’s angiogenic and innate immune response to malignant cells. The Journal of Immunology, 2011, 186: 000–000.

A n extensive number of studies demonstrate that TGF-β acts as a potent immune stimulatory factor in established carcinoma (1, 2). Cancer-derived TGF-β drives malignant progression by constitutively inducing epithelial to mesenchymal transition and tumor-associated angiogenesis and by suppressing tumor immunity, the sum of which effects are to promote tumor growth and metastasis (1, 3–6). There are three different TGF-β isoforms, TGF-β1, 2, 3. Among them, TGF-β1 is the most frequently overexpressed by carcinomas (7). In mammalian cells, TGF-β signals through heteromeric complexes of type I (activin receptor-like kinases, also known as TβRI) and type II (TβRII) transmembrane serine/threonine kinase receptors (8). Upon ligand binding, the constitutively active TβRII phosphorylates the type I receptor (ALK), which propagates signals into the cells by phosphorylating receptor-regulated SMADs (i.e., SMAD2 and SMAD3). Phosphorylated receptor-regulated SMADs form a heteromeric complex with the comediator SMAD4 (9) and translocate to the nucleus where this complex cooperates with transcription activators and repressors to regulate the expression of TGF-β target genes (10). Inhibitory SMADs (SMAD6 and SMAD7) act as negative regulators of TGF-β signaling pathway by preventing the interaction of TGF-βR complex with receptor-regulated SMADs and promoting the dephosphorylation and degradation of activated receptors (11, 12). As proangiogenic factors, TGF-β family members play an essential role in vasculoxygenation and tumor angiogenesis. TGF-β regulates the expression of various extracellular matrix components that play a key role in both the initiation and resolution phase of angiogenesis (13–16). Consistent with this notion, we hypothesize that the blockade of TGF-β signaling combined with cytokine-driven immune activation will couple anti-angiogenesis to an effective immune antitumor response, resulting in potent anticancer properties. In particular, IL-2 is a cytokine that can promote an innate antitumor response due to its effectiveness at inducing locoregional tumor rejection, acting as autocrine factor for T cells and supporting the development of cytotoxic T cells, stimulating NK cells proliferation and cytolytic activity (17–19). In addition, IL-2 was approved by the U.S. Food and Drug Administration in 1998 for immunotherapy of melanoma (20). We have found in a preclinical model of B16 melanoma that IL-2-driven antitumor activity is severely curtailed by tumor-derived active TGF-β. Several studies have reported that TGF-β inhibits the phosphorylation and activation of components of the JAK/STAT cascade downstream of IL-2R (21). Moreover, TGF-β inhibitory activity occurs at the nuclear level on a subset of IL-2 target genes including c-Myc, cyclin D2, and cyclin (22). Therefore, the blockade of TGF-β activity markedly enhances immunotherapy (23). Several approaches have been used to neutralize tumor-derived active TGF-β in vivo (24–30). In particular, soluble type II TGF-βR (sTβRII) treatment in mice bearing tumors was shown to significantly decrease tumor metastasis (3, 26). We therefore speculated
that IL-2 could serve as a rational cytokine partner of TβRII as part of a “sword and shield” pharmaceutical strategy. To test this concept, we created a novel recombinant protein fusion protein composed of IL-2 fused to the extracellular domain of TβRII and here demonstrate its biochemical effects on NK cells leading to potent antitumor activity in vivo.

Materials and Methods

Mice

B cell-deficient (μMT), NK, CD4, CD8 T cell, IFN-γ knockout (KO) mice, beige mice (C57BL/6-bg), C57BL/6, BALB/c immunocompetent, CXCX3 KO, NOD-SCID mice (The Jackson Laboratory, Bar Harbor, ME), and Rag2/γc KO mice (Taconic, Hudson Valley, NY) were female to 8 wk old. STAT1 KO mice were generously donated by Joan Durbin (Lady Davis Institute for Medical Research) (31).

FIST fusokine design, expression, and functionality

The mouse IL-2 cDNA (Invivogen, San Diego, CA) was modified by removing the 3′ nucleotide encoding the STOP codon, and subsequently cloning into the TGFβ receptor II ectodomain cDNA (Invivogen) to generate the cDNA for mouse IL-2/TβRII fusion protein (also known as fusion of IL-2 and the soluble extracellular domain of TGF-βRII; FIST). The stop codon and poly A were added at the C-terminal region. The vectors encoding FIST or control (IL-2 and TβRII) were used to transfect 293T cells. The supernatants of transfected cells were collected after 48 h, concentrated with centricon plus-70 (Millipore, Billerca, MA), and the molar concentration of FIST and IL-2 were quantified by ILISA, whereas TβRII protein was quantified by Western blot using a concentration versus OD curve of recombinant mouse TGFβ protein. For the proliferation assay, 105 CTL-2 cells (IL-2 dependent) per well were cultured in a 96-well plate and treated with 5 pmol FIST or controls for 72 h. Cell proliferation was assessed by MTT uptake. FIST and TGF-β complexes were precipitated using TGF-β–specific Abs bound to protein G beads, and the precipitated FIST protein was detected by Western blotting with IL-2–specific Abs bound to protein G beads, and the precipitated FIST protein was detected by Western blotting with IL-2–specific Abs. Infectious retroparticles encoding FIST were generated with 293-GP2 packaging cells (Clontech, Mountain View, CA) and used to modify genetically C57BL/6-derived B16F0 melanoma and pancreatic cancer Panc02 (duodenal adenocarcinoma of the pancreas) cell lines. CTL-2 cells were maintained in RPMI 1640 supplemented with 10% FBS; human NK cells (NK92) were maintained in 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, supplemented with 0.2 mM m- mercaptoethanol, 0.02 mM folic acid, 100–200 U/ml recombinant IL-2 and 50 U/ml penicillin and streptomycin, adjust to a final concentration of 12.5% horse serum and 12.5% FBS (Wisent Technologies, Rocklin, CA). B16F0 and Panc02 cell lines were maintained in DMEM medium supplemented with 10% FBS and 50 U/ml penicillin and streptomycin (Wisent Technologies).

Immune cell isolation and cytokine production

Enriched T, B, and NK cell populations were obtained from splenocytes of immunocompetent or STAT1 KO mice by magnetic separation according to the manufacturer’s recommendation (Stemcell Technologies, Vancouver, Canada). The purity of each population was assessed by flow cytometry (for B cells 94%, NK cells 96%, and T cells 96%) using specific conjugated Abs for cell markers (BD Biosciences, San Diego, CA). Human blood was drawn from healthy donors, after informed consent had been obtained, into heparin- or citrate-coated tubes (BD Biosciences, San Jose, CA), and PBMCs were isolated by density centrifugation. Cytokine concentrations were quantified by ELISA kits (eBioscience, San Diego, CA; and R&D Systems).

Cell signaling and receptor expression

CTL-2 cells (5 × 104) were stimulated with 5 pmol FIST or controls for 20 min before being lysed and probed by Western blot with rabbit phospho-specific and total SMAD2, SMAD3, STAT1, STAT3, and STAT5 (Cell Signaling Technology, Danvers, MA) Abs. CTL-2 cells were also stimulated with FIST or controls for 2 h to detect SMAD7 expression with rabbit-specific Abs (Santa Cruz Biotechnology, Santa Cruz, CA). For T-bet expression, cytokine-stimulated NK cells were treated with brefeldin A for 4 h, fixed, permeabilized, and stained with T-bet–conjugated Ab or isotype control for 1 h. For CXCR3 and IL-2RB expression, stimulated NK cells were incubated with FcR III/II blocking Ab for 30 min and labeled with conjugated Abs specific for CXCR3 and IL-2RB for 1 h. The mean fluorescence intensity was measured by flow cytometry, and the data were analyzed using CellQuest software (Becton Dickinson).

Immune cell infiltration in the tumor site

To analyze the immune cell types infiltrated in the tumor site, 106 genetically modified B16 cells expressing FIST or controls were mixed with 500 μl Matrigel (BD Biosciences) at 4°C and injected s.c. in immunocompetent C57BL/6 mice. Implants were surgically removed 6 d after implantation and enzymatically dissociated as reported previously (32). Infiltrated cells were collected and incubated with anti-FcR III/II Ab for 30 min and with specific cell type or isotypic control Abs for 1 h at 4°C. The expression of surface markers was determined as previously described.

Endothelial cell isolation, proliferation, migration, and in vitro and in vivo angiogenesis assays

Livers of immunocompetent mice were digested with collagenase for 40 min at 37°C to prepare a cell suspension. Cells were labeled with PE-conjugated CD31-specific Abs to isolate endothelial cells (EC) by positive selection of PE-labeled cells (Stemcell Technologies). For the in vitro capillary-like structure formation assay, 5 × 103 EC were seeded per well in a 96-well plate previously coated with a type IV collagen matrix (Chemicon, Billerca, MA). EC were incubated at 37°C for 24 h in the presence of conditioned media (CM) by NK cells previously stimulated with 5 pmol FIST or control for 72 h (CM NK cells). A numerical score was assigned to each condition according to the degree of angiogenesis progression based on the number and size of polygons formed, capillary thickness, and cell alignment and fusion, as previously reported (33). For the proliferation assay, 2 × 105 cells were cultured in a 0.1% gelatin-coated 96-well plate for 6 d in a low-serum EC media supplemented with EC growth supplement and epidermal growth factor (BD Biosciences) in the presence of CM by stimulated NK cells. Cell number was determined using the ViaLight Plus kit (Lonza, Rockland, ME). For migration assay, 24-well cell invasion assay (Millipore) was used to examine the migration of EC according to the manufacturer’s protocol. Briefly, this modified Boyden chamber is composed of a well (lower compartment) containing an insert (upper compartment). EC (8 × 104) were suspended in 0.5 ml serum-free medium and loaded into upper chambers of the Transwell. Lower chambers were loaded with 1% RPMI 1640 medium in presence or absence of FIST, IL-2, sTβRII, or IL-2 plus sTβRII-stimulated NK cell conditioned medium (NK cells were stimulated with 5 pmol FIST or control). After a 24-h incubation period at 37°C, cells remaining on the upper surface of the insert (nonmigrated cells) were removed gently and placed into a sterile 24-well plate containing a cell detachment solution for 80 min at 37°C. Cells were trypsinized with 0.025% Trypsin/0.002% EDTA for 4 h, fixed, permeabilized, and stained with the CyQuant GR dye for 15 min at room temperature. The mixture was transferred to a 96-well plate, and fluorescence was read at 480 nm. Invaded cell number was determined by running a fluorescent versus cell dose curve using 8000, 4000, 2000 EC (n = 3). To determine FIST angiostatic properties in vivo, 106 genetically modified B16 cells expressing FIST or controls were mixed with 500 μl Matrigel (BD Biosciences) at 4°C and injected s.c. in immunocompetent C57BL/6 mice. Implants were surgically removed 20 d later, and histological sections of the tumors were labeled with red fluorescence CD31 Abs to detect the formation of tumor-derived blood vessels.

Statistical analysis

We used the two-tailed unpaired Student t test to compare two experimental groups, Dunnett’s multiple comparisons to compare three or more test groups, and the log-rank test to compare two survival distributions.

Results

Generation and characterization of murine IL-2/sTβRII fusion protein: FIST

We generated a plasmid construct encoding for the fusion of IL-2 and the ectodomain of TβRII splicing variant B (sTβRIIB, from aa Leu 9 to Asp 184). The fusion transgene cDNA encodes for a single polypeptide chain of 328 aa (Fig. 1A) that migrates as an ~55 kDa protein in SDS-PAGE under reducing conditions (Fig. 1B, 1C). In a communoprecipitation assay, FIST precipitated...
active TGF-β1 (Fig. 1D), indicating that FIST acts as a decoy receptor trapping active TGF-β. As predicted, IL-2 as part of the fusion protein preserves the ability to recognize and bind IL-2R and induce the proliferation of IL-2–responsive cells (CTLL-2, Fig. 1E). The analysis of activation status of transcription factors downstream of TβRII (SMAD3 and SMAD2) indicate that FIST exerts a dominant negative effect on TGF-β canonical pathway not only by impairing the phosphorylation of SMAD2 and SMAD3 but also by inducing SMAD7 expression in CTLL-2 (Fig. 1F). The analysis of activation of transcription factors downstream of IL-2R signaling pathway indicates that FIST induces similar STAT5 phosphorylation levels compared with equimolar concentrations of controls but distinctive STAT3 and STAT1 activation levels. In particular, STAT1 is more greatly phosphorylated by FIST than by equimolar concentrations of controls (Fig. 1G). With regard to the activation levels of other signaling pathways downstream of IL-2R such as MAPK and PI3K/AKT pathways, no differences were observed between FIST and equimolar concentrations of controls (data not shown). FIST acts through the IL-2R, as blockade of IL-2R on CTLL-2 cells inhibits STAT5 phosphorylation (Supplemental Fig. 1A). Lymphocytes from STAT1 KO mice were used to investigate whether FIST induces SMAD7 expression via STAT1 activation. As a result, SMAD7 expression was significantly abrogated compared with lymphocytes from immunocompetent mice, yet we observed a preserved inhibition of SMAD2 phosphorylation compared with the controls (Fig. 1H). Based on the cross-reactivity between human and mouse IL-2 for its specific receptor, we tested the ability of mouse FIST to induce IFN-γ production by human immune cells, as well as its ability to antagonize TGF-β canonical pathway in human NK cells. FIST-stimulated human immune cells also produce significantly higher amounts of IFN-γ than that induced by equimolar concentrations of cytokine control. However, the levels of IFN-γ induced by FIST in human cells were lower than that produced by mouse immune cells (Supplemental Fig. 1B). Similar results were obtained with regard to Smad2 inhibition by mouse FIST-mediated Smad7 expression in human NK cells.
FIST desensitizes immune cells to TGF-β-mediated suppression

To test whether FIST can override TGF-β-mediated suppression of lymphocyte activation, lymphocytes were stimulated with 5 pmol FIST or control. FIST on its own primes $5 \times 10^6$ unfractionated splenocytes to produce a significant increase (>35%) of IFN-γ compared with equimolar concentration of IL-2 in 96 h. In contrast to IL-2 alone, this effect was not suppressed in the presence of 0.5 ng/ml of active TGF-β1 (Fig. 2A). In addition, we observed that FIST induces c-Myc upregulation, another gene suppressed by active TGF-β (Supplemental Fig. 2A, 2B). In an attempt to define the immune cell type responsive to FIST, $1 \times 10^6$ NK cells, T cells, or B cells were cultured with 5 pmol FIST or equimolar concentration of controls for 72 h, and it was determined by ELISA that FIST-stimulated T, B, and NK cells produce significantly higher amounts (>90% increase) of IFN-γ, TNF-α, and GM-CSF than those of controls (Fig. 2B–F).

**FIGURE 2.** FIST induces significant Th1 cytokine production by activated immune cells. A, Quantification by ELISA of IFN-γ produced by $5 \times 10^6$ unfractionated splenocytes from immunocompetent C57BL/6 mice cultured with 5 pmol FIST or controls for 96 h ($n = 4$; data are shown as mean ± SD). T, B, and NK cells were cultured with 5 pmol FIST or controls for 72 h, and the supernatants were collected and used to measure the concentration of IFN-γ, TNF-α, and GM-CSF by ELISA. B, IFN-γ produced by $1 \times 10^6$ FIST- or control-stimulated T cells ($n = 3$; data are shown as mean ± SD). C and D, TNF-α and IFN-γ produced by $1 \times 10^6$ FIST- or control-stimulated B cells ($n = 3$; data are shown as mean ± SD). E and F, GM-CSF and IFN-γ produced by $1 \times 10^6$ FIST- or control-stimulated NK cells ($n = 3$; data are shown as mean ± SD). For each image, the results are representative of three independent experiments performed in triplicate ($n = 3$) or quadruplicate ($n = 4$). See also Supplemental Fig. 2. *p < 0.05, **p < 0.005.
that of the control group implanted with $5 \times 10^5$ null B16 cells. In contrast, FIST-mediated bystander effect protected 60% of the injected mice with mixed cells (Fig. 3A). Although mice implanted with B16 cells secreting sTβRII (B16 sTβRII) show a significant delay of tumor growth, B16 sTβRII tumors grew exponentially over time, and after 80–100 d, mice were sacrificed because of large tumors (data not shown). After 80 d, mice previously implanted with B16 FIST or B16 IL-2 cells were challenged with $1 \times 10^6$ null B16 cells in the contralateral flank, and the percentage of survival was monitored over time. Fifty percent of mice previously treated with FIST rejected null B16 cells in contrast to the failure of IL-2 in conferring protective immunity (Fig. 3B). Similarly, immunocompetent mice implanted orthotopically with PANC02 cells in their pancreas expressing low concentrations of FIST do not develop tumors, whereas mice implanted with PANC02 cells expressing low concentrations of controls developed large tumors and were sacrificed (Fig. 3C). FIST-treated mice showed normal body weight and behavior and had normal blood counts (Supplemental Table I).

To assess the ability of FIST to promote tumor regression, mice with pre-established B16 tumors were s.c. injected with $1 \times 10^6$ irradiated (50 Gy) B16 FIST cells in the contralateral flank, and tumor volume was measured over time. As result, FIST-treated mice displayed a significantly lower rate of tumor growth compared with that of the control (nontreated group) and transient tumor regression. However, tumor regression was not complete, and the percentage of survival between the groups was not significant (data not shown).

FIST induces significant recruitment of lymphocytes to the tumor site

FIST induces significantly greater recruitment (>50% increase) of host-derived lymphocytes to Matrigel plugs (tumor site) than equimolar concentration of controls after 7 d of tumor implantation. Specifically, we observed a significant increase of NK (≥56%), NKT (≥70%), B (≥80%), and CD8⁺ T (≥85%) cells recruited in the tumor site (Fig. 4A, 4B).

**FIGURE 3.** FIST induces a potent antitumor response and immune bystander effect. A. $5 \times 10^5$ null or genetically modified B16 cells expressing FIST (B16 FIST cells) or controls were injected s.c. in C57BL/6 immunocompetent mice, and the percentage of survival was monitored over time. B. Mice previously implanted with B16 FIST or B16 IL-2 cells were challenged with $1 \times 10^6$ null B16 cells, and the percentage of survival was monitored over time. C. $5 \times 10^5$ null or genetically modified PANC02 cells expressing FIST (PANC02 FIST cells) or controls were orthotopically implanted in C57BL/6 immunocompetent mice, and the percentage of survival was monitored over time. The results displayed in A–C are representative of two independent experiments (n = 10 per group; data are shown as mean ± SD). See also Supplemental Table I. *p < 0.05, **p < 0.005.
To determine the main immune cell type implicated in FIST-dependent antitumor effect in immunodeficient mice, we injected s.c. into an array of immune defective mice, including CD8 KO, CD4 KO, B cell-deficient (μMT), NK-defective beige mice, NOD-SCID mice, and Rag2/γc KO mice, and survival was monitored over time. Surprisingly, B16 FIST cells do not form tumors in CD4 KO, CD8 KO, B cell-deficient (μMT), and NK-defective beige mice (Fig. 5A–D). However, 80% of NOD-SCID mice implanted with B16 FIST cells developed tumors and were sacrificed with a significant delay of 2 mo compared with the control, whereas Rag2/γc KO mice developed tumors at a rate indistinguishable from that of the control group (Fig. 5E, 5F). Based on the fact that NK cells from beige mice display a severe defect of natural killing capability but preserve an intact NK cytokine production (35), whereas NOD-SCID mice display defects in both NK cell number and function (36), it is suggested that FIST antitumor effect can be mediated by an NK cell-derived soluble factor. Notably, tumors expressing FIST implanted in NOD-SCID mice display very necrotic appearing, suggesting a defect in angiogenesis as well (data not shown). We investigated the contribution of IFN-γ in vivo because FIST-stimulated NK cells secrete substantial amounts of IFN-γ. However, B16 FIST cells also fail to form tumors in IFN-γ KO mice (Fig. 5G) as well as in IFN-γR KO mice (data not shown). The analysis in vitro of the expression profile of angiogenesis-related proteins secreted by FIST-stimulated NK cells reveals the presence of high levels of the angiostatic chemokines CXCL10 (IFN-γ inducible protein, also known as IP-10) and CXCL9 (monokine inducible by IFN-γ, also known as MIG), and absence of VEGF expression or other genes required for VEGF response such as angiopoietin-2, tissue inhibitor of matrix metalloproteinase-1, and basic fibroblast growth factor (data not shown). We measured by ELISA the concentration of CXCL10 in the secretome of NK cells stimulated by FIST or controls from immunocompetent, beige, IFN-γ KO, and NOD-SCID mice. FIST-stimulated NK cells from beige mice secrete similar CXCL10 amounts as immunocompetent mice, whereas FIST-stimulated NK cells from IFN-γ KO, although displaying a dramatic decrease of CXCL10 production, still have significantly higher CXCL10 production than that of NOD-SCID mice (Fig. 5H). In addition, FIST-stimulated NK cells from immunocompe-
and consequently upregulates the expression of STAT1 target genes essential for an effective Th1 cell-mediated cell immunity such as T-bet, SMAD7, and CXCL10. In turn, T-bet upregulates the expression of its target genes: CXCR3 and IL-2Rβ. Through SMAD7 expression, FIST inhibits active TGF-β, which exerts dramatic suppression of proinflammatory cytokine-mediated IFN-γ production indirectly by downregulating T-bet expression via SMAD-dependent mechanism and directly by T-bet–independent negative regulatory effect on the IFNγ promoter (43). IFN-γ may amplify FIST-dependent activation by binding to IFN-γR and creating a positive autocrine loop that enhances the expression of STAT1 target genes (Fig. 8).

Discussion

Upon IL-2 binding, the IL-2R activates two tyrosine kinases, JAK1 and JAK3, which interact with the cytoplasmic domains of the IL-2Rβ and γc subunits (38). Activated IL-2R becomes phosphorylated on specific tyrosine residues that serve as docking sites for proteins containing Src-homology 2 or phosphotyrosine binding domains including Shc adapter and STAT proteins (STAT1, STAT3, and STAT5). IL-2R recruits STAT proteins to different subdomains of the IL-2Rβ-chain. STAT5 associates with phosphorylated Tyr-510 of the IL-2Rβ C-terminal region. In contrast, STAT1 and STAT3 interaction with the IL-2Rβ-chain occurs through its acidic subdomain and may not require either phosphorylation of the receptor or even the presence of tyrosine residues of IL-2Rβ (39, 38). In addition, IL-2–mediated STATs activation is concentration sensitive, as low IL-2 concentration is sufficient to induce STAT5 activation, whereas STAT1 activation requires high IL-2 concentrations (39, 40).

Our new bifunctional pharmaceutical (FIST) is endowed with the ability to signal through the IL-2R inducing similar STAT5 activation and distinctive STAT3 and STAT1 activation compared with equimolar concentrations of IL-2 or IL-2 combined with sTβRII. Specifically, FIST induces a partial hyperagonist response in IL-2R–expressing cells characterized by a potent activation of STAT1 combined with contemporaneous inhibition of TGF-β signaling pathway through transcriptional induction of the inhibitory SMAD7. Based on previous studies, JAK1/STAT1 activation acts as a positive regulator of SMAD7 expression (41). STAT1 deletion completely abrogated FIST-dependent SMAD7 overexpression, but not the inhibition of SMAD2 phosphorylation, which indicates...
that the TβRII ectodomain moiety of FIST effectively functions as a decoy receptor trapping active TGF-β in solution. SMAD7 directly inhibits TGF-β signaling in the nucleus by interacting with transcriptional repressors or disrupting the formation of the TGF-β–induced functional Smad–DNA complexes (42). SMAD7 also recruits HECT type of E3 ubiquitin ligases, Smurf1 and Smurf2, to the activated TβRI leading to the degradation of the receptor through the proteasomal pathway (11).

TGF-β is also produced by immune cells including activated T and B cells, macrophages, dendritic cells, neutrophils, and NK cells, and its expression regulates the differentiation, proliferation, and activation state of these immune cells. In particular, resting NK cells have a constitutively active TGF-β–TGF-βR autocrine loop in the absence of proinflammatory immune stimulation (43–46).

In a STAT1-dependent mechanism, FIST acts intracellularly to inhibit TGF-β signaling pathway via SMAD7 expression. This property characterizes FIST’s mechanism of action and is responsible for the superiority of FIST as proinflammatory compound versus IL-2 or IL-2 combined with sTβRII. In contrast, total lymphocyte or purified immune cells stimulated by IL-2 plus sTβRII display similar activation as IL-2–stimulated cells indicating that although sTβRII traps active TGF-β secreted by immune cells, it does not operate intracellularly and does not enhance the activation status of STAT proteins downstream of IL-2R. Consequently, FIST-stimulated lymphocytes in vitro become

**FIGURE 6.** FIST inhibits angiogenesis in vivo and in vitro. A, Matrigel plugs sections stained with DAPI and red fluorescence conjugated CD31-specific Ab to detect blood vessel formation in Matrigel plugs removed from C57BL/6 immunocompetent mice previously injected with $5 \times 10^5$ B16 FIST or control cells embedded in Matrigel (original magnification $\times 40$). B, EC proliferation in the presence of CM by FIST- or controls-stimulated NK cells from immunocompetent mice. Cell number was quantified by running a luminescence versus cell dose curve ($n = 3$; data are shown as the mean ± SD). C, In vitro EC migration in response to the CM of FIST- or controls-stimulated NK cells from immunocompetent mice. Migrated cell number was quantified by running a fluorescence versus cell dose curve ($n = 3$; data are shown as the mean ± SD). D, In vitro angiogenesis assay of $5 \times 10^5$ EC cultured in the presence of the CM of FIST- or controls-stimulated NK cells from immunocompetent mice ($n = 3$; data are shown as the mean ± SD). E, Comparison of angiogenesis score between $5 \times 10^5$ wild-type and CXCR3 KO EC cultured in the presence of the CM of FIST- or control-stimulated NK cells from immunocompetent and STAT1 KO mice ($n = 3$; data are shown as the mean ± SD). F, Percentage of survival over time of CXCR3 KO mice s.c. injected with $5 \times 10^5$ B16 FIST or null cells ($n = 5$ per group; data are shown as the mean ± SD). *p < 0.05, **p < 0.005.
resistant to TGF-β–mediated suppression and produce significantly greater amounts of proinflammatory cytokines. However, IL-2–dependent cells (CTLL-2) cultured with FIST display a similar proliferation rate as CTLL-2 stimulated with equimolar concentrations of IL-2 or IL-2 combined with sTβRII. This confirms the observation that FIST-mediated STAT5 and PI3K activation levels are similar to the one induced by equimolar concentration of IL-2, as these transcription factors are implicated in T cell proliferation and survival (47, 48). FIST-dependent SMAD7 overexpression was only detected in the presence of active TGF-β, which can be explained by the reported SMAD binding elements in the SMAD7 promoter required for TGF-β–dependent transcriptional activation (49).

FIST-mediated hyperagonist signal transduction downstream of the IL-2R coupled to TGF-β blockade leads to resistance to TGF-β–mediated suppression and to a potent antitumor effect in vivo. Consequently, melanoma B16 FIST and PANC02 FIST cells fail to form tumors in immunocompetent mice. However, FIST secreted by irradiated B16 cells failed to induce complete tumor regression in mice with pre-established B16 tumors. The ineffectiveness of this whole-cell vaccine strategy may be due to rapid immune clearance of irradiated B16 FIST cells before they could secrete systemically effective amounts of FIST.

Because it is not possible to modify all preexisting tumor cells with suicide or proinflammatory cytokine genes in situ by any contemporary gene transfer technology, an important feature to consider for cancer immunotherapy is the bystander effect (50). The secretion of FIST by genetically modified B16 cells promotes a bystander anticancer effect that protected 60% of mice bearing non-genetically modified cancer cells from tumor development.

**FIGURE 7.** FIST mediates upregulation of STAT1 target genes. A and B, CXCL10 production over time by $1 \times 10^6$ FIST- or control-stimulated NK cells from immunocompetent and STAT1 KO mice, respectively. C and D, T-bet expression over time by $1 \times 10^6$ FIST- or controls-stimulated NK cells from immunocompetent or STAT1 KO mice, respectively. T-bet expression over time was determined by intracellular staining, and the mean fluorescence intensity (MFI) values were measured by flow cytometry. E and F, IFN-γ production over time by $1 \times 10^6$ FIST- or controls-stimulated NK cells from immunocompetent and STAT1 KO mice, respectively. G and H, Cell surface CXCR3 expression over time by $1 \times 10^6$ FIST- or controls-stimulated NK cells from immunocompetent and STAT1 KO mice, respectively. I and J, Cell surface IL-2Rβ expression over time by $1 \times 10^6$ FIST- or controls-stimulated NK cells from immunocompetent and STAT1 KO mice, respectively. For all the experiments, $n = 3$ per group; data are shown as the mean ± SD.
Unfortunately, we could not quantify tumor angiogenesis based on CD31 expression as readout of FIST-mediated bystander effect because of the very necrotic consistency of the tumors containing B16 FIST cells. Although FIST promoted significant recruitment of IL-2R-expressing immune cells such as NK, NKT, CD8+ T cells and B cells to the tumor site, FIST still prevents tumor formation in CD4 KO, CD8 KO, and B cell-deficient mice, which indicates that these immune cell types, although they may contribute, are not essential mediators of FIST antitumor activity. In contrast, B16 FIST cells were tumorigenic in NOD-SCID and Rag2/γc KO mice, which implicates by exclusion NK cells as the major immune mediator of FIST effects. One way to precisely evaluate the role of NK cells in this model would be to perform the NK cell depletion with anti-asialo GM1 Ab.

The necrotic appearance of tumors secreting FIST from NOD-SCID mice also suggests a profound defect of tumor-driven angiogenesis. FIST not only antagonizes TGF-β, an essential regulator of both phases of angiogenesis (16), but also stimulates virtually all lymphoid subsets, including NK cells, to secrete substantial amounts of IFN-γ, which may act as an angiostatic by suppressing the expression of genes required for VEGF response via STAT1 activation (51). However, B16 cells expressing FIST fail to form tumors in IFN-γ- and IFN-γ-R KO mice, suggesting that additional effector molecules are at play. FIST-stimulated NK cells produced significant amounts of CXCL10 and CXCL9. CXCL10 and CXCL9 as well as CXCL11 are chemokines induced by IFN-γ via STAT1 activation and are CXCR3 ligands that share potent angiostatic and chemotactic properties (52–54). In particular, high level of CXCL10 in the tumor is correlated with high amounts of tumor-infiltrating NK cells and better survival (55). FIST-stimulated NK cells from IFN-γ KO mice display a significant decrease of CXCL10 production, which supports the role of IFN-γ as the main inducer of CXCL10 expression. Notably, FIST-stimulated NK cells from IFN-γ KO mice still produce elevated levels of CXCL10 indicating that upon binding to IL-2R, FIST directly induces CXCL10 production via STAT1 hyper-activation. As expected, CXCL10 secretion was significantly reduced in FIST-stimulated NK cells from NOD-SCID mice, which display severe defect in NK cell functions, and was completely abrogated in FIST-stimulated NK cells from STAT1 KO mice. The specific angiostatic effect of FIST on tumor-derived angiogenesis is likely due to the transient CXCR3 expression restricted to newly formed blood vessels in vivo and in vitro (56). In concordance with previous studies, we found that CXCL10 behaves as an inhibitor of EC migration and differentiation into branching networks of tubular structures in vitro, a process that requires the interaction of EC with extracellular matrix components (53, 57, 58). However, the ability of CXCL10 to inhibit EC proliferation is controversial (53, 59). Despite this potent angiostatic effect observed in vitro and in vivo in immunocompetent mice, B16 FIST cells fail to form tumors in CXCR3 KO mice, which indicates that FIST-dependent CXCL10 induction is not essentially required for the FIST-mediated antitumor effect observed in these mice. Therefore, we speculate that other FIST-dependent mechanisms that prevent tumor formation are at play in CXCR3 KO mice such as the induction of a potent type 1 Th cell-mediated antitumor immunity triggered by STAT1 target genes or the up-regulation of other NK cell effector functions such as NK cell cytotoxicity against tumor cells. However, we cannot discard the possibility that several FIST-dependent mechanisms that rely on STAT1 activation may simultaneously operate to prevent cancer progression in immunocompetent mice.

Consequently, FIST-treated mice develop a robust anticancer adaptive immunity that protects 50% of mice from high burden tumor challenge. Notably, FIST-stimulated NK cells from STAT1 KO mice still produce significant higher amounts of IFN-γ than equimolar concentrations of controls. This suggests that the contemporaneous blockade of active TGF-β combined with an immune stimulation significantly enhances the activation level of NK cells, which constitutively produce active TGF-β1 as an autocrine/negative regulator of IFN-γ (43).

By targeting several immune cell types expressing the IL-2R, inhibiting active TGF-β, and upregulating the expression of STAT1 target genes, FIST conveys the host’s angiogenic and immune response against cancer cells. Therefore, cancer cells expressing FIST only form tumors in NOD-SCID and Rag2/γc double-KO mice, which lack most of these immune system components. Although other TGF-β antagonists like Fc:TBRII may neutralize large amounts of active TGF-β (60), Fc:TBRII treatment does not alter primary tumor growth of transplantable models of breast cancer metastases, which suggests that the antimitastatic effects of Fc:TBRII in vivo are independent of tumor cell proliferation. In addition, inhibition of tumor angiogenesis in vivo by Fc:TBRII treatment is controversial. Muraoka et al. (3) observed no reduction of vascular density in endogenously arising tumors.

FIST treatment could be delivered in humans systemically as recombinant protein, as low doses of the fusion (0.002 pmol) are effective in the treatment of carcinoma.

Alternatively, FIST could be delivered by vehicle cells such as genetically modified stem cells or autologous immune cells. In conclusion, FIST is a novel biopharmaceutical characterized by inhibiting TGF-β canonical pathway simultaneously with a distinctive STAT1 hyperactivation via IL-2R on immune cells, which conveys a robust upregulation of STAT1 target genes including key factors essential for an effective antitumor response. This first-in-class biological agent may represent a new paradigm in cancer immunotherapy contemporaneously modulating pro- and anti-inflammatory immune checkpoints operative on immune effector cells.
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C.P. and J.G. are named coinventors on a provisional U.S. patent for FIST technology.

References


