AAR

# **Cancer Research**

## Stat1 Deficiency in the Host Enhances Interleukin-12– Mediated Tumor Regression

Marina N. Torrero, Xueqing Xia, Williams Henk, et al.

Cancer Res 2006;66:4461-4467. Published online April 17, 2006.

Updated Version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-05-3554
Cited Articles	This article cites 55 articles, 23 of which you can access for free at: http://cancerres.aacrjournals.org/content/66/8/4461.full.html#ref-list-1
Citing Articles	This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/66/8/4461.full.html#related-urls
E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

### Stat1 Deficiency in the Host Enhances Interleukin-12–Mediated Tumor Regression

Marina N. Torrero,<sup>1</sup> Xueqing Xia,<sup>1</sup> Williams Henk,<sup>1</sup> Shry Yu,<sup>2</sup> and Shulin Li<sup>1</sup>

<sup>1</sup>Department of Comparative Biomedical Sciences, Louisiana State University, Baton Rouge, Louisiana and <sup>2</sup>Department of Biostatistics and Preventive Medicine, Vanderbilt University Medical Center, Nashville, Tennessee

#### Abstract

Signal transducer and activator of transcription 1 (Stat1) is considered a key transcription factor that inhibits tumorigenesis, and Stat1 activation in the host is required for interleukin-12 (IL-12)-mediated generation of CTL activity. Using syngeneic Stat1-/- C3H mice bearing SCCVII tumors in this study, we discovered opposite results. Stat1 deficiency in the host significantly enhances IL-12-mediated tumor regression, resulting in tumor eradication from 60% of SCCVII tumor-bearing mice and significant inhibition of tumor growth when compared with control treatment (P < 0.01). This effect is independent of both Stat1-activating cytokine IFN-y and Stat1-downstream effector molecule FasL because neither neutralization of IFN- $\gamma$  nor knocking out of FasL enhances or inhibits IL-12-mediated tumor regression. IL-12 induces a high intensity of tumor-specific CTL activity in Stat1-deficient mice (P < 0.01), increases the CD8 T-cell density in tumor bearing Stat1-/- mice, and induces a T-celldependent tumor regression. The increased CTL activity and the high-intensity infiltration of T cells into the tumors in IL-12-treated Stat1-/- mice are likely due to the longer survival than the same cells from wild-type mice. Together, the data show that inhibition of Stat1 expression in the host enhances tumor-local IL-12 gene therapy for regressing tumors. This conclusion provides a new concept for designing an effective treatment strategy. (Cancer Res 2006; 66(8): 4461-7)

#### Introduction

Interleukin-12 (IL-12) is primarily produced by antigen-presenting cells and the functional IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits (1). IL-12 has several distinctive functions, such as inducing Th1 response, enhancing the generation of allospecific CD8 T cells, augmenting the proliferation of activating T and natural killer (NK) cells, and inducing the production and stabilization of IFN- $\gamma$  (1). Both recombinant IL-12 protein therapy and *IL-12* gene therapy induce a significant inhibitory effect on the primary tumors or metastatic tumors in various tumor models and human clinical trial (2–14). *IL-12* gene therapy has resulted in less toxicity than recombinant IL-12 protein therapy as tested in the RENCA tumor model (15).

Induction of IFN- $\gamma$  by IL-12 in the NK cells is a hallmark of IL-12 function. IFN- $\gamma$  has been considered the main IL-12–induced secondary cytokine that triggers IL-12–mediated tumor regression (16). IFN- $\gamma$  binds two specific receptors, IFN- $\gamma$  receptor 1 and

©2006 American Association for Cancer Research.

IFN- $\gamma$  receptor 2, which are expressed in most cell membranes (17). The binding of these two receptors induces the oligomerization and activation of Jak1 and Jak2 by phosphorylation. The activated Jaks then activate IFN- $\gamma$  receptor 1 and signal transducer and activator of transcription 1 (Stat1) by phosphorylation. The activated Stat1 undergoes dimerization, translocation into the nucleus, and regulation of gene expression by binding the  $\gamma$ -activated sequence (18, 19). The regulation of gene expression by IFN- $\gamma$  via Stat1 has been validated using Stat1-/- mice and Stat1-/- cell lines (20).

Stat1 is the key transcription factor for IFN-y-mediated gene expression and its full transcriptional activation requires the serine 727 phosphorylation as well as the tyrosine 701 phosphorylation (21). The activation of Stat1 is critical for its ability to interact with other regulatory molecules such as MCM5, BRCA1, and p53 (22, 23). The activation of Stat1 is also critical for enhancing IFN- $\gamma$ -mediated apoptotic gene expression, such as the up-regulation of FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, p21, and p27 (23-27). The activated Stat1 is inhibited by PIAS1 (28) but PIAS1 also possibly targets p53 as a small ubiquitinrelated modifier (SUMO) protein (29). Stat1 expression in the tumor cells negatively regulates angiogenesis, tumorigenicity, and metastasis as determined from a Stat1-/- tumor cell line (30). Stat1 activation in the host is required for dendritic cell maturation and IL-12-mediated generation of CTL activity (31). Knockout of Stat1 in the host abrogates IFNa-mediated tumor regression and IL-12 tumor vaccine-mediated effect (31, 32). Stat1-/- mice are more susceptible to carcinogen-induced tumors than wild-type mice (32). Tumor patients with Stat1 phosphorylation have less risk of tumor recurrence and longer survival time than the ones without functional Stat1 expression (33). Our array analysis also indicates that Stat1 induction is associated with IL-12-mediated tumor regression (34). To validate this, we have generated C3H Stat1 knockout mice and found the same tumor growth kinetic in both Stat1+/+ C3H and Stat1-/- C3H mice. Surprisingly, we found that knocking out Stat1 from the host enhances IL-12-mediated tumor regression. This regression also occurs in mice that receive IFN- $\gamma$ neutralization antibody, indicating an IFN-y independent mechanism. Likewise, knocking out of the IL-12-induced and Stat1 activation-required effector molecule FasL does not affect IL-12mediated tumor regression. In agreement with the tumor regression result, IL-12 treatment induces a high level of CTL activity and a significant increased accumulation of CD8 T cells in tumors in Stat1–/– host. The data clearly suggest that IL-12 can overcome the FasL deficiency in the host for regressing tumors. Inhibition of Stat1 expression in the host enhances the IL-12mediated tumor regression.

#### Materials and Methods

Gene constructs and plasmid manufacture. The control plasmid DNA used for *in vitro* transfection was pcDNA3.1 (Invitrogen Corp., Carlsbad, CA).

4461

Downloaded from cancerres.aacrjournals.org on February 23, 2013 Copyright © 2006 American Association for Cancer Research

Requests for reprints: Shulin Li, Department of Comparative Biomedical Sciences, Louisiana State University, Skip Bertman Drive, Baton Rouge, LA 70803. Phone: 22-5578-9032; Fax: 22-5578-9895; E-mail: sli@vetmed.lsu.edu.

doi:10.1158/0008-5472.CAN-05-3554

The *IL-12* gene construct used for *in vivo* study was obtained from Valentis, Inc. (Burlingame, CA); the backbone of this construct was described in a previous publication (35). The control plasmid DNA used for *in vivo* study consisted of a deletion of the *IL-12* gene from the IL-12 construct. All plasmids were manufactured with the Qiagen EndoFree plasmid preparation kit (Valencia, CA).

**Cell growth, antibody production, and transfection.** SCCVII cells, known as SCCVII/SF, were derived from a spontaneously arising murine squamous cell carcinoma (36). The cells were maintained in DMEM with 10% fetal bovine serum (FBS; Life Technologies, Inc., Invitrogen, Carlsbad, CA).

For generation of IFN- $\gamma$  neutralization antibody, the hybridoma cell line HB170 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI medium containing 10% FBS. The medium containing the secreted IFN- $\gamma$  neutralization antibody was purified using ammonia sulfate precipitation at 45% saturated concentration. The precipitate was dialyzed to remove the ammonia sulfate and the antibody was diluted into 300 µg protein/100 µL for administration. Three hundred micrograms of protein, containing IFN- $\gamma$  neutralization antibody, were administered every 3 days.

Stat1–/– C3H and FasL–/– mice. The generation of Stat1–/– C3H mice was done at Taconic, Inc. (Hudson, NY) by backcross breeding 129 Stat1–/– mice and C3H mice. The Stat1–/– C3H mice used for this study were the sixth generation of backcross breeding. FasL–/– C3H mice (FasL<sup>gld/</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Generation of SCCVII tumors, measurement of tumor growth, and injection of plasmid DNA via electroporation. Six- to eight-week-old female Stat1+/+ C3H, Stat1-/- C3H, or FasL-/- C3H mice, weighing 18 to 20 g, from the in-house animal breeding facility were used for this study and were maintained under NIH guidelines, approved by the Institutional Animal Care and Use Committee of Louisiana State University.

Tumors were generated by s.c. inoculating the mice with  $2 \times 10^5$  SCCVII cells in a 30-µL volume. Tumor growth was measured with a caliper and tumor volume was calculated with the formula  $V = \pi / 8(a \times b^2)$ , where *V*, tumor volume; *a*, maximum tumor diameter; and *b*, diameter at 90° to *a* (37). Using the protocols previously described, IL-12 and control plasmid DNA were injected into muscles or tumors and each injected tissue was followed by electroporation (38, 39). The tumors were then examined to determine regression. In each experiment, five animals for each treatment or control group were used to study tumor regression. Two independent experiments were done for each experiment. The optimal electroporation variables for intratumoral injection, previously set at 450 V/cm and 20-ms pulse duration for two pulses, were used for gene injection into tumors (40).

**Fluorescent microscope-based CTL assay.** CTL activity was evaluated using a CyToxiLux kit (OncoImmunin, Inc., Gaithersburg, MD), a single-cell-based fluorogenic cytotoxicity assay (41, 42). Splenocytes were obtained 3 to 4 weeks after the treatment from SCCVII tumor-bearing mice. The effector cells from splenocytes were primed by coculture with mitomycin C-treated SCCVII tumor cells in a ratio of 25:1 for 4 days in RPMI 1640 with 20% FCS. Effector cells were incubated with red fluorescence-labeled target SCCVII and 4T1 cells in a ratio of 100:1 in a volume of 200  $\mu$ L for 3 hours and then incubated with the quenched green fluorescence-labeled caspase substrate for 1 hour. The apoptotic target cells (red/yellow color cells) were examined using an Olympus BX41 fluorescence microscope (Olympus, Melville, NY). The images were captured by a SPOT digital camera (model SP401-115, Diagnostic Instruments, Sterling Heights, MI). CTL activity was calculated using the following equation: % specific killing = 100  $\times$  (apoptotic target cells – spontaneous apoptotic target cells) / (total target cells).

**Immunostaining analysis of CD8 T-cell infiltration.** The procedures for frozen-block preparation, tissue sectioning, and immunostaining were the same as previously described (34, 43). The primary antibody applied to the sections was anti-CD8 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA).

**RNA isolation and Northern blot analysis of gene expression.** RNA isolation was done with TRIzol reagent as previously described (39). Probes for glyceraldehyde-3-phosphate dehydrogenase, Stat1, and IP-10 were derived from previous experiments (40). Northern blot analysis of gene expression was detailed in a previous publication (44). The Northern blot results were quantified by scanning the expression signal intensity with a PhosphorImager analyzer (model 445 SI, Molecular Dynamics, Sunnyvale, CA).

Analysis of IL-12 and IFN- $\gamma$  expression. The expression of IL-12 and IFN- $\gamma$  in tumors was determined using an IL-12 ELISA analysis kit from R&D Systems (Minneapolis, MN). Tissues for ELISA were obtained 3 days after intratumoral (tumor-local) administration of plasmid DNA via electric pulses.

**Statistical analysis.** When sample size was small (n = 5), the nonparametric permutation t test with Bonferroni adjustment was applied to the data analysis. In addition, the restricted/residual maximum likelihood-based mixed effect model was employed to test the overall trend effect between two treatment groups across time points. When sample size was relatively large (n > 10), standard t test was applied. All tests of significance were two sided and differences were considered statistically significant when P < 0.05. SAS version 9 and R were used for all analyses.

#### Results

Stat1 deficiency in the host mice enhances tumor regression by tumor-local IL-12 gene therapy. Stat1 is inactivated in a high number of patients bearing squamous cell carcinoma of the head and neck, and Stat1 inactivation in the tumors correlates to a poor prognosis for the treatment (33). Stat1 deficiency in the host is associated with immunodeficiency but IL-12 treatment is able to enhance the antitumor immune response (1, 45). However, it is unknown whether IL-12 treatment can overcome the host Stat1 deficiency to induce antitumor immune response because Stat1 is a key transcription factor for IL-12-induced, IFN-y-mediated signal transduction pathway. To address this issue, a syngeneic Stat1-/-C3H mouse model was generated for transplanting SCCVII tumors and for studying the response to IL-12 treatment. To ensure the absence of Stat1 expression in Stat1-/- mice, the expression of Stat1 was analyzed in the muscle tissues injected with control DNA or IL-12 gene via electric pulses. The electric pulse-based IL-12 gene delivery is referred to as IL-12 gene therapy in this study. IL-12 gene therapy induced a high level of Stat1 and IP-10 expression in the Stat1+/+ mice (Fig. 1). The same therapy did not induce any Stat1 expression but induced a low level of IP-10 expression in the Stat1-/- mice (Fig. 1), indicating that Stat1 is functionally



**Figure 1.** Demonstration of a defective Stat1 function in Stat1–/– C3H mice. Five micrograms of plasmid DNA were injected into tibialis muscles in the hind limbs via electric pulses (44). RNA was extracted from the injected muscles from both Stat1+/+ and Stat1–/– C3H mice 3 days after the administration (n = 3). Gene expression in the presence and absence of IL-12 was analyzed using Northern blot analysis. *pCtrl* and *plL-12*, control and IL-12–encoding plasmid DNA, respectively.

4462



**Figure 2.** IL-12 expression and IFN- $\gamma$  induction in Stat1-/- and Stat1+/+ mice after tumor-local IL-12 treatment. Tumor samples (n = 5) were obtained on day 3 after administration of 30 µg plasmid DNA per tumor via electric pulses. The level of gene expression was determined using ELISA.

defective in Stat1-/- mice, and failed to induce a high level of Stat1-dependent IP-10 gene expression.

To show that the lack of IP-10 induction in Stat1-/- mice by IL-12 treatment was not due to the absence of IFN- $\gamma$  induction, IL-12 expression and IFN- $\gamma$  induction in the Stat1-/- mice were determined after intratumorally injecting *IL-12* gene in both Stat1-/- and Stat1+/+ mice followed by electric pulses, as previously described (34, 39). The intratumoral administration approach via electroporation was defined as tumor-local *IL-12* gene therapy (46). Both IL-12 expression and IFN- $\gamma$  induction were observed in Stat1-/- and Stat1+/+ mice (Fig. 2), showing that knocking out Stat1 in the host does not affect Th1 response.

To show the effect of Stat1 deficiency in the host on IL-12mediated tumor regression, tumor-local *IL-12* gene therapy was done in the Stat1-/- mice bearing SCCVII transplant tumors. Tumor-local IL-12 gene therapy eradicated a relatively large number of established tumors from 60% of Stat1-/- mice with only two administrations (Fig. 3A and D). Surprisingly, all tumoreradicated Stat1-/- mice, referred to as tumor-free mice, rejected the monthly rechallenging tumor cells during a period of 8 months. In comparison, the same tumor-local IL-12 gene therapy with two administrations failed to eradicate any established tumors from Stat1+/+ mice, although tumor growth was significantly inhibited (Fig. 3B and D). To determine whether the effective tumor eradication by IL-12 in Stat1 - / - mice was due to our unique gene administration method-electroporation, recombinant IL-12 protein was also tested in the study. As expected, tumor regression was also found by recombinant IL-12 protein-treated Stat1-/- mice, although the regression was delayed compared with tumor-local *IL-12* gene therapy (Fig. 3C and D). Together, the data show that Stat1 deficiency in the host enhances IL-12-mediated tumor regression of relatively large volume tumors.

IFN-y-independent inhibition of tumor growth by tumorlocal IL-12 gene therapy in Stat1-/- and Stat1+/+ mice. IL-12 treatment induces IFN- $\gamma$ , which triggers the Stat1 signal transduction pathway. Tumor regression in 60% of Stat1-/- mice by tumor-local IL-12 gene therapy indicates an IFN- $\gamma$ - and Stat1 signal transduction-independent mechanism. However, it is possible that there might be a Stat1-independent, but IFN-y-dependent, signal transduction pathway that mediates tumor regression by IL-12 treatment. To test such a possibility, IFN- $\gamma$  neutralization antibody that was used by Spitalny and Havell (47) for neutralizing IFN-y was administered into the IL-12-treated mice. A similar level of tumor growth inhibition was observed with and without administration of IFN- $\gamma$  neutralization antibody (Fig. 4A), suggesting an IFN-y independent mechanism. The detailed analysis of tumor growth from individual mice indicates that administration of IFN-y neutralization antibody enhanced IL-12-mediated inhibition of tumor growth in a majority of the mice (Fig. 4B) except for

**Figure 3.** Comparison of therapeutic efficacy between Stat1-/- and Stat1+/+ mice by the same tumor-local *IL-12* gene therapy. Thirty micrograms of plasmid DNA were injected into tumors via electroporation. *Arrows*, injection times postinoculation. *Points*, mean (n = 5); *bars*, SE. *rIL-12*, recombinant IL-12 protein. *Stat1+/+* and *Stat1-/-*, wild-type and Stat1 knockout C3H mice, respectively. *A*, enhancement of tumor regression in Stat1-/- host by tumor-local IL-12-gene therapy (P < 0.05). *B*, inhibition of tumor growth in Stat1+/+ host by tumor-local *IL-12* gene therapy (P < 0.05). *C*, regression of tumors in Stat1-/- host by recombinant IL-12 protein therapy (P < 0.05). *D*, comparison of tumor eradication rate in different hosts (Stat1+/+ or Stat1-/- mice) using *IL-12* gene or protein for the treatment.



4463

Cancer Res 2006; 66: (8). April 15, 2006



**Figure 4.** Enhancement of tumor-local *IL-12* gene therapy-mediated tumor growth inhibition by depletion of IFN- $\gamma$ . See Fig. 3 legend for detailed description. *A*, demonstration of a similar level of overall IL-12– mediated tumor growth inhibition with and without IFN- $\gamma$  neutralization. *B*, tumor growth from individual mouse in the presence and absence of IFN- $\gamma$  depletion antibody. One individual mouse with very aggressive tumor growth inhibition in the mice that received IFN- $\gamma$  depletion antibody. *C*, aggressive tumor growth from one individual mouse in the presence of IFN- $\gamma$  depletion antibody. *C*, aggressive tumor growth from one individual mouse in the presence of IFN- $\gamma$  depletion antibody.

one individual mouse that developed an aggressive tumor (Fig. 4*C*). As a result, the tumor volume was about the same between IL-12 alone and IL-12 plus anti-IFN- $\gamma$  neutralization antibody. Likewise, the same observation was found in the Stat1–/– mice (data not shown). Taken together, the data indicate that neutralization of IFN- $\gamma$  may enhance the IL-12–mediated tumor growth inhibition in most mice.

A high level of CTL activity is induced by tumor-local IL-12 gene therapy and is enhanced by administration of IFN- $\gamma$ neutralization antibody. Stat1 activation in the host is required for IL-12-mediated generation of CTL activity (31). We hypothesize that this may not be the case in our tumor model as suggested from the therapeutic efficacy study above. To test this hypothesis, CTL activities were determined in both Stat1-/- and Stat1+/+ mice that receive tumor-local IL-12 gene therapy in the presence and absence of neutralization antibody administration. A higher level of CTL activity was detected from mice treated with IL-12 plus anti-IFN- $\gamma$  than from mice treated with IL-12 alone (Fig. 5A). The CTL activity was SCCVII tumor specific because it only induced a basal level of cytolysis to other tumor cell lines such as 4T1 (Fig. 5A). Likewise, a high level CTL activity was obtained in Stat1-/- mice that received IL-12 treatment (Fig. 5C), suggesting that Stat1 deficiency did not inhibit tumor-specific CTL induction. A similar level of CTL activity between IL-12-treated Stat1-/- mice and IL-12-treated Stat1+/+ mice receiving anti-IFN $\gamma$  antibody (Fig. 5B) further supports this notion.

FasL-independent inhibition of tumor growth by IL-12. The experiments above showed that either neutralization of Stat1activating ligand or knocking out Stat1 itself might be beneficial to IL-12-mediated tumor regression. Here, we are interested in determining the effect of FasL on IL-12-mediated tumor growth inhibition. We chose FasL because it is an effector molecule that enhances IL-12-mediated tumor regression (16) and the expression of FasL requires Stat1 (23-27). Moreover, TNF-related apoptosisinducing ligand, the other primary IL-12-downstream effector molecule, is undetectable in the tumors.<sup>3</sup> As a first step to establish the FasL effect, the expression of FasL was determined in Stat1+/+ and Stat1-/- mice. As expected, a high level of FasL was induced in the Stat1+/+ mice but a very low level of FasL was detected in the Stat1-/- mice by tumor-local *IL-12* gene therapy (Fig. 6A) and *B*). To determine that FasL is not required for IL-12-mediated tumor regression, the syngeneic tumor-bearing FasL knockout mice were treated with tumor-local IL-12 gene therapy. Indeed, a similar level of tumor growth inhibition was observed between

the wild-type (Fas+/+) and FasL knockout mice (Fig. 7*A*). The data suggest that the effector molecule FasL is not required for IL-12–mediated inhibition of SCCVII tumor growth. However, unlike Stat1 deficiency, FasL deficiency does not enhance IL-12–mediated inhibition of tumor growth.

Tumor-local IL-12 treatment induces a T-cell-dependent tumor regression in Stat1-/- mice but not in Stat1+/+ mice. To explore the possible mechanistic difference by which IL-12 induces a significant higher rate of tumor regression in Stat1-/- mice than in Stat1+/+ mice (Fig. 3), we determined CTL activity, CD8 T-cell infiltration, and T-cell depletion in IL-12-treated Stat1-/- and Stat1+/+ mice. The high level of CTL activity from IL-12-treated Stat1-/- mice may not be sufficient to explain the significant IL-12-mediated tumor eradication rate that occurs in Stat1-/- mice because the same strong CTL activity was also detected in Stat1+/+ wild-type mice receiving IL-12 plus IFNy antibody treatment (Fig. 5B). However, the difference in the total number of tumor infiltrated CD8 T cells between Stat1-/- and wild-type mice may account for the difference in tumor regression rate because a 4-fold increase in the number of CD8 T cells was detected in Stat1-/mice compared with wild-type mice (Fig. 5C).

There are two mechanisms by which an increased infiltration of CD8 T cells were detected (Fig. 5*C*): one is by an increased infiltration rate and the other is by an increased survival of the infiltrated T cells. In this case, it is not clear whether the former mechanism plays a role but it is certain that the survival mechanism works because Stat1-/- spleen cells survived longer than Stat1+/+ spleen cells (Fig. 5*D*).

To further support the notion that an increased infiltration of CD8 T is critical for IL-12–mediated tumor eradication in Stat1–/– mice, CD8 T cells were depleted in IL-12–treated Stat1–/– and Stat1+/+ mice by administration of CD8 T-cell depletion antibody. Administration of this antibody abrogated the IL-12–mediated tumor regression in Stat1–/– mice (Fig. 7*B*) but had little effect on IL-12–mediated tumor regression in wild-type Stat1+/+ mice (Fig. 7*C*). To determine whether CD4 T cells also play a role in IL-12–mediated tumor regression in Stat1–/– mice, CD4 T-cell depletion antibody was also administered in IL-12–treated Stat1–/– mice. Different from administration of CD8 T-cell depletion antibody, depletion of CD4 T cells failed to completely abrogate IL-12–mediated tumor regression (Fig. 7*D*).

#### Discussion

The most intriguing discovery in this study is that Stat1 deficiency in the host enhances IL-12–mediated tumor eradication as evidenced by the tumor regression in 60% of mice on average with initial tumor diameters of 6 to 7 mm. With such large tumors

Cancer Res 2006; 66: (8). April 15, 2006

4464

Downloaded from cancerres.aacrjournals.org on February 23, 2013 Copyright © 2006 American Association for Cancer Research

<sup>&</sup>lt;sup>3</sup> S. Li, unpublished data.

Figure 5. CTL activity and CD8 T-cell infiltration in Stat1+/+ and Stat1-/- mice mediated by tumor-local IL-12 gene therapy. CTL activity was determined against SCCVII tumor target cells and nonspecific 4T1 tumor cells and Yac 1 control cells. 4T1 cells are derived of mouse mammary malignancy and Yac 1 is the target cell for NK cellmediated cell death. See detailed legend description in Fig. 3. Mice were euthanized 3 days after the second administration for CTL activity assay or 10 days after the first administration for determining the tumor-infiltrated CD8 T cells. A. comparison of the difference in CTL activity from different treatment groups in Stat1+/+ C3H mice. plL-12 + anti-IFNy Ab, spleen cells derived from mice receiving tumor-local IL-12 gene therapy plus administration of IFN-y neutralization antibody. plL-12 + anti-IgG Ab, tumor local IL-12 gene therapy plus administration of mouse immunoglobulin G. B, comparison of CTL activity between Stat1 -/- C3H mice that received tumor-local IL-12 gene therapy (pIL-12) and Stat1+/mice that received pIL-12 plus administration of anti-IFNy antibody treatment. C, comparison of the difference in infiltration of CD8 T cells in tumors between Stat1-/- and wild-type mice after IL-12 treatment. The positive CD8 T cells were determined from five tumors and five sections of each tumor under a microscope at 40×. Tumors were collected on day 10 after the treatment. D, comparison of spleen cell survival difference between Stat1+/+ and Stat1-/- mice. The same number of spleen cells  $(2 \times 10^{6}/\text{mL})$  from both wild-type (Stat1+/+) C3H and Stat1-/- mice was cultured in RPMI complete medium for 6 days and cell survival was counted under a hemocytometer after staining with trypan blue.



in the Stat1+/+ mice, IL-12 failed to eradicate any tumors, but four administrations of IL-12 DNA eradicated small tumors of 4 to 5 mm in diameter in the Stat1+/+ mice (39). The high rate of tumor eradication by IL-12 in Stat1-/- mice and the subsequent rejection of the challenged tumor cells by the tumoreradicated mice indicate that the Stat1 deficiency also enhances the IL-12-mediated generation of antitumor immune memory. This effect is different from the result obtained by Fallarino and Gajewski (31) in P815 tumor model, in which Stat1 deficiency in the host mice completely reverses the effect of IL-12 vaccine and inhibits the generation of tumor-specific CTL activity. Such a discrepancy between Fallarino's result and ours is not entirely due to the therapeutic approaches because administration of IL-12 protein also leads to tumor regression (Fig. 3C and D). However, the electroporation-based tumor-local IL-12 gene therapy does expedite tumor eradication in Stat1-/- mice compared with



Figure 6. Induction of FasL expression in Stat1+/+ mice and Stat1-/- mice in the presence or absence of IL-12 treatment. See Fig. 3 legend for detailed description. *A*, induction of FasL in tumor-bearing Stat1+/+ mice by IL-12. Northern blot analysis was done. Each lane on the blots represents an individual tumor from a mouse that was administered with control plasmid DNA (pCtrl) or IL-12–encoding plasmid DNA (pIL-12) via electric pulses (n = 3). Tumors were obtained on the indicated dates. *B*, absence of FasL induction in Stat1-/- mice by IL-12.

administration of IL-12 protein (Fig. 3D), suggesting that the delivery approach does contribute to the IL-12 gene transfermediated tumor regression. The other contributing factor for the increased tumor eradication rate by IL-12 in Stat1-/- mice may be due to the presence of a large number of CD8 T cells that kill the tumor cells (Fig. 5C). The presence of a large number of T cells in tumors in Stat1 mice that was treated with IL-12 may be due to the increased survival of these cells (Fig. 5D) because Stat1 is required for the expression of apoptosis enzymes such as caspases Ice, Cpp32, and Ich-1, suggesting that deletion of Stat1 may inhibit the expression of these apoptosis-inducing genes. This statement is further supported by the fact that Stat1-deficient cells are resistant to TNF-α-mediated apoptosis (48). Moreover, knocking out of Stat1 activates Stat3 (49), which could promote survival gene expression. The other possible explanation for the presence of a large number of CD8 T cells in tumors bearing on Stat1-/- mice that have received IL-12 treatment is the abrogation of cell arrest because Stat1 expression inhibits cell proliferation (50). Finally, we will also not exclude the possibility that an increased infiltration rate may be responsible for the accumulation of a large number of CD8 T cells in tumors in Stat1-/- mice receiving IL-12 treatment, although there is no rationale for this possibility. Regardless, the result suggests that inhibition of host Stat1 expression enhances the CD8 T-cell accumulation in tumors and is associated with the significant tumor eradication (Fig. 3). Significantly, IL-12-mediated tumor eradication in Stat1-/- mice is abrogated when CD8 T cells, but not CD4 T cells, were depleted (Fig. 7D). However, IL-12-mediated tumor growth inhibition in the wild-type Stat1+/+ mice is not affected by the CD8 T-cell depletion (Fig. 7C and D), suggesting that CD8 T cells may greatly account for the difference from IL-12 treatment between Stat1 deficient and wild-type mice. It seems that CD8 T cells do not act alone because depletion of CD4 T cells also abrogates IL-12-mediated tumor eradication (Fig. 7D). Interestingly, depletion of either CD8 or CD4 T cells does not abrogate tumor eradication by IL-12 and only lack of both types of





T cells abrogates IL-12–mediated tumor eradication in Stat1+/+ mice (42), suggesting that a strong coordination between CD4 and CD8 T cells is required in wild-type mice for IL-12–mediated tumor growth inhibition. The cellular difference between the two different types of mice needs to be further explored to fully understand the contribution by each individual cell type.

The Stat1 deficiency-enhanced IL-12-mediated tumor regression suggests an IFN-y-independent mechanism. As expected, administration of IFN-y neutralization antibody tends to inhibit the tumor growth in most of the IL-12-treated Stat1+/+ mice, except for the one individual mouse (Fig. 4C), suggesting that neutralization of IFN- $\gamma$  during the treatment period may positively enhance the IL-12-mediated tumor regression, which is in agreement with the Stat1 deficiency-mediated effect (Fig. 3A-D). Such a result is in agreement with the result from IFN- $\gamma$  knockout mice, in which IL-12-mediated tumor regression was achieved (51). However, this result is against the popular view of the IFN- $\gamma$ -dependent IL-12 inhibition of tumor growth, as suggested by multiple investigators (16), because IFN- $\gamma$  has been known to inhibit tumor growth by increasing antiangiogenic gene expression and activating T and macrophage cells (1). Why does inhibition of IFN-γ signal transduction pathway enhance IL-12-mediated tumor regression? One logical explanation is that IFN-y may enhance expression of genes that inhibit IL-12-mediated tumor eradication. Several lines of evidence support this explanation. First, neutralization of IFN- $\gamma$  enhances the IL-12-mediated therapeutic efficacy (Fig. 4). Second, neutralization of IFN- $\gamma$  during IL-12 treatment clearly increases the tumor-specific CTL activity (Fig. 5A). Third, others reported that IFN- $\gamma$  inhibits the antigen presentation by  $CD8\alpha^{-}$  dendritic cells (52). Finally, IFN induces and activates Stat3, which promotes the expression of oncogenes and tumor growth factors (34, 53). However, more work is needed to determine the exact role of IFN- $\gamma$  in IL-12-mediated tumor regression.

FasL is reported as an effector molecule that enhances the IL-12-mediated tumor regression (16, 54). Expression of FasL requires Stat1 activation and is enhanced by IL-12 (26). Indeed, FasL expression in the tumors is inhibited in the Stat1-/- mice but induced in the Stat1+/+ mice (Fig. 6A and B). However, knocking out FasL did not abrogate the IL-12-mediated inhibition of tumor growth in the SCCVII model (Fig. 6C), further supporting the notion that Stat1 signaling pathway is not required for IL-12mediated tumor growth inhibition. However, this does not exclude the use of FasL/Fas signaling pathway in other tumor models for inhibiting tumor growth and metastasis (16, 55), suggesting that IL-12 is capable of operating multiple pathways to induce tumor regression. Depletion of T cells and NK cells did abrogate IL-12mediated tumor regression (42), suggesting that the effector molecules granzymes and perforin play roles because these molecules are defective in T-cell- and NK cell-deficient mice. Our speculation is that the tumor regression by IL-12 may depend on the availability of signal pathways in the host and tumors.

In conclusion, Stat1 deficiency does not inhibit the IL-12-mediated tumor regression, induction of the Th1 response, or generation of tumor-specific CTL activity. Moreover, Stat1 deficiency enhances tumor-local IL-12-mediated SCCVII tumor regression. This discovery suggests that a therapeutic strategy that inhibits Stat1 plus IL-12 treatment may be highly effective for treating SCC malignancy.

#### Acknowledgments

Received 10/3/2005; revised 1/18/2006; accepted 2/8/2006.

Grant support: National Cancer Institute/NIH grant RO1CA98928 and National Institute of Dental and Craniofacial Research/NIH grant R21 DE14682 (S. Li).

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

We thank Ryan Craig and Katie Watson for their assistance in the preparation of this manuscript.

Downloaded from cancerres.aacrjournals.org on February 23, 2013 Copyright © 2006 American Association for Cancer Research

#### References

- 1. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv Immunol 1998;70: 83–243.
- **2.** Yoon SJ, Heo DS, Kang JO, et al. Combination gene therapy of IL-12 and allogeneic MHC class I gene via stimulating NK cytolytic activity. Anticancer Res 1999; 19:4337–42.
- **3.** DiMeco F, Rhines LD, Hanes J, et al. Paracrine delivery of IL-12 against intracranial 9l gliosarcoma in rats. J Neurosurg 2000;92:419–27.
- Lechanteur C, Moutschen M, Princen F, et al. Antitumoral vaccination with granulocyte-macrophage colony-stimulating factor or interleukin-12-expressing dhd/k12 colon adenocarcinoma cells. Cancer Gene Ther 2000;7:676–82.
- 5. Karpoff HM, Kooby D, D'Angelica M, et al. Efficient cotransduction of tumors by multiple herpes simplex vectors: implications for tumor vaccine production. Cancer Gene Ther 2000;7:581–8.
- 6. Gautam SC, Xu YX, Dumaguin M, et al. Interleukin-12 (IL-12) gene therapy of leukemia: immune and antileukemic effects of IL-12-transduced hematopoietic progenitor cells. Cancer Gene Ther 2000;7:1060–8.
- Divino CM, Chen SH, Yang W, et al. Anti-tumor immunity induced by interleukin-12 gene therapy in a metastatic model of breast cancer is mediated by natural killer cells. Breast Cancer Res Treat 2000;60:129–34.
- Worth LL, Jia SF, Zhou Z, et al. Intranasal therapy with an adenoviral vector containing the murine interleukin-12 gene eradicates osteosarcoma lung metastases. Clin Cancer Res 2000;6:3713–8.
- Zhang HQ, He NG, Xue SB. Effect of bleomycin-a5 with calmodulin inhibitor on the proliferation of s-180 cells. In Vitro 1990;11:470–3.
- **10.** Chen L, Chen D, Block E, et al. Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cdnas for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. J Immunol 1997;159:351–9.
- Rakhmilevich AL, Hooper AT, Hicklin DJ, et al. Treatment of experimental breast cancer using interleukin-12 gene therapy combined with anti-vascular endothelial growth factor receptor-2 antibody. Mol Cancer Ther 2004;3:969–76.
- **12.** Dow SW, Elmslie RE, Fradkin LG, et al. Intravenous cytokine gene delivery by lipid-DNA complexes controls the growth of established lung metastases. Hum Gene Ther 1999;10:2961–72.
- 13. Rakhmilevich AL, Turner J, Ford MJ, et al. Gene gunmediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. Proc Natl Acad Sci U S A 1996;93:6291–6.
- **14.** Yamamoto M, Kobayashi Y, Li M, et al. *In vivo* gene electroporation of glial cell line-derived neurotrophic factor (GDNF) into skeletal muscle of sod1 mutant mice. Neurochem Res 2001;26:1201–7.
- **15.** Tan J, Newton CA, Djeu JY, et al. Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. Cancer Res 1996;56:3399–403.
- 16. Wigginton JM, Gruys E, Geiselhart L, et al. IFN- $\gamma$  and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. J Clin Invest 2001;108:51–62.
- 17. Rosenzweig SD, Schwartz OM, Brown MR, et al. Characterization of a dipeptide motif regulating IFN- $\gamma$  receptor 2 plasma membrane accumulation and IFN- $\gamma$  responsiveness. J Immunol 2004;173:3991–9.

**18.** Darnell JE, Jr. Stats and gene regulation. Science 1997; 277:1630–5.

- Ramana CV, Chatterjee-Kishore M, Nguyen H, et al. Complex roles of STAT1 in regulating gene expression. Oncogene 2000;19:2619–27.
- 20. Durbin JE, Hackenmiller R, Simon MC, et al. Targeted disruption of the mouse STAT1 gene results in compromised innate immunity to viral disease. Cell 1996;84:443–50.
- **21.** Jove R. Preface: Stat signaling. Oncogene 2000;19: 2466–7.
- 22. Townsend PA, Scarabelli TM, Davidson SM, et al. Stat-1 interacts with p53 to enhance DNA damageinduced apoptosis. J Biol Chem 2004;279:5811–20.
- 23. Ouchi T, Lee SW, Ouchi M, et al. Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-y target genes. Proc Natl Acad Sci U S A 2000; 97:5208–13.
- 24. Agrawal S, Agarwal ML, Chatterjee-Kishore M, et al. Stat1-dependent, p53-independent expression of p21(waf1) modulates oxysterol-induced apoptosis. Mol Cell Biol 2002;22:1981–92.
- 25. Lee JK, Sayers TJ, Brooks AD, et al. IFN-γdependent delay of *in vivo* tumor progression by Fas overexpression on murine renal cancer cells. J Immunol 2000;164:231–9.
- **26.** Xu X, Fu XY, Plate J, et al. IFN- $\gamma$  induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. Cancer Res 1998;58:2832–7.
- 27. Choi EA, Lei H, Maron DJ, et al. Stat1-dependent induction of tumor necrosis factor-related apoptosisinducing ligand and the cell-surface death signaling pathway by interferon β in human cancer cells. Cancer Res 2003;63:5299–307.
- 28. Liu B, Liao J, Rao X, et al. Inhibition of STAT1mediated gene activation by pias1. Proc Natl Acad Sci U S A 1998;95:10626–31.
- 29. Kahyo T, Nishida T, Yasuda H. Involvement of piasl in the sumoylation of tumor suppressor p53. Mol Cell 2001;8:713–8.
- **30.** Huang S, Bucana CD, Van Arsdall M, et al. Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells. Oncogene 2002;21:2504–12.
- Fallarino F, Gajewski TF. Cutting edge: Differentiation of antitumor CTL *in vivo* requires host expression of STAT1. J Immunol 1999;163:4109–13.
- **32.** Lesinski GB, Anghelina M, Zimmerer J, et al. The antitumor effects of IFN-α are abrogated in a STAT1-deficient mouse. J Clin Invest 2003;112:170–80.
- **33.** Widschwendter A, Tonko-Geymayer S, Welte T, et al. Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. Clin Cancer Res 2002;8:3065–74.
- **34.** Li S, Xia X, Mellieon FM, et al. Candidate genes associated with tumor regression mediated by intratumoral IL-12 electroporation gene therapy. Mol Ther 2004;9:347–54.
- 35. Coleman M, Muller S, Quezada A, et al. Nonviral interferon  $\alpha$  gene therapy inhibits growth of established tumors by eliciting a systemic immune response. Hum Gene Ther 1998;9:2223–30.
- 36. O'Malley BW, Jr., Cope KA, Johnson CS, et al. A new immunocompetent murine model for oral cancer. Arch Otolaryngol Head Neck Surg 1997;123:20–4.
- **37.** Puisieux I, Odin L, Poujol D, et al. Canarypox virusmediated interleukin 12 gene transfer into murine mammary adenocarcinoma induces tumor suppression and long-term antitumoral immunity. Hum Gene Ther 1998;9:2481–92.

- **38.** Li S, MacLaughlin F, Fewell J, et al. Muscle-specific enhancement of gene expression by incorporation of sv40 enhancer in the expression plasmid. Gene Ther 2001;8:1–7.
- 39. Li S, Zhang X, Xia X. Regression of tumor growth and induction of long-term antitumor memory by interleukin 12 electro-gene therapy. J Natl Cancer Inst 2002;94: 762–8.
- **40.** Li S, Xia X, Zhang X, et al. Regression of tumors by IFN- $\alpha$  electroporation gene therapy and analysis of the responsible genes by cdna array. Gene Ther 2002; 9:390–7.
- **41.** Liu L, Chahroudi A, Silvestri G, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nat Med 2002;8:185–9.
- **42.** Li S, Zhang L, Torrero M, et al. Administration routeand immune cell activation-dependent tumor eradication by ill2 electrotransfer. Mol Ther 2005;12:942–9.
- **43.** Li S, Wilkinson M, Xia X, et al. Induction of IFNregulated factors and antitumoral surveillance by transfected placebo plasmid DNA. Mol Ther 2005;11: 112–9.
- 44. Li S, Zhang X, Xia X, et al. Intramuscular electroporation delivery of IFN- $\alpha$  gene therapy for inhibition of tumor growth located at a distant site. Gene Ther 2001; 8:400–7.
- **45.** Meraz MA, White JM, Sheehan KC, et al. Targeted disruption of the STAT1 gene in mice reveals unexpected physiologic specificity in the Jak-STAT signaling pathway. Cell 1996;84:431–42.
- **46.** Biagi E, Dotti G, Yvon E, et al. Molecular transfer of CD40 and OX40 ligands to leukemic human B cells induces expansion of autologous tumor-reactive cytotoxic t lymphocytes. Blood 2005;105:2436–42.
- 47. Spitalny GL, Havell EA. Monoclonal antibody to murine  $\gamma$  interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J Exp Med 1984;159:1560–5.
- **48.** Kumar A, Commane M, Flickinger TW, et al. Defective TNF-α-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science 1997; 278:1630–2.
- **49.** Qing Y, Stark GR. Alternative activation of STAT1 and STAT3 in response to interferon-γ. J Biol Chem 2004;279: 41679–85.
- 50. Kortylewski M, Komyod W, Kauffmann ME, et al. Interferon-γ-mediated growth regulation of melanoma cells: Involvement of STAT1-dependent and STAT1independent signals. J Invest Dermatol 2004;122:414–22.
- 51. Zilocchi C, Stoppacciaro A, Chiodoni C, et al. Interferon  $\gamma$ -independent rejection of interleukin 12transduced carcinoma cells requires CD4+ T cells and granulocyte/macrophage colony-stimulating factor. J Exp Med 1998;188:133–43.
- 52. Ramana CV, Grammatikakis N, Chernov M, et al. Regulation of c-myc expression by IFN- $\gamma$  through STAT1-dependent and -independent pathways. Embo J 2000;19:263–72.
- **53.** Jung JE, Lee HG, Cho IH, et al. STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB J 2005;19:1296–8.
- 54. Brockstedt DG, Diagana M, Zhang Y, et al. Development of anti-tumor immunity against a non-immunogenic mammary carcinoma through *in vivo* somatic GM-CSF, II-2, and HSVtk combination gene therapy. Mol Ther 2002;6:627–36.
- 55. Lafleur EA, Jia SF, Worth LL, et al. Interleukin (IL)-12 and IL-12 gene transfer up-regulate Fas expression in human osteosarcoma and breast cancer cells. Cancer Res 2001;61:4066–71.

Copyright © 2006 American Association for Cancer Research