Twist mediates suppression of inflammation by type I IFNs and Axl

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Type I interferons (IFNs) are pleiotropic cytokines with antiviral and immunomodulatory properties. The immunosuppressive actions of type I IFNs are poorly understood, but IFN-mediated suppression of TNFα production has been implicated in the regulation of inflammation and contributes to the effectiveness of type I IFNs in the treatment of certain autoimmune and inflammatory diseases. In this study, we investigated mechanisms by which type I IFNs suppress induction of TNFα production by immune complexes, Fc receptors, and Toll-like receptors. Suppression of TNFα production was mediated by induction and activation of the Axl receptor tyrosine kinase and downstream induction of Twist transcriptional repressors that bind to E box elements in the TNF promoter and suppress NF-κB–dependent transcription. Twist expression was activated by the Axl ligand Gas6 and by protein S and apoptotic cells. These results implicate Twist proteins in regulation of TNFα production by antiinflammatory factors and pathways, and provide a mechanism by which type I IFNs and Axl receptors suppress inflammatory cytokine production.

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Abbreviations used: bHLH, basic helix loop helix; ChIP, chromatin immunoprecipitation; ERK, extracellular signal–regulated kinase; MAPK, mitogen-activated protein kinase; SLE, systemic lupus erythematosus.

Type I IFNs, IFNα and IFNβ, are pleiotropic cytokines that are induced in many cell types by viral or bacterial products (such as double-stranded RNA and LPS) and have potent antiviral effects (1). In addition to antiviral effects, type I IFNs exhibit immunomodulatory properties and enhance innate and acquired immune responses, promote the transition from innate to acquired immunity, and promote certain autoimmune disorders (1–5). Mechanisms by which type I IFNs enhance immunity include stimulation of cytokine and chemokine production, activation of NK cells, maturation of DCs, and promoting Th1 responses and increased B cell class switching and antibody production.

Like most cytokines, type I IFNs are pleiotropic and also have suppressive effects on immunity and inflammation. The suppressive effects of type I IFNs can predominate in certain (patho)physiological settings. For example, type I IFNs suppress delayed type hypersensitivity reactions, host defense to Listeria monocytogenes and Mycobacteria tuberculosis, and can suppress endotoxin-induced mortality under certain conditions (6–11). Type I IFNs suppress inflammation and disease activity in experimental allergic encephalitis, arthritis, colitis, and osteolysis (6, 12–15), and are effective in the treatment of multiple sclerosis and possibly ulcerative colitis (16, 17). The mechanisms underlying the suppressive effects of type I IFNs are not well understood but include suppression of cell proliferation, IL-12 and TNFα production, and, under specific conditions, IFNγ production (5). IFN-induced suppression of TNFα production was required for the suppressive effects of type I IFNs in experimental allergic encephalitis, and treatment with type I IFNs suppressed TNFα production in vivo in humans (15, 18). Thus, IFN-mediated suppression of TNFα production is important in disease pathophysiology, but mechanisms by which type I IFNs suppress TNFα production and inflammation have not been clarified.

TNFα is a potent inflammatory cytokine that is important for host defense and is expressed at sites of inflammation where it activates resident tissue cells, infiltrating immune cells and endothelial cells in surrounding blood vessels (19). TNFα has been implicated in the pathogenesis of many acute and chronic inflammatory conditions, including septic shock, rheumatoid arthritis, and inflammatory bowel disease. TNFα also appears to play a role in

promoting end organ inflammation, such as nephritis, in systemic lupus erythematosus (SLE), although TNFα suppresses the development of autoimmunity in murine models of this disease (4, 20). Major sources of TNFα are activated macrophages and DCs, and major inducers of TNF include pathogen-associated molecular patterns (such as TLR ligands), inflammatory cytokines (such as IL-1), cell surface and secreted factors expressed by activated T cells, and immune complexes that activate cells via Fc receptors. Fc receptors are key mediators of TNF production in acute models of immune complex-induced inflammation, including the Arthus reaction and experimental arthritis, and likely contribute to TNFα production in chronic inflammatory diseases such as rheumatoid arthritis and SLE (21).

TNFα production needs to be tightly controlled to avoid excessive inflammation, tissue damage, and toxicity. NF-κB is an important inducer of TNFα transcription, and activation of the p38 mitogen-activated protein kinase (MAPK) is required for stabilization and efficient translation of TNFα mRNA (19). Major immunosuppressive and antiinflammatory factors, such as IL-10 and corticosteroids, suppress TNFα production by inhibiting activation of NF-κB and p38. How antiinflammatory factors suppress NF-κB activation is not well understood and mechanisms that suppress activation of IκB kinases, degradation of I-κB, and nuclear translocation of NF-κB subunits are under intense investigation. Recent work has identified an alternative mechanism of inhibiting NF-κB function, namely inhibition of p65/RelA-mediated transactivation in the nucleus by Twist proteins (22). Twist proteins (Twist1 and Twist2) are basic helix loop helix (bHLH) transcriptional repressors that bind to E boxes in gene promoters, including the TNFα promoter, and repress transcription. Twist proteins are key regulators of NF-κB–mediated inflammation, as Twist2 deficiency, or haploinsufficiency of Twist1 and Twist2, results in a lethal systemic inflammatory syndrome linked to enhanced proinflammatory cytokine production (22). TNFα production is also suppressed by receptor tyrosine kinases of the Tyro 3 family, which is comprised of Tyro 3, Mer, and Axl (23). Tyro 3 family kinases contribute to the antiinflammatory effects of apoptotic cell phagocytosis and suppress TNFα production by unknown mechanisms (23–26). In this study, we investigated mechanisms by which type I IFNs suppress immune complex/Fc receptor-induced TNFα production, which is relevant for the modulation of chronic inflammation by IFNs. We found that IFNα suppressed TNFα production by inducing a cascade of gene activation that resulted in repression of the TNFα promoter by Twist.

RESULTS

IFNα suppresses FcγR- and TLR-induced TNFα production

We determined the effects of preculture with IFNα on FcγR-induced production of TNFα. Primary human macrophages were cultured for 2 d with low priming concentrations (250 pg/ml = 100 antiviral U/ml) or saturating concentrations (7.5 ng/ml = 3,000 U/ml) of IFNα, stimulated by cross-linking of FcγRs, and TNFα production was measured. As expected, cross-linking of FcγRs on control macrophages resulted in the secretion of substantial levels of TNFα (Fig. 1 A). In contrast, preculture with IFNα dramatically suppressed FcγR-induced secretion of TNFα protein (Fig. 1 A). In addition, IFNα strongly suppressed FcγR-induced increases in TNFα mRNA expression (Fig. 1 B). Inhibition of FcγR-induced TNFα production by IFNα was highly reproducible

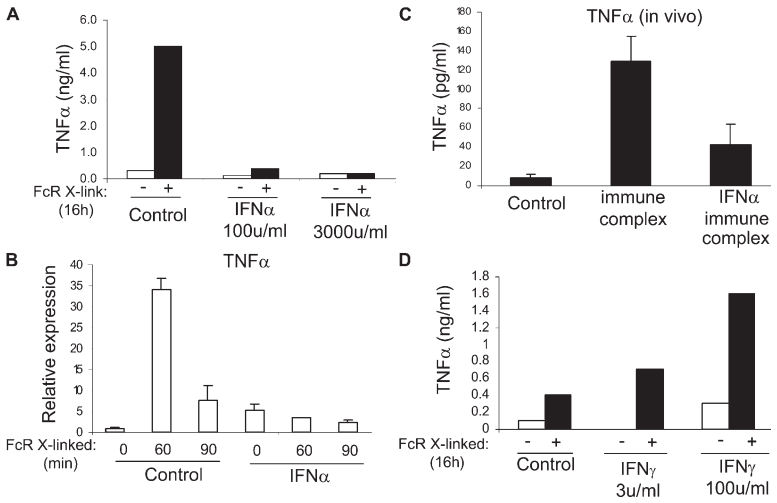

Figure 1. IFNα suppresses FcγR-induced TNFα production. Macrophages were cultured for 2 d without IFNs or with IFNα (A and B) or IFNγ (D). FcγRs were then cross-linked using plate bound IgG (20 μg/ml). In A and D, supernatants were harvested 16 h later, and TNFα was measured using ELISA. In B, cells were harvested 60 and 90 min after FcγR cross-linking, and TNFα mRNA levels measured using real-time PCR. For IFNα, 100 antiviral U/ml = 250 pg/ml and 3,000 U/ml = 7.5 ng/ml; for IFNγ, 100 U/ml = 10 ng/ml. (C) Groups of three to four mice were injected i.p. with PBS or 10^5 U of IFNα, and 2 d later they were injected i.v. with 300 μg (100 μl) of immune complexes formed by mixing rabbit anti–human F(ab′) with human IgG. Serum was obtained 1 h later, and TNFα was measured using ELISA. Results from three independent experiments are shown.

and has been observed in experiments with more than 20 different blood donors. IFNα also effectively inhibited immune complex-induced TNFα production in vivo (Fig. 1 C). In contrast to the effects of IFNα, preculture of macrophages with IFNγ resulted in increased TNFα production after FcγR ligation (as expected) (Fig. 1 D), thus demonstrating the specificity of IFNα-mediated inhibition.

We then investigated whether IFNα suppressed the production of cytokines other than TNFα and the effects of IFNα on the activation of macrophages by TLR ligands. In addition to suppressing TNFα production (Fig. 1), IFNα effectively suppressed FcγR-mediated induction of CCL3/MIP-1α, IL-8, and COX2, proinflammatory factors whose induction is NF-κB dependent (similar to TNFα) (Fig. 2 A). FcγR cross-linking did not induce IL-12 expression in our system, and no additional effects of IFNα were observed (unpublished data). Similar to the strong and highly reproducible inhibition of FcγR-induced cytokine expression, IFNα effectively suppressed TLR2-induced cytokine production (Fig. 2 B, left and middle; $n = 4$). In contrast, suppression of TLR4-induced TNFα expression was relatively weak (20–35%) and variable among different blood donors, even when low concentrations of LPS (0.1 ng/ml) were used (Fig. 2 B, right; $n = 12$). However, the partial suppression of LPS-induced TNFα production was statistically significant ($P \leq 0.02$ for all concentrations of LPS and IFNα that were used, paired Student t test; Fig. 2 B). These results demonstrate that IFNα suppressed the activation of macrophages by several key inflammatory stimuli. The weak suppression of low concentrations of LPS (Fig. 2 B), in contrast to the strong suppression of FcγR-mediated effects (Fig. 1), suggested that low levels of LPS contamination of immune complexes did not contribute to effects observed in Fig. 1. The issue of LPS contamination of immune complexes was further addressed using C3H/HeJ mice that express a mutated nonfunctional TLR4. FcγR-mediated induction of TNFα production was comparable in macrophages from C3H/HeJ and genetically matched control C3H/OuJ mice, and was comparably suppressed by IFNα (Fig. 2 C), thus indicating contaminating endotoxin did not contribute significantly to the regulation of TNFα production by immune complexes and IFNα.

IFNα effects on FcγR expression and proximal signaling

We investigated whether IFNα suppressed FcγR-induced TNFα production by down-regulating FcγR expression or signal transduction. Cell surface expression of the three

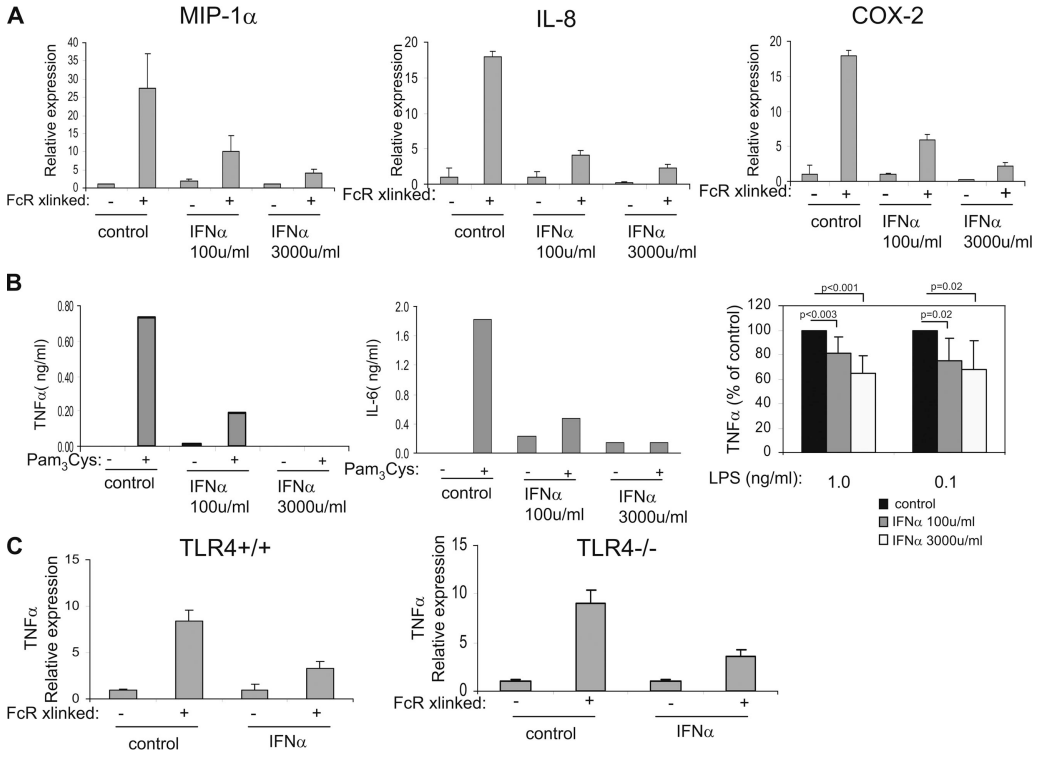

Figure 2. Suppression of FcγR-, TLR2-, and TLR4-induced cytokine expression by IFNα. (A) Human macrophages were cultured for 2 d with or without IFNα, FcγRs were cross-linked for 90 min using plate-bound IgG (20 μg/ml), and mRNA levels were measured using real-time PCR and normalized relative to GAPDH. (B) Cells were stimulated overnight with the TLR2 ligand Pam_3Cys (100 ng/ml) or the TLR4 ligand LPS (1 ng/ml or 0.1 ng/ml), and culture supernatants were analyzed using ELISA. For experiments using Pam_3Cys, a representative experiment out of four is shown. For LPS experiments, cumulative data is depicted (for 1 ng/ml LPS, $n = 12$; for 0.1 ng/ml LPS, $n = 8$). Differences were statistically significant (Students paired t test) at both concentrations of IFNα and both concentrations of LPS as marked. (C) Murine bone marrow–derived macrophages from C3H/HeJ mice that harbor a loss of function TLR4 mutation and from control C3H/OuJ mice were used.

FcγRs that are expressed on macrophages, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), was analyzed using flow cytometry. Consistent with previous reports (21), IFNα had only modest effects on cell surface FcγR expression (Fig. 3 A). These results indicate that the inhibition of FcγR-induced TNFα production was not secondary to global suppression of FcγR expression. Ligation of FcγRs activates NF-κB and MAPK pathways that are important for TNFα production (19). We assessed the effects of IFNα on FcγR-activated signal transduction by analyzing activation of NF-κB and the extracellular signal–regulated kinase (ERK) family of MAPKs. The activation of ERKs by FcγR ligation was comparable in control and IFNα-treated macrophages at each time point tested (Fig. 3 B). These results indicate that IFNα did not globally block FcγR signal transduction and are consistent with comparable cell surface FcγR expression in control and IFN-treated cells (Fig. 3 A). Activation of the NF-κB pathway was assessed by measuring phosphorylation and degradation of I-κB at several time points after FcγR ligation of control and IFNα-primed macrophages. I-κB phosphorylation and degradation after FcγR ligation were modest (Fig. 3 C) relative to an LPS-positive control (unpublished data), and both phosphorylation and degradation of I-κB were partially suppressed in IFNα-treated cells relative to control cells (Fig. 3 C, a representative experiment out of three is shown). The modest suppressive effect of IFNα on NF-κB activation is consistent with previous results in a different system (27) but is unlikely to explain the strong suppression of TNFα production that was observed in IFNα-treated cells (Fig. 1, A and B). Therefore, we investigated additional IFNα-inducible mechanisms that may inhibit TNFα production.

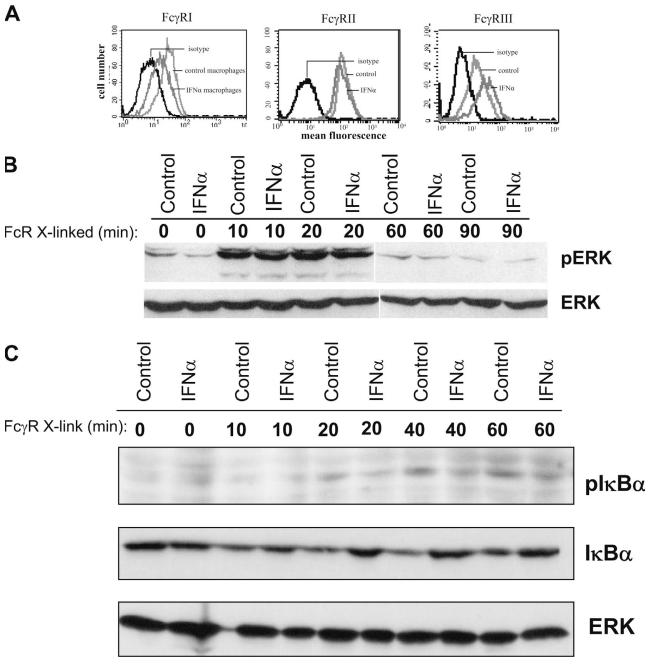

Figure 3. IFNα regulation of FcγR expression and signaling. (A) Flow cytometry was used to measure cell surface FcγR expression on control and IFNα-treated macrophages. IFNα was used at 3,000 U/ml, and similar results were obtained over a range of IFNα concentrations from 100 U/ml to 3,000 U/ml. (B) Macrophages were cultured for 2 d with 3,000 U/ml of IFNα, FcγRs were cross-linked using plate-bound IgG (20 μg/ml), and activation of ERKs was measured using immunoblotting. (C) Control macrophages or cells cultured for 2 d with IFNα (3,000 U/ml) were added to control or IgG-coated (20 μg/ml) wells. Cell extracts were analyzed by immunoblotting of replicate filters.

Twist proteins mediate IFNα suppression of TNFα production

We reasoned that IFNα induced the expression of inhibitors of NF-κB activation or function and tested several candidate inhibitors, including Twist proteins (22). Twist1 and Twist2 are bHLH transcriptional repressors that bind to an E-box in the TNFα promoter and repress TNFα transcription by inhibiting transcriptional activation by the p65 subunit of NF-κB (22). We first tested if Twist expression was induced by IFNα. Interestingly, Twist1 and Twist2 mRNA expression was strongly induced by IFNα (Fig. 4, A and B). The kinetics

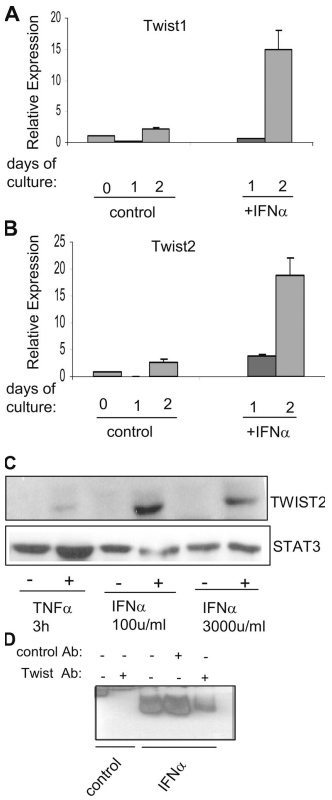

Figure 4. IFNα induces expression of Twist. (A and B) Twist1 and Twist2 mRNA levels were measured in control and IFNα-primed macrophages using real-time PCR and were normalized relative to GAPDH levels. (C) Twist2 expression was measured using immunoblotting. (D) Nuclear extracts from control and IFNα-stimulated cells were used in an EMSA assay with a radiolabeled E box containing oligonucleotide. Control and Twist antibodies (2 μg) were added to the binding reaction 20 min before adding radiolabeled oligonucleotide.

of Twist induction were delayed such that elevated Twist expression was observed after 2 d of IFNα stimulation, which corresponds to the time point when TNFα expression was suppressed. Twist protein levels increased in parallel with mRNA levels (Fig. 4 C). Interestingly, treatment with IFNα for 2 d induced higher Twist expression than did treatment with TNFα for 3 h, which was previously shown to rapidly induce Twist expression in a feedback inhibition loop (22) and was used as a positive control (Fig. 4 C, compare lane 2 to lanes 4 and 6). We then used gel shift assays to investigate whether IFNα-induced Twist proteins bind to DNA. Twist bHLH transcription factors bind to promoter elements termed E boxes and have been shown to bind to an E box–containing oligonucleotide derived from the TNFα promoter (22). IFNα induced a nuclear DNA-binding complex that bound to an E box–containing oligonucleotide derived from the TNFα promoter (Fig. 4 D), consistent with previously reported results (22). Similar results were obtained using a consensus E-box oligonucleotide (unpublished data). Formation of this IFNα-inducible DNA-binding complex was disrupted by Twist antibodies but not by control antibodies, indicating that this complex contained Twist proteins (Fig. 4 D). Collectively, the results establish that IFNα induces the expression of Twist proteins.

Next, we used macrophages from Twist-deficient mice to determine the role of Twist in the regulation of TNFα production by IFNα. Twist1 deficiency is embryonal lethal, whereas *twist2*$^{-/-}$ and *twist1*$^{+/-}$ *twist2*$^{+/-}$ mice develop a severe spontaneous neonatal inflammatory syndrome (22, 28). *twist1*$^{-/-}$ macrophages were not available, and therefore we used bone marrow–derived macrophages from *twist2*$^{-/-}$ and *twist1*$^{+/-}$ *twist2*$^{+/-}$ mice and genetically matched controls. Macrophages were cultured with or without IFNα for 2 d, plated onto control or IgG-coated wells, and TNFα protein and mRNA levels were measured using ELISA and qPCR. As observed previously with human macrophages (Fig. 1), IFNα suppressed FcγR-mediated induction of TNFα protein and mRNA in wild-type murine macrophages (Fig. 5, A and B). In contrast, in *twist2*$^{-/-}$ macrophages, IFNα was unable to suppress FcγR induction of TNFα protein (Fig. 5 A) or mRNA (Fig. 5 B). Comparable results were obtained when *twist1*$^{+/-}$ *twist2*$^{+/-}$ macrophages were used (Fig. 5, C and D). These results demonstrate that Twist transcriptional repressors are required for IFNα suppression of FcγR-induced TNFα production, and suggest that suppression is mediated by binding of Twist to the TNFα promoter.

We performed transient transfection and chromatin immunoprecipitation (ChIP) assays to gain greater insight into the regulation of the TNFα promoter by Twist proteins. RAW267.4 macrophage cells were cotransfected with a reporter construct driven by the TNFα promoter and increasing amounts of Twist1 and Twist2, and FcγRs were ligated and reporter gene activity measured. Twist1 and Twist2 suppressed TNFα promoter activity in a dose-dependent

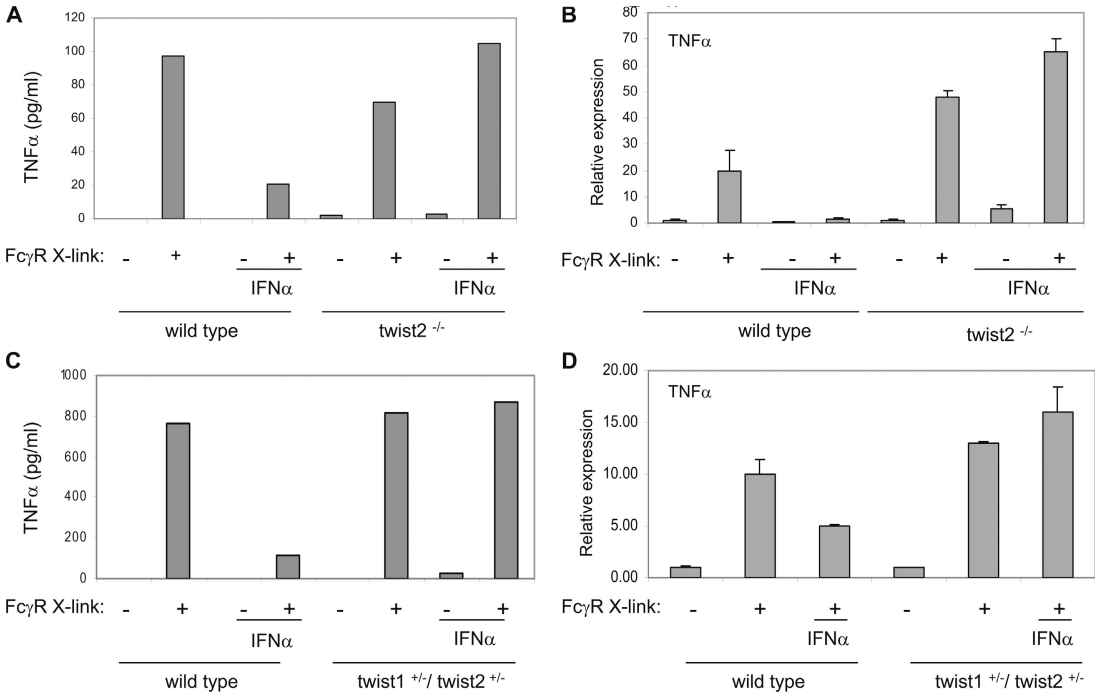

Figure 5. Twist mediates IFNα inhibition of FcγR-induced TNF production. (A and B) Bone marrow–derived macrophages from genetically matched control mice and *twist2*$^{-/-}$ mice were cultured with or without IFNα for 2 d and plated onto control or IgG-coated wells for 6 h (A) or 1 h (B). TNFα in culture supernatants was measured using ELISA (A), and TNFα mRNA levels were determined using real-time PCR and normalized relative to GAPDH (B). (C and D) Macrophages from genetically matched control mice and *twist1*$^{+/-}$*twist2*$^{-/-}$ mice were treated as in A and B, and TNFα protein and mRNA were measured.

manner (Fig. 6 A). Furthermore, TNFα promoter activity was effectively suppressed by the combination of Twist1 and Twist2, consistent with the abrogation of IFNα effects in compound heterozygote macrophages (Figs. 5, C and D). When a reporter construct harboring mutations in two out of the three E box motifs in the TNFα promoter was used, suppression of TNFα promoter activity by Twist proteins was attenuated (Fig. 6 B), thereby suggesting that suppression of TNFα expression by Twist is dependent on its interaction with the TNFα promoter E boxes. Twist and NF-κB p65 have been shown to interact when overexpressed in COS cells, but an association between these proteins was not detected in primary macrophages (unpublished data), possibly because of lower expression. Next, we determined whether Twist proteins induced by IFNα bind to the endogenous TNFα promoter. ChIP assays demonstrated that IFNα induced the binding of Twist to a TNFα promoter fragment that contains an E box (22) (Fig. 6 C). Negative controls included lack of Twist binding to GAS sites (Fig. 6 C) and lack of Stat1 binding to E box sites (unpublished data). Collectively, these results show that IFNα suppression of TNFα expression is mediated, at least in part, by induction of Twist binding to the TNFα promoter.

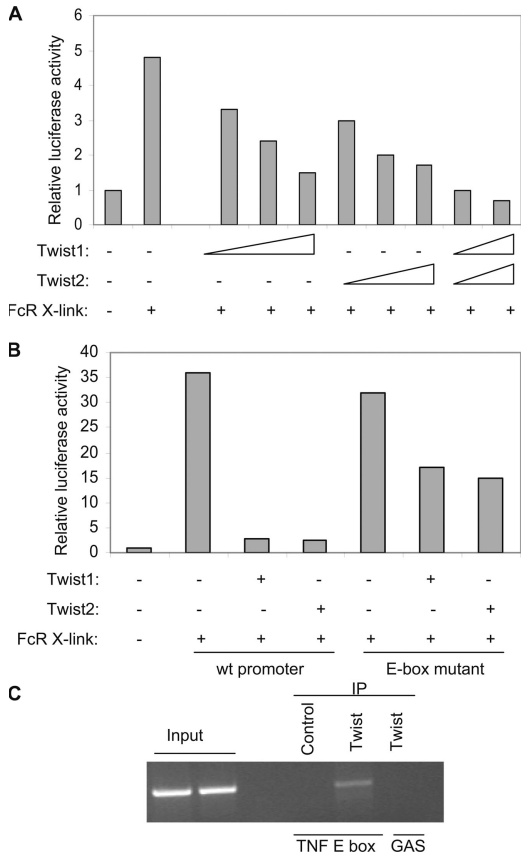

Figure 6. Twist represses TNFα gene expression. (A) RAW267.4 macrophage cells were transfected in duplicate with a TNFα promoter-driven luciferase reporter construct, increasing amounts of Twist1 and Twist2 expression vectors, and a Renilla luciferase-encoding internal control plasmid. Cells were stimulated by FcγR ligation for 4 h, and luciferase activity was measured and normalized relative to Renilla luciferase. A representative experiment out of five is shown. (B) Transfection experiments were performed as in A, except a TNFα reporter construct in which 2 E boxes had been mutated was used. (C) Soluble chromatin was prepared from IFNα-treated primary macrophages and immunoprecipitated with control or anti-Twist antibodies. Extracted DNA was amplified using primers that span the E box of the TNFα gene or the GAS site of the IRF-1 gene promoter.

IFNα induction of Twist is mediated by the Axl tyrosine kinase

The delayed kinetics of Twist induction by IFNα (Fig. 4) suggested indirect activation of Twist by IFNα-induced intermediates. To identify such intermediates we performed microarray analysis of IFNα-induced gene expression to screen for induction of genes known to regulate the expression of TNF or Twist. IFNα induced expression of the Axl member of the Tyro 3 family of receptor tyrosine kinases that are preferentially expressed in myeloid, neuronal, and reproductive system cells (23; unpublished data). Tyro 3 receptor kinases have been implicated in suppression of TNFα production by unknown mechanisms (24), and their ligands protein S (29, 30) and apoptotic macrophages (25, 26) are constitutively present in our system, and expression of the Axl ligand Gas6 was induced by IFNα (unpublished data). Therefore, we tested whether IFNα-induced Axl expression contributed to IFNα-mediated induction of Twist and thereby suppression of TNFα production. First, we used qPCR to confirm the microarray results that IFNα, but not IL-10, induced Axl expression in macrophages (Fig. 7 A). Then, we investigated the role of Axl in mediating Twist expression by blocking the interaction of Axl with endogenous ligands present in our cultures. Blocking of the interaction of Axl with endogenous ligands using either soluble Axl-Fc or neutralizing anti-Axl antibodies effectively suppressed IFNα-induced Twist expression (Fig. 7 B), thereby implicating Axl in the induction of Twist expression. We then tested the effects of stimulating Axl and related kinases in control macrophages that expressed low levels of Axl and related Tyro 3 family receptors. The Tyro 3 family receptor ligands protein S and Gas6, and also apoptotic cells that are bound by protein S and Gas6, induced expression of Twist1 (Fig. 7 C), thus further supporting a role for Tyro 3 kinases in inducing Twist expression. Induction of Twist2 by Tyro 3 receptor ligands in non-IFNα– treated macrophages was less robust (unpublished data), suggesting that induction of Twist2 is specifically mediated by Axl or requires additional IFNα-inducible factors. To test the role of Axl in mediating IFNα suppression of TNFα expression, we measured the effects of blocking Axl interaction with endogenous ligands on FcγR-induced TNFα production. Consistent with the down-regulation of Twist expression when Axl function was blocked (Fig. 7 B), Axl blockade also reversed IFNα-mediated suppression of TNFα production (Fig. 7 D). Although IFNα induction of Axl expression in murine bone

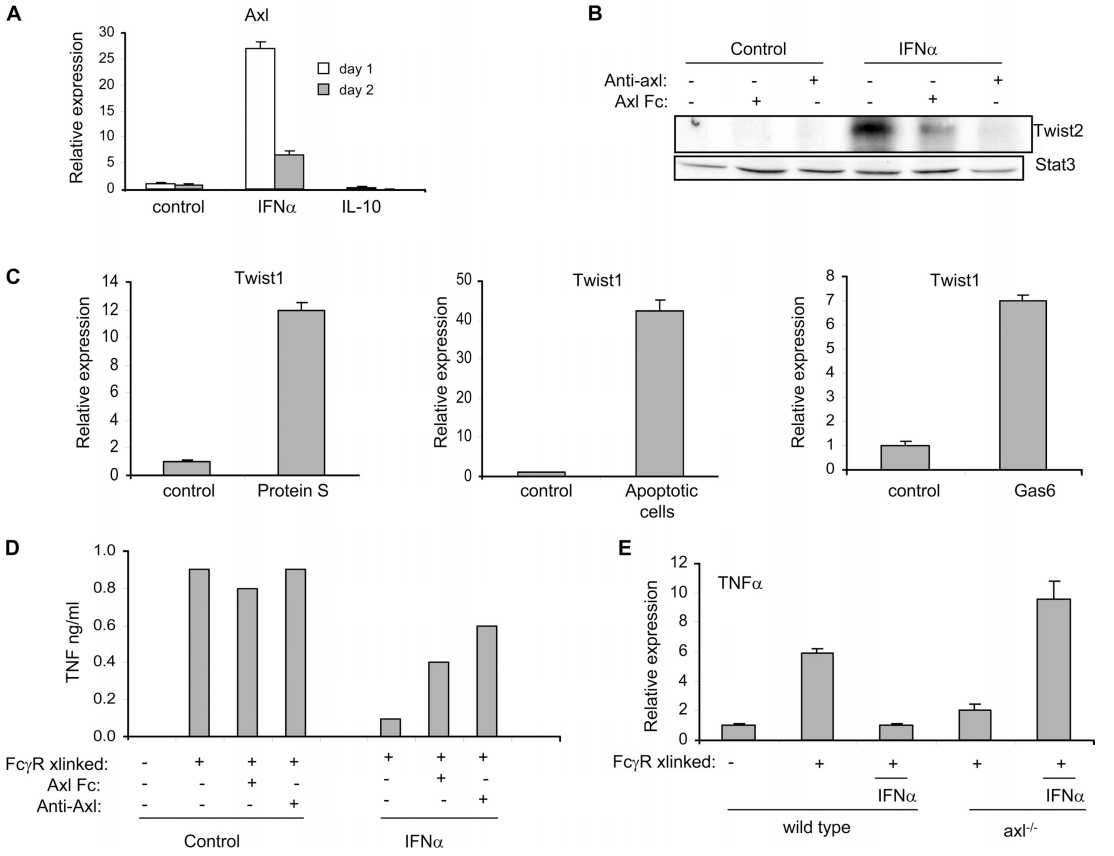

Figure 7. IFNα–induced Axl mediates induction of Twist expression. (A) Macrophages were stimulated for 1 or 2 d with IFNα (3,000 U/ml) or IL-10 (100 ng/ml), and Axl mRNA was measured using real-time PCR. (B) Macrophages were cultured for 2 d with IFNα (3,000 U/ml) in the presence of isotype control or anti-Axl antibodies (20 μg/ml) or with Axl-Fc (20 μg/ml), and Twist2 was measured using immunoblotting. (C) Macrophages were transferred into serum-free media for 6 h and then stimulated for 3 h with protein S (40 μg/ml), Gas6 (5 μg/ml), or apoptotic Jurkat cells (ratio of apoptotic cells to macrophages of 10:1), and Twist1 mRNA was measured using real-time PCR. Twist1 mRNA was normalized relative to GAPDH (protein S and Gas6 stimulations) or CD14 (apoptotic Jurkat stimulation). (D) Macrophages were cultured for 2 d with IFNα (3,000 U/ml) in the presence of isotype control or anti-Axl antibodies (20 μg/ml) or with Axl-Fc (20 μg/ml), FcγRs were cross-linked, and TNFα production was measured using ELISA. (E) Bone marrow–derived macrophages from genetically matched control mice and *axl*$^{-/-}$ mice were cultured with or without IFNα for 2 d, plated onto control or IgG-coated wells for 3 h, and TNFα mRNA levels were determined using real-time PCR and normalized relative to GAPDH. A representative experiment out of three is shown; similar results were obtained in a fourth experiment using macrophages triply deficient in Axl, Mer, and Tyro 3.

marrow–derived macrophages was more modest than in human monocyte-derived macrophages (unpublished data), a substantial role for Axl in mediating IFNα suppression of TNFα production was confirmed using *axl*$^{-/-}$ murine macrophages (Fig. 7 E). Collectively, the results demonstrate that IFNα suppresses TNFα production by increasing macrophage expression of Axl and responsiveness to Axl ligands, thereby inducing Twist expression and Twist-mediated repression of the TNFα promoter.

DISCUSSION

The antiinflammatory effects of type I IFNs are important for suppression of disease activity in several animal models of autoimmune disease and for the therapeutic efficacy of IFNs in multiple sclerosis, but mechanisms underlying their antiinflammatory activity are poorly understood. In this study, we have identified a mechanism by which type I IFNs suppress production of the potent inflammatory cytokine TNFα. Inhibition is mediated by induction of the receptor tyrosine kinase Axl, and activation of Axl leads to expression of Twist transcriptional repressors that suppress TNFα expression (Fig. 8). These results extend our understanding of the regulation of Twist proteins and their role in modulating cytokine production and provide insight into mechanisms that mediate the antiinflammatory action of the Tyro 3 family of receptor tyrosine kinases. Manipulation of Axl expression and downstream activation of Twist represent a new approach to regulating cytokine production and inflammation.

An important mechanism of type I IFN action is regulation of cellular responses to other extracellular factors (31). In the current paradigm, type I IFNs sensitize cells to

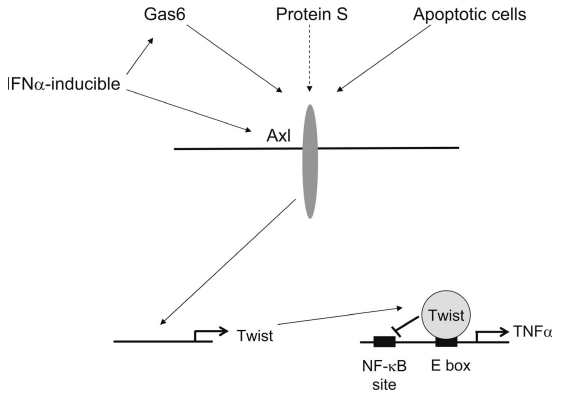

Figure 8. IFNα inhibits TNFα production by induction of Axl and Twist. IFNα induces expression of the Tyro 3 family receptor tyrosine kinase Axl. Axl, in turn, is activated by Gas6, protein S, and apoptotic cells. Protein S is present at high levels in serum, Gas6 can be induced by IFNα, and apoptotic cells bind Gas6 and protein S. Axl activation leads to expression of Twist, which binds to the E box in the TNFα promoter and inhibits NF-κB–dependent transcription.

activation by inflammatory cytokines and viruses, a phenomenon that has been termed priming (31–36). Several mechanisms of priming have been described, including increased expression of activating receptors, such as Toll-like receptor 3, that senses dsRNA and thereby viral infection (37). We show that type I IFNs also enhance cellular responses to antiinflammatory factors, in this case protein S, Gas6, and apoptotic cells, by inducing expression of their receptor Axl. Thus, cells that have been primed by type I IFNs are not necessarily programmed for activation, but their fate and activation status are determined by the balance of pro- and antiinflammatory factors present in the microenvironment. Type I IFN-induced enhanced responsiveness to both pro- and antiinflammatory factors extends our understanding of the concept of priming and helps explain the pleiotropic nature and homeostatic functions of type I IFNs. In contrast, the sole type II IFN, IFNγ, does not increase expression of inhibitory receptors (unpublished data) and subsumes a predominantly activating role.

Axl was the only member of the Tyro 3 family that was induced by IFNα. The Tyro 3 kinases are preferentially expressed in myeloid, neuronal, and reproductive system cells, and all three kinases are constitutively expressed in macrophages in our system (unpublished data). Since all three receptor kinases in this family share ligands, such as protein S, Gas6, and apoptotic cells, it is likely that Mer and Tyro 3 contribute to the induction of Twist expression in control non-IFN–treated macrophages that were stimulated with these ligands (Fig. 8). Twist1 was induced more effectively than Twist2 by Tyro 3 receptor ligands in control macrophages. As Axl expression increased after IFNα stimulation, Axl subsumed a major role in mediating the induction of Twist proteins, and both Twist1 and Twist2 were strongly induced (Figs. 4 and 8). The effective induction of Twist2 in IFNα-treated but not in control macrophages can be explained by specific induction of Twist2 by Axl relative to Mer or Tyro 3, or by IFNα-dependent induction of additional molecules or pathways that regulate Twist2 expression. Twist1 and Twist2 have both redundant and unique functions, and differential regulation of the expression of these genes would represent one example of divergence in function between the two genes. Axl ligands are expressed constitutively; for example, the normal serum concentration of protein S is ∼25 μg/ml and apoptotic cells are continuously generated in remodeling tissues and in our culture system (29, 38). Furthermore, numbers of apoptotic cells increase during immune and inflammatory reactions, and expression of the Gas6 ligand is regulated by many factors, pathways, and cytokines, including IFNα itself (unpublished data). Thus, the IFN-Axl–mediated homeostatic mechanism is potentiated not only by increased Axl expression but also by increased expression of Axl ligands.

The tissue-specific expression pattern of Axl expression suggests that IFN induction of Twist and concomitant suppression of TNFα production occurs preferentially in cells that express Axl, such as myeloid cells. Indeed, IFNα did not induce Twist expression in nonmyeloid cell lines that were tested (unpublished data). Suppression of TNFα production in myeloid cells by Tyro 3 family kinases and their immunosuppressive and antiinflammatory properties are well established (24–26), and several signaling pathways activated downstream of these kinases have been identified (39–41). However, signaling pathways downstream of Tyro 3 family kinases that mediate inhibition of inflammation are not known. Tyro 3 kinases contain a variant immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domains, and it has been proposed that this immunoreceptor tyrosine-based inhibitory motif–like motif may mediate a suppressive signal by recruiting SH2-containing protein tyrosine phosphatases to these receptors (23). Our results provide a mechanism by which Tyro 3 family receptors suppress macrophage inflammatory function, namely, the induction of Twist proteins that suppress NF-κB–dependent TNFα gene activation.

Twist expression can be induced by NF-κB, and it has been previously shown that Twist participates in a negative feedback loop in which Twist expression is activated by inflammatory factors via activation of NF-κB (22). Twist, in turn, binds to E boxes present in the promoters of several NF-κB–dependent inflammatory cytokines, such as TNFα, IL-1, and IL-6, and suppresses cytokine production by blocking NF-κB–dependent transcriptional activation (22). The induction of Twist by noninflammatory factors, such as Tyro 3 kinases, establishes that Twist functions not only in feedback inhibition but also in negative crossregulation of TNFα expression by antiinflammatory pathways. Induction of Twist by the sequential action of IFNα and Axl results in the binding of Twist to the TNFα promoter (Fig. 6), where it is poised to suppress TNFα expression when cells and NF-κB are subsequently activated by FcγR ligation. Similar to NF-κB–induced Twist, IFNα-induced Twist likely binds to E

boxes present in the promoters of several genes other than TNFα, including inflammatory cytokines such as IL-1 and IL-8, and thus may more broadly inhibit NF-κB–dependent transcription. This prediction is supported by results that pretreatment with IFNα suppressed FcγR-mediated induction of several cytokine and chemokine genes (Fig. 2). In addition, IFNα suppressed induction of cytokine expression by TLR2 and TLR4, suggesting a more broad role in the inhibition of NF-κB–dependent cytokine expression. Further work will be required to explain the weaker inhibition of TLR4 relative to FcγRs and TLR2. The relative resistance of TLR4 to inhibition, even when low concentrations of LPS were used, suggests that TLR4 may activate an additional signaling pathway, such as the Trif-dependent pathway, that overrides Twist-dependent inhibitory mechanisms. Twist also interacts with and modulates the function of several other transcription factors expressed in macrophages, including bHLH E proteins, MEF2, Runx, and SREBP1 (42–45), and therefore has the potential to broadly regulate the primed macrophage phenotype beyond the inhibition of NF-κB responses.

MATERIALS AND METHODS

Cell culture, animals, and reagents. Human macrophages were derived from CD14+ blood monocytes cultured with 10 ng/ml of M-CSF (Peprotech). CD14+ monocytes were purified from peripheral blood mononuclear cells obtained from disease-free volunteers using anti-CD14 magnetic beads (Miltenyi Biotec) as previously described (39). Purity of monocytes was >97% as verified by FACS. Murine bone marrow–derived macrophages were obtained as described (39) and maintained in media containing 10 ng/ml of M-CSF. Macrophage cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone). The generation of Twist- and Axl-deficient mice has been previously described (22, 26). The experiments using human and murine cells were approved by, respectively, the Hospital for Special Surgery Institutional Review Board and Institutional Animal Care and Use Committee. Human IFN-α and mouse IFN-α were purchased from Roche Applied Science and Peprotech, respectively. IFN-αA/D, a universal mammalian type I IFN, was from R&D Systems. Human and mouse IgG were from Sigma-Aldrich, human protein S was from Enzyme Research Laboratories, and Gas6 was purified from 293T cells transfected with a Gas6 expression plasmid.

ELISA and flow cytometry. Paired TNFα and IL-6 capture and detection antibodies were purchased from R&D Systems and used in a sandwich ELISA according to the instructions of the manufacturer. Murine TNFα ELISA was performed by using BD OptEIA ELISA set for mouse TNFα from BD Biosciences. Staining for cell surface expression of FcγRs, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) was performed using monoclonal anti–human CD64, CD32, and CD16 (BD Biosciences). A FACScan flow cytometer with CELLQuest software (Becton Dickinson) were used as previously described.

mRNA expression analysis. For real-time PCR, total RNA was extracted using a RNeasy Mini kit (Qiagen), and 1 μg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas). Real-time, quantitative PCR was performed using iQ SYBR-Green Supermix and iCycler iQ thermal cycler (Bio-Rad Laboratories) following the manufacturer's protocols. Relative expression was normalized for levels of GAPDH. The generation of only the correct size amplification products was confirmed using agarose gel electrophoresis.

Immunoblotting. Whole cell extracts were obtained, and 10 μg of cell lysates were analyzed by immunoblotting as described (39). Polyclonal rabbit anti-Twist2 antibodies were generated (unpublished data) and used as previously described (22). Monoclonal antibodies against Stat3 and IκBα were from BD Transduction Laboratories and Cell Signaling Technology, respectively. Phosphorylation-specific antibodies against Erk (p44/42) and IκBα (Ser32) were from Cell Signaling Technology, and antibodies against Erk2 were from Santa Cruz Biotechnology, Inc.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were performed essentially as described (22) using either a TNFα promoter fragment that contains an E box (CACATG) that lies 122 bp upstream of the transcription initiation site, or an oligonucleotide containing a consensus E box (5′-CCCCAACACCTGCCTGCCTGA-3′). 5 or 10 μg of nuclear extract were incubated for 20 min at room temperature in a 15-μl binding reaction with 2 ng radiolabeled oligonucleotide and 1 μg poly(dI-dC) (GE Healthcare) in binding buffer containing 20 mM HEPES (pH 7.9), 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, and 10% glycerol. For elecrophoresis, 5% polyacrylamide (30:1) gels containing 0.5 × Tris-borate-EDTA (TBE) with 2.5% glycerol were prerun at 100 V for 2 h at 4°C in 0.5 × TBE buffer with 2.5% glycerol. Samples were then loaded and gels were run at 4°C in 0.5 × TBE buffer. For supershift experiments, nuclear extracts were incubated with 2 μg Twist or control antibodies (Santa Cruz Biotechnology, Inc.) for 20 min at 4°C before the addition of radiolabeled oligonucleotide.

Cell transfections. Luciferase reporter constructs containing a 1.3-kb fragment of the TNFα promoter (−1163 to +125 relative to transcription initiation site) (22) were used. A reporter construct mutated in two out of the three E box sequences present in this promoter fragment was generated using the Quickchange site directed mutagenesis kit (Stratagene); the mutations introduced were $^{-498}$CACTTG → CCATAC and $^{-122}$CACATG → CTCCAG. RAW267.4 macrophage cells were transfected in duplicate in 60-mm tissue culture dishes using Effectene (Qiagen) and a total of 2 or 4 μg plasmid DNA per dish according to manufacturer's instructions. Cells were cotransfected with luciferase reporter constructs, expression plasmids encoding Twist1, Twist2, or an empty control expression vector, and a Renilla luciferase-encoding internal control plasmid. 2 d after transfection, cells were stimulated by FcγR ligation for 4 h and luciferase activity was measured and normalized relative to the Renilla luciferase internal control. Representative experiments out of five (wild-type reporter) or four (mutated reporter) are shown.

ChIP assay. ChIP assay was performed using a ChIP kit (17–295; Upstate) according to the manufacturer's instructions using a polyclonal anti-Twist rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) as previously described (22). In brief, 10^7 primary macrophages were used per condition to prepare soluble chromatin which was then immunoprecipitated with either Twist or control (anti-Stat1) antibodies. Extracted DNA was amplified by PCR using primer pairs specific for the E box of the TNFα gene promoter (5′-GGGAGCTATTTCCAAGATGTTCTGG-AG-3′ and 5′-GGGTTTCAGTTCTCAGGGTCCTATACA-3′) or the GAS site of the IRF-1 gene promoter (5′-CTTCGCCGCTAGCTCTAC-AACAG-3′ and 5′-GCTCCGGTGGCCTCGGTTCG-3′) as previously described (22).

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