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Cancer Res. Author manuscript; available in PMC 2012 June 1.

Published in final edited form as:

Cancer Res. 2011 June 1; 71(11): 3772–3780. doi:10.1158/0008-5472.CAN-10-3304.

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, promoting 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 anti-tumor immunity despite an elevated level of NF-κB in tumors. Here 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 the promoters. A large number of NF-κB downstream genes associated with oncogenesis and chronic inflammation contain STAT3 DNA-binding site(s). In contrast, many genes frequently associated with anti-tumor immunity lack STAT3 DNA-binding site(s) and can only be activated by NF-κB when STAT3 is inhibited in tumors. Introducing 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 important for tumor growth while inhibiting its binding to Th-1 immunostimulatory genes in growing tumors including tumor-infiltrating immune cells. Our results provide insight into how some of the oncogenic/inflammatory and Th-1 immunostimulatory genes are differentially regulated in cancer.

Keywords

STAT3; NF-κB; Gene regulation; Cancer; Inflammation

Introduction

The importance of our host immune system in both suppressing tumor incidence/growth and promoting malignant transformation has been well supported (1-4). However, the underlying molecular mechanisms that coordinate such complex and opposing immune regulations are only beginning to emerge. Signal transducer and activator of transcription (STAT) proteins are central in regulating both anti-tumor and cancer-promoting immune responses (4). One of the STAT protein family members, STAT3, is critical in supporting cancer inflammation and in suppressing anti-tumor immunity (4-8). As a signal transducer, STAT3 is a key point of convergence for numerous oncogenic tyrosine kinase signaling pathways (7, 9). STAT3 is also a well-known transcription activator necessary for upregulating a large number of genes

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No potential conflicts of interest were disclosed.

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encoding factors essential for tumor cell proliferation, survival, invasion, and cancer-promoting inflammation (4, 7, 9). Additionally, STAT3 can inhibit expression of several tumor-suppressor genes and many Th-1 immunostimulatory molecules (4, 5, 10-13). While some studies indicated that STAT3 can suppress expression of certain tumor suppressor genes by mediating DNA methylation (14), how STAT3 might inhibit transcription activity of Th-1 immunostimulatory genes remains to be elucidated.

Parallel to STAT3, Nuclear factor-κB (NF-κB) has been recognized as a core transcription factor for pro-oncogenic and pro-cancer inflammatory genes (4, 15, 16). In fact, the two transcription factors regulate many of the same oncogenic/pro-cancer 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 both normal immune cells as well as in tumor cells and tumor-infiltrating immune cells (4-7, 12, 13). STAT3 has been shown to suppress 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 activity is elevated in both tumor cells and in tumor-infiltrating immune cells (4, 17), raising the question how STAT3 differentially regulates oncogenic/inflammatory *vs*. Th-1 immunostimulatory genes inducible by NF-κB.

During tumorigenesis and cancer-related chronic inflammation, STAT3 and NF-κB reciprocally regulates each other at multiple levels (4, 8, 12, 13, 18, 19). Continuous STAT3 activation in tumor cells and especially in immune cells in the tumor microenvironments can be maintained by several factors induced by NF-κB, such as IL-6, Cox2 and IL-23 (4, 8, 16, 20-22). On the other hand, STAT3 mediates constitutive NF-κB activation in tumors by promoting nuclear accumulation of NF-κB through p300-mediated RelA acetylation (4, 16, 17). However, the question remains how STAT3 promotes one set while inhibiting another set of NF-κB-regulated genes in tumor cells and tumor-infiltrating immune cells. In this study, we have identified a molecular mechanism by which concurrent activation of STAT3 and NF-κB in tumors exerts distinct regulation on expression of Th-1 immunostimulatory *vs*. pro-oncogenic/immunosuppressive genes. We show in both tumor cells and tumor-infiltrating myeloid cells that STAT3 DNA-binding is crucial for activation of NF-κB inducible genes. The lack of STAT3 DNA-binding sites(s) in many of the genes generally associated with Th-1 immune responses preclude their expression in tumor setting where STAT3 is activated and is in the same complex with NF-κB and p300. Only by either freeing NF-κB from the STAT3/p300 complex or by introducing STAT3 DNA-binding site(s) to the promoter of a Th-1 immunostimulatory gene can confer transcriptional activation of the immunostimulatory genes in tumor setting. Our studies provide insight on how a number of cancer-promoting and anti-tumor immunostimulatory genes are differentially regulated by STAT3.

Material and Methods

Materials

A2058 human melanoma cell line was obtained from ATCC. The DC 2.4 mouse dendritic cell line was a generous gift from Dr. K. L. Rock (University of Massachusetts Medical School). C-4 mouse melanoma cells were kindly provided by Dr. I. Fidler (University of Texas, M.D. Anderson Cancer Center). Cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum. Tumor conditioned medium (TMC) was prepared from C-4 cells as described previously (17) and added to culture media for overnight to 10% final concentration.

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Polyclonal antibodies recognizing STAT3, RELA, p50, STAT1 and HDAC1, and siRNA targeting *STAT3* and *RELA* were purchased from Santa Cruz Biotechnology; p-STAT3 (Y705) and p-RELA (S536) antibodies from Cell Signaling; anti-HA (12CA5) from Roche. TNFα was from Endogen and IL-10 from PeptoTech. Both of them were used at 20 ng/ml. Recombinant STAT3 (full length) and RELA (14 amino acids truncation at the C-teminus) proteins were obtained from Active Motif.

Chromatin immunoprecipitation (ChIP) assays

Cells were seeded in 150 mm plates at the density of 1×10^6 then transfected with 20 nM siRNA using Lipofectamine 2000 (Invitrogen). Forty-eight hour after transfection, formaldehyde was added into culture media to a final concentration of 1% and incubated at room temperature for 10 min. For ChIP assay using spleen, tumor-draining lymph node (TDLN) and tumor, tissue was ground in liquid nitrogen in mortar and pestle. Nuclei were isolated from tissue powder then formaldehyde was added into nuclei solution for 30 min. Chromatin immunoprecipitation was performed based on the protocol provided by Millipore-Upstate Biotechnology. Primers for PCR analysis only amplify the region of κB site. Primer sequences are listed in Supplementary Table S1. Experimental details are provided in Supplementary Methods.

Promoter activity assay

Partial STAT3 site (TTCC) or hSIE consensus sequence was introduced into the human *CCL5* (*RANTES*) promoter using PCR based site-directed mutagenesis. The primers used were 5′-TTGCTATTTTGGAAATTCCCCTTAGGGGATGCCCCTCAACTGGCCCTA-3′ for the partial STAT3 site and 5′-TGGAAATTCCCGTAAGGGGATGCCCCTCAACTGG-3′ for *hSIE* (complete STAT3 site). Amplified PCR products were subcloned into pGL3 basic vector then verified by DNA sequencing. Promoter activity assay was carried out as described in Supplementary Methods. All statistical analysis was performed using GraphPad Prism version 4.0 software (GraphPad). Unpaired *t*-test was used to calculate one-tailed *P* value.

In vitro translation/antibody-pull down assay

In vitro translated p300 proteins were prepared by TnT® T7/Sp6 coupled wheat germ extract system (Promega) using pCMVβ-HA-p300 (Millipore) as template. 10 μl of the translation mixture was incubated with either recombinant protein (50 ng) or nuclear extracts from A2058 cells (5 μg) in 500 μl binding buffer (20 mM Tris, pH 8.0, 60 mM NaCl, 1 mM EDTA, 6 mM MgCl2, 1 mM DTT, 8% Glycerol and 0.05% NP-40). Immune complexes were pulled down with anti-HA antibody then washed three times with binding buffer, followed by SDS-PAGE. p300-bound proteins were visualized by western blotting analysis with the indicated antibodies.

Oligo-binding assay

Nuclear extracts prepared from A2058 cells (23) were incubated with oligonucleotides labeled with biotin in 500 μl oligo-binding buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 12% Glycerol, 150 mM KCl, 1 mM EDTA, 1 mM DTT and 10 μg poly (dI-dC)). The oligonucleotide sequences used in this study are shown in Fig. 2*A*. Protein complexes bound to each oligonucleotide were pulled down with Streptavidin beads. After extensive washing with oligo-binding buffer, proteins were separated on SDS-PAGE gels, blotted and probed with antibodies, and visualized with the ECL detection method.

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In vivo experiments

Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at City of Hope Medical Center. *Mx1-Cre* mice were obtained from Jackson Laboratory and $Stat3^{flox/flox}$ 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 Supplementary Methods.

Isolation of immune cells

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

Real-time RT-PCR

Total RNA was prepared from isolated immune cells or whole tumors for real-time PCR analysis as indicated. RNA (0.5 to 1 μg) was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad), and real-time PCR reactions were performed 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 of three 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 κB 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 κB sites and how that might affect NF-κB binding to various oncogenic/inflammatory genes. Chromatin immunoprecipitation (ChIP) assays were performed 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 anti-apoptotic 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-1*β) 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 (IL-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 κB sites within these genes. ChIP assays indicated that in contrast to the oncogenic/inflammatory 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 κB-site(s) within these Th-1 immunostimulatory genes. To test this, we prepared chromatin fractions from A2058 tumor cells transfected with either control or *STAT3* siRNA in the presence or absence of TNFα to

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compare STAT3/NF-κB-DNA binding in the *BIRC5* and *CCL5* promoters. Previous studies demonstrated that treatment of A2058 tumor cells with TNFα further stimulates NF-κB activity but has no effect on STAT3 activation (26). TNFα had no effects on STAT3 protein expression level (Supplementary Fig. S1B), and STAT3 was not associated with TNFα-activated NF-κB complex bound to the *CCL5* promoter (17, 27). Consistent with these findings, STAT3 did not interact with TNFα-activated RELA bound to the *CCL5* promoter in A2058 tumor cells (Fig. 1C). In the absence of *STAT3*, RELA was bound to *CCL5* promoter and could be further induced upon TNFα 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 now 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 TNFα 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 κB-site stably (17). Based on these findings, we considered the possibility that once in 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, p300/STAT3/RelA complex was not detectable on the promoter of the immunostimulatory gene, *Cxcl10*, in DC2.4 dendritic cells treated with TNFα, 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 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, NF-κB/p300/STAT3 protein complex preferentially binds to the promoters of many genes involved in tumor growth/inflammation but not in 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 *vs*. Th-1 immuostimulatory 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 the many of the Th-1-immunostimulatory genes when STAT3 is inhibited. One possible explanation for the observations is that STAT3/NF-κB/p300 can only activate genes if they have STAT3 DNA-binding site(s) in their promoter regions. To test this idea, we performed computational analyses to include all NF-κB target genes associated with oncogenesis listed in a review (15), compared to LPS-inducible NF-κB target genes often associated with acute infection and anti-tumor 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. We next assessed whether the RelA-regulated genes have STAT3-binding site(s), and also whether their expression is regulated by STAT3, based on published reports and computational sequence analysis.

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

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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 *NOS2* (*iNOS*), *COX2, IL6, IL1B, IL8, CCL2* (*MCP-1*), *CSF1, CXCL2* (*MIP-2*), and *ICAM*. The majority of the above mentioned 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 *Nos2* and *Il6*, can also be upregulated when STAT3 is inhibited, especially in the presence of microbial TLR ligand stimulation (11). We also noted that *IFNB, CCL5, CD80, CD86, CIITA* (*MHC Class II*), *IL12A* (*IL-12(p35)*), *CXCL10* and *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), suggesting that the role of various immune factors in procarcinogenic *vs*. anti-carcinogenic responses is complex. Nevertheless, 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 viewed important for cancer progression (15), genes 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 transcription activity. To further test the idea experimentally, we introduced mutations in the well-characterized human *CCL5* (*RANTES*) promoter (29) which contains two adjacent consensus NF-κB DNA-binding sites (Fig. 2A). Conversion of either one or three nucleotides resulted in two 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 κB site still allowed binding of RELA through the second κB site (Fig. 2B), indicating that introducing STAT3 binding site does not interfere 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 then western blot analysis. As shown in Fig. 2C, while STAT3 bound poorly to the oligonucleotide containing only NF-κB sites within the *CCL5* promoter, STAT3 effectively bound to the modified *CCL5* promoters with one or three nucleotide modifications (Fig. 2C). Moreover, phosphorylated STAT3 (p-STAT3)/ phosphorylated RELA (p-RELA) only interacted with the oligonucleotide containing both STAT3 and NF-κB sites (mt-2) (Fig. 2C). EMSA assays also indicated that p-STAT3/p-RelA/p50 formed complex 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, while 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

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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, while the activity of the wild-type *CCL5* promoter was low in tumor cells, it was greatly increased by silencing *STAT3* with siRNA but decreased by *RELA* knockdown (Fig. 2F), consistent with the observation that endogenous *CCL5* gene expression is suppressed by STAT3 (5). These promoter conversion experiments support that STAT3 binding to promoters can influence differential regulation of NF-κB downstream genes. We note that, since these experiments were performed with an overexpressed promoter construct, their physiological significance remains to be further tested.

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

We next assessed whether STAT3 DNA-binding contributes to differential NF-κB transcriptional activity *in vivo*. We performed ChIP assays using chromatin prepared from mouse spleens and B16 mouse melanoma tumors grown in mice with $Stat3^{+/+}$ and $Stat3^{-/-}$ myeloid cells (Fig. 3A). While 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 Stat3/NF-κB complex similarly affects their transcriptional specificity in tumor-infiltrating immune cells, we performed additional ChIP assays in immune cells isolated from either B16 tumor-draining lymph node (TDLN) or control lymph node from naive mice. Whereas NF-κB and Stat3 binding to the *Bcl2l1* promoter was increased in immune cells from TDLN, Stat3/NF-κB 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-κB binding to the *Ccl5* promoter (Supplementary Fig. S4 and Fig. 3C). In contrast, Stat3 activation by tumor-derived factors further increased NF-κB binding on the *Birc5* and *Vegf* promoters (Fig. 3C). TNFα induced NF-κB association with the *Ccl5* promoter (Fig. 3C). Collectively, these *in vivo* data substantiated our conclusion that NF-κB 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-κB on gene promoter regulates expression of NF-κB downstream targets *in vivo*, real-time RT-PCR was performed in either $CD11b^+$ cells isolated from B16 tumors or from the 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 anti-tumor immune responses, including *Il12a, Ifng, Cxcl10, Ccl5 and Ifnb* (Fig. 4A). By contrast, expression of the pro-survival genes, *Birc5* and *Bcl2l1*, as well as 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 level of the immunostimulatory genes *Ccl5* and *Il12a* was increased while the pro-carcinogenic genes *Bcl2l1, Birc5 and Il6* was 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-κB binding/activity to pro-oncogenic vs. immunostimulatory genes include *Il1rn* (*IL-1ra*) gene regulation (31). *Il1rn* contains STAT3 DNA-binding sites in the promoter. Although LPS induces nuclear translocation of NF-κB in human monocytes, LPS can not induce NF-κB binding to the κB sites in *Il1rn* promoter alone. However, together with IL-10 stimulation, which activates STAT3, NF-κB could interact with the promoter (32). Furthermore, NF-κB is not recruited to the *Il1rn* promoter in HIES patients carrying defective STAT3 (31). While

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our studies focused on phosphorylated STAT3, other reports have shown an important role of unphosphorylated STAT3 interacting with unphosphorylated NF-κB, leading gene regulation (19), suggesting the complexity of STAT3 and NF-κB interactions in regulating oncogenic *vs*. anti-tumor gene expression.

It appears that p300 is only associated with NF-κB on the promoters of pro-oncogenic genes in tumor cells, but not on those of immunostimulatory genes in immune cells (Fig. 1E). Since STAT3 interaction with p300 is critical for constitutive NF-κB activity in tumor cells and immune cells in the tumor microenvironment (17), p300 may bridge STAT3 to NF-κB in the promoter of NF-κB target genes with pro-oncogenic property. This interaction may lead to further increases in NF-κB 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 two 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the staff members of DNA Synthesis Core, and the Animal Facility of the Beckman Research Institute at City of Hope for their superb technical support.

This work is funded by National Institutes of Health (R01CA115815, R01CA122976 and R01CA146092).

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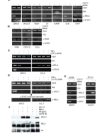

Figure 1.

STAT3-associated NF-κB preferentially binds to oncogenic over immunostimulatory gene promoters in tumors. A, ChIP analyses of STAT3 and NF-κB in A2058 tumor cells at the promoter of NF-κB target genes containing STAT3-binding sites. Pre-immune serum (PIS) was used as antibody control at the same concentration. B, ChIP assays at the NF-κB downstream gene promoters devoid of STAT3 binding site(s). C, ChIP assays at the κB site of *BIRC5* or *CCL5* promoter from *STAT3* siRNA transfected A2058 tumor cells treated with TNF-α for 6 hours. D, ChIP assay to detect STAT3/NF-κB binding to the indicated promoters. STAT3 complex was depleted in A2058 tumor cell chromatin fractions with anti-STAT3 antibody (αSTAT3-unbound chromatin). E, *Left*, p300 interaction with STAT3/NF-κB in A2058 tumor cells shown by ChIP assay. *Right*, Absence of p300 on the promoter of immunostimulatory gene in DC2.4 dendritic cells stimulated with TNFα. F, Western blotting of *in vitro* translated p300 interaction with either recombinant STAT3/RELA proteins or nuclear STAT3/RELA complex in A2058 tumor cells (A2058 NE). Arrow indicates the location of recombinant RELA proteins that have 14 amino acids truncation at the C-terminus. Data shown represent one of three independent experiments in all cases.

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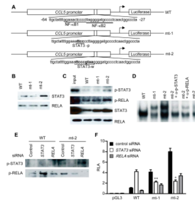

Figure 2.

Introducing STAT3 binding sites in the *CCL5* promoter facilitates its activation in tumor cells. A, Schematic diagram of NF-κB binding sequences within the human *CCL5* promoter-luciferase reporter construct. NF-κB and STAT3 binding sites are underlined. WT contains two κB elements; mt-1, a partial STAT3 site (STAT3-p, ttcc); mt-2, a putative STAT3 binding site (STAT3-w). B, Western blotting measuring recombinant STAT3 and RELA proteins captured on a biotinylated oligonucleotides. C, Western blotting to show p-STAT3/ p-RELA in A2058 tumor cells on the oligonucleotides containing STAT3 binding site (mt-2). A2058 nuclear extract was used as input. D, EMSA to detect p-STAT3/p-RELA/p50 complexes on the oligonucleotides with STAT3 binding site. E, Western blotting analysis using nuclear extracts from A2058 cells transfected with the indicated siRNA to measure p-STAT3/ p-RELA binding to different oligonucleotides. F, Luciferase assay showing the requirement of STAT3 for the modified *CCL5* promoter activity in A2058 tumor cells. Results are shown as mean ± SEM of RLA performed in triplicates. Asterisk indicates significant difference between the promoter activity of mt-1 or mt-2 *vs*. WT following STAT3 knockdown (Unpaired *t*-test: ***P* value for mt-1 *vs*. WT = 0.0075; **P* value for mt-2 *vs*. WT = 0.0141).

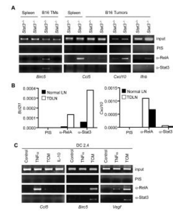

Figure 3.

Tumor STAT3/NF-κB preferentially interacts with the promoter of oncogenic genes *in vivo*. A, STAT3 impacts on NF-κB differential binding to a promoter *in vivo*. Spleens or B16 tumors pooled from 6 mice were used to prepare chromatin immunoprecipitates, followed by PCR analysis for the detection of RelA or Stat3 on a κB site within the indicated promoter. B, ChIP precipitates prepared from cells of tumor-draining lymph nodes (TDLN), pooled from 6 mice, and detected by quantitative real-time RT-PCR using primers specific for the indicated gene. C, STAT3/NF-κB binding to the oncogenic genes was mediated by tumor-derived factors, which activate STAT3. ChIP analysis to show interaction of STAT3/NF-κB to the promoter of pro-oncognic/immunosuppressive genes in DC2.4 dendritic cells stimulated with tumor-conditioned medium (TCM). Data represent one of three independent experiments in all cases.

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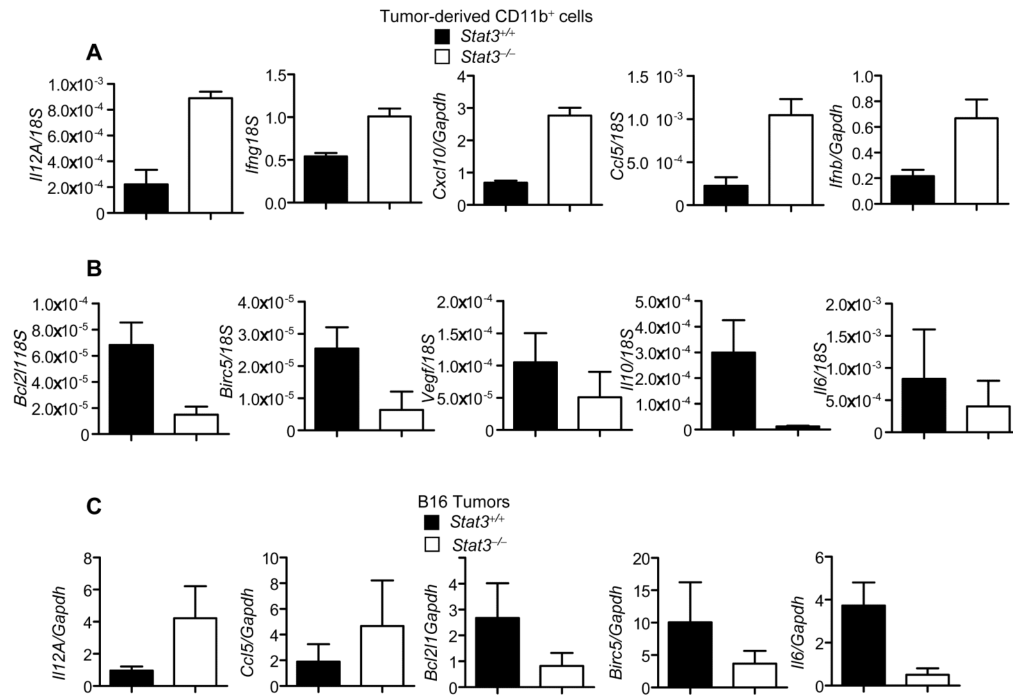

Figure 4.

Persistent STAT3 activation in immune cells in the tumor microenvironment and in tumors modulates NF-κB target gene expression. A and B, Quantification of NF-κB target gene mRNA expression by real-time RT-PCR in $CD11b^{+}$ myeloid cells from B16 tumors grown in mice with *Stat3*$^{+/+}$ or *Stat3*$^{-/-}$ hematopoietic cells (n=6). Inhibition of *Stat3* in tumor-associated myeloid cells upregulates NF-κB downstream genes involved in pro-inflammatory/anti-tumor immune responses (A) or downregulates NF-κB-mediated pro-oncogenic genes (B). C, Real-time RT-PCR using RNA from B16 tumors consisting of both tumor cells and stromal cells in the tumor microenvironment to measure STAT3-dependent NF-κB target gene regulation in tumors. Shown are representative data from three independent experiments in all cases; Mean ± SEM.