Interferon- γ Is Induced in Human Peripheral Blood Immune Cells *In Vitro* by Sodium Stibogluconate/Interleukin-2 and Mediates Its Antitumor Activity *In Vivo*

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Sodium stibogluconate (SSG), an inhibitor of SHP-1 that negatively regulates cytokine signaling and immunity, suppressed growth of murine Renca tumors in combination with interleukin-2 (IL-2) via a T-cell-dependent mechanism. The ability of SSG to interact with IL-2 in activating primary human immune cells was evaluated herein by assessing its induction of interferon (IFN)- γ^+ TH1 cells in human peripheral blood *in vitro*. The significance of IFN- γ^+ cells was also investigated by assessing SSG/IL-2 antitumor activity in wild-type and IFN- $\gamma^{-/-}$ mice. IFN- γ^+ cells but not IL-5 $^+$ cells were induced markedly (9.1 $\times$) in healthy peripheral blood by SSG/IL-2 in contrast to the modest induction by SSG alone (2.1 $\times$) at its clinically achievable dose (20 μ g/mL) or by IL-2 (3.1 $\times$) at its C_{max} of low-dose schedule (30 IU/mL). SSG at a higher dose (100 μ g/mL) was less effective alone (1.5 $\times$) or in combination with IL-2 (7.8 $\times$). Peripheral IFN- γ^+ cells were induced after 4 or 16 h treatment with SSG/IL-2 within CD4 $^+$ and CD8 $^+$ lymphocytes coincided with heightened CD69 expression ($\sim$ 3–4 $\times$). SSG/IL-2 was also more effective than the single agents in inducing IFN- γ^+ cells in the peripheral blood of melanoma patients, whose basal IFN- γ^+ cell levels were $\sim$ 5% of healthy controls. Renca tumor growth was inhibited by SSG/IL-2 in wild-type but not IFN- $\gamma^{-/-}$ mice. These results demonstrate SSG interactions with IL-2 *in vitro* to activate key antitumor immune cells in peripheral blood of healthy and melanoma donors, providing further evidence for proof of concept clinical trials for effecting augmentation of IL-2 through inhibiting negative regulatory protein tyrosine phosphatases.

Introduction

PROTEIN TYROSINE KINASES (PTKs) are important cancer therapeutic targets as demonstrated by the clinical efficacy of PTK inhibitors for both solid tumors and hematopoietic malignancies (Druker and others 2001a, 2001b; Strumberg and others 2005; Motzer and others 2006). Like PTKs, protein tyrosine phosphatases (PTPases) are also key regulators of intracellular signal transduction and, as such, might have potential as molecular targets for developing novel anticancer agents (Tonks 2006). Among the PTPases, SHP-1 is a pivotal negative regulator of cytokine signaling and immune cell activation (Zhang and others 1999; Tsui and others 2006) and might be targeted by small chemical inhibitors to augment anticancer efficacy of cytokine therapy and immunotherapy. Cytokine therapy of interleukin-2 (IL-2) or interferon (IFN)- α is standard of care for patients with advanced melanoma, inducing modest response rates

(10–20%) (Borden 2000, 2005). Combination of the cytokines with an inhibitor of negative regulatory PTPases would be expected to enhance cytokine signaling and might lead to increased response rates. Moreover, oncogenic PTPases (eg, SHP-2) and tumor suppressor PTPases have also been identified and are also attractive cancer targets (Tartaglia and others 2001; Araki and others 2004; Easty and others 2006; Tonks 2006). However, proof of concept for targeting PTPases in cancer treatment remains to be established. This task has been hampered by the lack of clinical usable PTPase inhibitors and by the limited evidence of anticancer activity of PTPase inhibitory compounds.

Sodium stibogluconate (SSG), a therapeutic for leishmaniasis with an undefined mechanism of action (Berman 1988, 1997), was identified in our recent studies as a clinically usable inhibitor of selective PTPases (Pathak and Yi 2001) that include SHP-1 and SHP-2. Consistent with inhibition of

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intracellular SHP-1, SSG treatment enhanced tyrosine phosphorylation induced by cytokines in hematopoietic cells in culture (Pathak and Yi 2001; Yi and others 2002) and augmented IL-2-induced IFN- γ ⁺ cells in murine splenocytes *in vitro* (Fan and others 2005). In synergy with IFN- α , SSG induced cure of established melanoma in mice that was only partially responsive to the agents individually (Yi and others 2002). SSG also augmented IL-2-induced growth inhibition of murine renal tumors in a T-cell-dependent manner, accompanied by induction of IFN- γ ⁺ T cells (Fan and others 2005). In addition, SSG was capable of direct growth inhibition of cancer cells *in vitro* and prostate cancer tumors in mice (Li and others 2006), consistent with targeting an oncogenic PTPase like SHP-2 (Pathak and Yi 2001). These preclinical evidences suggest the potential of SSG as a novel anticancer agent that targets PTPases to activate antitumor immune cells and directly inhibit tumor growth. It was unclear whether SSG possesses similar activities in human cells.

Given that IFN- γ ⁺ T cells are activated TH1 cells critical in antitumor immunity (Becker 2006) and apparently important in SSG/IL-2 antitumor action in mouse models (Fan and others 2005), we investigated in this study the capacity of SSG to interact with IL-2 in inducing IFN- γ ⁺ T cells in human peripheral blood *in vitro*. We demonstrated that SSG augmented IL-2-induced IFN- γ ⁺ T cells in peripheral blood of healthy donors and melanoma patients *in vitro*. Our results suggest that SSG might have potential to improve the efficacy of IL-2 therapy and provide further support for the concept that chemical inhibitors of PTPases could be developed as safe and efficacious cancer therapeutics.

Materials and Methods

Reagents and human peripheral blood samples

SSG has been described previously (Yi and others 2002) and was stored at 4°C prior to use. Recombinant human IL-2 (Proleukin, 22 million IU/1.3 mg, Chiron, Emeryville, CA) and recombinant human IFN- α (IFN- α 2b, specific activity 2×10^8 units/mg protein, Intron A, Schering-Plough) were purchased from Cleveland Clinic Pharmacy. Human IFN- γ ELISPOT Kit (R & D System, Minneapolis, MN), Human IL-5 ELISPOT Kit (R & D System), CD4⁺ Cell Intracellular IFN- γ Detection Kit (BD Bioscience, San Jose, CA), and CD8⁺ Cell Intracellular IFN- γ Detection Kit (BD Bioscience) were purchased from commercial sources. The murine renal cancer cell line Renca was maintained in culture as described previously (Fan and others 2005).

Heparinized peripheral blood samples were obtained by vein puncture from healthy volunteers and melanoma patients following an established protocol approved by the Institutional Review Board (IRB) of Cleveland Clinic. All patients in the study had regressed/stabilized disease and had not been on active treatment for >3 months. Blood samples were treated and processed immediately for experiments described below.

Treatment and processing of human peripheral blood samples *in vitro* for ELISPOT assays

To mimic *in vivo* drug exposure, human peripheral blood samples were directly treated with different reagents

without preseparation of blood cells from other blood components. Reagents were mixed with blood samples and incubated at 37°C for the designated amounts of times prior to isolation of white blood cells (WBCs) through RBC depletion. Briefly, blood samples after treatment (0.1 or 0.5 mL/tube as indicated) were diluted with five volumes of hypotonic solution (10 mM Tris, pH 7.4; 10 mM NaCl) to lyse RBC and centrifuged to pellet WBCs. The WBC pellets were then washed with hypotonic solution one time, resuspended in RPMI 1640 culture medium, supplemented with 10% fetal calf serum, and used immediately for ELISPOT assays.

Quantification of human peripheral IFN- γ ⁺ cells and IL-5⁺ cells by ELISPOT assays

ELISPOT assays were performed using commercial kits following the manufacturers' instructions. In brief, freshly prepared WBCs from human peripheral blood samples were seeded into flat-bottom 96-well plates coated with monoclonal antibodies specific for human IFN- γ (Human IFN- γ ELISPOT Kit, R&D System) or for human IL-5 (Human IL-5 ELISPOT Kit, R&D System). The plates were then incubated in a humidified CO₂ incubator at 37°C for different times as indicated prior to *in situ* detection of spot-forming cells (SFCs) by ELISA. Scanning and counting of SFCs in the plates were accomplished using an automatic ELISPOT reader with Immunospot2 software (Cellular Technology Ltd). For each donor, duplicates of peripheral blood samples were evaluated for control and each of the treatments to derive means and standard variations. The differences between control/treatment or between different treatments were analyzed using Student's *t*-test to calculate corresponding *P*-values.

Quantification of human peripheral CD4⁺ and CD8⁺ cells expressing IFN- γ by FACS analysis

Human peripheral blood samples were treated as indicated, mixed with five volume of FACS lysing solution (BD Bioscience), set at RT for 10 min, and then stored at -80°C prior to quantification of IFN- γ ⁺ cells using the Intracellular IFN- γ Detection Kit (BD Bioscience) following the manufacturer's procedure. Briefly, frozen blood samples were thawed and washed with washing buffer, treated with BD FACS Permeabilizing Solution at room temperature for 10 min. After centrifugation, the cell pellets were washed three times, incubated with 20 μ L of antibody cocktail (eg, anti-IFN- γ -FITC/CD69-PE/PerCP-Cy5.5-CD4 or isotype control) in darkness at room temperature for 30 min. Following staining, the samples were washed three times, resuspended in 200 μ L of 1% paraformaldehyde solution, and analyzed (20,000 cells/sample) using a BD FACS Caliburs cytometer. Data were analyzed using WinList software.

Animals and animal studies

BALB/c mice (~10 weeks old, female; Taconic Farms, Germantown, NY) and IFN- γ -deficient Balb/c mice (IFN- γ ^{-/-}, ~10 weeks old, female; Jackson Lab, Bar Harbor, ME) were inoculated (s.c.) at the flanks with Renca cells (10⁶ cells/site). Four days post-inoculation, the mice were treated with PBS (control) or IL-2 (10⁵ IU/daily for 5 days, i.p.) and SSG (12 mg/daily for 5 days, i.m. at hip regions) as reported

previously (Fan and others 2005). Tumor volume was measured during the study period and calculated using the formula for a prolate spheroid ($V = 4/3 \pi a^2 b$) (Lindner and others 1997). Student's *t*-test was used for assessing the significance of tumor volume differences among differential treatment groups. Mouse viability (daily) and body weights (weekly) were also recorded during the study period. All studies involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic.

Results

Induction of IFN- γ ⁺ cells in human peripheral blood by SSG and SSG/IL-2 combination

SSG had antitumor activity in combination with IL-2 in mice that was likely mediated in part through the induction of IFN- γ ⁺ T cells (Fan and others 2005). As an initial step to investigate SSG activity in human cells, we determined the capacity of SSG, IL-2, or the combination to induce IFN- γ ⁺ cells in human peripheral blood *in vitro*. To mimic *in vivo* drug exposure, heparinized fresh whole blood from healthy donors were used directly, without prior separation of blood cells from other blood components, for treatment using clinically achievable doses of SSG and IL-2. Following treatment, IFN- γ ⁺ cells in blood samples were quantified using ELISPOT assays.

SSG induced IFN- γ ⁺ cells modestly (~2.2-folds, $P > 0.05$) as a single agent at 20 $\mu\text{g/mL}$ (Fig. 1A), a concentration comparable to the established SSG therapeutic dose (20 mg/kg) (Berman, 1988) and achievable in circulation during SSG therapy (Rees and others 1980). SSG at a higher dose (100 $\mu\text{g/mL}$) did not further increase the number of IFN- γ ⁺ cells (Fig. 1A). At its C_{max} (~30 IU/mL) of the low-dose schedule approved for cancer treatment (Kirchner and others 1998), IL-2 induced a modest increase in IFN- γ ⁺ cells (~3.4-fold, P

< 0.05 , Fig. 1B) consistent with previous reports (Chang and Rosenberg 1989). Interestingly, IL-2-induced IFN- γ ⁺ cells were markedly further increased in the presence of SSG at 20 $\mu\text{g/mL}$ (~9.2-fold) or 100 $\mu\text{g/mL}$ (~7.6-fold) (Fig. 1B). Under comparable conditions, cells expressing IL-5, a cytokine marker of TH2 cells (Becker 2006), were not induced by SSG, IL-2, or the combination (Fig. 1C).

Thus at its therapeutic dose, SSG modestly induced IFN- γ ⁺ cells but significantly and selectively augmented the levels of IFN- γ ⁺ cells induced by IL-2 in human peripheral blood *in vitro*.

SSG accelerates the induction of human peripheral IFN- γ ⁺ cells with minimal 4 h exposure

The induction of human peripheral IFN- γ ⁺ cells described earlier was measured after treating the blood samples with the agents for 16 h. However, the circulation levels of SSG (Rees and others 1980) or IL-2 (Kirchner and others 1998) *in vivo* during therapy are not at consistent levels for long duration as a result of drug clearance. We therefore determined the minimal treatment time required for IFN- γ ⁺ cell induction by these agents. Peripheral blood from a healthy donor was treated *in vitro* with the agents individually or in combination for 1, 2, or 4 h prior to quantification of IFN- γ ⁺ cells by ELISPOT assays.

Both SSG (20 $\mu\text{g/mL}$) and IL-2 (30 U/mL) as single agents failed to induce IFN- γ ⁺ cells after 1, 2, or 4 h of treatment (Fig. 1D). In contrast, the combination markedly induced IFN- γ ⁺ cells after 4 h treatment (Fig. 1D), resulting in approximately a 6.1-fold increase of IFN- γ ⁺ cells in comparison to the untreated control ($P < 0.01$). The combination failed to induce IFN- γ ⁺ cells when used for only 1 or 2 h (Fig. 1D).

These results establish that 4 h of treatment was required and sufficient for induction of IFN- γ ⁺ cells in human peripheral blood by SSG/IL-2 *in vitro*. Since IL-2 or SSG as single

FIG. 1. Induction of interferon (IFN)- γ ⁺ cells but not interleukin (IL)-5⁺ cells in healthy donor peripheral blood *in vitro* by sodium stibogluconate (SSG), IL-2, or SSG/IL-2 combination. (A–C) Peripheral blood samples (0.1 mL/sample) of a healthy donor were treated *in vitro* with SSG, IL-2, or SSG/IL-2 combination for 16 h prior to quantification of IFN- γ ⁺ SFCs (spot-forming cells) or IL-5⁺ SFCs by ELISPOT assays. (D) Peripheral blood samples (0.1 mL/sample, two samples/treatment condition) of a healthy donor were untreated (0) or treated *in vitro* with SSG (20 $\mu\text{g/mL}$), IL-2 (30 U/mL), or SSG/IL-2 combination for 1, 2, or 4 h prior to quantification of IFN- γ ⁺ cells (IFN- γ SFCs) by ELISPOT assays. Data represent mean $\pm$ SD of duplicate samples.

agents failed to induce IFN- γ ⁺ cells after 4 h of treatment (Fig. 1D), the activity of SSG/IL-2 at this time point to induce IFN- γ ⁺ cells (Fig. 1D) demonstrates an acceleration of IFN- γ ⁺ cell induction by IL-2 in the presence of SSG.

Human peripheral IFN- γ ⁺ cells induced by SSG/IL-2 are within the CD4⁺ and CD8⁺ subpopulations of T cells

IFN- γ is expressed by activated TH1 cells (CD4⁺) and cytotoxic T cells (CD8⁺), two immune cell populations essential in antitumor immunity (Becker 2006), as well as by other peripheral white blood cells (Schroder and others 2004). To identify the types of IFN- γ ⁺ cells induced by SSG/IL-2, peripheral blood samples from two healthy donors were treated with SSG/IL-2 for 16 h, co-stained for intracellular IFN- γ plus surface CD4 or CD8, and then subjected to FACS analysis. Activation marker CD69 was also stained and analyzed.

The number of IFN- γ ⁺ cells within the CD4⁺ cell population (Fig. 2A) and CD8⁺ cell population (Fig. 2B) were significantly increased following SSG/IL-2 treatment. The average increases in the blood samples of the two donors were ~5-fold in both cell populations (Fig. 2C), significant ($P < 0.01$) in comparison to controls. These increases occurred independent of the expression of CD69 surface marker, since IFN- γ ⁺ cell induction were detected within CD69⁺ and CD69⁻ cells (Fig. 2A and B). Interestingly, CD69⁺ cells were also induced by SSG/IL-2 treatment (Fig. 3A), particularly within the CD4⁺ population (~5-fold, $P < 0.05$) (Fig. 3B).

These results demonstrate that SSG/IL-2 induced IFN- γ ⁺ cells within peripheral CD4⁺ and CD8⁺ T-cell populations in addition to CD69 induction, providing evidence that the combination treatment was capable of activating these key antitumor immune cells.

SSG enhances IL-2-induced IFN- γ ⁺ cells in peripheral blood of melanoma patients

The induction of IFN- γ ⁺ T cells in healthy peripheral blood by SSG/IL-2 prompted us to investigate the effects of the agents in peripheral blood from melanoma patients. Our initial analysis of 10 melanoma samples failed to detect significant numbers of IFN- γ ⁺ cells (data not shown) either before or after SSG/IL-2 treatment using conditions we had established for studying healthy individuals (0.1 mL blood/sample) for the ELISPOT assay. However, meaningful data for melanoma patients were obtained by using a larger blood volume per assay (0.5 mL blood/sample). For comparison, the effects of the agents in the peripheral blood for six healthy donors were also determined in parallel using the standard 0.1 mL blood/sample for the ELISPOT assays.

In melanoma peripheral blood, IL-2 at 30 U/mL induced modest increases of IFN- γ ⁺ cells in all of the samples (Fig. 4) that were statistically significant ($P < 0.05$) while SSG as a single agent had only minor effects (Fig. 4). The combination of SSG/IL-2 was more effective than the agents individually in inducing IFN- γ ⁺ cells in the melanoma peripheral blood of case 1 ($P < 0.005$) and case 4 ($P < 0.02$) (Fig. 4). SSG/IL-2 also showed an apparently positive interaction for the other cases that was statistically insignificant ($P > 0.05$) (Fig. 4). An increased effectiveness of the combination as compared to the individual agents was evident in the peripheral blood samples of several of the healthy donors evaluated ($P < 0.03$ for individuals 3 and 6; $P > 0.05$ for individuals 1, 2, 4, and 5) (Fig. 5).

Interestingly, the numbers of IFN- γ ⁺ cells in the melanoma samples were substantially lower than those in the healthy controls prior to and after treatment (comparing Figs 4 and 5). The basal levels of IFN- γ ⁺ cells in melanoma (average 1.4/0.1 mL blood) were only ~5% of that observed

FIG. 2. Sodium stibogluconate/interleukin-2 (SSG/IL-2)-induced interferon (IFN)- γ ⁺ cells are detectable within CD4⁺ and CD8⁺ cell populations. Peripheral blood samples of two healthy volunteers were treated with SSG (20 μ g/mL) and IL-2 (30 U/mL) combination for 16 h prior to co-staining for intracellular IFN- γ and surface CD69, CD4, or CD8 followed by FACS analysis. Representative data of FACS analysis of CD4⁺IFN- γ ⁺ cells (A) and CD8⁺IFN- γ ⁺ cells (B) are shown. Induction of CD4⁺IFN- γ ⁺ cells or CD8⁺IFN- γ ⁺ cells in the peripheral blood of the two healthy donors are summarized (C).

FIG. 3. Induction of CD69-expressing cells in human peripheral blood. Peripheral blood samples from two healthy human donors were treated with sodium stibogluconate (SSG) (20 μ g/mL) and interleukin-2 (IL-2) (30 U/mL) combination for 16 h and then subjected to FACS analysis. CD69 expression in lymphocytes (A) and in CD4⁺ or CD8⁺ cell populations (B) are summarized (replicate samples).

in the healthy controls (average 26/0.1 mL blood). In addition, peripheral blood from melanoma patients as a group displayed markedly variable responses to IL-2 or SSG/IL-2 combination (Fig. 4).

These results demonstrate that the SSG/IL-2 combination was capable of inducing IFN- γ ⁺ cells in melanoma peripheral blood and revealed low basal levels of IFN- γ ⁺ cells in melanoma peripheral blood that suggested the preexistence of an IFN- γ ⁺ cell deficiency.

IFN- γ is required for SSG/IL-2 anti-Renca tumor activity in vivo

IFN- γ ⁺ T cells were previously implicated in the anti-Renca tumor action of SSG/IL-2 in mice based upon their induction by the agents and the requirement of T cells for the anti-Renca tumor effect of SSG/IL-2 (Fan and others 2005). Having found that IFN- γ ⁺ T cells were also inducible by the SSG/IL-2 in healthy and melanoma human peripheral blood (Figs 1–5), we assessed the requirement for IFN- γ for the anti-Renca tumor activity of SSG/IL-2 combination using an IFN- γ ^{-/-} mouse model.

Consistent with our previous report (Fan and others 2005), SSG/IL-2 had significant anti-Renca tumor activity ($P < 0.01$), inducing ~76% growth inhibition of Renca tumors in wild-type Balb/c mice (Fig. 6A). In contrast, Renca tumors showed comparable growth in Balb/c IFN- γ ^{-/-} mice untreated or treated with SSG/IL-2 (Fig. 6B), demonstrating a lack of anti-Renca tumor activity of the combination in the absence of the cytokine. In comparison to those in wild-type mice, the Renca tumors in IFN- γ ^{-/-} mice exhibited an apparently slower growth due to an undefined reason but might have been resulted from experimental variations given that

the wild-type mice IFN- γ ^{-/-} ones were inoculated and evaluated in two separate studies.

These results established a requirement for IFN- γ for the anti-Renca tumor activity of SSG/IL-2 in mice and provided additional evidence supporting a key role for activated immune cells, particularly IFN- γ ⁺ T cells, as antitumor mediators of the agents. Additional studies of longer treatment durations will lead to a more defined role for IFN- γ in the antitumor action of the combination treatment.

Discussion

Our previous studies have implicated IFN- γ ⁺ T cells, activated TH1 cells critical for antitumor immunity (Becker 2006), as significant mediators of SSG antitumor action in mice. In mouse models, anti-Renca tumor activity of the SSG/IL-2 combination was mediated by T cells (Fan and others 2005) and that IFN- γ ⁺ T cells were markedly induced in SSG/IL-2-treated mice (Fan and others 2005). The importance of this cell type has now been further underscored by the requirement of IFN- γ for anti-Renca tumor activity of the combination (Fig. 6).

Our work herein provided evidence for the first time that, as in mice, human peripheral IFN- γ ⁺ T cells were also significantly inducible by SSG via interacting with IL-2 *in vitro*. Whereas modestly effective as a single agent (Fig. 1A), SSG significantly augmented IL-2-induced IFN- γ ⁺ cells in human peripheral blood (Fig. 1B) that were verified to be CD4⁺ or CD8⁺ T lymphocytes (Fig. 2). This SSG activity was apparently selective for TH1 cells, since the numbers of cells expressing TH2 cytokine (IL-5⁺ cells) were not affected under comparable conditions (Fig. 1C). Importantly, it was effective in peripheral blood of healthy donors (Fig. 5) and

FIG. 4. Induction of interferon (IFN)- γ^+ cells in peripheral blood of melanoma patients. Peripheral blood from six melanoma patients (Cases 1–6) were untreated or treated with sodium stibogluconate (SSG), interleukin-2 (IL-2), or the combination for 16 h *in vitro* prior to quantification of IFN- γ^+ cells by ELISPOT assays. Data represent mean $\pm$ SD of replicate samples.

melanoma patients (Fig. 4). Since the activity was detectable under conditions that closely mimic *in vivo* drug exposure (Fig. 1), our results suggested that SSG might augment IL-2-induced IFN- γ^+ T cells and antitumor immunity in melanoma patients in a similar manner. Therefore, this work represents a significant extension of our previous studies in mouse models and provides additional supporting evidence for clinical evaluation of SSG as a novel anticancer agent that might improve the efficacy of IL-2 therapy and other cytokine therapy or immunotherapy. In this regard, it is interesting that SSG also augmented IFN- α -induced human peripheral IFN- γ^+ cells under comparable conditions (our unpublished data).

Our finding of the SSG activity to induce human peripheral IFN- γ^+ T cells has other implications as well. It designates human peripheral IFN- γ^+ T cells as a potentially important biomarker for SSG clinical investigations that could be monitored for assessing *in vivo* biological effects of the drug. Given the established role of immune cells in SSG antitumor action in mouse models (Fan and others 2005), levels of immune cell activation induced by SSG in cancer patients might correlate with clinical responses or side effects. For instance, cancer patients with more marked immune cell activation in response to SSG might show better disease response as well. Accordingly, induction of human peripheral IFN- γ^+ T cells *in vitro* could be exploited for pre-screening and identification of cases that might be more responsive

to the drug. In support of this notion, we detected substantial individual variations in the levels of peripheral IFN- γ^+ T cells induced by SSG/IL-2 *in vitro* (Figs. 4 and 5). Moreover, it also indicates that induction of human peripheral IFN- γ^+ T cells might be of value as a strategy to identify refined candidate cancer therapeutics from SSG analogs and other SHP-1-targeted molecules.

Since SSG was capable of augmenting cytokine activity to induce IFN- γ^+ T cells of both mouse (Fan and others 2005) and human origins (Figs. 1–5), it likely acted via a comparable mechanism of action in both species. The ability of SSG to augment cytokine activity is consistent with targeting a negatively regulatory PTPase like SHP-1 (Zhang and others 1999), which is known to down-regulate intracellular signaling of IL-2 and other cytokines (Yi and Ihle 1993; Yi and others 1993; David and others 1995; Klingmuller and others 1995; Yi and others 1995; Jiao and others 1997; Migone and others 1998). The notion of targeting SHP-1 by SSG to induce human peripheral IFN- γ^+ T cells is also supported by the heightened TH1 responses (Deng and others 2002; Kamata and others 2003; Yu and others 2005) in SHP-1-deficient mice (Shultz and others 1993; Tsui and others 1993). Comparative analysis of SSG-induced phosphotyrosine proteins, particularly those regulated by SHP-1, in murine and human immune cells will help to further define the mechanism of action of SSG in the future. It was noticed that SSG at a higher dose (100 μ g/mL) was less effective than its therapeutic

FIG. 5. Induction of interferon (IFN)- γ ⁺ cells in peripheral blood of healthy controls. Peripheral blood from six healthy volunteers (Donors 1–6) were untreated or treated with sodium stibogluconate (SSG), interleukin-2 (IL-2), or the combination for 16 h *in vitro* prior to quantification of IFN- γ ⁺ cells by ELISPOT assays. Data represent mean $\pm$ SD of replicate samples.

dose (20 μ g/mL) in augmenting IL-2-induced IFN- γ ⁺ cells (Fig. 1). Although the underlying mechanism has not been determined and might be related to the cytotoxicity of high doses of SSG (Yi and others 2002), it suggests a lack or limited benefit of SSG dose escalation in clinical applications. Interestingly, the capacity of SSG to induce human IFN- γ ⁺ T cells also reinforces the concept that SSG anti-*Leishmania* action might be mediated in significant part, if not exclusively, via activating host immune cells (Fan and others

2005). Accordingly, other PTPase-targeted compounds more effective in immune cell activation might be developed as improved anti-*Leishmania* therapeutics. Small chemicals capable of inducing human peripheral IFN- γ ⁺ cells might be particularly attractive candidates.

Another interesting result was an apparent deficiency in all 16 melanoma patients, who had low levels of peripheral IFN- γ ⁺ cells ($\sim$ 5% of the healthy controls) (Figs. 4 and 5). This deficiency was likely a preexisting condition of the

FIG. 6. Interferon (IFN)- γ is required for growth inhibition of murine Renca tumors by sodium stibogluconate/interleukin-2 (SSG/IL-2) combination. Balb/c mice of (A) wild-type or (B) IFN- γ -deficiency (IFN- γ ^{-/-}) with 4-day-established s.c. Renca tumors ($n = 8$ tumors, 2 tumors/mouse) were treated with SSG/IL-2 combination. Renca tumor volumes (mean $\pm$ SD) in these mice were recorded as indicated.

melanoma rather than immune suppression induced by clinical treatments or aggressive malignancy (Kim and others 2006), since the patients in the study had been off treatment for months with durable regression or stabilized disease. It might be a significant common defect in melanoma given its presence in all of the 16 patients in our study but not in the healthy controls. Immune deficiency is a contributing factor in the pathogenesis of several types of human malignancies, best illustrated by AIDS-associated Kaposi sarcoma and lymphoma (Cheung and others 2005). The presence of peripheral IFN- γ ⁺ cell deficiency in melanoma suggests its potential involvement in the pathogenesis and/or progression of melanoma that needs to be investigated in future study.

It is of interest that the melanoma peripheral IFN- γ ⁺ cell deficiency was partially corrected *in vitro* by IL-2, and more effectively by SSG/IL-2 combination (Fig. 4), demonstrating responsiveness to the cytokine and SSG despite the deficiency. This responsiveness might be intrinsic for individual patients and related to the favorable treatment outcome of the patients in our study. Cytokine therapy using IL-2 or IFN- α induces durable regression of melanoma (Borden 2000) and renal cancer (McDermott and Atkins 2006) in only small patient populations. Identification of patients likely to have positive responses prior to or at the early stages of treatment may improve the value and benefit of cytokine therapy. Future comparative evaluation of peripheral IFN- γ ⁺ cell responsiveness of patients with differential treatment outcomes will help to determine its significance as a biomarker for early identification of cases that would benefit from cytokine treatment.

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Author Disclosure Statement

Authors K.F. and E.B. declare no conflict; T.Y. has patent rights on SSG.

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