Microenvironment and Immunology

A Requirement of STAT3 DNA Binding Precludes Th-1 Immunostimulatory Gene Expression by NF-κB in Tumors

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Abstract

Both STAT3 and NF-κB are persistently activated in diverse cancers and promote tumor cell proliferation, survival, angiogenesis, and metastasis through transcriptional activation of multiple common genes. Paradoxically, STAT3 also suppresses many NF-κB–inducible genes involved in innate and adaptive antitumor immunity in spite of elevated levels of NF-κB in tumors. In this study, we show that expression of many NF-κB downstream target genes in tumors depends on STAT3 DNA binding. When STAT3 is elevated in tumor cells and tumor-infiltrating immune cells, persistently activated NF-κB interacts with STAT3 and preferentially binds to genes with STAT3-binding site(s) in promoters. A large number of NF-κB downstream genes associated with oncogenesis and chronic inflammation contain STAT3 DNA-binding site(s). However, in contrast, many genes frequently associated with antitumor immunity lack STAT3 DNA-binding site(s) and can only be activated by NF-κB when STAT3 is inhibited in tumors. The introduction of STAT3 DNA-binding sequences by site-specific mutagenesis in an immunostimulatory gene promoter allows its transcriptional activation by NF-κB in tumor cells. Furthermore, STAT3 facilitates NF-κB binding to genes that are important for tumor growth while inhibiting its binding to Th-1 immunostimulatory genes in growing tumors, including in tumor-infiltrating immune cells. The results of this study provide insight into how some of the oncogenic/inflammatory and Th-1 immunostimulatory genes are differentially regulated in cancer. Cancer Res; 71(11); 3772–80. ©2011 AACR.

Introduction

The importance of the human host immune system in both suppressing tumor incidence/growth and promoting malignant transformation has been well established (1–4). However, the underlying molecular mechanisms that coordinate such complex and opposing immune regulations have only begun to emerge. STAT proteins are central in regulating both complex and opposing immune regulations have only begun to emerge. STAT proteins are central in regulating both antagonistic pathways of inflammation (4, 7, 9). Additionally, STAT3 can inhibit expression of several tumor suppressor genes and many Th-1 immunostimulatory molecules (4, 5, 10–13). Although some studies have indicated that STAT3 can suppress expression of certain tumor suppressor genes by mediating DNA methylation (14), the mechanism by which STAT3 might inhibit transcriptional activity of Th-1 immunostimulatory genes remains to be elucidated.

Parallel to STAT3, NF-κB has been recognized as a core transcription factor for pro-oncogenic and procancer inflammatory genes (4, 15, 16). In fact, the 2 transcription factors regulate many of the same oncogenic/procancer inflammatory genes (4, 16). However, in contrast to STAT3, NF-κB is also fundamental for inducing expression of Th-1 mediators important for antitumor immune responses (4, 7, 16). Interestingly, many of the Th-1 immunostimulatory genes regulated by NF-κB are inhibited by STAT3, in normal immune cells as well as in tumor cells and tumor-infiltrating immune cells (4–7, 12, 13). STAT3 has been shown to suppress IkB kinase (IKK) activity, which contributes to inhibition of NF-κB transcriptional activity in both immune cells and tumor cells (12, 17). However, STAT3 and NF-κB activities are elevated in tumor cells as well as in tumor-infiltrating immune cells (4, 17), raising the question of how STAT3 differentially regulates oncogenic/inflammatory versus Th-1 immunostimulatory genes inducible by NF-κB.

During tumorigenesis and cancer-related chronic inflammation, STAT3 and NF-κB reciprocally regulate each other at multiple levels (4, 8, 12, 13, 18, 19). Continuous STAT3 activation in tumor cells and, in particular, in immune cells in the
tumor microenvironments can be maintained by several factors induced by NF-kB, such as interleukin (IL)-6, COX-2, and IL-23 (4, 8, 16, 20–22). On the other hand, STAT3 mediates constitutive NF-kB activation in tumors by promoting nuclear accumulation of NF-kB through p300-mediated RelA acetylation (4, 16, 17). However, the question remains with regard to how STAT3 promotes a set of NF-kB–regulated genes while inhibiting another set of NF-kB–regulated genes in tumor cells and tumor-infiltrating immune cells. In this study, we identified a molecular mechanism by which concurrent activation of STAT3 and NF-kB in tumors exerts distinct regulation on expression of Th-1 immunostimulatory versus pro-oncogenic/immunosuppressive genes. We show, in both tumor cells and tumor-infiltrating myeloid cells, that STAT3 DNA binding is crucial for the activation of NF-kB inducible genes. The lack of STAT3 DNA-binding sites(s) in many of the genes generally associated with Th-1 immune responses precludes their expression in tumor settings where STAT3 is activated and is in the same complex with NF-kB and p300. Only by either freeing NF-kB from the STAT3–p300 complex or introducing STAT3 DNA-binding site(s) to the promoter of a Th-1 immunostimulatory gene can transcriptional activation of the immunostimulatory genes be conferred in the tumor setting. Our studies provide insight into how a number of cancer-promoting and antitumor immunostimulatory genes are differentially regulated by STAT3.

Materials and Methods

Materials

An A2058 human melanoma cell line was obtained from the American Type Cell Culture. The DC-2.4 mouse dendritic cell line was provided as a generous gift by Dr. K.L. Rock (University of Massachusetts Medical School, Worcester, MA). C-4 mouse melanoma cells were kindly provided by Dr. I. Fidler (University of Texas, MD Anderson Cancer Center, Houston, TX). Cells were maintained in RPMI 1640 supplemented with 10% FBS. The DC-2.4 mouse dendritic cell line was maintained under pathogen-free conditions in accordance with procedures were carried out under pathogen-free conditions in accordance with procedures.

In vitro translated p300 proteins were prepared by TnT T7/Sp6-Coupled Wheat Germ Extract System (Promega), using pCMVβ-HA-p300 (Millipore) as the template. Ten microliters of the translation mixture was incubated with either recombinant protein (50 ng) or nuclear extracts from A2058 cells (5 μg) in 500 μL of binding buffer [20 mmol/L Tris, pH 8.0; 60 mmol/L NaCl, 1 mmol/L EDTA, 6 mmol/L MgCl₂, 1 mmol/L dithiothreitol (DTT), 8% glyceral, and 0.05% NP-40]. Immune complexes were pulled down with anti-HA antibody and then washed 3 times with binding buffer, followed by SDS-PAGE. Furthermore, p300-bound proteins were visualized by Western blotting analysis with the indicated antibodies.

In vivo experiments

Mouse care and experimental procedures were carried out under pathogen-free conditions in accordance with
established institutional guidelines and approved protocols from the Institutional Animal Care and Use Committee of the Beckman Research Institute at the City of Hope Medical Center, Duarte, CA. Mx1-Cre mice were obtained from Jackson Laboratory, and Stat3floxflox mice were generously provided by Drs. Shizuo Akira and Kiyoshi Takeda. Stat3+/− or Stat3−/− mice were generated and challenged with B16 tumor cells as described previously (6, 17). Experimental details are described in Supplemental Methods.

Isolation of immune cells
Purification of specific immune subsets was described previously (6, 17), and detailed experimental procedures are also provided in the Supplemental Methods.

Real-time reverse transcriptase-PCR
Total RNA was prepared from isolated immune cells or whole tumors for real-time PCR analysis as indicated. RNA (0.5–1 μg) was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad), and real-time PCR reactions were conducted using iQ SYBR Green Supermix (Bio-Rad) on DNA Engine Thermal Cycler equipped with Chromo4 detector (Bio-Rad). Gene-specific primer sets were purchased from SA Bioscience. Either 18S rRNA or Gapdh housekeeping genes were used as internal controls to normalize mRNA expression. Each independent experiment was pooled to calculate average and SEM. Representative data for 3 independent experiments are shown in figures.

Results and Discussion

Interaction with STAT3 allows NF-κB binding to oncogenic genes while preventing its binding to immunostimulatory genes
Although STAT3 and NF-κB (RelA) are known to regulate many of the same genes involved in proliferation, survival, invasion, and chronic inflammation (4, 7, 15, 16), whether binding of NF-κB to the kB sites in these genes generally depends on STAT3 has not been directly examined. To address this question, we explored whether STAT3 could complex with NF-κB at kB sites and how that might affect NF-κB binding to various oncogenic/immunostimulatory genes. ChIP assays were carried out in A2058 human melanoma cells transfected with either control or STAT3 siRNA, which efficiently reduced protein expression levels of STAT3 but not of RelA and STAT1 (Supplementary Fig. S1A). We analyzed the promoters of antiapoptotic genes BIRC5 (SURVIVIN), BCL2L1 (BCL-x), angiogenic/immunosuppressive gene VEGF, and genes associated with both innate immunity and inflammation-induced carcinogenesis, Il6 (Il-6), ICAM, IL1B (Il-1β), and CSF1 (M-CSF). Both STAT3 and RelA interacted with these promoters and knocking down STAT3 decreased RelA interaction with the promoters (Fig. 1A).

Many NF-κB downstream Th-1 immunostimulatory genes, such as IFNB (IFN-β), CXCL10 (IP-10), IFNG (IFN-γ), CD86, IL12 (II-12), and CCL5 (RANTES), are inhibited in both tumor cells and tumor-infiltrating immune cells by STAT3 (4–7, 13, 24, 25). We, therefore, assessed how STAT3 might impact NF-κB (RelA) binding to the kB sites within these genes. ChIP assays indicated that, in contrast to the oncogenic/immunostimulatory genes, STAT3 did not interact with the promoters of IFNB, CXCL10, and CCL5 (Fig. 1B). Furthermore, STAT3 knockdown increased RelA binding to IFNB, CXCL10, and CCL5 promoters (Fig. 1B), suggesting that STAT3 inhibits RelA binding to the kB site(s) within these Th-1 immunostimulatory genes. To test this hypothesis, we prepared chromatin fractions from A2058 tumor cells transfected with either control or STAT3 siRNA in the presence or absence of TNF-α to compare STAT3–NF-κB DNA binding in the BIRC5 and CCL5 promoters. Previous studies have shown that treatment of A2058 tumor cells with TNF-α further stimulates NF-κB activity but has no effect on STAT3 activation (26). TNF-α had no effects on STAT3 protein expression level (Supplementary Fig. S1B), and STAT3 was not associated with TNF-α-activated NF-κB complex bound to the CCL5 promoter (17, 27). Consistent with these findings, STAT3 did not interact with TNF-α-activated RelA bound to the CCL5 promoter in A2058 tumor cells (Fig. 1C). In the absence of STAT3, RelA was bound to the CCL5 promoter and could be further induced following TNF-α treatment (Fig. 1C). On the other hand, RelA and STAT3 were continuously bound to BIRC5 promoter, and silencing STAT3 with siRNA decreased STAT3–RelA recruitment to the promoter (Fig. 1C). To test whether, in the absence of STAT3 protein complexes, NF-κB (RelA) could bind to the CCL5 promoter, we depleted STAT3 protein complexes in chromatin fractions by anti-STAT3 antibody (STAT3-unbound chromatin) before ChIP assay. Results showed that NF-κB could only be detected in the "STAT3-unbound" chromatin fraction in TNF-α-treated A2058 tumor cells transfected with STAT3 siRNA (Fig. 1D). In contrast, RelA binding to the BIRC5 promoter was only detectable with STAT3 complex but not in the "STAT3-unbound" chromatin fraction (Fig. 1D).

STAT3 and NF-κB interact with p300, which is important for binding of NF-κB to kB-site stably (17). Based on these findings, we considered the possibility that, once incorporated into the complex with STAT3 and p300, RelA might not bind efficiently to the Th-1 immunostimulatory genes. In A2058 tumor cells, p300 was physically associated with STAT3–NF-κB in the promoter of BIRC5 (Fig. 1E). However, when STAT3 gene was silenced, neither p300 nor RelA could bind to the BIRC5 promoter. In contrast, the STAT3–RelA–p300 complex was not detectable on the promoter of the immunostimulatory gene Cxcl10 in DC2.4 dendritic cells treated with TNF-α, although RelA could bind to the Cxcl10 promoter, without p300 (Fig. 1E). Furthermore, recombinant RelA protein did not directly interact with p300 unless STAT3 protein was present (Fig. 1F), whereas the STAT3–RelA complex was bound to p300 in the nuclei of A2058 tumor cells (Fig. 1F). Taken together, in tumor cells with persistently activated STAT3, the STAT3–NF-κB–p300 protein complex preferentially binds to the promoters of many genes involved in tumor growth/inflammation but not with those that are mainly associated with Th-1 immunostimulation. The importance of CBP–p300 in promoting RelA-mediated expression of genes involved in inflammation has been previously reported (28). Our results confirmed these findings and further suggest the importance
of STAT3 in determining the preferential regulation of pro-
cancer inflammatory versus Th-1 immunostimulatory genes.

**NF-κB–induced gene transcription requires STAT3**

**DNA-binding site(s)**

Results shown so far indicated that many genes that are
upregulated by NF-κB in tumor cells require STAT3 binding
to the promoters. In sharp contrast, NF-κB can only bind to
many of the Th-1-immunostimulatory genes when STAT3 is
inhibited. One possible explanation for the observations is
that the STAT3–NF-κB–p300 can only activate genes if they
have STAT3 DNA-binding site(s) in their promoter regions.

To test this hypothesis, we conducted computational ana-
lyses to include all NF-κB target genes associated with
oncogenesis listed in a review (15) and compared them with
lipopolysaccharide (LPS)-inducible NF-κB target genes often
associated with acute infection and antitumor immunity.
We first determined, using information from previous
reports, whether these NF-κB target genes are regulated
by RelA–p65 or by other NF-κB subunits. Next, we assessed
whether the RelA-regulated genes have STAT3-binding site
(s) and whether their expression is regulated by STAT3,
based on published reports and computational sequence
analysis.
The majority of the analyzed NF-κB RelA (p65/p50) target genes that have STAT3-binding sites are frequently associated with tumorigenesis (Supplementary Table S2). Moreover, most of the oncogenic NF-κB RelA (p65/p50) target genes with STAT3-binding sites have been reported to require STAT3 for expression (Supplementary Table S2). Among the group of RelA target genes, there are a number of LPS-inducible genes, including \textit{NOS2 (iNOS)}, \textit{COX2}, \textit{IL6}, \textit{IL1B}, \textit{IL8}, \textit{CCL2 (MCP-1)}, \textit{CSF1}, \textit{CXCL2 (MIP-2)}, and \textit{ICAM}. The majority of the abovementioned genes have functional STAT3 DNA-binding sites in their promoters, and many of them have been shown to depend on STAT3 for their expression (Supplementary Table S3). Each of these genes encodes protein product reported to promote carcinogenesis or growth of established cancers (15). Some of the genes important for innate immunity against infection, such as \textit{Nos2} and \textit{Il6}, can also be upregulated when STAT3 is inhibited, particularly in the presence of microbial toll-like receptor (TLR) ligand stimulation (11). Furthermore, we noted that \textit{IFNB}, \textit{CCL5}, \textit{CD80}, \textit{CD86}, \textit{CIITA (MHC class II)}, \textit{ILI2A [IL-12 (p35)]}, \textit{CXCL10}, and \textit{IFNG} lack STAT3-binding sites and can be inhibited by STAT3 in cancer cells and/or in immune cells (Supplementary Table S4). Many of these genes are associated with Th-1 type immune responses critical for inducing immune-mediated antitumor effects. However, in the tumor milieu, CCL5 can be elevated, enhancing cell motility, which leads to invasion and metastasis (26), thus suggesting that the role of various immune factors in procarcinogenic versus anti-carcinogenic responses is complex. Nonetheless, it is clear that their effects are context dependent, which is impacted by whether it is during Th-1 acute inflammation or chronic inflammation/excessive wound healing akin to cancer. The genes we
analyzed are the ones frequently cited as either oncogenic or Th-1 immunostimulatory. Although we have not excluded any particular genes that are considered important for cancer progression (15), genes included in our analyses only represent a small fraction of all NF-κB downstream genes, whose functions are diverse and regulation complicated.

Introducing STAT3 DNA-binding sequences confers promoter transcriptional activation by NF-κB in cancer cells

The ChIP assays and computational analyses suggest that the presence of STAT3-binding site(s) within a promoter is important for NF-κB promoter binding and transcriptional activity. To further test this idea experimentally, we introduced mutations in the well-characterized human CCL5 (RANTES) promoter (29), which contains 2 adjacent consensus NF-κB DNA-binding sites (Fig. 2A). Conversion of either 1 or 3 nucleotides resulted in 2 modified CCL5 promoter constructs containing either partial STAT3 consensus sequences (mt-1) or a whole STAT3 site (mt-2) adjacent to an intact NF-κB binding site (Fig. 2A). Generating mutation in the first NF-κB site still allowed binding of RelA through the second NF-κB site (Fig. 2B), indicating that introduction of a STAT3-binding site does not interfere with RelA recruitment to DNA. To test whether the modified sequences within the CCL5 promoter could allow direct interaction with STAT3 protein in addition to NF-κB, biotin-labeled oligonucleotides were incubated with nuclear extracts prepared from A2058 melanoma cells, followed by streptavidin–agarose pull-down assay and Western blot analysis. As shown in Figure 2C, while STAT3 bound poorly to the oligonucleotide containing only NF-κB sites within the CCL5 promoter, it effectively bound to the modified CCL5 promoters with 1 or 3 nucleotide modifications (Fig. 2C). Moreover, p-STAT3/p-RelA only interacted with the

![Graphical representation](image-url)
oligonucleotide containing both STAT3 and NF-κB sites (mt-2; Fig. 2C). Furthermore, electrophoretic mobility shift assay (EMSA) indicated that p-STAT3–p-RelA–p50 formed complexes in the promoter with both STAT3 and κB-binding sites (Fig. 2D). STAT3 binding to mt-2 oligonucleotide was not affected by silencing RelA in A2058 tumor cells, whereas knocking down STAT3 decreased RelA binding. These observations suggest that STAT3 is required for RelA binding to κB sites in the promoter containing STAT3-binding sites (Fig. 2E).

This in vitro system allowed us to further assess whether sequence conversion to STAT3 binding confers transcriptional activity of the CCL5 promoter, which is normally silent in the tumor cells with constitutively activated STAT3 (5). Luciferase reporter assays indicated that the modified CCL5 promoters, which contain sequences capable of STAT3 binding, showed a significant induction of CCL5 promoter activity in the tumor cells (Fig. 2F). In addition, although the activity of the wild-type (WT) CCL5 promoter was low in tumor cells, it was greatly increased by silencing STAT3 with siRNA but decreased by RelA knockdown (Fig. 2F), which is consistent with the observation that endogenous CCL5 gene expression is suppressed by STAT3 (5). These promoter conversion experiments support the hypothesis that STAT3 binding to promoters can influence differential regulation of NF-κB downstream genes. We note that, because these experiments were done with an overexpressed promoter construct, their physiologic significance remains to be further tested.

**STAT3 and NF-κB coregulate oncogenic gene expression within the tumor milieu in vivo**

Next, we assessed whether STAT3 DNA binding contributes to differential NF-κB transcriptional activity in vivo. We conducted ChIP assays using chromatin prepared from mouse spleens and B16 mouse melanoma tumors grown in mice with Stat3+/+ and Stat3−/− myeloid cells (Fig. 3A). Although both Stat3 and NF-κB bound to the Birc5 promoter in the freshly harvested tumors, NF-κB could only bind to the promoter of Ccl5, Cxcl10, and Ifnb when Stat3 was ablated in tumor-infiltrating myeloid cells (Fig. 3A and Supplementary Fig. S3). To further confirm that the STAT3–NF-κB complex
similarly affects their transcriptional specificity in tumor-infiltrating immune cells, we conducted additional ChIP assays in immune cells isolated from either B16 TDLN or control lymph node from naive mice. Whereas NF-kB and Stat3 binding to the Bcl2l1 promoter was increased in immune cells from TDLN, Stat3–NF-kB binding was decreased on the promoter of Cxcl10 relative to controls (Fig. 3B). In addition, Stat3 activation induced by either tumor-derived factors or immunosuppressive cytokine IL-10, which is a STAT3 activator, did not lead to NF-kB binding to the Ccl5 promoter (Supplementary Fig. S4 and Fig. 3C). In contrast, Stat3 activation by tumor-derived factors further increased NF-kB binding on the Birc5 and Vegf promoters (Fig. 3C). TNF-α induced NF-kB association with the Ccl5 promoter (Fig. 3C). Collectively, these in vivo data substantiated our conclusion that NF-kB binding to oncogenic/immunosuppressive genes is favored by the presence of activated Stat3 in the tumor milieu.

To assess whether differential binding of Stat3–NF-kB on gene promoter regulates expression of NF-kB downstream targets in vivo, real-time reverse transcriptase-PCR (RT-PCR) assay was conducted in either CD11b+ cells isolated from B16 tumors or from whole tumors with Stat3+/− or Stat3−/− hematopoietic cells. Targeted gene ablation of Stat3 in the myeloid compartment reduced the activity of Stat3 and RelA in tumor-associated CD11b+ cells and the entire tumors (17, 30). Ablation of Stat3 in tumor-infiltrating myeloid cells resulted in an increased expression of genes involved in antitumor immune responses, including Il12a, Il10, Cxcl10, Ccl5, and Ifnb (Fig. 4A). In contrast, expression of prosurvival genes, Birc5 and Bcl2l1, and of immunosuppressive genes, Vegf and Il10, was upregulated by persistent Stat3 activity in tumor-infiltrating myeloid cells (Fig. 4B). Consistent with the data in tumor-infiltrating myeloid cells, mRNA levels of the immunostimulatory genes Ccl5 and Il12a were increased whereas those of the procarcinogenic genes Bcl2l1, Birc5, and Il6 were decreased in tumors in mice without Stat3 in myeloid cells (Fig. 4C).

Other studies support that STAT3 DNA-binding site(s) is a determinant of NF-kB binding/activity to pro-oncogenic versus immunostimulatory genes, including Il1rn (IL-1ra) gene regulation (31). Il1rn contains STAT3 DNA-binding sites in the promoter. Although LPS induces nuclear translocation of NF-kB in human monocytes, it cannot induce NF-kB binding to the kB sites in Il1rn promoter alone. However, together with IL-10 stimulation, which activates STAT3, NF-kB could interact with the promoter (32). Furthermore, NF-kB is not recruited to the Il1rn promoter in hIES (hyper-IgE syndrome) patients carrying defective STAT3 (31). Although our studies focused on p-STAT3, other reports have shown an important role of unphosphorylated STAT3 interacting with unphosphorylated NF-kB, leading gene regulation (19), which suggests the complexity of STAT3 and NF-kB interactions in regulating oncogenic versus antitumor gene expression.

It appears that p300 is only associated with NF-kB on the promoters of pro-oncogenic genes in tumor cells but not on those of immunomodulatory genes in immune cells (Fig. 1E). Because STAT3 interaction with p300 is critical for constitutive NF-kB activity in tumor cells and immune cells in the tumor microenvironment (17), p300 may bridge STAT3 to NF-kB in the promoter of NF-kB target genes with pro-oncogenic property. This interaction may lead to further increases in NF-kB transcriptional activity that is accompanied by the change in chromatin structure. Although much remains to be explored and learned, our findings shed light on how 2 key transcription factors activated in cancer coordinate distinct gene regulation to promote oncogenesis and identify a molecular mechanism by which our immune system plays a dual role in both immune surveillance and tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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