The Journal of Immunology

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J Immunol 2007; 179:7215-7219; ;
doi: 10.4049/jimmunol.179.11.7215
http://www.jimmunol.org/content/179/11/7215

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: A Transcriptional Repressor and Corepressor Induced by the STAT3-Regulated Anti-Inflammatory Signaling Pathway[1]

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***IL-10 regulates anti-inflammatory signaling via the activation of STAT3, which in turn controls the induction of a gene expression program whose products execute inhibitory effects on proinflammatory mediator production. In this study we show that IL-10 induces the expression of an ETS family transcriptional repressor, ETV3, and a helicase family corepressor, Strawberry notch homologue 2 (SBNO2), in mouse and human macrophages. IL-10-mediated induction of ETV3 and SBNO2 expression was dependent upon both STAT3 and a stimulus through the TLR pathway. We also observed that ETV3 expression was strongly induced by the STAT3 pathway regulated by IL-10 but not by STAT3 signaling activated by IL-6, which cannot activate the anti-inflammatory signaling pathway. ETV3 and SBNO2 repressed NF-κB- but not IFN regulatory factor 7 (IRF7)-activated transcriptional reporters. Collectively our data suggest that ETV3 and SBNO2 are components of the pathways that contribute to the downstream anti-inflammatory effects of IL-10. The Journal of Immunology,* 2007, 179: 7215–7219.**

Although discovered >16 years ago and characterized as an inhibitor of activated macrophages and APCs, the mechanistic basis of the anti-inflammatory effects of IL-10 remain unresolved (1, 2). The IL-10R activates STAT3, which is both necessary and sufficient for the generation of the anti-inflammatory response (AIR)[4] (3–5). By contrast, other receptors that activate STAT3 such as the IL-6R are inhibited from generating the AIR (6, 7). STAT3 signaling can be manipulated to generate the AIR by removing inhibitory components such as suppressor of cytokine signaling 3 (SOCS3)-mediated regulation of the IL-6R (3, 8). These data argue that AIR generation in macrophages is specific to the IL-10R. Following STAT3 activation by the IL-10R, the expression of induced inflammatory genes is inhibited. The AIR is selective, and targets subsets of inflammatory genes accounting for ~10–15% of the genes induced by TLR signaling after LPS stimulation (9). Within the inhibited mRNA pool are targets such as TNF-α, IL-6, IL-12p40, IL-1α, IL-1β, and chemokine mRNAs. The IL-10-mediated inhibition of these genes is essential because in the absence of IL-10 production, excessive and often lethal, inflammation results. How does the AIR selectively inhibit inflammatory gene expression? A key piece of evidence is that STAT3 acts indirectly, inducing one or more genes whose products selectively target inflammatory gene transcription (10). Transcriptional regulation is the primary target of IL-10 and therefore the focus of efforts to isolate STAT3-regulated genes that mediate the AIR.

Existing screening efforts have failed to isolate a single gene whose product mediates the AIR, leading to the suggestion that there is no single master gene but rather the products of multiple IL-10-regulated genes combine to mediate the AIR (11). Conversely, it remains possible that a handful of undiscovered gene products induced by IL-10 mediate the key steps that inhibit inflammatory gene transcription. Previous attempts to isolate IL-10-induced, STAT3-dependent genes whose products are implicated in the AIR include SOCS3, BCL3, ABIN-3/LIND/TNIP3, and IκBNS, each of which has subsequently been shown to have limited or no direct function or no species selectivity in the activation and propagation of the AIR (8, 11–17). At this stage however, mice bearing combined mutations in these genes have yet to be generated and evaluated for their

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Received for publication August 7, 2007. Accepted for publication October 5, 2007.

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[1] This work was supported by grants to P.J.M. from the National Institutes of Health, the Sandler Program for Asthma Research, and the American Lebanese Syrian Associated Charities and by Cancer Center CORE Grant P30 CA 21765; by grants to A.P. from National Institutes of Health (T32-CA-09598-16) and the American Legion (American Legion Auxiliary Award); and by grants to S.S.W. from the American Heart Association Texas Affiliate (0455143Y), the Gillson Longenbaugh Foundation, and the MD Anderson Cancer Center Institutional Grants Program.

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[4] Abbreviations used in this paper: AIR, anti-inflammatory response; BMDM, bone marrow-derived macrophage; IRF7, IFN regulatory factor 7; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; SBNO2, Strawberry notch homologue 2; SOCS3, suppressor of cytokine signaling 3.

functions in the AIR. In this article, we report the isolation of a transcriptional repressor, ETV3 and a transcriptional corepressor, Strawberry notch homologue 2 (SBNO2), regulated specifically by the IL-10 signaling pathway.

Materials and Methods

Affymetrix analysis

Expression analyses were performed using the Affymetrix MOE-430v2 GeneChip array. To identify differentially expressed genes at each time point, the local pooled error *t* test (18) was used. The method of Benjamini and colleagues (19) was used to estimate the false discovery rate.

Mice

C57BL/6 and *Il10*$^{-/-}$ (C57BL/6 background) were from The Jackson Laboratories. *Stat3*$^{flox/-}$*;Tie2cre* mice (20) and *Socs3*$^{flox/flox}$*;LysMcre* mice have been described (21). The latter were used to generate *Socs3*$^{flox/flox}$;LysMcre;Il10$^{-/-}$ mice by intercrossing.

RNA and protein analysis

Northern blotting was performed using a cDNA probe for mouse ETV3 (10). Quantitative RT-PCR analysis for ETV3 and SBNO2 was normalized to GAPDH. Immunoblotting was performed as described (22) using rabbit polyclonal Abs to ETV3 raised against a peptide common to mouse and human ETV3 (RLKRRWNDDPEAREL).

Small interfering (siRNA) analysis in human macrophages

Primary monocyte-derived macrophages were transfected with siRNAs against human STAT3 (Dharmacon) using the Amaxa system. Control siRNAs and siRNAs against BTK were used as negative controls. Following transfection, amounts of STAT3 were detected by immunoblotting. Independent pools from the same transfections were stimulated with IL-10 and SBNO2 and BCL3 mRNAs were detected by quantitative RT-PCR (qRT-PCR) normalized to GAPDH mRNA.

Reporter analysis

293T cells were transfected by Lipofectamine with expression constructs (all in variants of pcDNA3) encoding NF-κB p65, IFN regulatory factor 7 (IRF7), ETV3, or SBNO2. The luciferase reporters used have been described (23). COS cells were transfected by the DEAE dextran method (24).

Results

Isolation of ETV3 and SBNO2 as targets of IL-10 signaling

We reasoned that we could isolate new classes of IL-10-regulated genes by: 1) screening in early time increments following IL-10 signaling; 2) using IL-10-deficient macrophages to circumvent autocrine/paracrine effects of IL-10; 3) isolating RNA from macrophages stimulated with IL-10 and an inflammatory costimulus (in these experiments, LPS); and 4) using denser microarrays that could reveal targets missed by earlier arrays. We isolated total RNA from *Il10*$^{-/-}$ bone marrow-derived macrophages (BMDMs) treated with LPS or IL-10 plus LPS for 10, 20, or 30 min. Three complete independent experiments were performed and collectively analyzed for specific IL-10-induced gene expression in *Il10*$^{-/-}$ macrophages. We observed the IL-10-specific induction of few genes above the LPS background (Fig. 1*a*), which itself induces and represses the expression of hundreds of genes by 30 min. Two previously characterized IL-10-regulated genes were detected (9, 25) (*Socs3* and *Gadd45g*) and thus served to validate the screening methodology. Of the remaining targets, two were notable for their potential in transcriptional regulation, the primary mechanism used by IL-10 to inhibit inflammatory gene expression. These targets were *Etv3*, an ETS family transcriptional repressor expressed in macrophages (26), and BC019206, an EST derived from *Sbno2*, a member of the Strawberry notch family of helicase transcriptional corepressors (27).

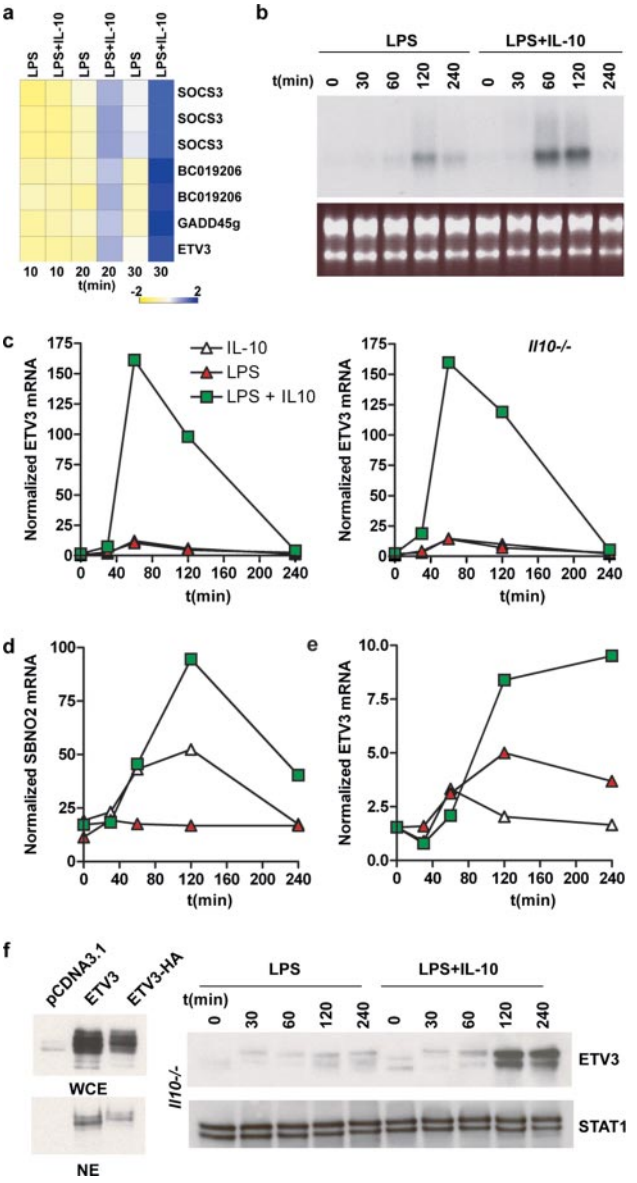

FIGURE 1. *Etv3* and *Sbno2* are IL-10-regulated genes. *a*, Summary of the microarray screening data. Probe sets for genes whose expression is increased by IL-10 in the presence of LPS as a costimulus are shown. Hierarchical clustering was performed to correlate profiles among the differentially expressed probe sets. The probe sets with the greatest correlation to SOCS3 are shown in a heat map (the scale bar indicates SD values from the mean in $\log_2$ scale). *b*, Northern blotting analysis of ETV3 mRNA in C57BL/6 BMDMs. Ethidium bromide staining of the gel before blotting indicates equal loading of RNA. *c*, *left panel*, qRT-PCR analysis of ETV3 mRNA in BMDMs stimulated with LPS, IL-10, or LPS plus IL-10 over time. *c*, *right panel*, Kinetic analysis of ETV3 mRNA in *Il10*$^{-/-}$ BMDMs stimulated with LPS (gray triangle) or LPS plus IL-10 (gray square). *d*, qRT-PCR analysis of SBNO2 mRNA in BMDMs stimulated as in *c*. *e*, qRT-PCR analysis of human ETV3 mRNA in monocyte-derived macrophages stimulated as shown in *c*. Data in *c* and *d* are from representative experiments using duplicate samples and then averaged. Data in *e* are from a single donor of four examined. *f*, Specificity of anti-ETV3 polyclonal Abs used in this study. Affinity-purified Abs were used to probe whole cell extracts (WCE) or nuclear extracts (NE) from 293T cells transfected with pcDNA3.1-ETV3 or pcDNA3.1-ETV3-HA. The size range of the ETV3 bands is ~50–57 kDa. *f*, *right panel*, Kinetic analysis of ETV3 in *Il10*$^{-/-}$ BMDMS stimulated as in *c*.

ETV3 expression was synergistically induced by LPS plus IL-10 (Fig. 1, *b* and *c*). In wild-type macrophages ETV3 mRNA is induced by LPS alone but was delayed relative to

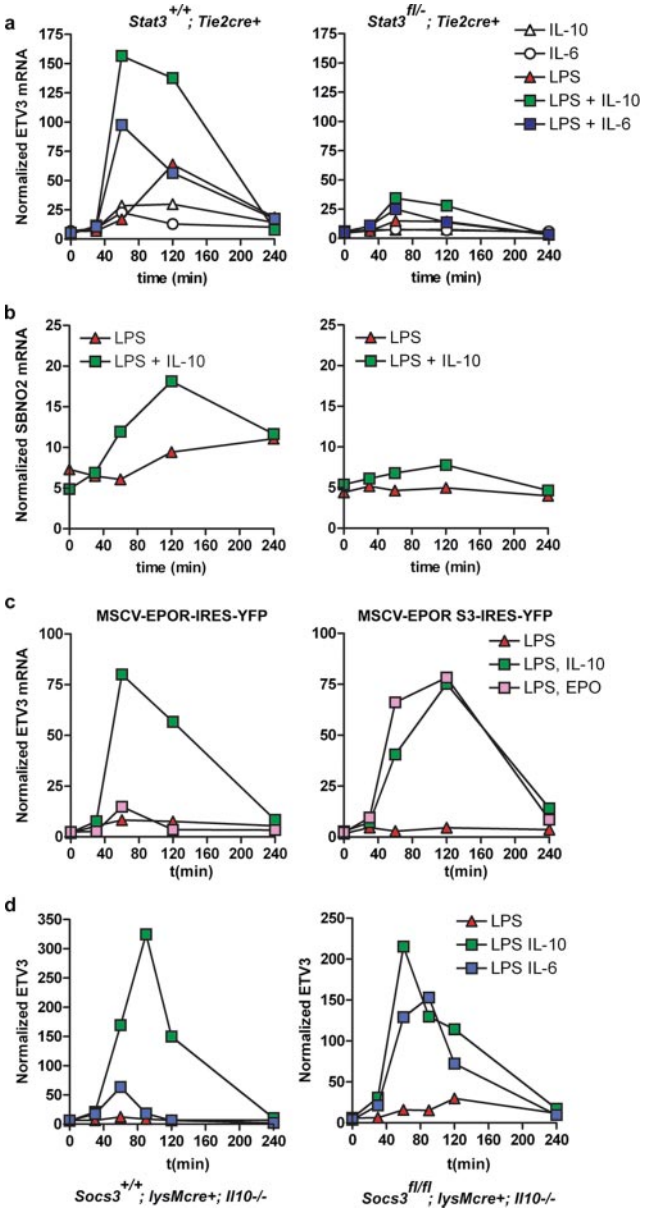

FIGURE 2. IL-10 regulates *Etv3* and *Sbno2* gene expression via STAT3. *a*, Kinetic analysis of ETV3 mRNA in control BMDMs (*left panel*) or STAT3-deficient BMDMs (*right panel*) stimulated as in Fig. 1*c*. *b*, Kinetic analysis of SBNO2 mRNA in control BMDMs (*left panel*) or STAT3-deficient BMDMs (*right panel*) stimulated with LPS or LPS plus IL-10. *c*, ETV3 mRNA is induced by a synthetic STAT3-activating receptor. C57BL/6 bone marrow stem cells were transduced with MSCV-EPOR-IRES-YFP or MSCV-EPOR-S3-IRES-YFP and selected for yellow fluorescent protein (YFP) by flow cytometry. Subsequently, BMDMs were generated and stimulated with LPS, LPS plus IL-10, or LPS plus EPO, and ETV3 mRNA was analyzed by qRT-PCR. Data are representative averaged samples from two complete independent transductions and selections. *d*, ETV3 expression is induced by IL-6 in the absence of SOCS3. BMDMs were isolated from individual *Socs3*$^{flox/flox}$*;LysMcre;Il10*$^{-/-}$ mice (*right panel*) or control mice (*left panel*) and stimulated with LPS, LPS plus IL-10, or LPS plus IL-6 as described for *a*. Data are representative of experiments performed with three independent mice derived from crosses of *Socs3*$^{flox/+}$*; LysMcre;Il10*$^{+/-}$ mice ($n = 156$). *Socs3*$^{flox/flox}$*;LysMcre;Il10*$^{-/-}$ mice were not produced in a Mendelian ratio from this cross.

the expression kinetics observed when LPS plus IL-10 was used as a stimulus, therefore suggesting that LPS-responsive ETV3 expression was due to the autocrine/paracrine effects of IL-10 induced by LPS (Fig. 1*b*; compare 60-min time points).

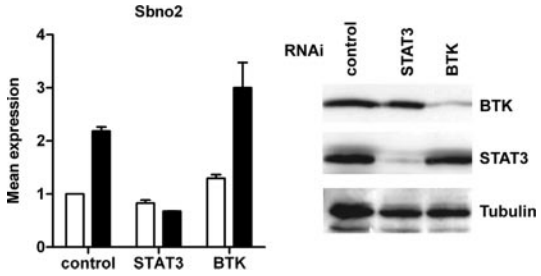

FIGURE 3. siRNA-mediated knockdown of STAT3 in human monocyte-derived macrophages. siRNAs against BTK (negative control) and STAT3 or no siRNAs were transfected into macrophages and stimulated with IL-10 (black bars) or not stimulated (open bars). The efficiency of knockdown is shown in the immunoblot. SBNO2 mRNA was measured by qRT-PCR ($n = 3$, error bars are SD) and expressed as relative means. Data are representative of three independent experiments.

The synergistic induction of ETV3 mRNA was confirmed by qRT-PCR in C57BL/6 or *Il10*$^{-/-}$ BMDMs (Fig. 1*c*). SBNO2 mRNA expression was also synergistically induced by LPS plus IL-10 (Fig. 1*d*). However, in contrast to ETV3 mRNA, SBNO2 mRNA was detected in resting macrophages and induced by IL-10 alone, yet it was not regulated significantly by LPS alone. Human ETV3 mRNA was increased by LPS plus IL-10 stimulation of monocyte-derived macrophages (Fig. 1*e*), although we noted that ETV3 expression at the protein level was variable in magnitude from donor to donor (data not shown). ETV3 expression was induced by LPS plus IL-10 and followed kinetically ETV3 mRNA expression (Fig. 1, *f* and *g*). The mobility of the multiple forms of ETV3 on SDS-PAGE was changed in the presence of LPS regardless of the presence of IL-10, suggesting that LPS induces modifications to ETV3 that we have yet to characterize. Therefore, ETV3 is present in macrophages at low amounts but is induced by LPS plus IL-10.

Etv3 and Sbno2 gene expression is regulated by the IL-10 arm of STAT3 signaling

Using STAT3-deficient macrophages isolated from *Stat3*$^{flox/-}$*; Tie2cre* mice, we observed that LPS plus IL-10-mediated induction of both ETV3 and SNBO2 was ablated in the absence of STAT3 (Fig. 2, *a* and *b*). We noted that the constitutive expression of SBNO2 mRNA detected previously (Fig. 1*d*) was unaffected by the absence of STAT3, suggesting that a STAT3-independent pathway regulates SBNO2 expression in unstimulated cells. We extended these observations by asking whether STAT3 was sufficient for ETV3 mRNA expression. We used a synthetic EPOR that activates STAT3 but not STAT5 (EPOR-S3). EPOR-S3, when expressed ectopically in macrophages, activates an AIR indistinguishable to that of the IL-10R (3). We stimulated primary macrophages transduced with the EPOR or EPOR-S3 with LPS plus IL-10 or LPS plus EPO. ETV3 expression was induced by LPS plus IL-10 in both cases (Fig. 2*c*). By contrast, EPO did not induce ETV3 expression in EPOR-transduced BMDMs but did so in macrophages expressing the EPOR-S3 (Fig. 2*c*). Therefore, STAT3 activation, independent of any other IL-10R specific signals, is sufficient to induce ETV3 expression.

We next tested whether the IL-6R could be manipulated to induce ETV3 expression. Previous studies have shown that the IL-6R can generate an AIR but only when the negative regulator SOCS3 is absent or cannot bind to the gp130 signaling

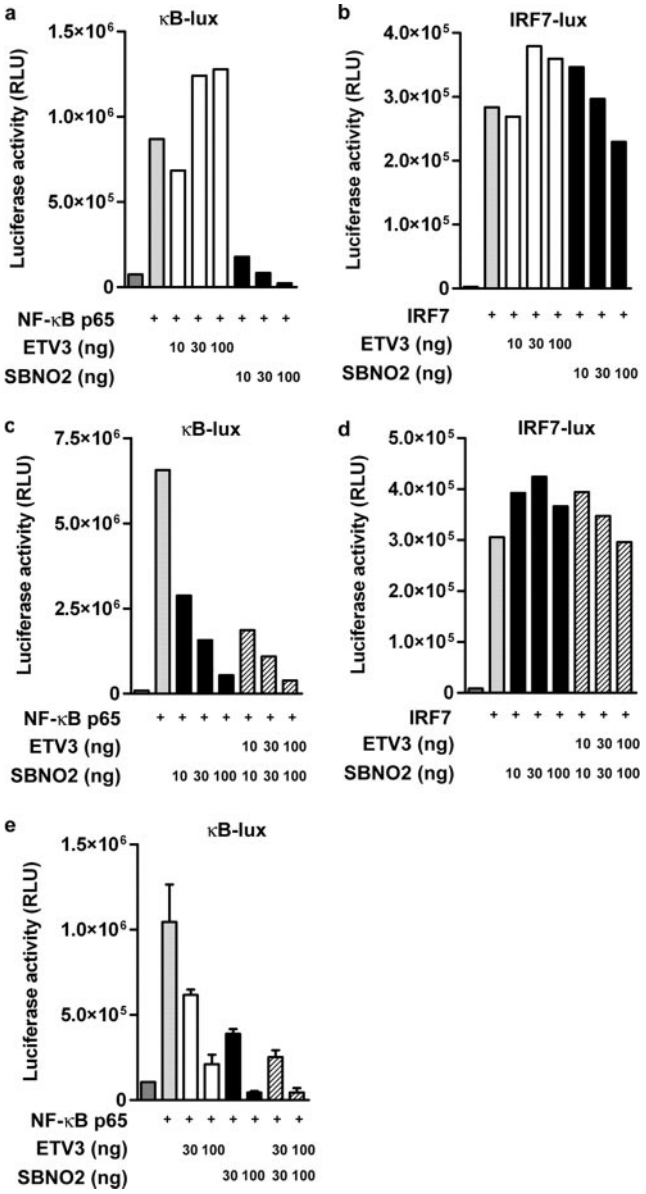

FIGURE 4. ETV3 and SBNO2 have functional transcriptional repression activity. 293T cells (*a–d*) or COS cells (*e*) were transfected with NF-κB p65 or IRF7 expression constructs, κB luciferase (lux) reporter, IRF7 luciferase reporter, a *Renilla* luciferase normalization construct, or expression constructs for ETV3 or SBNO2 as shown under each graph and in the quantities shown (ng). Luciferase activity was read from cell lysates after 24 h and presented as normalized relative values. Data are representative of three (*a–d*) or four (*e*) independent transfection experiments.

subunit of the IL-6R (6, 11). We therefore stimulated SOCS3-deficient BMDMs generated from *Socs3*$^{flox/flox}$*;LysMcre;Il10*$^{-/-}$ mice or control mice (*Socs3*$^{+/+}$*;lysMcre;Il10*$^{-/-}$), with LPS, LPS plus IL-10, or LPS plus IL-6. We found that IL-6 induced ETV3 mRNA expression with similar kinetics and magnitude to IL-10 when costimulated with LPS, but only in the absence of SOCS3 (Fig. 2, *d* and *e*). We also noted that IL-6 (in the presence of LPS) can induce the ETV3 mRNA in wild-type BMDMs, but the mRNA expression was short lived compared with that induced by LPS plus IL-10 (e.g., LPS plus IL-6 in Fig. 2*d*). These data argue that ETV3 expression in murine macrophages is linked to the STAT3 signaling mechanism that controls the AIR rather than being linked to STAT3 signaling in general.

Finally, we asked whether the expression of human SBNO2 was dependent on STAT3. Using siRNA-mediated depletion of STAT3 in primary human macrophages, we demonstrated that the expression of SBNO2, in addition to a known STAT3 target gene (*Bcl3*), was dependent on STAT3 (Fig. 3). Corresponding studies for human ETV3 were inconclusive because of the variability in ETV3 expression from one donor to the other as noted above.

ETV3 and SBNO2 function as transcriptional repressors

Previous studies have shown ETV3 functions as a repressor when overexpressed during macrophage development (26, 28) and forms a complex with DDX20, a helicase corepressor. SBNO2, in contrast, has not been characterized in mammals, but an ortholog in *Drosophila*, Strawberry notch, appears to function as a corepressor in the Notch pathway (27, 29). We therefore tested the ability of ETV3 and/or SBNO2 for their capacity to regulate transcription mediated by different pathways central to innate immune responses, namely NF-κB and IRF7. In 293T cells, SBNO2 was a potent and titratable inhibitor of NF-κB- but not IRF7-mediated transcription (Fig. 4, *a–d*). In 293 T cells, ETV3 did not have inhibitory effects on either NF-κB- or IRF7-mediated reporter activity. However, ETV3 did inhibit NF-κB reporter activity in COS cells (Fig. 4*e*), suggesting cell type-specific differences in ETV3 activity. When ETV3 and SBNO2 were cotransfected, slight additive inhibitory effects were observed, especially in COS cells (Fig. 4, *c* and *e*).

Discussion

Our studies have uncovered two targets of the IL-10 signaling pathway linked to transcriptional repression. IL-10-regulates the expression of both *Etv3* and *Sbno2* in a STAT3-dependent way. Sustained and robust ETV3 expression is specific to the anti-inflammatory arm of STAT3 signaling. By contrast, ETV3 and SBNO2 expression was not substantially activated by STAT3 signaling activated by non-AIR activating receptors such as the IL-6R. We also found that SBNO2 was able to strongly repress NF-κB-mediated transcription but not IRF7 transcription. Collectively, these data argue that ETV3 and SBNO2 function as components of the IL-10-regulated pathway that represses inflammatory gene expression.

ETV3 was discovered as an ETS family repressor of gene expression during macrophage development from bone marrow stem cells (26) and forms complexes with DDX20, a helicase family corepressor. In the same studies, enforced ETV3 expression in hemopoietic stem cells retarded macrophage growth and differentiation. Using a similar system of retroviral-mediated gene transfer, we were able to reproduce these findings: therefore, the derivation of sufficient numbers of primary macrophages ectopically expressing ETV3 precludes, for the moment, the determination of whether ETV3 is sufficient to mediate the AIR (our unpublished data).

We found that SBNO2 has strong repressive activity for NF-κB but not IRF7-mediated transcriptional activity. We propose that SBNO2 likely interacts with a range of other proteins that selectively inhibit transcription in macrophages. In mammals there are two Strawberry notch-like genes, *Sbno1* and *Sbno2*, located on different chromosomes. Both genes encode large ~170-kDa proteins containing canonical DExD/H helicase domains. Although closely related, the functions of

SBNO2 cannot be covered by SBNO1 because a complete loss-of-function mutant in *Sbno2* is lethal at E6-E7 (P. J. Murray, J. Kennedy, unpublished data), suggesting that, in addition to its role in IL-10 signaling, SBNO2 has other essential functions.

The IL-10-STAT3-ETV3/SBNO2 pathway is consistent with the hypothesis that IL-10 selectively controls inflammatory gene expression through a regulated repression mechanism. Rigorous testing of the precise functions of ETV3 and SBNO2 in inflammatory transcription will require the generation of mice lacking ETV3 and mice bearing conditional alleles of *Sbno2*.

Acknowledgments

We thank Doug Hilton and Warren Alexander (Walter and Eliza Hall Institute of Medical Research) for the gift of *Socs3*$^{flox/flox}$ mice and Lilian Moreira for help with the figures.

Disclosures

An unrelated research project in the laboratory of S.S.W. is supported by a Preclinical Research Agreement with Amgen, Inc. The other authors have no financial conflicts of interest.

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