Cancer Immunol Immunother (2007) 56:1845–1852
DOI 10.1007/s00262-007-0329-9

ORIGINAL ARTICLE

STAT1-dependent and STAT1-independent gene expression in murine immune cells following stimulation with interferon-alpha

Jason M. Zimmerer · Gregory B. Lesinski · Michael D. Radmacher · Amy Ruppert · William E. Carson III

Received: 29 January 2007 / Accepted: 11 April 2007 / Published online: 15 May 2007

Abstract
Purpose The precise molecular targets of interferon-alpha (IFN-α) therapy of melanoma are unknown but likely involve signal transducer and activator of transcription 1 (STAT1) signal transduction within host immune effector cells. We hypothesized that microarray analysis could be utilized to identify candidate molecular targets important for mediating the anti-tumor effect of exogenously administered IFN-α.
Experimental Methods To identify the STAT1-dependent genes regulated by IFN-α, the gene expression profile of splenocytes from wild type (WT) and STAT1$^{-/-}$ mice was characterized.
Results This analysis identified 30 genes that required STAT1 signal transduction for optimal expression in response to IFN-α ($p < 0.001$). These genes include granzyme b (Gzmb), interferon regulatory factor 7 (Irf7), Fas death domain-associated protein (Daxx), and lymphocyte antigen 6 complex, locus C (Ly6c). The expression of 20 genes was found to be suppressed in the presence of STAT1 including chemokine ligand 2 (Ccl2), Ccl5, and Ccl7. Nineteen genes were significantly upregulated in murine splenocytes following treatment with IFN-α regardless of the presence of STAT1 including CD86, lymphocyte antigen 6 complex, locus A (Ly6a), and Tap binding protein (Tapbp). The expression of representative IFN-responsive genes was confirmed at the transcriptional level by Real Time PCR.
Conclusion This report is the first to demonstrate that STAT1-mediated signal transduction plays a major role in the transcriptional response of murine immune cells to IFNα.

Keywords Interferon-alpha · Oligonucleotide microarray analysis · STAT1 · Immune cells

J. M. Zimmerer
Integrated Biological Sciences Graduate Program,
The Ohio State University Comprehensive Cancer Center,
Columbus, OH 43210, USA

J. M. Zimmerer · G. B. Lesinski · W. E. Carson III
Human Cancer Genetics Program Department of Molecular Virology, Immunology, and Medical Genetics,
The Ohio State University Comprehensive Cancer Center,
Columbus, OH 43210, USA

M. D. Radmacher · A. Ruppert
Center for Biostastistics,
The Ohio State University Comprehensive Cancer Center,
Columbus, OH 43210, USA

W. E. Carson III (✉)
Division of Surgical Oncology, Department of Surgery,
The Ohio State University Comprehensive Cancer Center,
N924 Doan Hall, 410 West 10th Ave., Columbus, OH 43210, USA
e-mail: william.carson@osumc.edu

Introduction

Interferon-alpha (IFN-α) is used as an adjuvant therapy in patients with malignant melanoma following surgical resection of high-risk lesions (lymph node metastases or primary tumor thickness >4 mm). However, the precise molecular targets of exogenously administered IFN-α are unknown. IFN-α exerts direct anti-proliferative, pro-apoptotic, and anti-angiogenic effects on melanoma cells in culture, and has potent immunologic actions when administered in vivo [2, 3, 14, 28]. The binding of IFN-α to its heterodimeric receptor (IFNAR) activates Janus kinase 1 (Jak1) and Tyrosine kinase 2 (Tyk2) that in turn phosphorylate tyrosine residues on the cytoplasmic region of the receptor. These phosphotyrosine residues provide docking sites for the signal transducer and activator of transcription (STAT) family of proteins that are phosphorylated on tyrosine and serine residues by the activated Janus kinases [19]. The

prototypical IFN-α signaling reaction results in the formation of a DNA binding complex known as the interferon-stimulated gene factor 3 (ISGF3) that consists of tyrosine phosphorylated STAT1 (STAT1α or STAT1β), tyrosine phosphorylated STAT2, and a p48 binding protein, known as interferon regulatory factor 9 (IRF9) [9]. This complex then translocates to the cell nucleus and activates the transcription of IFN-responsive genes [25].

Our group has previously demonstrated that the anti-tumor effects of IFN-α are critically dependent on STAT1-mediated signal transduction within host immune cells [1, 23]. In these reports, tumoral expression of STAT1 had no bearing on the ability of IFN-α to prolong the survival of tumor-bearing mice. In distinct contrast, $STAT1^{-/-}$ mice could not utilize exogenous IFN-α to inhibit the growth of $STAT1^{+/+}$ melanoma cells. Thus, STAT1-mediated gene regulation within the host was most important to the anti-tumor effects of IFN-α in this experimental system. There is also compelling evidence from other groups to suggest that the immunostimulatory effects of IFN-α are a critical component of its anti-tumor activity [3-5, 11, 17, 18]. In fact, recent data have shown that the occurrence of autoimmune sequelae and the presence of tumor-infiltrating lymphocytes correlate with clinical response in patients receiving IFN-α [22, 30]. Together, these data suggest that the immunomodulatory actions are critical to the anti-tumor actions of this cytokine. However, a comprehensive analysis of gene regulation within immune effector cells following IFN-α treatment has not been reported. We have previously shown that activation of STAT1 within host immune cells is critical to the anti-tumor activity of IFN-α. With this in mind, we wished to identify the interferon-stimulated genes whose transcription in immune cells was dependent on STAT1 activity. We theorized that elucidation of these genes might provide insight into the mechanisms whereby IFN-α exerts its anti-tumor activity.

In the present study, microarray analysis was utilized to examine the differential gene expression within splenocytes from wild type (WT) and STAT1-deficient mice following in vitro treatment with IFN-α. These studies provide a transcriptional profile of IFN-α-stimulated murine immune effector cells and demonstrate that STAT1 is involved in both the induction and repression of multiple genes. This data may aid in understanding the mechanism by which IFN-α exerts its anti-tumor activity.

Materials and methods

Reagents

Universal Type I IFN (IFN-A/D, specific activity of 1.1×10^8 U/mg) was purchased from R&D Systems Inc., Minneapolis, MN, and used in murine experiments.

Animals

C57BL/6 mice were purchased from Taconic Farms Inc., Germantown, NY. $STAT1^{-/-}$ mice (C57BL/6 background) provided by the Dr. Durbin laboratory were generated by homologous recombination as previously described and housed in a pathogen-free environment [10].

Murine in vitro studies

All experiments were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Female mice (5-6 weeks of age) were used in all experiments. Spleens from C57BL/6 and $STAT1^{-/-}$ mice were removed aseptically and dispersed through 70 μM cell strainers. Splenocytes were washed with PBS, pelleted by centrifugation, and resuspended in RPMI-1640 supplemented with 10% FBS. Previous studies conducted in our laboratory indicated that IFN-α-induced signal transduction in immune cells was maximal following stimulation with 10^4 U/ml [24]. In addition, expression of well-characterized IFN-α-responsive genes (IFIT2 and ISG15) were greater following stimulation with IFN-α for 12 h vs. 6 h (data not shown). Thus, the in vitro studies employed a 12-h stimulation. Purified splenocytes were stimulated with either 10^4 U/ml IFN-A/D or PBS (negative control). Cells were harvested, lysed with TRIzol reagent (Invitrogen, Carlsbad, CA) and then processed for RNA extraction.

cRNA preparation and array hybridization

Mouse Genome U74Av2 Set GeneChips (Affymetrix, Santa Clara, CA), which query ~6,000 murine genes, were used for these analyses. The cRNA was synthesized as suggested by Affymetrix. Briefly, total RNA from cells was prepared in TRIzol (Invitrogen) followed by RNeasy purification (Qiagen, Valencia, CA). Double stranded cDNA was generated from 8 μg of total RNA using the Superscript Choice System kit according to the manufacturer's instructions (Invitrogen). Biotinylated cRNA was generated by in vitro transcription using the Bio Array High Yield RNA Transcript Labeling System (Enzo Life Sciences Inc., Farmingdale, NY). The cRNA was purified using the RNeasy RNA purification kit (Qiagen). cRNA was fragmented according to the Affymetrix protocol and the biotinylated cRNA was hybridized to U133A or U74va2 microarrays [29]. The arrays were then scanned (Affymetrix GMS418) and analyzed (GenePix Pro 4.0) according to Affymetrix protocols.

Data analysis

Raw data were collected with a confocal laser scanner (Hewlett Packard, Palo Alto, CA) and probe level data

was analyzed using dChip Version 1.3 [26]. Array normalization was performed using the invariant set procedure. Then, model-based expression indices (MBEI) were computed using the perfect match only model. Probe-set level data that was identified as an "array outlier" by dChip was omitted and considered to be missing data in subsequent analyses. Array quality characteristics (including percent array outliers, percent present calls and median intensity) were examined. After MBEI computation and log-transformation of the values, data were imported into BRB-ArrayTools Version 3.22 for subsequent statistical analysis. Probe sets receiving an Affymetrix "Absent" call for more than 50% of the specimens were omitted. Univariate paired *t*-tests were used to make comparisons between saline and IFN-α treatment conditions. A nominal significance level of 0.001 was employed. To determine whether specific genes were differentially regulated in the two groups of mice, the random variance *t*- and *F*-tests were utilized. The *F*-test assumes that different genes have different variances, but that these variances can be regarded statistically as independent samples from the same distribution. Using this assumption, we performed a comparison of the gene expression profiles of the STAT1-deficient and WT mice using a 0.001 level of significance.

Real time PCR

Gene expression estimates from the microarray experiments were validated by Real Time PCR for select genes. Following TRIzol extraction and RNeasy purification for microarray analyses, 2 μg of total RNA was reverse transcribed and the resulting cDNA was used as a template to measure gene expression by Real Time PCR using predesigned primer/probe sets (Assays On Demand; Applied Biosystems, Foster City, CA) and 2X Taqman Universal PCR Master Mix (Applied Biosystems) according to manufacturer's recommendations as previously described [8]. Pre-designed primer/probe sets for human β-actin were used as an internal control in each reaction well (Applied Biosystems). Real Time PCR reactions were performed in triplicate in a capped 96-well optical plate. Real Time PCR data was analyzed using the ABI PRISM® 7900 Sequence Detection System (Applied Biosystems).

Results

STAT1-mediated gene regulation in murine splenocytes

To identify candidate genes within host immune cells involved in mediating the STAT1-dependent immunomodulary effects of IFN-α, the gene expression profile of splenocytes from individual WT and STAT1$^{-/-}$ mice ($n = 3$) was examined following 12-h treatment with IFN-α (10^4 U/ml) or PBS (negative control; 6 mice and 12 oligonucleotide arrays were utilized). Microarray analysis of gene expression indicated that STAT1-deficiency within the host resulted in the impaired or altered expression of many genes involved in immune function and the response to viral pathogens. From these studies, three categories of genes were identified based on the importance of STAT1 in controlling their expression. Of note, the splenocytes used in this experiment were not pooled but were analyzed individually following treatment with either PBS or IFN-α. The profile of gene expression of individual STAT1$^{-/-}$ mice was quite similar (i.e., not significantly different) as determined by the paired *t*-test. Thus, the gene profiles were specific to the STAT1 genotype and were not unduly influenced by inter-individual variation.

STAT1-enhanced genes

Thirty genes were significantly up-regulated to a greater degree in response to IFN-α in WT mice as compared to STAT1$^{-/-}$ mice such that the ratio of expression in WT mice versus knockout (KO) mice was >2.0 ($P < 0.001$; Table 1). Included in this category were genes involved in the regulation of T-cell adhesion (Ly6c), natural killer (NK) cell and T-cell cytotoxicity (Gzmb), chemotaxis (Ccl3 and Ccrl2), and several genes involved in regulating the immune response (Ifit2, Isg20, and Irf7).

STAT1-suppressed genes

Twenty genes were significantly up-regulated to a greater degree in response to IFN-α in STAT1$^{-/-}$ mice as compared to WT mice such that the ratio of expression in WT mice versus KO mice was <0.5 ($P < 0.001$; Table 2). Included were genes that encoded negative regulators of Jak-STAT signal transduction (Socs3), genes involved in the suppression of alloreactive T-cell function (Arg-1), and genes contributing to chemotaxis (Ccl2, Ccl7, and Ccr5).

STAT1-independent genes

Nineteen genes were up-regulated to a similar degree in response to IFN-α in WT mice and STAT1$^{-/-}$ mice (such that the ratio of expression in WT mice versus KO mice was <2.0 and >0.5; Table 3). Included in this category were genes involved in transcriptional regulation (Ifi204), class I MHC antigen processing (Psmb9 and Tapbp), and genes involved in the ubiquitination of proteins (Ube2l6 and Zubr1). Of note, the majority of the genes in this group were expressed to a somewhat greater degree in WT mice.

Table 1 STAT1-enhanced genes: augmented up-regulation (>2-fold; $P < 0.001$) in WT splenocytes following IFN-α

Gene	Function	Geometric mean fold			Probe set
		KO	WT	WT/KO**	
Csprs	G-protein coupled receptor	1.3	5	3.85	101845_s_at
Gzmb	Cytolysis by natural killer cells and T cells	2	7.4	3.70	102877_at
Slfn3	Negative regulation of cell proliferation	2.2	7.1	3.23	98299_s_at
Ly6c	Positive regulation of CD8 T-cell adhesion	1	3.2	3.21	93077_s_at
Camk2b	Enhances T-cell proliferation/cytotoxicity	0.7	2.2	3.14	103562_f_at
LOC625360	Unknown	1.8	5.4	3.00	102254_f_at
Isg20	Antiviral resposne, exonuclease activity	2.7	7.9	2.92	103432_at
Sgcb	Membrane organization and biogenesis	1.2	3.4	2.83	93898_at
Ifit2	Immune response	3	8.5	2.83	103639_at
Csrp1	Actin cytoskeleton organization and biogenesis	0.8	2.2	2.75	160065_s_at*
Tcstv1	Unknown	1.2	3.3	2.75	94727_f_at
Daxx	Apoptosis, transcriptional repressor	1.5	4	2.67	96125_at
AF067061	Unknown	1.1	2.8	2.54	94749_f_at*
LOC673100	Unknown	0.8	2	2.50	160799_at
Ddit3	Regulation of transcription/apoptosis	0.9	2.2	2.44	101429_at
Slfn4	Negative regulation of cell proliferation	3.5	8.5	2.43	92315_at
Tcstv3	Unknown	1.1	2.6	2.36	96584_f_at
Lgals3bp	Scavenger receptor activity	1.7	4	2.35	97507_at
H2-T24	Endogenous antigen presentation	1.2	2.8	2.33	94746_at
Ccrl2	Chemotaxis, inflammation	1	2.3	2.30	93617_at
Lgals9	Cell adhesion, galactose binding	1.3	2.8	2.16	103335_at
Irf7	Transcription factor	4.8	10.1	2.11	104669_at*
G1p2	Chemotaxis, ubiquitin cycle	5.7	11.9	2.09	98822_at
Sh3bp2	NK cell-mediated cytotoxicity, TCR-mediated signaling	1.1	2.2	2.00	92975_at
Tor3a	Protein folding, ATP binding	2.1	4.1	1.95	96533_at
TRAFD1	Unknown	1.4	2.7	1.93	103254_at*
Irf1	Immune response, transcription factor	1.1	2.1	1.91	102401_at
Ube1l	Ubiquitin cycle, apoptosis	1.4	2.6	1.86	102279_at
Trim21	Ubiquitin-protein ligase activity	1.3	2.4	1.85	102678_at*
LOC673370	Unknown	1.9	3.3	1.74	93779_at

* Significant gene up-regulation was observed in multiple probe sets

** Significant difference in fold expression between mouse strains (random variance *t* and F tests; $P < 0.001$)

The expression of representative IFN-α-induced genes from each category was validated in WT and STAT1$^{-/-}$ splenocytes via Real Time PCR. The following genes were evaluated: Gzmb, Ifit2, Irf7, Ly6c, Sh3bp2 (STAT1-enhanced genes), Ccr5, Gadd45g, Ifi30, Nfil3, Socs3 (STAT1-suppressed genes), CD86, Ifi204, Igtp, Ly6a, and Tapbp (STAT1-independent genes). In response to IFN-α, the expressions of Gzmb, Ifit2, Irf7, Ly6c, and Sh3bp2 were less in STAT1$^{-/-}$ mice as compared to WT mice. Conversely, Ccr5, Gadd45g, Ifi30, Nfil3, and Socs3 were induced to a greater degree by IFN-α in STAT1$^{-/-}$ mice. Finally, CD86, Ifi204, Igtp, Ly6a, and Tapbp were induced to a similar degree in WT and STAT1$^{-/-}$ mice (Fig. 1a–c).

Discussion

The gene expression profile elicited by IFN-α in murine splenocytes was investigated using microarray analysis. These studies were conducted in an effort to identify genes that might be instrumental in mediating the STAT1-dependent anti-tumor effects of IFN-α [23]. Murine studies identified a panel of genes whose expression was enhanced by (or suppressed by) STAT1 signal transduction. In addition, a number of genes were found to be up-regulated independently of STAT1, that is, they were significantly induced in murine splenocytes regardless of the presence or absence of STAT1.

Table 2 STAT1-suppressed genes: augmented up-regulation (>2-fold; $P < 0.001$) in STAT1 KO splenocytes following IFN-α

Gene	Function	Geometric mean fold			Probe set
		KO	WT	KO/WT**	
Arg1	Alloreactive T-cell suppression	7.5	0.7	10.71	93097_at
Ccl7	Chemotaxis, inflammation	11.9	1.2	9.92	94761_at
Ccl2	Chemotaxis, inflammation	8.9	1.4	6.36	102736_at
Tgfbi	Cell cycle regulation, cellular adhesion	2	0.4	5.00	92877_at
Il18r1	Immune response	3.2	0.7	4.57	101144_at
Ccr5	Chemotaxis, inflammation	7.6	1.7	4.47	161968_f_at*
Gadd45g	Apoptosis, T-helper 1 cell differentiation	4.9	1.1	4.46	101979_at
SOCS3	Inhibition of Jak/STAT signaling	5.5	1.3	4.23	162206_f_at*
Dab2	Regulation of cell cycle	2	0.5	4.00	98045_s_at
AW061234	Unknown	2.2	0.6	3.67	103697_at
Eps8	Electron transport	2	0.6	3.33	103222_at
Ifi27	Antiviral response	8.3	2.6	3.19	92718_at
Il1rl1	Immune response	2.6	0.9	2.89	98500_at
Il1r2	ATP-binding cassette (ABC) transporter	2.2	0.8	2.75	102658_at
Il2ra	T-cell proliferation	2.4	0.9	2.67	101917_at
EST sequence	Unknown	3.1	1.2	2.58	92779_f_at
EST sequence	Unknown	2.8	1.1	2.55	92778_i_at
Ifi30	Immune response	2	0.8	2.50	97444_at
Tgtp	GTPase	9.9	4.6	2.15	102906_at
Nfil3	Cellular survival	2.5	1.2	2.08	102955_at

* Significant gene up-regulation was observed in multiple probe sets

** Significant difference in fold expression between mouse strains (random variance *t* and *F*-tests; $P < 0.001$)

Table 3 STAT1-independent genes: up-regulation (>2-fold; $P < 0.001$) in WT and/or STAT1 KO splenocytes following IFN-α

Gene	Function	Geometric mean fold			Probe set
		KO	WT	WT/KO	
Igtp	Immune response	2.6	5.0	1.92	160933_at
Ube2I6	Ubiquitin cycle	1.2	2.2	1.83	102279_at*
Zubr1	Ubiquitin cycle metabolism	1.2	2.2	1.83	104041_at
Ifi204	Transcriptional co-activator, antiviral	2.4	4.3	1.79	98466_r_at
Wars	Protein biosynthesis	1.2	2.1	1.75	98605_at*
Tapbp	MHC class I molecules linkage to TAP	1.2	2.1	1.75	102689_at
Tyki	Thymidylate kinase activity	2.8	4.7	1.68	103066_at
Stxbp3	Glut4 translocation	1.2	2.0	1.67	92648_at
Tpst1	Inflammatory response	1.4	2.2	1.57	103032_at
BC094916	Unknown	1.8	2.8	1.56	103615_at
AI481105	Transcription regulation	2.0	3.1	1.55	102965_at
Ms4a4c	Signal transduction	2.0	3.1	1.55	98373_at
Cd86	T-cell stimulation, inflammatory response	1.3	2.0	1.54	102831_s_at
EST sequence	Unknown	1.3	2.0	1.54	103517_at
Kif9	Microtubule motor	1.3	2.0	1.54	161035_at
Pml	Regulation of transcription	1.9	2.9	1.53	99015_at
Psmb9	Protein catabolism, processing of class I MHC peptides	1.8	2.5	1.39	93085_at
Samhd1	Immune response	1.9	2.6	1.37	103080_at
Ly6a	Cellular adhesion	3.1	2.2	0.71	93078_at

* Significant gene up-regulation was observed in multiple probe sets

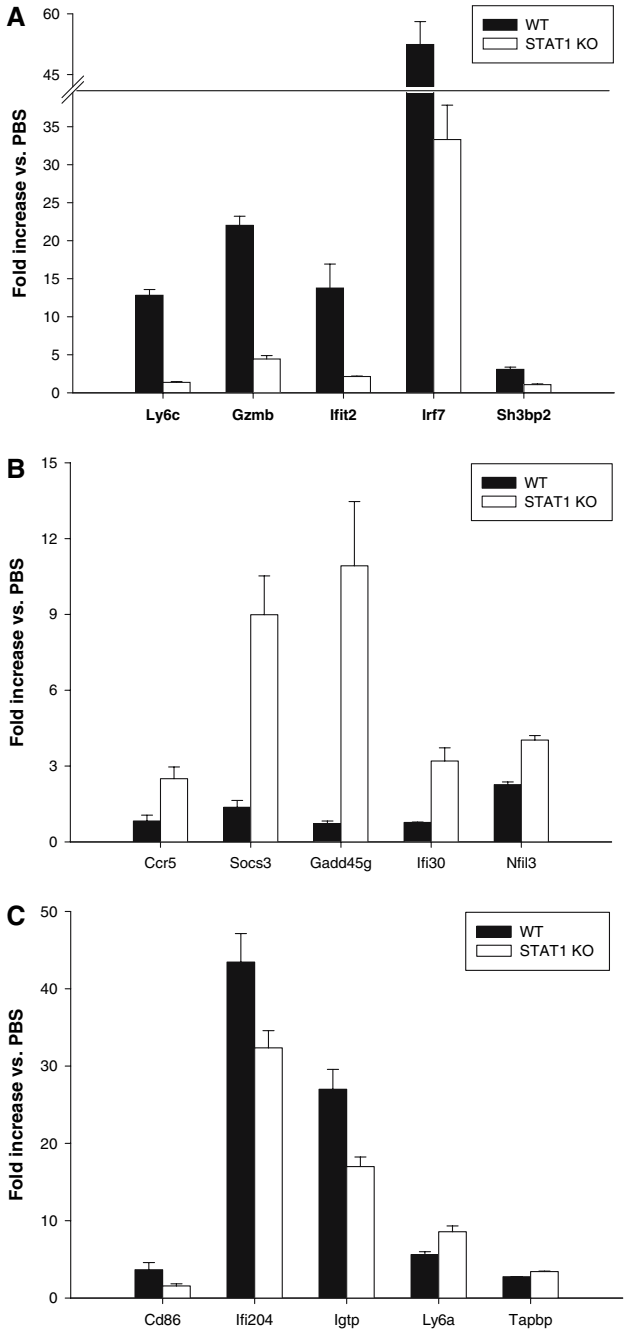

Fig. 1 Real time PCR validation of murine microarray data. Real Time PCR analysis was used to validate the expression of **A** Gzmb, Ifit2, Irf7, Ly6c, and Sh3bp2 (STAT1-enhanced genes), **B** Ccr5, Gadd45g, Ifi30, Nfil3, and Socs3 (STAT1-suppressed genes), and **C** CD86, Ifi204, Igtp, Ly6a, and Tapbp (STAT1-independent genes) in WT and STAT1$^{-/-}$ splenocytes following treatment with IFN-α. Data were expressed as the mean fold increase relative to baseline levels (PBS treatment). All real time PCR data were normalized to the level of β-actin mRNA (housekeeping gene). *Error bars* denote the standard deviations of triplicate experiments

Mice with genetic deficiencies are an important tool for analyzing the role of specific transcription factors and signaling pathways in the response of immune effectors to cytokine stimulation [15, 33]. Previous studies from our laboratory have demonstrated that elimination of STAT1 signal transduction within mouse immune effector cells completely abrogated the anti-tumor effects of IFN-α in a murine model of malignant melanoma [23]. Thus, it is likely that the interferon-stimulated genes identified in this study as being significantly induced or repressed by STAT1 play a role in the elimination of tumor cells by activated immune effectors. Both NK cells and T cells have been implicated as mediators of the anti-tumor effects of IFN-α [6, 7, 32]. Thus in order to further characterize the immunostimulatory effects of IFN-α and the ability of this cytokine to promote the elimination of malignant cells, it will be important in future studies to examine the IFN-α-induced gene expression profile of specific immune compartments over time in both murine tumor models and patients with cancer. Importantly, our studies of IFN-α gene expression in human cancer patients indicate significant overlap with the present study [37]. Of the 50 murine genes whose regulation was dependent on STAT1 (30 STAT1-enhanced and 20 STAT1-suppressed), 19 genes have been identified in the PBMCs of patients receiving IFN-α [36, 37]. These included human homologues of Csprs, G1p2, Gzmb, Ifi27, Ifi30, Ifi204, Ifit2, Igtp, Isg20, Lgals3bp, Lgals9, Irf1, Irf7, Nfil3, Pml, Socs3, Tgtp, Trim21, and Wars.

In the present study, defects in STAT1 signal transduction led to the decreased transcription of 30 genes pertaining to immune cell function, including genes with well-documented effects on cytolytic T-cell function (Ly6c) and the cytolytic activity of T cells and NK cells (granzyme B). Ly6c is a hemopoietic cell differentiation antigen that is expressed on a subset of peripheral CD8$^+$ T cells. It is involved in cytolytic T-cell elimination of target cells, enhances T-cell receptor-induced production of IL-2 and IFN-γ in CD8$^+$ T cells, and regulates homing of CD8$^+$ T cells in vivo. Jaakkola et al. have shown that cross-linking of Ly6c causes clustering of LFA-1 (CD11a/CD18) on the surface of CD8$^+$ T cells and thereby augments lymphocyte adhesion to endothelium and trafficking to lymph nodes [21]. Granzyme B is essential for the cytolysis of malignant and virally infected cells by T cells and NK cells [31]. The exocytosis of death-inducing granzymes that are stored in the granules of cytotoxic lymphocytes allows the immune system to rapidly eliminate transformed cells. The membrane-disrupting protein perforin permits the entry of granzymes into the target cell, where they induce mitochondrial dysfunction and subsequent apoptosis by cleaving target proteins in the cytoplasm and nucleus. Further studies will be needed to determine whether the reduced expression of other STAT1-dependent genes listed in Table 1 amplifies the immune deficiency that comes with the reduced expression of Ly6c and granzyme B in IFN-stimulated STAT1$^{-/-}$ splenocytes.

We have shown that IFN-α induces several genes involved in the ubiquitin cycle (Ube1l, Ube2l6, Trim21, and Zurbr1), MHC class I expression (Tapbp and Psmb9), and the co-stimulation and clonal expansion of antigen specific T cells (CD86 and Camk2b). The ubiquitination of cellular proteins is a critical first step in the presentation of antigens within the context of MHC class I [16]. Similarly, Psmb9 is a protease that cleaves proteins into peptides of appropriate length for loading onto MHC class I [20]. Tapbp is a key regulator of antigen transport and is associated with MHC class I in the endoplasmic reticulum [34]. Additional co-stimulatory signals are typically required for proper activation of antigen specific T cells. Co-stimulation of T cells via CD86 (B7.2) expressed on antigen-presenting cells can enhance the activation of effector T cells [13]. Upon antigen recognition, Camk2b is involved in T-cell receptor signaling and the autocrine production of IL-2 production for the clonal expansion of activated T cells [27]. IFN-α also activates NK cell cytotoxicity and proliferation. This is likely mediated in part by up-regulation of Sh3bp2, which associates with CD244 and potentiates NK cell cytotoxicity [35].

Interferon-alpha was also able to inhibit the expression of multiple genes in murine splenocytes in a STAT1-dependent fashion, most notably arginase I. L-Arginine plays a central role in several biological systems including the regulation of T-cell function. The release of arginase I by myeloid suppressor cells leads to depletion of L-Arginine from the tumor microenvironment which in turn exerts a suppressive effect on T-cell proliferation and cytokine synthesis [12]. Reduced expression of arginase I following exposure of immune effector cells to IFN-α might therefore be expected to have an overall stimulatory effect on specific immunity.

We observed that several genes were regulated in a STAT1-independent fashion by IFN-α. This has been previously demonstrated for IFN-γ. Gil et al. and Ramana et al. compared the ability of WT and STAT1-null mouse bone-marrow-derived macrophages or embryonic fibroblast cell lines to respond to IFN-γ. They demonstrated that a 1-h treatment with IFN-γ induced the expression of over 51 genes independently of STAT1 [15]. Two of these genes, Gadd45g (regulator of apoptosis) and SOCS3 (an inhibitor of Jak-STAT signal transduction) were also induced by IFN-α in both WT and STAT1-deficient splenocytes [15, 33].

The present study has demonstrated the role of STAT1 signaling in the transcriptional profile of murine immune cells in response to IFN-α. While the transcription of many genes are not affected by STAT1 expression, some genes are induced or suppressed in the absence of STAT1 following IFN-α stimulation. Genes that require STAT1 for optimal response are likely critical for the anti-tumor response of IFN-α. We are currently using murine models to evaluate the role that these species might play in the anti-tumor effects of IFN-α.

Acknowledgments The authors thank The Ohio State University Comprehensive Cancer Center (CCC) Microarray Core Facility for performing cRNA hybridization and the raw data collection. We also thank The Ohio State University CCC Real Time Core Facility for assisting in the operation of the ABI PRISM 7900 Sequence Detection System. Some statistical analyses were performed using BRB-ArrayTools Version 3.22 developed by Dr. Richard Simon and Amy Peng Lam. This work was supported by National Institutes of Health (NIH) Grants K24 CA93670, P30-CA16058 (to WE Carson) and a Career Development Award from the Melanoma Research Foundation (to GB Lesinski).

References

1. Badgwell B, Lesinski GB, Magro C, Abood G, Skaf A, Carson W III (2004) The antitumor effects of interferon-alpha are maintained in mice challenged with a STAT1-deficient murine melanoma cell line. J Surg Res 116:129–136
2. Bauer JA, Morrison BH, Grane RW, Jacobs BS, Borden EC, Lindner DJ (2003) IFN-alpha2b and thalidomide synergistically inhibit tumor-induced angiogenesis. J Interferon Cytokine Res 23:3–10
3. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM (2002) Interferon-alpha in tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13:119–134
4. Belardelli F, Gresser I, Maury C, Maunoury MT (1982) Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. I. Int J Cancer 30:813–820
5. Belardelli F, Gresser I, Maury C, Maunoury MT (1982) Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. II. Role of host mechanisms. Int J Cancer 30:821–825
6. Biron CA (2001) Interferons alpha and beta as immune regulators–a new look. Immunity 14:661–664
7. Brassard DL, Grace MJ, Bordens RW (2002) Interferon-alpha as an immunotherapeutic protein. J Leukoc Biol 71:565–581
8. Carr DJ, Chodosh J, Ash J, Lane TE (2003) Effect of anti-CXCL10 monoclonal antibody on herpes simplex virus type 1 keratitis and retinal infection. J Virol 77:10037–10046
9. Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421
10. Durbin JE, Hackenmiller R, Simon MC, Levy DE (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 84:443–450
11. Fallarino F, Gajewski TF (1999) Cutting edge: differentiation of antitumor CTL in vivo requires host expression of Stat1. J Immunol 163:4109–4113
12. Frey AB (2006) Myeloid suppressor cells regulate the adaptive immune response to cancer. J Clin Invest 116:2587–2590
13. Fuse S, Obar JJ, Bellfy S, Leung EK, Zhang W, Usherwood EJ (2006) CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68. J Virol 80:9159–9170
14. Garbe C, Krasagakis K, Zouboulis CC, Schroder K, Kruger S, Stadler R, Orfanos CE (1990) Antitumor activities of interferon alpha, beta, and gamma and their combinations on human melanoma cells in vitro: changes of proliferation, melanin synthesis, and immunophenotype. J Invest Dermatol 95:231S–237S

15. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, Virgin HW, Schreiber RD (2001) Biologic consequences of Stat1-independent IFN signaling. Proc Natl Acad Sci USA 98:6680–6685
16. Grant EP, Michalek MT, Goldberg AL, Rock KL (1995) Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. J Immunol 155:3750–3758
17. Gresser I, Kaido T, Maury C, Woodrow D, Moss J, Belardelli F (1994) Interaction of IFN alpha/beta with host cells essential to the early inhibition of Friend erythroleukemia visceral metastases in mice. Int J Cancer 57:604–611
18. Gresser I, Maury C, Carnaud C, De Maeyer E, Maunoury MT, Belardelli F (1990) Anti-tumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend erythroleukemia cells. VIII. Role of the immune system in the inhibition of visceral metastases. Int J Cancer 46:468–474
19. Haque SJ, Williams BR (1998) Signal transduction in the interferon system. Semin Oncol 25:14–22
20. Imanishi T, Kamigaki T, Nakamura T, Hayashi S, Yasuda T, Kawasaki K, Takase S, Ajiki T, Kuroda Y (2006) Correlation between expression of major histocompatibility complex class I and that of antigen presenting machineries in carcinoma cell lines of the pancreas, biliary tract and colon. Kobe J Med Sci 52:85–95
21. Jaakkola I, Merinen M, Jalkanen S, Hanninen A (2003) Ly6C induces clustering of LFA-1 (CD11a/CD18) and is involved in subtype-specific adhesion of CD8 T cells. J Immunol 170:1283–1290
22. Koon H, Atkins M (2006) Autoimmunity and immunotherapy for cancer. N Engl J Med 354:758–760
23. Lesinski GB, Anghelina M, Zimmerer J, Bakalakos T, Badgwell B, Parihar R, Hu Y, Becknell B, Abood G, Chaudhury AR, Magro C, Durbin J, Carson WE III (2003) The antitumor effects of IFN-alpha are abrogated in a STAT1-deficient mouse. J Clin Invest 112:170–180
24. Lesinski GB, Kondadasula SV, Crespin T, Shen L, Kendra K, Walker M, Carson WE III (2004) Multiparametric flow cytometric analysis of inter-patient variation in STAT1 phosphorylation following interferon Alfa immunotherapy. J Natl Cancer Inst 96:1331–1342
25. Levy D, Reich N, Kessler D, Pine R, Darnell JE Jr (1988) Transcriptional regulation of interferon-stimulated genes: a DNA response element and induced proteins that recognize it. Cold Spring Harb Symp Quant Biol 53(Pt 2):799–802
26. Li C, Wong WH (2003) The analysis of gene expression data: methods and software. DNA-Chip Analyzer (dChip)
27. Lin MY, Zal T, Ch'en IL, Gascoigne NR, Hedrick SM (2005) A pivotal role for the multifunctional calcium/calmodulin-dependent protein kinase II in T cells: from activation to unresponsiveness. J Immunol 174:5583–5592
28. Maellaro E, Pacenti L, Del Bello B, Valentini MA, Mangiavacchi P, De Felice C, Rubegni P, Luzi P, Miracco C (2003) Different effects of interferon-alpha on melanoma cell lines: a study on telomerase reverse transcriptase, telomerase activity and apoptosis. Br J Dermatol 148:1115–1124
29. Moschella F, Bisikirska B, Maffei A, Papadopoulos KP, Skerrett D, Liu Z, Hesdorffer CS, Harris PE (2003) Gene expression profiling and functional activity of human dendritic cells induced with IFN-alpha-2b: implications for cancer immunotherapy. Clin Cancer Res 9:2022–2031
30. Moschos SJ, Edington HD, Land SR, Rao UN, Jukic D, Shipe-Spotloe J, Kirkwood JM (2006) Neoadjuvant treatment of regional stage IIIB melanoma with high-dose interferon alfa-2b induces objective tumor regression in association with modulation of tumor infiltrating host cellular immune responses. J Clin Oncol 24:3164–3171
31. Mullbacher A, Waring P, Tha Hla R, Tran T, Chin S, Stehle T, Museteanu C, Simon MM (1999) Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc Natl Acad Sci USA 96:13950–13955
32. Parronchi P, Mohapatra S, Sampognaro S, Giannarini L, Wahn U, Chong P, Maggi E, Renz H, Romagnani S (1996) Effects of interferon-alpha on cytokine profile, T cell receptor repertoire and peptide reactivity of human allergen-specific T cells. Eur J Immunol 26:697–703
33. Ramana CV, Gil MP, Han Y, Ransohoff RM, Schreiber RD, Stark GR (2001) Stat1-independent regulation of gene expression in response to IFN-gamma. Proc Natl Acad Sci USA 98:6674–6679
34. Rizvi SM, Raghavan M (2006) Direct peptide-regulatable interactions between MHC class I molecules and tapasin. Proc Natl Acad Sci USA 103:18220–18225
35. Saborit-Villarroya I, Del Valle JM, Romero X, Esplugues E, Lauzurica P, Engel P, Martin M (2005) The adaptor protein 3BP2 binds human CD244 and links this receptor to Vav signaling, ERK activation, and NK cell killing. J Immunol 175:4226–4235
36. Zimmerer JM, Lesinski GB, Kondadasula SV, Karpa VI, Lehman A, Raychaudhury A, Becknell B, Carson WE III (2007) IFN-{alpha}-Induced signal transduction, gene expression, and antitumor activity of immune effector cells are negatively regulated by suppressor of cytokine signaling proteins. J Immunol 178:4832–4845
37. Zimmerer JM, Lesinski GB, Kornacker K, Shen L, Liyanarachichi S, Durbin J, Kendra K, Walker M, Carson WE (2003) Gene expression profiling of the response to interferon-alpha immunotherapy. American Association for Cancer Research Annual Meeting, Orlando, FL [Abstract no. 4679]