Combination Therapy with AG-490 and Interleukin 12 Achieves Greater Antitumor Effects than Either Agent Alone


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Abstract
Constitutive activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) occurs at very high frequency in various hematopoietic malignancies and solid tumors. It has been demonstrated that the tyrosine kinase inhibitor, AG-490, selectively blocks JAK activity and completely eliminates leukemia cells in a severe combined immunodeficient (SCID) mouse model. Because many cytokines, including interleukin (IL)-12, have been shown to signal through JAK/STAT pathways, AG-490 may inhibit cytokine-based cancer therapy. In this study, we evaluated the effects of AG-490 on IL-12 functional signaling and IL-12-mediated antitumor response in vivo. Previous studies have established the critical roles of macrophages and IFN-γ in mediating IL-12-induced antitumor effects. Our results show that in vivo administration of AG-490 causes tumor cell apoptosis but does not inhibit IL-12-mediated macrophage activation and IFN-γ production by lymphocytes. Furthermore, our data indicate that combined therapy with AG-490 and IL-12 induces greater antitumor effects than either agent alone in a murine myeloma tumor model. These results suggest that JAK/STAT inhibitors deserve further investigation for use with IL-12 therapy in treating human cancers with elevated JAK/STAT activity.

Introduction
Constitutive activation of JAKs and STATs has been identified in increasing numbers of human cancers, including various leukemias, lymphomas, multiple myeloma, head and neck squamous cell carcinoma, and breast cancer (1–12). Recently, genetic evidence for the oncogenic potential of one STAT family member, Stat3, has been presented (13). In the case of myeloma, IL-6 is the major survival factor for myeloma cells and signals through JAK and STAT proteins (14). One STAT family member, Stat3, is constitutively activated in bone marrow tumor cells from patients with multiple myeloma and in the IL-6-dependent human myeloma cell line U266 (6). It was shown that constitutive activation of Stat3 in U266 cells induced overexpression of the antiapoptotic protein Bcl-XL and conferred protection from Fas-mediated apoptosis (6). Blocking JAK/STAT signaling in U266 cells by a JAK-specific inhibitor, AG-490, or by the enforced expression of a dominant-negative Stat3 protein induced apoptosis and sensitized these cells to Fas-mediated apoptosis (6). Similarly, blocking JAK activity with AG-490 led to the inhibition of Stat3 signaling and growth arrest/apoptosis of breast carcinoma cells (15). These results raise the possibility that the JAK-Stat3 signaling pathway can be a target for the treatment of several types of cancers.

As a tyrosine kinase inhibitor, AG-490 is selective for the JAK family kinases, whereas other lymphocytic tyrosine kinases, including Lck, Lyn, Btk, Syk, and Src, are not targets (16, 17). A previous study (16) demonstrated that systemic administration of AG-490 in SCID mice with disseminated human leukemic cells that depended on Jak2 for survival caused tumor cell apoptosis, leading to complete tumor regression. However, the antitumor efficacy of AG-490 remains to be fully evaluated because apoptosis-based therapy frequently results in only transient tumor growth suppression. In contrast to apoptosis-based therapy, immunotherapy often has little effect on established tumors but can induce long-term antitumor immunity when tumor volume is greatly reduced. IL-12 has been shown to be one of the most effective cytokines for cancer therapy (18). In addition to inducing antitumor immune responses, IL-12 is also known to inhibit tumor growth by suppressing tumor angiogenesis (19). These findings collectively suggest that combining inhibitors of JAK/STAT signaling like AG-490 with IL-12 cytokine therapy may have more potent antitumor activity than either treatment alone. However, IL-12 has been shown to signal through JAK kinases and STAT transcription factors (20–23). It is important, therefore, to investigate whether the JAK inhibitor interferes with IL-12 functional signaling and its ability to induce antitumor responses in vivo.

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3 The abbreviations used are: JAK, Janus kinase; STAT, signal transducer(s) and activator(s) of transcription; IL, interleukin; SCID, severe combined immunodeficient; EMSA, electrophoretic mobility shift assay; rIL, recombinant IL; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling; hSIE, high-affinity mutant of the sis-inducible element; pTyr, phosphotyrosine; NK, natural killer.

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It has been established that IL-12 induces lymphocytes to produce IFN-γ, which is critical for the antitumor immunity of IL-12 as well as its antiangiogenesis effects (19, 24). In addition to IFN-γ, macrophages also have a critical role in IL-12-mediated antitumor effects (25). In this study, we investigated the effects of AG-490 on the survival of murine myeloma cells, as well as the effects of AG-490 on IL-12-induced lymphocyte IFN-γ production and macrophage activation/cytotoxicity. We also evaluated whether interrupting JAK/STAT signaling by AG-490 would inhibit the antitumor effects of IL-12 in vivo. Our findings demonstrate that AG-490 suppresses Stat3 DNA-binding activity and induces apoptosis of the murine myeloma cells in vitro and in vivo. In addition, in vivo systemic AG-490 treatment does not inhibit the IL-12-activated cytotoxicity of peritoneal macrophages, nor does it reduce IL-12-induced IFN-γ production by lymphocytes. Furthermore, although tumor regression induced by AG-490 is transient and IL-12 treatment alone has only slight tumor suppressive activity, combinational treatment with AG-490 and IL-12 significantly prolongs tumor regression.

Materials and Methods

Cell Lines. The murine myeloma (plasmacytoma) cell lines MPC11, MOPC, S194, and J558 were obtained from American Type Culture Collection and the murine sarcoma cell line MethA was kindly provided by Dr. Ning-Sun Yang of the California Institute of Technology (Pasadena, CA). All of the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin.

Nuclear Extracts and EMSA. Tumor cells were treated with 50 μM AG-490 as described previously (16) before the isolation of nuclei. Nuclear extract preparation and EMSA were performed essentially as described previously (6, 26).

In Vitro Apoptosis Assay. After a 24-h incubation with DMEM containing 0, 25, or 50 μM AG-490, cells were stained with phycoerythrin (PE)-Annexin V (PharMingen, San Diego, CA). Dual-color fluorescence was measured on a FACScan flow cytometer and analyzed using CellQuest software (Becton Dickinson, Mountain View, CA).

Splenic Interferon-γ Production. Mice were treated daily with i.p. injections of 100 μl of AG-490 (0.5 mg) or DMSO vehicle (50%) for a total of 4 days. During the last 2 days of AG-490 or DMSO treatment, a daily i.p. injection of 400 ng of rIL-12 (kindly provided by Genetics Institute, Cambridge, MA) was also given i.p. simultaneously with either AG-490 or DMSO. Two days after the last treatment with AG-490, single-cell suspensions of splenocytes were prepared from individual mice. The splenocytes were cultured in medium supplemented with 2.5 μg/ml ConA and 100 units/ml rIL-2 to stimulate IFN-γ production (27, 28). IFN-γ ELISA (Genzyme, Cambridge, MA) was performed as described previously (29).

Peritoneal Macrophage Preparation and Cytostatic Assay. Peritoneal cells were prepared from the same mice treated with either AG-490/rIL-12 or DMSO/rIL-12 as described above. The peritoneal macrophage population was enriched by adhesion on plastic plates followed by washing and aspiration of nonadherent cells. On the basis of morphological criteria using Giemsa staining and by CD11b (Mac-1) antibody staining, greater than 95% of the remaining cells were macrophages. Antitumor cytostatic activity of macrophages was determined by the inhibition of DNA synthesis of target tumor cells (J558 myeloma cells). Briefly, macrophage-sensitive J558 cells (2 × 10⁵/well) were cocultured for 48 h with and without macrophages (2 × 10⁵/well) prepared from individual mice. To estimate DNA synthesis, the cells were pulsed with [³H]thymidine (0.25 μCi/well) during the last 6 h of incubation. [³H]Thymidine incorporation was determined using a liquid scintillation β-counter. Results are expressed as percentage inhibition of [³H]thymidine incorporation by J558 cells incubated with macrophages compared with [³H]thymidine incorporation by J558 cells incubated in medium alone.

Mice and Tumor Formation in Vivo. Six-to-8-week-old female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD) and housed in the accredited animal facility at H. Lee Moffitt Cancer Center and Research Institute. Cohorts of 3–5 mice per group were used for these experiments. Mice were shaved on the right flank and were given injections s.c. with 5 × 10⁵ of either MOPC or MPC11 cells in 100 μl of PBS to induce tumors.

In Vivo Treatment with AG-490 and IL-12. When tumors reached ~5 mm in diameter, AG-490 treatment of tumors was initiated. For MOPC tumors, injections of 0.85 mg/day of AG-490 were given peritumorally, supplemented with 0.5 mg/day AG-490 i.p. for 10 days. For MPC11 tumors, the peritumoral dose was halved, whereas the i.p. dose remained the same (5 days). Control mice received 50% DMSO vehicle alone in the same volume as the AG-490 treatment group. rIL-12 was given peritumorally at indicated concentrations every other day. Tumor growth was monitored daily by measuring two perpendicular tumor diameters with a caliper, and tumor volume was calculated according to the formula \( V = 0.52 \times a \times b \times (a+b)/2 \), where \( a \) is smallest superficial diameter, and \( b \) is largest superficial diameter.

TUNEL Assay. MOPC tumors that received either AG-490 or 50% DMSO treatment were used for this assay. Three-μm sections from paraffinized tissues were dewaxed and rehydrated according to standard protocols. After incubation with proteinase K (30 min at 21°C), the TUNEL reaction mixture (Boehringer Mannheim, Indianapolis, IN) was added to rinsed slides, which were incubated in a humidified chamber for 60 s at 37°C. This was followed by incubating with Conferter-AP (50 μl) and substrate solution (50 μl). The reaction was visualized by light microscopy.

Immunohistochemical Detection of Phospho-Stat3 in MPC11 Tumor Sections. AG-490 and vehicle-treated tumors (treatment schedule and dose are as described above) were fixed in 10% neutral-buffered formalin and embedded in serial 3- to 4-mm paraffin blocks. Consecutive sections were cut 5 μm thick, and one of them was stained with H&E for histological identification of the tumor. Tumor tissue sections were also immunostained using a phospho-Tyr705-Stat3 antibody (Cell Signaling, Beverly, MA) to localize activated Stat3. As a negative control, rabbit immunoglobulins

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to a lesser extent, S194 cells. The murine MethA sarcoma cells contain no detectable STAT activation, whereas J558 cells contain low levels of activated Stat1 (data not shown).

The JAK-specific Inhibitor, AG-490, Blocks Stat3 DNA-binding and Induces Apoptosis in Murine Myeloma Cells in Vivo. Stat3 activation in human multiple myeloma and breast cancer cell lines was shown to be dependent on JAK family kinases (6, 15). We evaluated whether constitutive activation of Stat3 in the mouse myeloma cell lines also required the activity of JAK family kinases by using the tyrophostin compound, AG-490, a selective inhibitor of JAK family kinases (16). Stat3 DNA-binding activity was greatly inhibited by AG-490 treatment within 24 h in all of the three myeloma cell lines (Fig. 1). These results suggest that constitutive activation of Stat3 in MOPC, MPC11, and S194 cells is mediated predominantly through JAK family kinases.

We next examined whether inhibition of Stat3 activation could cause apoptosis of the murine myeloma cells in vitro. After 24-h exposure to AG-490, the MOPC, S194, MPC11, J558, and MethA cell lines were examined for apoptosis by Annexin V–PE staining, followed by flow cytometric analysis. AG-490 treatment of myeloma cell lines displaying constitutively activated Stat3 resulted in a dramatic dose-dependent increase in the levels of Annexin V–positive cells, which indicated significantly higher numbers of apoptotic cells (Table 1). In contrast, both J558 myeloma and MethA sarcoma cells, which do not display activated Stat3, exhibited relatively small increases in apoptosis (Table 1).

AG-490 Treatment Results in Inhibition of Stat3 Activity, Tumor Growth, and Induction of Tumor Cell Apoptosis in Vivo. Mice with 5-mm preexisting murine myeloma tumors were treated with either AG-490 or DMSO vehicle as described in “Materials and Methods.” Tumor sections were prepared from regressing tumors, followed by immunohistochemical analysis with a pTyr-Stat3-specific antibody, which detects only activated Stat3 phosphorylated on Tyr-705. As shown in Fig. 2A, although tumor sections from vehicle-treated mice were strongly positive for pTyr-Stat3, AG-490-treated tumor cells were negative for pTyr-Stat3. At the same time, AG-490 treatment also caused death of the tumor cells. Interestingly, several pTyr-Stat3-negative tumor cells were still viable in the AG-490-treated specimen, which suggested that the inhibition of Stat3 activity in the myeloma cells in vivo precedes tumor cell death. All of the AG-490-treated tumors completely regressed within 3–8 days (Fig. 2B). However, tumor regrowth was observed in all of the AG-490 treated mice except for one, which developed metastatic foci 2 months after the treatment (data not shown). To investigate whether AG-490-induced tumor growth inhibition was associated with tumor cell apoptosis, regressing MOPC tumors were examined by TUNEL assay to detect apoptotic tumor cells. As shown in Fig. 2C, although control tumor specimens had no clearly apoptotic cells (Fig. 2C, top panel), tumor sections prepared from AG-490-treated mice contained high numbers of apoptotic cells (Fig. 2C, lower panel).

**Results**

Stat3 Is Constitutively Activated in Murine Myeloma Cell Lines. Nuclear extracts prepared from murine tumor cell lines were examined by EMSA for DNA-binding to a hSIE oligonucleotide probe specific for activated Stat1 and Stat3 (Fig. 1). To confirm the identity of STAT family members activated in the murine tumor cell lines, the human myeloma cell line U266, shown previously to contain constitutively activated Stat3 (6), was used as a positive control in the EMSA. Additional confirmation of Stat3 activation in MOPC, MPC11, and S194 murine myeloma (plasmacytoma) cell lines was provided by Stat3 antibody supershift analysis (data not shown). Results from these experiments indicated that Stat3 is constitutively activated in MOPC, MPC11, and,

![Fig. 1. Stat3 is activated in murine myeloma/plasmacytoma cell lines, and AG-490 treatment inhibits Stat3 DNA-binding activity. Nuclear extracts prepared from the indicated mouse myeloma cell lines and human U266 myeloma cells were incubated with the 32P-labeled hSIE oligonucleotide probe and analyzed by EMSA to detect activated Stat3. EMSA analysis of nuclear extracts prepared from mouse myeloma cells after 24-h incubation with 50 μM of AG-490 was also performed. ST3/3, activated Stat3 homodimers bound to hSIE probe.](Image)
treatment for better antitumor effect, we analyzed the effect of AG-490 on IL-12-activated immune responses. In vivo treatment of mice with IL-12 has been shown to induce NK and T cells to produce IFN-γ, which is required for IL-12-mediated antitumor effects (24, 29, 30). We, therefore, examined whether AG-490 administration could affect rIL-12-induced IFN-γ production by splenocytes ex vivo. For these experiments, AG-490 was administered simultaneously with rIL-12 at the same site. As shown in Table 2, although a slight suppression of IL-2-induced IFN-γ production by splenocytes from AG-490-treated mice was observed in all of the experiments, the increase in IFN-γ production by splenocytes as a result of rIL-12 administration was the same in both the AG-490- and the DMSO-treated animals.

It has been demonstrated that macrophages are effector cells that mediate IL-12-induced antitumor immune responses (25). We, therefore, examined the effect of in vivo treatment with AG-490 on the ability of peritoneal macro-

### Table 1  AG-490 induces apoptosis in murine myeloma cell lines harboring activated Stat3

Data shown are the percentage of Annexin V-positive apoptotic cells as determined by flow cytometry and are average ± SD of at least three experiments for each cell line.

<table>
<thead>
<tr>
<th>AG-490 (μM)</th>
<th>MOPC</th>
<th>MPC11</th>
<th>S194</th>
<th>J558</th>
<th>MethA</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>77.0 ± 1.5</td>
<td>79.3 ± 14.7</td>
<td>46.5 ± 4.6</td>
<td>20.5 ± 1.5</td>
<td>28.2 ± 1.7*</td>
</tr>
<tr>
<td>25</td>
<td>39.8 ± 12.7</td>
<td>45.1 ± 10.2</td>
<td>25.3 ± 4.6</td>
<td>11.1 ± 1.0*</td>
<td>24.2 ± 1.6*</td>
</tr>
<tr>
<td>0</td>
<td>17.2 ± 4.0</td>
<td>14.2 ± 3.2</td>
<td>12.6 ± 3.4</td>
<td>9.7 ± 0.4</td>
<td>22.6 ± 3.6</td>
</tr>
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* Statistical difference from the control is not significant in the Stat3-negative J558 and MethA cells (P > 0.05).

Fig. 2. Administration of AG-490 inhibits activated Stat3, causes transient regression of murine myeloma/plasmacytoma tumors, and induces apoptosis of tumor cells in vivo. A, AG-490 treatment of MPC11 tumors results in a dramatic reduction of activated Stat3 as determined by immunohistochemical analysis with a pTyr-Stat3-specific antibody. Top panels, control vehicle-treated tumor sections, ×10 and ×40, respectively. Lower panels, AG-490-treated tumor sections (×10 and ×40, respectively). The pY-Stat3 positive cells in AG-490 treated tumor sections are endothelial cells. B, results of three independent experiments are expressed as the means of tumor volumes ± SD. AG-490-treated mice, n = 13; DMSO-treated mice, n = 10. Last day of treatment was day 10 and tumor regrowth and/or metastasis occurred in all of the AG-490-treated mice after 2 months. C, TUNEL assays of tissue sections prepared from MOPC tumors after treatment with either DMSO vehicle (top panel) or AG-490 (lower panel).
independent experiments were performed with similar results. Using syngeneic murine tumor models, we have shown that the inhibition of JAK/STAT signaling pathways by a specific JAK inhibitor, the tyrphostin AG-490, resulted in apoptosis of tumor cells in vitro and in vivo. Furthermore, our data show that the AG-490-induced antitumor effect strongly correlated with the inhibition of Stat3 activity in myeloma cells in vivo. These findings confirm previous studies that Stat3 signaling is critical for the survival of tumor cells that depend on JAK activity for growth (6, 15). Thus, selective inhibition of JAK/STAT signaling has potential therapeutic value in reducing the size of Stat3-positive tumors. At the same time, AG-490 did not cause cell death of Stat3 DNA-binding negative MethA and J558 tumor cells (Table 1), nor did it kill normal peritoneal macrophages (data not shown). The lack of cell death in macrophages is consistent with a previous study in which AG-490 had no significant effect on the growth of normal B and T cells in vivo (16). These results suggest that targeting JAK/STAT3 signaling does not markedly affect the survival of normal cells.

Combining inhibitors of JAK/STAT signaling like AG-490 with immunotherapy such as IL-12 appears attractive, because the latter can help eliminate minimal residual disease and/or induce long-term antitumor immunity. Our present study demonstrates that treatment with AG-490 does not affect the abilities of IL-12 to stimulate IFN-γ production in vivo and to activate resident peritoneal macrophages. These findings are somewhat unexpected in that IL-12 is known to signal through Stat3 and Stat4. Nevertheless, it has been shown that IL-12 induces stronger phosphorylation of Stat4 than of Stat3, and AG-490 preferentially inhibits Stat3 phosphorylation more than Stat4 phosphorylation (23, 31).

**Discussion**

Using syngeneic murine tumor models, we have shown that the inhibition of JAK/STAT signaling pathways by a specific JAK inhibitor, the tyrphostin AG-490, resulted in apoptosis of tumor cells in vitro and in vivo. Furthermore, our data show that the AG-490-induced antitumor effect strongly correlated with the inhibition of Stat3 activity in myeloma cells in vivo. These findings confirm previous studies that Stat3 signaling is critical for the survival of tumor cells that depend on JAK activity for growth (6, 15). Thus, selective inhibition of JAK/STAT signaling has potential therapeutic value in reducing the size of Stat3-positive tumors. At the same time, AG-490 did not cause cell death of Stat3 DNA-binding negative MethA and J558 tumor cells (Table 1), nor did it kill normal peritoneal macrophages (data not shown). The lack of cell death in macrophages is consistent with a previous study in which AG-490 had no significant effect on the growth of normal B and T cells in vivo (16). These results suggest that targeting JAK/STAT3 signaling does not markedly affect the survival of normal cells.

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**Table 2** Activation of macrophages and splenocytes by rIL-12 is not affected in mice treated with AG-490.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mph cytotoxic activity (%)</th>
<th>IFN-γ production* (pg/ml)</th>
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<tbody>
<tr>
<td>AG490</td>
<td>66.1 ± 0.14</td>
<td>1500 ± 122</td>
</tr>
<tr>
<td>DMSO</td>
<td>64.9 ± 2.53</td>
<td>2458 ± 48</td>
</tr>
<tr>
<td>AG490 + IL-12</td>
<td>92.2 ± 0.75</td>
<td>5763 ± 443</td>
</tr>
<tr>
<td>DMSO + IL-12</td>
<td>90.3 ± 1.11</td>
<td>5315 ± 669</td>
</tr>
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* Spleenocytes derived from each group were treated with 100 units/ml rIL-2.

![Fig. 3](image)

**Fig. 3.** Treatment with rIL-12 has a small inhibitory effect on MPC11 tumors. Thirteen days after tumor implantation (10^6 MPC11 cells), mice were treated with rIL-12 peritumorally with the indicated doses every 2nd day for a total of seven times, n = 4 for each group.

![Fig. 4](image)

**Fig. 4.** rIL-12 prolongs the AG-490-mediated antitumor effect. Ten days after tumor implantation (10^6 MPC11 cells), mice were treated with AG-490 or DMSO vehicle alone for 5 days. All of the tumors in AG-490-treated mice regressed 4 days after AG-490 treatment. On day 15, both DMSO- and AG-490-treated mice began receiving peritumoral administration of rIL-12 (200 ng every 2nd day for five times) until day 25. Secondary tumor growth and metastasis (large lymph nodes or paralytic symptoms) were determined by palpation and visual observation. Mice with either secondary tumor growth or metastasis were scored as mice with tumors. DMSO + PBS, AG-490 + PBS, or DMSO + IL-12, n = 10; AG-490 + IL-12, n = 8 (from two independent experiments). AG-490 treatment followed by 100-ng rIL-12 treatment every 2nd day was also performed with results similar to AG-490 followed by 200 ng of rIL-12.
gether with these earlier findings, our results suggest that AG-490 inhibits predominantly Stat3, which is not the principal and/or only mediator involved in IL-12 signaling. Consistent with our finding that AG-490 does not block the function of immune cells, an earlier study showed that in vivo AG-490 treatment did not inhibit the proliferation of antigen-stimulated peripheral lymph node cells (32). In addition, our results indicate that AG-490 treatment stimulates the ability of macrophages to present antigens in vitro. Combined together, these findings suggest that blocking JAK/STAT signaling pathways in immune cells does not negatively affect the functions of immune cells, and, under certain circumstances, it may enhance their functions.

Systemic AG-490 therapy alone has been shown to completely eliminate disseminated human leukemia in a SCID mouse model (16). However, AG-490 treatment of MPC11 tumors in our study failed to induce complete tumor regression because of the rapid regrowth of the murine tumor cells tumors in our study. Nevertheless, because of the rapid regrowth of the murine tumor cells in this tumor model. Additional experiments are also required to determine the role of IL-12 in inducing antiangiogenesis in the AG-490/IL-12 therapeutic model. Nevertheless, we have shown that blocking JAK/Stat3 signaling in tumors in vivo does not significantly interfere with IFN-γ production, which is fundamental for IL-12-induced antitumor immunity and antiangiogenesis. In summary, our findings suggest the potential use of JAK/STAT inhibitors, such as AG-490, in combination with IL-12 therapy for more effective treatment of human cancers displaying elevated JAK/STAT activity.

Acknowledgments

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