Doxorubicin Directs the Accumulation of Interleukin-12–Induced IFNγ into Tumors for Enhancing STAT1–Dependent Antitumor Effect

Shiguo Zhu, Marian Waguespack, Steven A. Barker, and Shulin Li

Abstract

Purpose: To examine the mechanism by which doxorubicin plus interleukin-12 (IL-12) gene transfer induces enhanced therapeutic efficacy against tumors.

Experimental Design: Tumor-bearing mice were treated with doxorubicin, IL-12–encoding plasmid DNA, doxorubicin plus IL-12–encoding plasmid DNA, or plasmid DNA control. Doxorubicin was systemically given via i.p. injection, and IL-12 was systemically expressed via i.m. injection. To show that doxorubicin enhances the accumulation of IL-12–induced IFNγ into tumors and the signal transducer and activator of transcription 1 (Stat1)–dependent antitumor efficacy, the distribution of IFNγ and the therapeutic end points, such as T-cell infiltration, inhibition of tumor vessel density, tumor growth inhibition, and inhibition of spontaneous tumor metastasis in wild-type and Stat1−/− host and tumors were determined after the treatment at the indicated time points.

Results: In this study, a novel mechanism was unveiled. We discovered that doxorubicin enhances the accumulation of IL-12–induced IFNγ in tumors. The doxorubicin-mediated accumulation of IFNγ in tumors is caused by an increased accumulation of IFNγ-secreting immune cells and not by a direct translocation of IFNγ protein into tumors. Depletion of immune cells reverses the doxorubicin-mediated accumulation of IFNγ into tumors and reverses the inhibition of tumor vessel density induced by coadministration of doxorubicin and IL-12 DNA. Knocking out IFNγ signaling in the tumor host reverses the significant inhibition of tumor growth by coadministration of doxorubicin and IL-12.

Conclusions: The enhanced antitumor efficacy by coadministration of doxorubicin and IL-12 is dependent on the accumulation of IFNγ in tumors. This discovery provides a possible strategy to reduce side effects caused by IL-12.

Interleukin-12 (IL-12) is primarily produced by antigen-presenting cells, and the biologically active IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits (1, 2). IL-12 induces Th1 response, enhances the generation of allospecific CD8 T cells, augments the proliferation of activating T and natural killer (NK) cells, and induces the production and stabilization of IFNγ (1). These distinctive biological functions of IL-12 result in the inhibition of primary and metastatic tumors in various tumor models and human clinical trial (3–15). However, IL-12 is toxic when an acute dose of IL-12 protein is given systemically (16). Systemic delivery is not as effective as local tumor delivery, but in some circumstances, such as after surgery, systemic delivery is the only choice. Because IL-12–induced IFNγ is the primary source for triggering the systemic toxicity after systemic delivery (17), discovering a strategy for reducing the systemic IFNγ distribution may minimize the side effects of systemic IL-12 therapy.

Combination therapy has been used to increase the antitumor efficacy, reduce the systemic toxicity, and reduce the absolute dose of IL-12. For example, a combination of doxorubicin and IL-12 protein therapy has boosted the inhibition of tumor growth in bladder carcinoma, B16 melanoma, and L1210 leukemia models (18, 19). However, the mechanism for the enhanced effect by coadministration of IL-12 and doxorubicin is largely unknown. In this report, authors reveal that coadministration of doxorubicin and IL-12 gene enhances accumulation of IFNγ in tumors and triggers a doxorubicin-mediated, IFNγ signaling-dependent, and IFNγ accumulation in tumor-associated inhibition of tumor growth. This novel mechanism will be significant for designing an optimal administration approach for treating tumors.

Materials and Methods

Gene constructs and plasmid manufacture. The IL-12 gene construct used for in vivo study was obtained from Valentis, Inc.; the backbone of this construct was described in a previous publication (20). The control plasmid DNA used for in vivo study consisted of a deletion of the IL-12

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Authors’ Affiliation: Department of Comparative Biomedical Sciences, Louisiana State University, Baton Rouge, Louisiana

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Requests for reprints: Shulin Li, Department of Comparative Biomedical Sciences, Louisiana State University, Skip Bertman Drive, Baton Rouge, LA 70803. Phone: 225-578-9032; Fax: 225-578-9895; E-mail: sli@vetmed.lsu.edu.

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gene from the IL-12 construct. All plasmids were manufactured with the Qiagen EndoFree plasmid preparation kit.

**Reagents and chemicals.** Doxorubicin hydrochloride (D1515, 98% purity) and daunorubicin hydrochloride (30450, 90% purity) for high-pressure liquid chromatography (HPLC) analysis were purchased from Sigma. Monobasic, anhydrous sodium phosphate (reagent grade) was obtained from Amresco, and a-phosphoric acid (85%) was obtained (certified ACS) from Fisher Scientific. Water and methanol, both Optima grade, were purchased from Fisher Scientific. Recombinant IFNγ protein was obtained from R&D Systems, Inc. Doxorubicin used for animal study was the same as used in clinical treatment (Bedford Laboratories) and was purchased from the pharmacy at the Louisiana State University School of Veterinary Medicine. Cyclophosphamide used for animal study was manufactured by Bristol-Myers Squibb Co.

**Cell culture.** SCCVII cells, known as SCCVII/SF, were derived from a spontaneously arising murine squamous cell carcinoma (21). 4T1 adenocarcinoma cell line was purchased from American Type Culture Collection and was originally derived from mammary tumors. AGS-MCS cells [signal transducer and activator of transcription 1 (Stat1)–deficient cells] and Stat1-reconstituted isogenic cells AGS-Stat1 are a generous gift from Dr. William E. Carson, III and Gregory B. Lesinski (Ohio State University). The syngeneic Stat1−/− C3H mice for establishing SCCVII tumors were obtained by backcross breeding 129 Stat1−/− mice and C3H mice for six generations (22).

Tumors were generated by s.c. inoculating C3H mice with 2 × 105 SCCVII cells, C57BL/6 mice with 2 × 105 Stat1-deficient AGS-MCS cells or Stat1-reconstituted AGS-Stat1 cells, and BALB/c mice with 1 × 105 4T1 cells in a 30-μL volume. Spontaneous metastatic tumors were developed in lungs from 4T1 s.c. tumors. S.c. tumor growth was measured with a caliper and tumor volume was calculated with the formula: $V = \frac{\pi}{6} a b^2$, where $V$ is the tumor volume, $a$ is the maximum tumor diameter, and $b$ is the diameter at 90° to $a$ (23). Metastatic tumors in lungs were counted under a dissecting microscope.

Using the protocols described previously, IL-12– encoding DNA or control plasmid DNA was injected into tibialis muscles, and each injection was followed by electroporation (24–26). Ten micrograms for each BALB/c, 5 μg for each C57BL/6, and 1 μg for each C3H mouse were given. Different doses were used for different mouse strains to avoid mortality of the mice from the treatments. The electroporation variables for i.m. injection were 350 V/cm and 20 ms pulse duration for two pulses, and they were applied after gene injection into muscles or tumors (24). Doxorubicin was given via i.p. injection at a dose of 5 mg/kg per mouse. For each mouse, two administrations were done 10 days apart. In each experiment, five animals for each treatment or control group were used to study tumor regression. Each experiment was repeated at least twice.

**Analysis of IL-12 and IFNγ expression.** The expression of IL-12 and IFNγ in tumors and blood was determined using the corresponding ELISA analysis kits from R&D Systems. Tissues and serum for ELISA were obtained 1 day after i.m. administration of plasmid DNA via electric pulses. Tissues were homogenized as described previously in cell lysis buffer purchased from Promega (26).

**Immunohistochemistry of tumor vessel density and infiltration of CD8 T cells.** The procedures for frozen-block preparation, tissue sectioning, and immunostaining were the same as described previously (24, 26–28). The primary antibodies applied to the sections were anti-CD8 and anti-CD31 (1:400; Santa Cruz Biotechnology). Five fields from each tumor (n = 5) were examined to count the number of vessels or CD8 T cells per field under an Olympus microscope. Five tumors from each treatment group were used.

**Immune cell depletion study.** Tumor-bearing mice were grouped before the therapeutic treatment, and murine IgG and antibodies against murine NK cells (PK136), CD8 T cells (2.43), and CD4 (GK1.5) were given to different corresponding treatment groups 1 day before the treatment and every 3 days thereafter. Each mouse received 50 μg IgG or one of the corresponding antibodies in a volume of 50 μL via i.p. injection.

**Determination of doxorubicin concentration in blood and tumor lysates using HPLC.** Serum and tumor samples were collected from 4T1 tumor-bearing mice receiving doxorubicin plus control or IL-12 – encoding plasmid DNA. Tissues were homogenized as described previously (26). Each serum or tissue homogenate sample in a volume of 50 μL was aliquoted along with 50 μL blank water into the assay plate. Working standards were prepared from the purchased standard by dilution in ice-cold methanol to produce 0.1 and 1.0 μg/mL (free

![Fig. 1. Therapeutic efficacy of doxorubicin (DOX) plus systemic IL-12 electroporation gene therapy. Control plasmid DNA (pCtr) and plasmid DNA encoding IL-12 (pIL12) were injected i.m. via electroporation in separate test groups. The injected muscles for expressing IL-12 gene product were distal from the tumor location to show the systemic effect. A total of 10 μg DNA was given into each mouse. Doxorubicin at a dose of 5 mg/kg per mouse was given i.p. and simultaneously with DNA administration. A total of two administrations were done for both doxorubicin and DNA with 10 d between the first and second administrations of each (see arrow for administration time). Mouse strain is indicated on top of each panel. Five mice were used for each treatment group and each experiment was repeated at least twice. *, significantly different. A and B, attenuation of metastatic tumor growth in lungs by coadministration of doxorubicin and IL-12 gene. C, enhanced inhibition of s.c. 4T1 tumor growth by coadministration of doxorubicin and the IL-12 gene.](image-url)
base) concentrations. Calibration standards were prepared in 50 μL of blank mouse serum or blank mouse tissue homogenate. The internal standard, daunorubicin, also diluted in cold methanol, was added in all standards and test samples to yield a final concentration of 500 ng/mL and a final volume of 150 μL. Tubes were mixed for 1 min on a vortex mixer equipped with a microcentrifuge tube insert and centrifuged for 10 min at 15,600 × g. The supernatants were transferred to amber autosampler vials with microvolume inserts for HPLC analysis.

HPLC analyses were done according to the method of Zhou et al. (29), with the following changes: samples were analyzed by reversed-phase liquid chromatography on an Agilent 1100 Series HPLC with an 1100 Series fluorescence detector (Agilent Technologies, Inc.). A 50-μL aliquot of each sample was injected on an Eclipse XDB-C18 analytic column (5 μm, 4.6 mm × 150 mm) with an Eclipse XDB-C18 analytic guard cartridge (5 μm, 12.5 mm × 4.6 mm) from Agilent Technologies at ambient temperature (25°C). Samples were isocratically eluted with 50 mmol/L sodium phosphate buffer (pH 2.0) at a flow rate of 1 mL/min at ambient temperature (25°C). The compounds were detected by fluorescence with excitation at 480 nm and emission at 560 nm. Data analysis and peak integration were done using Agilent ChemStation software (Agilent Technologies). Concentrations were determined by producing a calibration curve using the peak area ratios of the analyte to the internal standard.

Statistical analysis. A two-sided Student’s t test was used to compare the means of individual treatments. P values <0.05 were considered statistically significant.

Results

Systemic coadministration of doxorubicin and IL-12–encoding plasmid DNA induces enhanced inhibition of tumor growth. Development of a systemic treatment strategy is important for treating residual microscopic malignancy, inhibiting metastatic...
tumor development, and preventing tumor recurrence after surgery. Others reported that systemic coadministration of doxorubicin and IL-12 recombinant protein inhibits bladder carcinoma and B16 melanoma growth and extends the survival of L1210 leukemia-bearing mice (19), but the mechanism is unknown. To discern the underlying mechanism, we established an IL-12 and doxorubicin combination treatment using i.m. IL-12 electroporation gene therapy and simultaneous i.p. administration of doxorubicin. i.m. injection of IL-12 gene via electric pulses was used as a systemic treatment approach because our previous study showed the production and secretion of gene product into blood circulation from the muscle receiving DNA administration, inhibiting distal tumor growth (24, 25). To show whether the established approach is proper for determining the mechanism of the doxorubicin and IL-12 combination therapy, the therapeutic difference between coadministration of doxorubicin and IL-12 gene, and both IL-12 plus control plasmid DNA and doxorubicin plus control plasmid DNA were compared. Systemic coadministration of IL-12 and doxorubicin completely abrogates the metastatic tumor development in the highly malignant breast tumor model (P < 0.05; Fig. 1A and B). This systemic coadministration enhances the inhibition of s.c. tumor growth in the 4T1 (adenocarcinoma) tumor model (P < 0.01; Fig. 1C). Taken together, these results suggest that the used animal models and this therapeutic approach are proper for discerning the mechanism of combination therapy for inducing the doxorubicin-enhanced antitumor efficacy.

Doxorubicin enhances the accumulation of IL-12–induced IFNγ in tumors but not in livers. To determine the underlying

![Image](https://example.com/image.png)

**Fig. 3.** Illustration of the mechanism for doxorubicin-mediated IFNγ accumulation into tumors. See Fig. 1 legend for details of doses and administrations. A. Linkage between doxorubicin-mediated accumulation of IFNγ in tumors and infiltration of CD8 T cells. Tumors were collected from 4T1 tumor-bearing mice (n = 5) 4 d after receiving the second administration. Tumor sections were stained with anti-CD8 antibody (see Materials and Methods). B. Depletion of potent IFNγ-secreting immune cells reversed the accumulation of IFNγ in tumors (n = 5). C. Doxorubicin did not induce infiltration of recombinant IFNγ protein into tumors directly. The murine recombinant IFNγ protein (400 ng/mouse) was directly given i.v. into 4T1 tumor-bearing mice and the levels of IFNγ in tumors and blood were determined 4 h after administration (n = 3). D. An equal amount of IFNγ was detected in blood from administration of IFNγ alone or coadministration of both IFNγ and doxorubicin. E. Images of CD8 T-cell infiltration under different treatments. Tumors were collected the same as described in (A). Magnification, ×20.
mechanism by which coadministration of doxorubicin and IL-12 increases the therapeutic efficacy, we tested the distribution of both IL-12 and IFNγ between tumors and blood in both 4T1 and SCCVII tumor models. Distribution of IL-12 was analyzed because a high level of local tumor IL-12 expression accounts for effective tumor eradication (30–32). We reasoned that systemic coadministration of doxorubicin and IL-12 may favor the IL-12 accumulation into tumors. Likewise, distribution of IFNγ was analyzed because IFNγ is the main IL-12–induced cytokine that inhibits tumor angiogenesis. Coadministration of doxorubicin and IL-12 increased the total level of IL-12 expression in blood by 30% to 50% (P > 0.05; Fig. 2A) but did not change the distribution ratio of IL-12 between tumors and blood (Fig. 2A and B).

Surprisingly, coadministration of doxorubicin and IL-12 only increased the total level of IFNγ production by 4% to 30% in blood (P > 0.05; Fig. 2C) but significantly increased IFNγ accumulation in tumors by 2- to 3-fold (P = 0.002; Fig. 2D). This increased accumulation of IFNγ into tumors is associated with the enhanced antitumor efficacy of coadministration of IL-12 and doxorubicin (Fig. 2D versus Fig. 1). The increased accumulation of IFNγ (P < 0.05) in tumors was found in independent experiments, in independent tumor models, and from both the first and second administration (Fig. 2D-G).

An increased accumulation of antitumor cytokines in tumors may enhance antitumor efficacy (30, 31) but an increased accumulation of IFNs in IFN-sensitive organs, such as the liver, may cause toxicity (16, 17, 33). Therefore, the distribution of IFNγ was also analyzed in livers after both the first and the second administration. The results indicate that coadministration of IL-12 and doxorubicin did not enhance the accumulation of IFNγ into liver (P = 0.54) but did increase the accumulation of IFNγ into tumors (P = 0.04) in the same experimental setting (Fig. 2E-H).

To determine whether other drugs also potentiated the accumulation of IL-12–induced IFNγ in tumors, a distribution study was done after coadministration of cyclophosphamide and IL-12 or IL-12–encoding DNA alone. The same administration methods as described for coadministering doxorubicin and IL-12 were used. Cyclophosphamide was selected instead of other drugs because this drug is also known to enhance antitumor efficacy when coadministered with IL-12. Unexpectedly, IFNγ was only slightly increased in tumors by coadministration, and no increase was found in livers (Fig. 2I and J).

**Infiltration of lymphocyte cells may account for the accumulation of IFNγ in tumors by coadministration of doxorubicin and the IL-12 gene.** Two possible mechanisms could account for doxorubicin-mediated accumulation of IL-12–induced IFNγ into tumors: an increased infiltration of IFNγ-secreting immune cells into tumors or direct infiltration of soluble IFNγ protein into tumors. To determine whether the former mechanism accounted for the accumulation, the infiltration of T cells was analyzed because T cells are one of the primary types of cells that secrete IFNγ following IL-12 stimulation. The immunostaining of tumor sections supports this hypothesis because an ~30% increase in CD8 T-cell infiltration was detected in tumors receiving systemic coadministration of doxorubicin and the IL-12 gene versus administration of the IL-12 gene alone (P = 0.015; Fig. 3A). This result is supported by the independent CD8 T-cell density image analysis (Fig. 3E). Due to the lack of a proper murine antibody to determine the NK cell infiltration using an immunostaining method, a NK cell depletion study was done (as described below in this section) because NK cells are another primary source of IL-12–induced IFNγ expression.

To further support the concept that infiltration of immune cells accounted for the doxorubicin-mediated accumulation of IL-12–induced IFNγ into tumors and to determine whether NK cells were a source that contributed to the accumulation of IFNγ in tumors, IFNγ level was compared among different immune cell-depleted mice in the IL-12 plus doxorubicin treatment group. The result clearly shows that all of these cells might contribute to the accumulation of IFNγ in tumors, and NK cells might be the most important cells to produce this cytokine in tumors because depletion of any one of these cell types impaired IFNγ accumulation in tumors (Fig. 3B).

To determine that the increased accumulation of IFNγ into tumors was not due to a direct doxorubicin-mediated infiltration of soluble IFNγ into tumors, the biologically active recombinant IFNγ was i.p. coadministered with and without doxorubicin. The level of recombinant IFNγ in tumors was not increased by coadministering doxorubicin and IFNγ protein in either blood (P = 0.97) or tumors (P = 0.64; Fig. 3C and D). Taken together, the results indicate that the increased infiltration of immune cells might account for the increased IFNγ distribution in tumors by coadministration of IL-12 and doxorubicin.
Doxorubicin is not increased in tumors with or without coadministration with the IL-12 gene via i.m. electroporation. Others found that coadministration of tumor vessel-targeted tumor necrosis factor-α increases the accumulation of antiangiogenesis drugs into tumors, and this increase is IFN-γ dependent (34). To determine whether delivery of the IL-12 gene via i.m. electroporation induces doxorubicin accumulation in tumors, both blood samples and tumor lysates were collected to detect the doxorubicin using HPLC. The same or similar levels of doxorubicin were detected between doxorubicin administration plus administration of control plasmid DNA and the coadministration of doxorubicin and IL-12 gene in blood (P = 0.87) and tumors (P = 0.88; Fig. 4A and B). Clearly, the distribution of doxorubicin into tumors is IL-12 administration independent.

Coadministration of doxorubicin and IL-12 reduces tumor vessel density that is reversed by turning off IFN-γ signaling pathway in the host. One of the primary roles of IFN-γ is to reduce tumor vessel density by regulating antiangiogenic gene expression, such as induction of IP-10 and Mig (26, 35, 36). To show whether an increased accumulation of IFN-γ in tumors by coadministration of IL-12 and doxorubicin enhances the inhibition of tumor angiogenesis, tumor vessel density was examined. Coadministration of doxorubicin and the IL-12 gene reduced tumor vessel density by 58%, whereas the IL-12 gene alone and doxorubicin alone reduced tumor vessel density by 37% and 12%, respectively (Fig. 5A). These results suggest that coadministration of doxorubicin and IL-12 enhances the inhibition of tumor vessel development (P = 0.004).

To confirm that doxorubicin-mediated IFN-γ accumulation in tumors accounts for inhibition of tumor vessel density and increases the therapeutic effect, we determined whether turning off IFN-γ signaling pathway reversed the change in tumor vessel density by the same coadministration. To show this effect, tumor vessel density was compared in the Stat1-deficient mice because Stat1 is the key downstream transcription factor in executing IFN-γ-mediated signaling. The result supports the notion that doxorubicin-mediated accumulation of effector cells secreting IFN-γ in tumors may account for the reduced vessel density because tumor vessel density was not altered in the Stat1-deficient mice (P = 0.68) but was greatly altered in the wild-type (WT) mice (Fig. 5B). Likewise, depletion of IFN-γ-producing cells in the mice receiving coadministration of doxorubicin and IL-12 increased tumor vessel density to a level similar to IL-12 treatment (P < 0.01; Fig. 5C).

Turning off IFN-γ signaling or depletion of IFN-γ in the host reverses doxorubicin-enhanced inhibition of tumor growth by IL-12. To further show that the doxorubicin-potentiated accumulation of IFN-γ in tumors accounts for the mechanism of the enhanced antitumor effect by coadministration of doxorubicin and IL-12, we also determined whether turning off IFN-γ signaling pathway and depletion of IFN-γ in the host would reverse the significant inhibition of tumor growth in SCCVII tumor-bearing mice. As was found in 4T1 tumor-bearing mice (Fig. 1A), treatment of SCCVII tumors with IL-12 plus doxorubicin yielded an enhanced inhibition of tumor growth in the WT C3H mice (P = 0.02; Fig. 6A). The same coadministration did not inhibit tumor growth in the non-IFN-γ signaling mice (P = 0.69; Fig. 6B). Likewise, depletion of IFN-γ also reversed the coadministration of doxorubicin and IL-12–mediated inhibition of tumor growth (P < 0.01; Fig. 6C), further suggesting that the doxorubicin-potentiated IL-12–mediated inhibition of tumor growth is dependent on IFN-γ signaling.

Turning off IFN-γ signaling in the tumor cells without interfering with the same signaling cascade in the host also reverses the enhanced inhibition of tumor growth by coadministration of doxorubicin and IL-12. To determine whether IFN-γ accumulation-enhanced inhibition of tumor growth by coadministration of doxorubicin and IL-12 is solely dependent on IFN-γ signaling cascade in the host, we also investigated tumor growth inhibition in WT mice bearing Stat1-deficient tumor cells. Abrogation of IFN-γ signaling cascade in the tumor cells while leaving this signaling intact in the host mice almost reversed the enhanced tumor growth inhibition by coadministration of doxorubicin and IL-12 (Fig. 7A). Reconstitution of the Stat1-deficient tumor cells with WT Stat1 increased the inhibition of tumor growth but was not as effective as was found in WT mice (Fig. 7B versus Figs. 6A and 1C), suggesting that IFN-γ signaling in the host is more important than in tumor cells for inducing the enhanced inhibition of tumor growth by coadministration of doxorubicin and IL-12 DNA.

![Fig. 5](image.png)

Inhibition of tumor vessel density in WT mice, Stat1-deficient mice, and immune cell-depleted mice after receiving different treatments. See Fig. 1 legend for details of labels, doses, and administrations. Tumor vessel densities were determined 4 d after the second administration with the corresponding treatments. A, maximum inhibition of tumor vessel density was found in mice receiving coadministration of doxorubicin and IL-12 DNA (pL12; n = 5). B, inhibition of tumor vessel density was found in WT C3H mice but not in Stat1-deficient mice by the same coadministration of doxorubicin and pL12 (n = 5). Wt and Stat1−/−, WT and Stat1-deficient C3H mice, respectively. C, the inhibition of tumor vessel density was reversed by depleting either T or NK cells (n = 5). NS, nonspecific murine immunoglobulin. NS, not significantly different.
Discussion

Combinations of doxorubicin with various therapeutic proteins, such as IL-2, tumor necrosis factor-α, and CNGRC-tumor necrosis factor-α, or therapeutic genes, such as TRAIL, for treating tumors were tested previously (37–41), but only a few reports examined the robust antitumor efficacy by coadministration of doxorubicin and IL-12 recombinant protein (18, 19, 42). Coadministration of doxorubicin and the IL-12 gene has not been explored previously. This study indicates that coadministration of doxorubicin and the IL-12 gene induces a similar robust inhibition of tumor growth as recombinant IL-12 protein plus doxorubicin in two different tumor models (18, 19). Importantly, the development of spontaneous metastatic tumors was completely inhibited by the systemic coadministration of doxorubicin via i.p. injection and the IL-12 gene via i.m. electroporation (Fig. 1).

The underlying mechanism for the enhanced antitumor efficacy by coadministration of CNGRC-tumor necrosis factor-α and doxorubicin is due to the tumor necrosis factor-α-mediated accumulation of doxorubicin in tumors (34). This report reveals a similar but opposite mechanism in which doxorubicin enhances the accumulation of IL-12–induced IFNγ into tumors but not in the IFNγ-sensitive livers (Fig. 2). This innovative discovery is significant because doxorubicin-enhanced accumulation of IFNγ in tumors reduces the IFNγ-mediated systemic side effects (16, 33). This innovative discovery also perfectly explains why tumor vessel density is reduced significantly by coadministration of doxorubicin and IL-12–encoding DNA because an increased level of IFNγ in tumors may also enhance the expression of antiangiogenic molecules, such as IP-10 and Mig (35, 43). The doxorubicin-mediated tumor-specific accumulation of IL-12–induced IFNγ also perfectly explains an increased antitumor immune response because an increased level of IFNγ may stimulate the tumor-specific immune response. It would be interesting to test in the future whether the effect of doxorubicin on IL-12–induced IFNγ accumulation in tumors is cytokine specific and whether the enhanced accumulation is drug specific. The current report is the first demonstration of an antineoplasm drug, doxorubicin, changing the distribution of IL-12–induced IFNγ in tumor-bearing mice. This novel discovery may be useful in designing the proper administration scheme for a drug and an immunostimulatory gene in a clinical trial. The dependence and independence of IFNγ by IL-12–mediated inhibition of tumor growth remains debatable (43–47), but the current study clearly indicates that the doxorubicin-enhanced IL-12 therapeutic effect is dependent on IFNγ and is associated with the accumulation of IFNγ in tumors. The first line of evidence to support this notion is the 2- to 3-fold increase in the accumulation of IFNγ in tumors by coadministration of doxorubicin and IL-12.

Fig. 6. Inhibition of IFNγ-mediated signaling pathway reversed the doxorubicin-enhanced IL-12–mediated inhibition of tumor growth. See Fig. 1 legend for details in labels, doses, and administrations (n = 5). SCCVII tumors were used for this experiment. A, inhibition of tumor growth in WT mice by coadministration of doxorubicin and IL-12 (pIL12). B, inhibition of tumor growth by coadministration of doxorubicin and pIL12 was reversed in Stat1−/− deficient mice (Stat1−/−). C, depletion of IFNγ reversed the inhibition of tumor growth by the combination of doxorubicin and IL-12 therapy. NS, not significantly different.

Fig. 7. Stat1 deficiency in tumors reverses doxorubicin-enhanced IL-12–mediated synergistic tumor growth inhibition. See Fig. 1 legend for details in labels, doses, and administrations (n = 5). A, doxorubicin plus IL-12–mediated tumor growth inhibition was almost the same as IL-12 alone in WT mice bearing Stat1−/− deficient tumor cells (AGS-MCV). B, doxorubicin plus IL-12 was superior to IL-12 treatment alone in the WT mice bearing Stat1−/− reconstituted tumors (AGS-Stat1). NS, not significantly different.
over the administration of the IL-12 gene alone (Fig. 2). Second, the increased level of IFN-γ accumulation in tumors is associated with the enhanced inhibition of tumor growth by coadministration of doxorubicin and IL-12 (Fig. 1) and the enhanced reduction of tumor vessel density (Fig. 5A). In agreement with this evidence, turning off IFN-γ-mediated signaling dependence not only occurs in the host but also in tumor vessel density (Fig. 5C). The doxorubicin-mediated IFN-γ signaling dependence not only occurs in the host but also in tumors because knocking out Stat1 in the tumor cells also partially abrogates the significant tumor growth inhibition by the coadministration of doxorubicin and IL-12 (Fig. 6C). Likewise, depletion of IFN-γ-secreting immune cells reverses the doxorubicin-enhanced inhibition of tumor vessel density (Fig. 5C). The doxorubicin-mediated IFN-γ signaling dependence not only occurs in the host but also in tumors because knocking out Stat1 in the tumor cells also partially abrogates the significant tumor growth inhibition by the coadministration of doxorubicin and IL-12 (Fig. 7A).

Although we found that Stat1 deficiency in the host enhances intratumoral IL-12 gene therapy-mediated tumor eradication, the muscle-based systemic IL-12 gene therapy failed to yield the same effect (Fig. 6B). This discrepancy between systemic and intratumoral administration is most likely due to the localization of IL-12, as has been reported by us and others (30, 31). Only when IL-12 is localized in tumors was Stat1 deficiency-associated maximum therapeutic efficacy obtained by IL-12 treatment (22). When IL-12 is not localized in tumors but is present primarily in the blood as shown in this study (Fig. 2A versus B), Stat1 deficiency in the host does not promote any IL-12 or coadministration of IL-12 and doxorubicin—mediated inhibition of tumor growth (Fig. 6C). This type of result is important for intelligently selecting patient populations for doing this coadministration.

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