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Regulation of the IL-23 and IL-12 Balance by Stat3 Signaling in the Tumor Microenvironment

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SUMMARY

Interactions between tumor and immune cells either enhance or inhibit cancer progression. We show here that Stat3 signaling within the tumor microenvironment induces a procarcinogenic cytokine, IL-23, while inhibiting a central anticarcinogenic cytokine, IL-12, thereby shifting the balance of tumor immunity toward carcinogenesis. Stat3 induces expression of IL-23, which is mainly produced by tumor-associated macrophages, via direct transcriptional activation of the *IL-23/p19* gene. Furthermore, Stat3 inhibits NF-κB/c-Rel-dependent *IL-12/p35* gene expression in tumor-associated dendritic cells. Tumor-associated regulatory T cells (Tregs) express IL-23 receptor, which activates Stat3 in this cell type, leading to upregulation of the Treg-specific transcription factor Foxp3 and the immunosuppressive cytokine IL-10. These results demonstrate that Stat3 promotes IL-23-mediated procarcinogenic immune responses while inhibiting IL-12-dependent antitumor immunity.

INTRODUCTION

The transcription factor Stat3 is constitutively activated in diverse cancers (Yu and Jove, 2004), and its activation in tumors enhances transcription of genes associated with cell-cycle progression, cell survival, angiogenesis, and immune evasion (Yu and Jove, 2004; Yu et al., 2007). In addition to tumor cells, Stat3 is constitutively activated within many immune cell types in the tumor microenvironment, including dendritic cells (DCs) and macrophages (Kortylewski et al., 2005). Ablating *Stat3* in myeloid cells allows efficient $CD8^+$ T cell infiltration into tumors while inhibiting accumulation of regulatory T cells (Tregs) (Kortylewski et al., 2005). Activated Stat3 suppresses antitumor immunity by inhibiting the expression of many cytokines and chemokines important for stimulating antitumor immunity and by upregulating production of several immunosuppressive factors, including IL-10 and VEGF (Takeda et al., 1999; Wang et al., 2004). These immunosuppressive factors are not only Stat3 target genes but also Stat3 activators (Yu et al., 2007). In order to further explore the mechanisms by which Stat3 mediates tumor immunosuppression, we evaluated its role in the regulation of two closely related cytokines, IL-23 and IL-12, which play critical but opposing roles in tumor immunity.

IL-12, a heterodimer composed of α and β subunits (termed p35 and p40, respectively), promotes antitumor immunity via activation of natural killer (NK) cells and T helper 1 (Th1) T cells and is characterized by production of interferon-γ, another important cytokine in antitumor immunity (Gerosa et al., 2002; Kaplan et al., 1998; Shankaran et al., 2001; Trinchieri, 2003). IL-12 further promotes the expansion and activity of cytotoxic T lymphocytes (CTLs) both directly and indirectly by Th1 cells (Colombo and Trinchieri, 2002). IL-23, a more recently discovered IL-12 family member, is composed of the p40 subunit in common with IL-12, paired with a unique p19 subunit (Oppmann et al., 2000). Similarly, IL-12 receptor (IL-12R) and IL-23 receptor (IL-23R) share a common β subunit, which is paired with a unique α subunit for

SIGNIFICANCE

Recent studies suggest that two related cytokines, IL-23 and IL-12, play opposite roles in carcinogenesis. However, the underlying mechanisms regulating the balance between these cytokines in the tumor microenvironment have not been elucidated. Mechanisms by which IL-23 promotes tumor immune evasion also remain to be explored. Our results reveal that Stat3 signaling in the tumor microenvironment regulates the IL-12/IL-23 balance and, furthermore, that IL-23 enhances the immunosuppressive activity of regulatory T cells within the tumor microenvironment, in part via IL-23 receptor-dependent Stat3 activation. Because Stat3 is a point of convergence for signaling pathways commonly activated in cancer, our data reveal a mechanism by which oncogenic pathways regulate the immune microenvironment to promote tumor development.

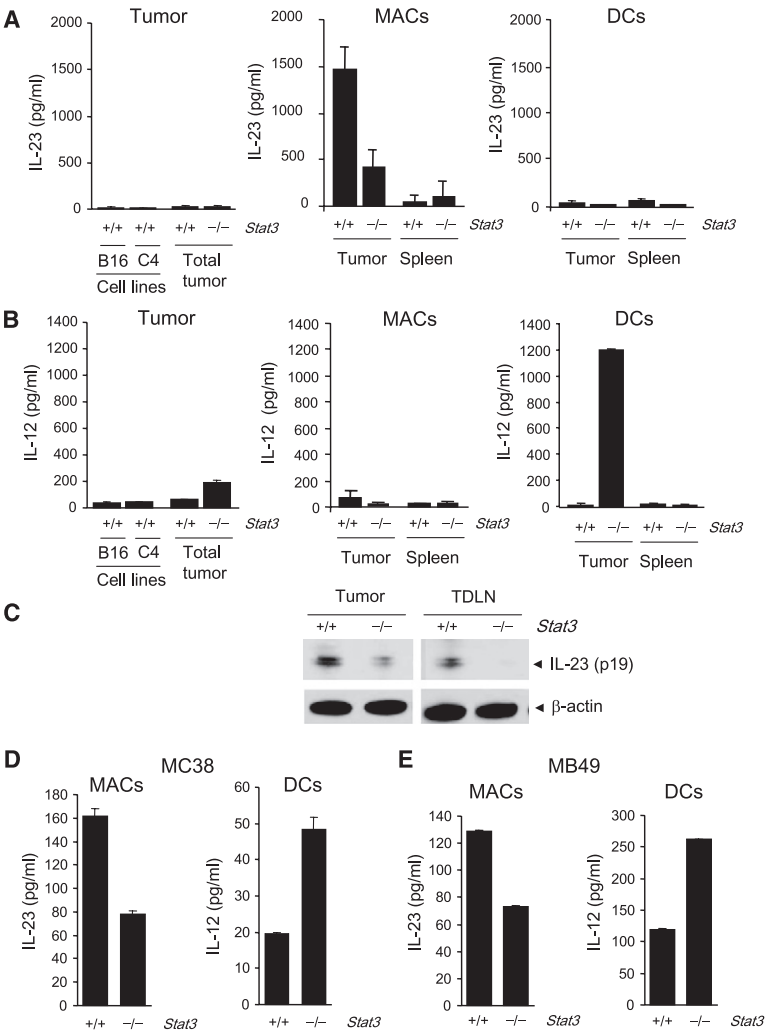

Figure 1. IL-23 and IL-12 Expression in Tumor-Infiltrating Myeloid Cells Is Compartmentalized and Oppositely Regulated by Stat3

(A and B) ELISA measurement of protein levels of IL-23 (A) and IL-12 (B) in supernatants of tumor cell lines (B16 and C4 melanoma cells), B16 whole tumor cell suspensions (total tumor), enriched tumor-infiltrating $CD11b^+CD11c^-$ macrophages (MACs), and $CD11c^+$ dendritic cells (DCs) cultured in vitro for 24 hr from mice with $Stat3^{+/+}$ or $Stat3^{-/-}$ hematopoietic cells. Data shown are mean values ± SD from one of three separate experiments performed on cells pooled from four animals per group analyzed.

(C) Western blot detection of IL-23/p19 protein levels in $CD11b^+$ cells isolated from whole B16 tumors and tumor-draining lymph nodes (TDLN) of $Stat3^{+/+}$ or $Stat3^{-/-}$ mice.

(D and E) IL-23 and IL-12 protein levels measured by ELISA as in (A), in supernatants from enriched tumor-infiltrating $CD11b^+CD11c^-$ MACs and $CD11c^+$ DCs isolated from whole MC38 colon carcinoma (D) and MB49 bladder carcinoma (E) tumors. Data are mean ± SEM (n = 3).

each receptor (Parham et al., 2002). While IL-23 was originally thought to possess proinflammatory properties similar to IL-12, analysis of mice with selective knockout of the *IL-12/p35* gene versus the *IL-23/p40* gene revealed distinct functions for these two cytokines. In particular, a number of experimental autoimmune diseases were shown to be dependent on IL-23 and not on IL-12 (Cua et al., 2003; Ghilardi et al., 2004; Langrish et al., 2005; Murphy et al., 2003). IL-23 has also been shown to promote the expansion of a distinct lineage of helper T cell, termed Th17 (Langrish et al., 2005). Th17 cells are characterized by production of a number of specific cytokines not produced by Th1 or Th2 cells, including IL-17A, IL-17F, IL-21, and IL-22. Interestingly, Stat3 has also been documented to be an essential transcriptional regulator of IL-17, IL-21, and IL-22 production by Th17 cells (Chen et al., 2006; Harris et al., 2007; Laurence et al., 2007; O'Shea and Murray, 2008; Wei et al., 2007; Zheng et al., 2007; Zhou et al., 2007).

An opposite role in carcinogenesis has recently been reported for IL-23 compared to IL-12 (Langowski et al., 2006, 2007). Carcinogen-induced tumor formation was greater in *IL-12/p35* KO mice, confirming the physiologic antitumor role of IL-12; however the opposite effect was observed in *IL-23/p19* KO mice (Langowski et al., 2006). Whereas IL-12 facilitated tumor infiltration of $CD8^+$ T cells, IL-23 reduced $CD8^+$ T cells in tumor and promoted tumor angiogenesis (Langowski et al., 2006). While these results suggest a procarcinogenic role for IL-23 production within tumors, its mechanisms of action in tumor regulation have not been elucidated. We therefore investigated how IL-23 and IL-12 expression are differentially regulated in the tumor microenvironment and how IL-23 further propagates tumor immunosuppressive effects.

RESULTS

Role of Stat3 in the Differential Expression of IL-23 and IL-12 in Tumors

We assessed IL-23 and IL-12 protein secretion by B16 tumor cells in vitro and B16 tumors growing in vivo after implantation into C57BL/6 mice. B16 tumor cells and a second melanoma tumor line, C4, cultured in vitro secreted very little of either cytokine as detected by ELISA (Figure 1A, left). B16 tumors growing in vivo were dissociated into single-cell suspensions that consisted of tumor cells as well as hematopoietically derived cells comprising the tumor microenvironment. Similar to in vitro results, only a small amount of IL-23 or IL-12 secretion was detected by ELISA when the unfractionated cell suspensions were cultured (Figures 1A and 1B, left). When the tumor-associated myeloid component was sorted by fluorescence-activated cell sorting (FACS) into macrophages ($CD11b^+c^-$) and DCs ($CD11b^+c^+$), high levels of IL-23 were found to be produced by the tumor-associated macrophages (MACs; Figure 1A, middle), with much lower levels produced by tumor-infiltrating DCs (Figure 1A, right). Western blot data demonstrated that IL-23 was also detectable in $CD11b^+$ cells isolated from tumors and tumor-draining lymph nodes (Figure 1C). Virtually no IL-12 was secreted either by unfractionated tumors or by purified tumor-associated macrophages or splenic DCs as determined by ELISA (Figure 1B).

The role of Stat3 signaling in IL-23 and IL-12 regulation within the tumor microenvironment was initially determined by

conditional knockout of the *Stat3* gene in the hematopoietic compartment. To accomplish this, *Mx1-Cre/Stat3$^{flox/flox}$* mice were treated with polyinosinic:polycytidylic acid (poly(I:C)). We have previously demonstrated that *Stat3* is efficiently knocked out in myeloid cells under these conditions (Kortylewski et al., 2005). By 24 hr after poly(I:C) treatment, there was no evidence of residual activation of either DCs or macrophages as measured by either cell membrane markers or cytokine production (Kortylewski et al., 2005). To avoid any confounding effects of immunostimulation by poly(I:C) treatment, *Mx1-Cre/Stat3$^{flox/flox}$* and control *Stat3$^{flox/flox}$* littermate mice were not challenged with tumors until 4 days after completion of poly(I:C) treatment. IL-12 and IL-23 production in tumors was analyzed 2–3 weeks later. We purified macrophages and DCs in tumors grown in poly(I:C)-treated *Mx1-Cre/Stat3$^{flox/flox}$* mice with induced *Stat3* ablation, as well as poly(I:C)-treated *Stat3$^{flox/flox}$* control mice. Hematopoietic *Stat3* ablation significantly reduced IL-23 production by B16 tumor-associated macrophages (Figure 1A). In contrast, IL-12 production was upregulated in B16 tumors from mice with hematopoietic *Stat3* knockout. The majority of IL-12 expression came from the tumor-associated DCs infiltrating B16 tumors (Figure 1B). Thus, these data implied that Stat3 signaling drives IL-23 production from the tumor-associated macrophages while restraining IL-12 production by tumor-associated DCs. This effect was specific for the tumor microenvironment since there was no significant production of either IL-12 or IL-23 by splenic macrophages or DCs regardless of whether the *Stat3* gene was ablated.

To test the generality of our findings that Stat3 in tumor stromal myeloid cells contributed to regulating IL-23 and IL-12 expression in tumors, we performed similar experiments using MC38 colon carcinoma (Figure 1D) and MB49 bladder carcinoma (Figure 1E) models. In vivo experiments showed that the growth of MC38 tumors was inhibited by *Stat3* ablation in myeloid cells (see Figure S1 available online), confirming previous data in B16 and MB49 tumor models (Kortylewski et al., 2005). Consistent with our results in the B16 tumor model, ablating *Stat3* in myeloid cells decreased production of IL-23 by macrophages within MC38 and MB49 tumors (Figures 1D and 1E). While the overall level of IL-23 secretion by MC38 and MB49 tumor-infiltrating macrophages in control *Stat3$^{flox/flox}$* mice was lower than that observed in B16 tumors, the IL-23 that was produced was clearly Stat3 dependent. Likewise, the DCs isolated from MC38 and MB49 tumors showed increased secretion of IL-12 following induced *Stat3* ablation. The level of IL-12 secretion by DCs isolated from MC38 and MB49 tumors did not reach the levels observed in the B16 tumor model, but the increases observed with hematopoietic *Stat3* knockout were highly reproducible. Multiple factors likely contribute to the differences in magnitude of IL-23 and IL-12 production by tumor-associated macrophages and *Stat3$^{-/-}$* tumor-associated DCs, respectively, in the different tumors. Previous studies indicated that IL-12 expression can be induced in *Stat3$^{-/-}$* splenic DCs by Toll-like receptor (TLR) activation (Kortylewski et al., 2005). Dying tumor cells have also been shown to stimulate DC IL-12 production (Apetoh et al., 2007). The presence of large necroses (up to 89% of tumor area) within B16 tumors (Garcia-Hernandez et al., 2002), which was not observed in MC38 or MB49 tumors (M. Kortylewski and H. Yu, unpublished data), might provide cell debris capable of costimulating intratumoral DCs to produce IL-12 after deletion of *Stat3*.

Stat3 Directly Regulates *IL-23/p19* Transcription

In order to determine the mechanism for regulation of these cytokines by Stat3 in the tumor microenvironment, we first analyzed *IL-23/p19* gene regulation. *Stat3* ablation indeed reduced *IL-23/p19* mRNA in tumor-associated macrophages by roughly 10- fold, with virtually no *IL-23/p19* mRNA detectable in *Stat3$^{+/+}$* or *Stat3$^{-/-}$* splenic macrophages (Figure 2A). Despite low levels (26- and 79-fold lower in B16 and C4 cells, respectively, relative to tumor-infiltrating macrophages), *IL-23/p19* mRNA in the tumor cells was reduced more than 80% by *Stat3* siRNA knockdown (Figure S2A). These results suggested that Stat3 positively regulates IL-23 at the transcriptional level in different cell types. In order to determine whether this transcriptional regulation was direct, we performed luciferase reporter and chromatin immunoprecipitation (ChIP) analyses. First, 3T3 cells were transfected with a luciferase expression vector driven by the *IL-23/p19* promoter (−1159 to +160). Dual-luciferase activity was enhanced significantly by cotransfection with a constitutively active Stat3 mutant, Stat3C (Figure 2B). B16 cell transfection with the *IL-23/p19* promoter-driven luciferase reporter gene resulted in progressively decreased luciferase activity with successively increasing amounts of *Stat3* siRNA (Figure 2C, top). Cotransfection with *NF-κB/p65* siRNA also resulted in decreased luciferase activity, with the greatest suppression occurring upon cotransfection with both *Stat3* and *NF-κB* siRNAs (Figure 2C, bottom). These results indicate that NF-κB/p65, which is also constitutively activated in B16 like in many other cancers, participates in *IL-23/p19* transcription.

We next performed ChIP analysis on B16 cells using primers for the *IL-23/p19* promoter. Both Stat3 and NF-κB/p65 were associated with the *IL-23/p19* promoter (Figure 3A). While it was difficult to obtain enough tumor-associated macrophages to perform ChIP analysis, ChIP of total tumors growing in vivo as well as tumor-draining lymph nodes demonstrated Stat3 binding to the *IL-23/p19* promoter along with NF-κB/p65 (Figures 3B and 3C). NF-κB/p65 binding to the *IL-23/p19* promoter was also higher in tumor-draining lymph nodes relative to nondraining lymph nodes (Figure 3B). Hematopoietic *Stat3* ablation resulted in a decreased ChIP signal for both Stat3 and NF-κB/p65 on the *IL-23/p19* promoter in growing B16 tumors (Figure 3C).

Stat3 Inhibits NF-κB/c-Rel-Mediated *IL-12/p35* Gene Expression

Real-time PCR demonstrated that Stat3 reciprocally downregulated *IL-12/p35* transcription in whole B16 tumors growing in hematopoietic *Stat3* KO mice (Figure 4A, left), in tumor-associated myeloid cells (Figure 4A, right), and in cultured B16 tumor cells after siRNA transfection (Figure S2B). The NF-κB family transcription factor c-Rel has been demonstrated to be essential for transcription of *IL-12/p35* (Grumont et al., 2001) and other genes important for DC activation (Wang et al., 2007). However, we found that levels of active c-Rel were low in growing tumors (Figure 4B). Upon hematopoietic *Stat3* ablation, there was increased c-Rel activity (phospho-c-Rel) in tumors (Figure 4B, left), as well as increased binding to the *IL-12/p35* promoter of

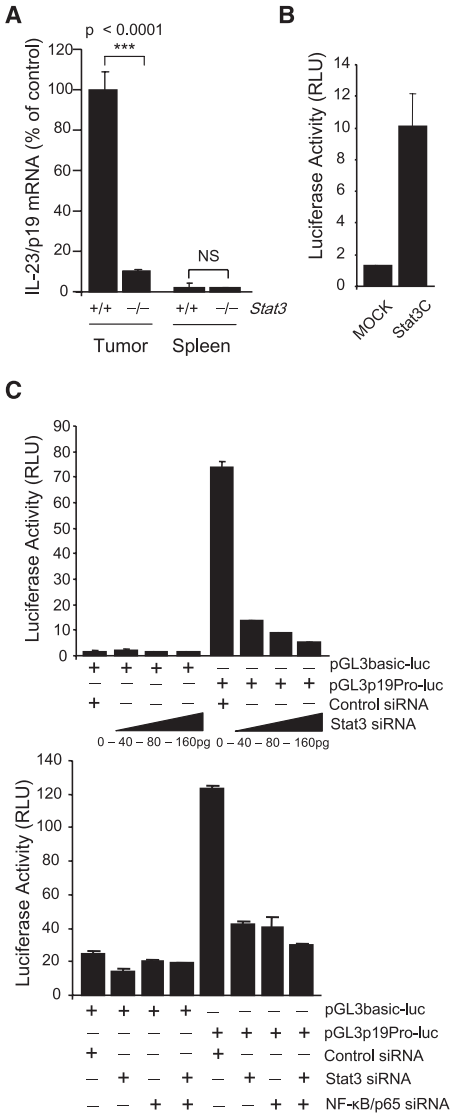

Figure 2. Stat3 and NF-κB/p65 Synergistically Enhance *IL-23* Expression by Directly Binding to the *IL-23/p19* Promoter

(A) Stat3 upregulates expression of *IL-23/p19* mRNA in CD11b$^+$ myeloid cells freshly isolated from B16 tumors. Shown are the results from one of three independent experiments analyzed by real-time PCR, normalized to 18S rRNA.

(B) Overexpression of a constitutively active Stat3 mutant (Stat3C) activates transcription by the *IL-23/p19* promoter. The fragment from the mouse *IL-23/p19* promoter (−1159 to +160) was cloned into pGL3 vector with a luciferase reporter gene. Dual-luciferase activity was determined 24 hr after transfection of various sets of expression vectors into 3T3 fibroblasts.

(C) Stat3 and NF-κB/p65 regulate the *p19* promoter. Top: Stat3 silencing downregulates the activity of the *IL-23/p19* promoter. Dual-luciferase activity was measured in lysates of B16 cells 24 hr after transfection with various concentrations of *Stat3* siRNA or with scrambled RNA control. Bottom: both Stat3 and NF-κB/p65 transcription factors are required for the transcriptional activity of the *IL-23/p19* promoter. *Stat3* siRNA, *NF-κB/p65* siRNA, or both were transfected into B16 cells together with *IL-23/p19* promoter-luciferase construct. Dual-luciferase activity was measured as described above.

Data shown are mean values ± SD from experiments performed in triplicate.

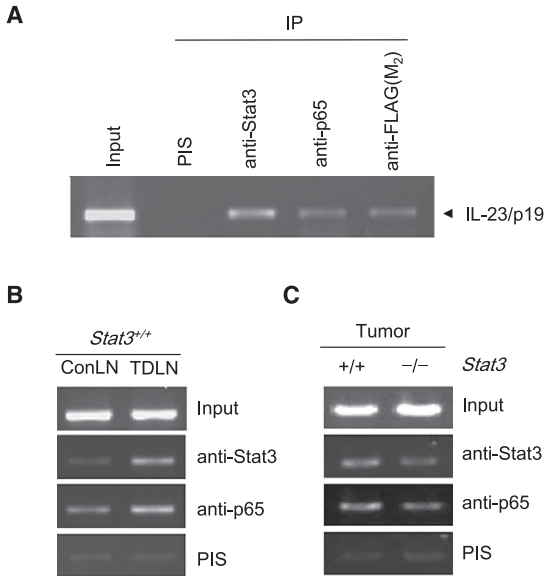

Figure 3. Both Stat3 and NF-κB/p65 Bind to the *IL-23/p19* Promoter

(A) B16 tumor cells were transfected with pRV-CMV Stat3-FLAG or control vector, and antibodies specific for control IgG (PIS), Stat3, NF-κB/p65, or FLAG-M_2 as indicated were used for chromatin immunoprecipitation (ChIP). Chromatin was purified and amplified by PCR using primers specific for Stat3 and NF-κB binding sequence of the mouse *IL-23/p19* promoter.

(B) Binding of Stat3 and NF-κB/p65 to the *IL-23/p19* promoter is elevated in tumor-draining lymph nodes (TDLN), but not in distant lymph nodes. Cells isolated from pooled tumor-draining or contralateral lymph nodes were fixed with formaldehyde and subjected to ChIP assay performed as described above.

(C) Tumors from mice with myeloid cell-specific *Stat3* ablation show reduced Stat3 and NF-κB/p65 binding to the *IL-23/p19* promoter as assessed by ChIP assay.

NF-κB/c-Rel (Figure 4B, right). It is noteworthy that *Stat3* ablation did not affect p65 binding to the *IL-12/p35* promoter.

IL-23R and Stat3 Signaling in Tumor-Infiltrating Tregs

An immunosuppressive role for Stat3 in myeloid cells in the tumor microenvironment has been demonstrated in *Mx1-Cre/Stat3$^{flox/flox}$* mice (Kortylewski et al., 2005). However, whether intrinsic Stat3 signaling in T cells mediates tumor immunosuppression has not been shown. To address this question, we next determined the role of Stat3 and IL-23 in T cell physiology within the tumor microenvironment. Tumor growth is enhanced by the CD4$^+$Foxp3$^+$ Treg subset, which produces cytokines such as IL-10 and TGF-β and antagonizes Th1- and CD8-mediated antitumor responses (Zou, 2006). As has been found in several other tumors, the major subset of CD4$^+$ T cells infiltrating growing B16 tumors (>50%) are Foxp3$^+$ Treg cells (Kortylewski et al., 2005). Analysis of B16 tumor Tregs by intracellular staining of phospho-Stat3 (pStat3) demonstrated that Stat3 was constitutively activated in tumor-infiltrating Tregs relative to their splenic counterparts from the same mice (Figure 5A, top). Since IL-23R is known to signal through Stat3, we tested whether tumor CD4$^+$Foxp3$^+$ Tregs express detectable IL-23R in B16 tumors. Whereas splenic Tregs did not express detectable IL-23R, Tregs from both tumors and tumor-draining lymph nodes were positive for IL-23R (Figure 5A, bottom). Elevated expression of IL-23R in Tregs from tumors and tumor-draining lymph

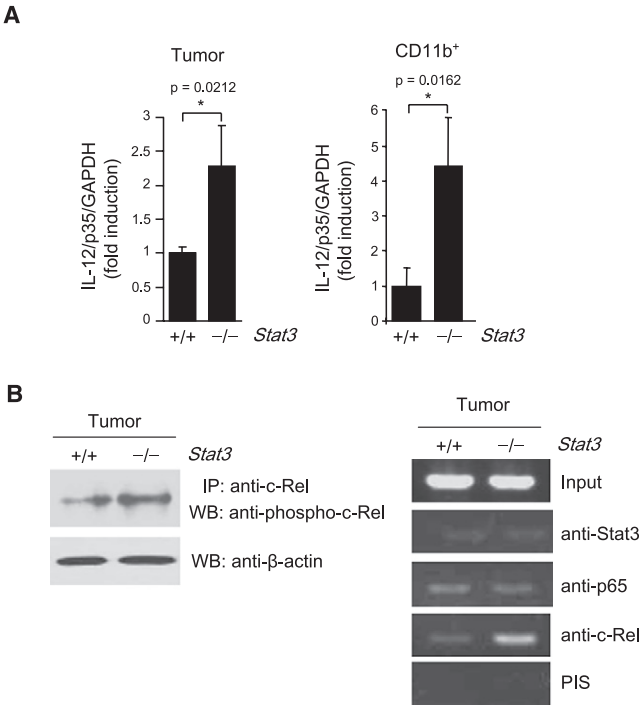

Figure 4. Stat3 Inhibits *IL-12/p35* Gene Expression

(A) *Stat3* knockdown or ablation augments *IL-12/p35* expression in whole tumors (left) and in tumor-infiltrating myeloid cells (right). Data are mean ± SD (n = 3).

(B) Stat3 inhibits c-Rel activity and binding of c-Rel to the *IL-12/p35* promoter in tumors. Left: western blot analysis of c-Rel phosphorylation after immunoprecipitation of total c-Rel from spleens and tumors of mice with $Stat3^{+/+}$ and $Stat3^{-/-}$ hematopoietic compartments. Right: ablating *Stat3* in hematopoietic cells increases binding of NF-κB/c-Rel, but not NF-κB/p65, to the *IL-12/p35* promoter in whole tumor preparations as measured by ChIP assay.

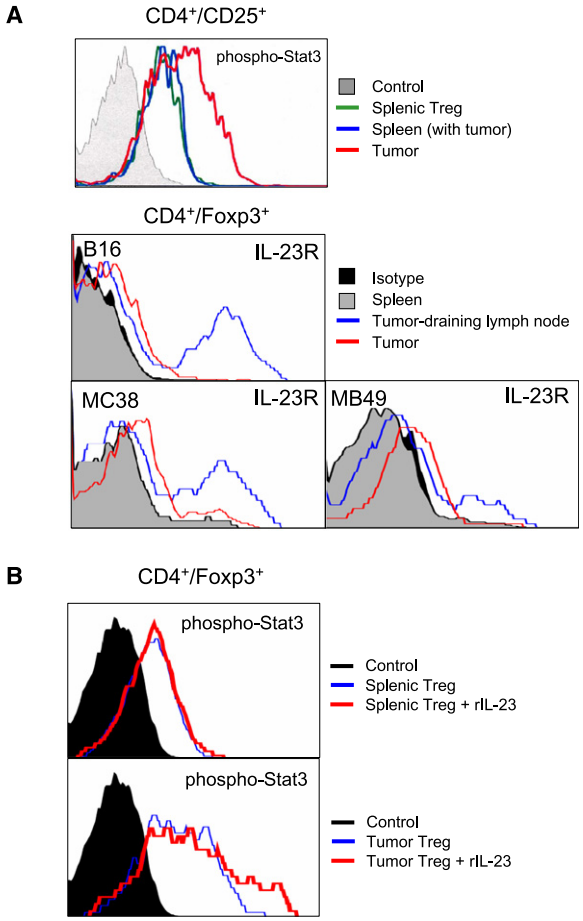

Figure 5. IL-23 Receptor and Stat3 Signaling in Tumor Regulatory T Cells

(A) Stat3 is activated in tumor regulatory T cells (Tregs), which are IL-23R positive. Cell suspensions prepared from spleens and from tumors as well as tumor-draining lymph nodes were subjected to flow analyses for phospho-Stat3 (top panel) and IL-23R (bottom three panels).

(B) Recombinant IL-23 can further activate Stat3 in $CD4^+Foxp3^+$ lymphocytes derived from tumors but not spleens. Shown are representative results from 2–3 independent experiments.

nodes was further confirmed in both the MB49 and MC38 tumor models (Figure 5A, bottom). Furthermore, although recombinant IL-23 did not induce Stat3 activation in splenic Tregs, the already evident endogenous pStat3 of the tumor Tregs could be further upregulated by addition of recombinant IL-23 in vitro (Figure 5B). These results demonstrated that tumor-associated Tregs are distinct from peripheral Tregs in that they express functional IL-23R and that IL-23R signals in part through Stat3.

In order to assess the role of Stat3 activity in tumor-infiltrating T cells in vivo, we introduced B16 tumors into *CD4-Cre/*$Stat3^{flox/flox}$ mice, which selectively ablate *Stat3* in the T cell compartment. Figure 6A shows that tumor-infiltrating Tregs from wild-type mice contained significant amounts of pStat3, while no pStat3 was detected in tumor-infiltrating Tregs from *CD4-Cre/*$Stat3^{flox/flox}$ mice. Consistent with an important role for Stat5 in maintaining Tregs (Yao et al., 2007), we found that B16 tumor-infiltrating Tregs also displayed high Stat5 activity (Figure 6A). Since IL-23 is known to stimulate both Stat3 and Stat5 (Parham et al., 2002), the finding that tumor Tregs express functional IL-23R suggests that Stat3-dependent expression of IL-23 in the myeloid compartment can promote tumor growth through an autoamplification mechanism involving activation of both Stat3 and Stat5 in $IL\text{-}23R^+$ tumor-infiltrating Tregs. In mice with $Stat3^{-/-}$ T cells, $Foxp3^+$ Tregs were still the dominant tumor-infiltrating CD4 subset; however Foxp3 levels were reduced relative to tumor-bearing wild-type mice (Figure 6B). Furthermore, tumor-infiltrating Tregs from *CD4-Cre/*$Stat3^{flox/flox}$ mice produced significantly less IL-10 than tumor-infiltrating Tregs from their $Stat3^{+/+}$ counterparts (Figure 6C). Consistent with previous reports on the absolute requirement for Stat3 in Th17 development in vivo (Harris et al., 2007; Wei et al., 2007; Zhou et al., 2007), Th17 cells (as defined by intracellular cytokine staining for IL-17) were completely absent in *CD4-Cre/*$Stat3^{flox/flox}$ mice, while Th1 cells (as defined by intracellular cytokine staining for IFN-γ) were unaffected (Figure 6D).

Effects of IL-23R Blockade on Tumor Tregs and Tumor Growth

Because IL-23R signals through Stat3 (Parham et al., 2002) and Stat3 can affect expression of Foxp3 and IL-10 in $CD4^+$ T cells (Kinjyo et al., 2006; Pallandre et al., 2007; Yao et al., 2007), we

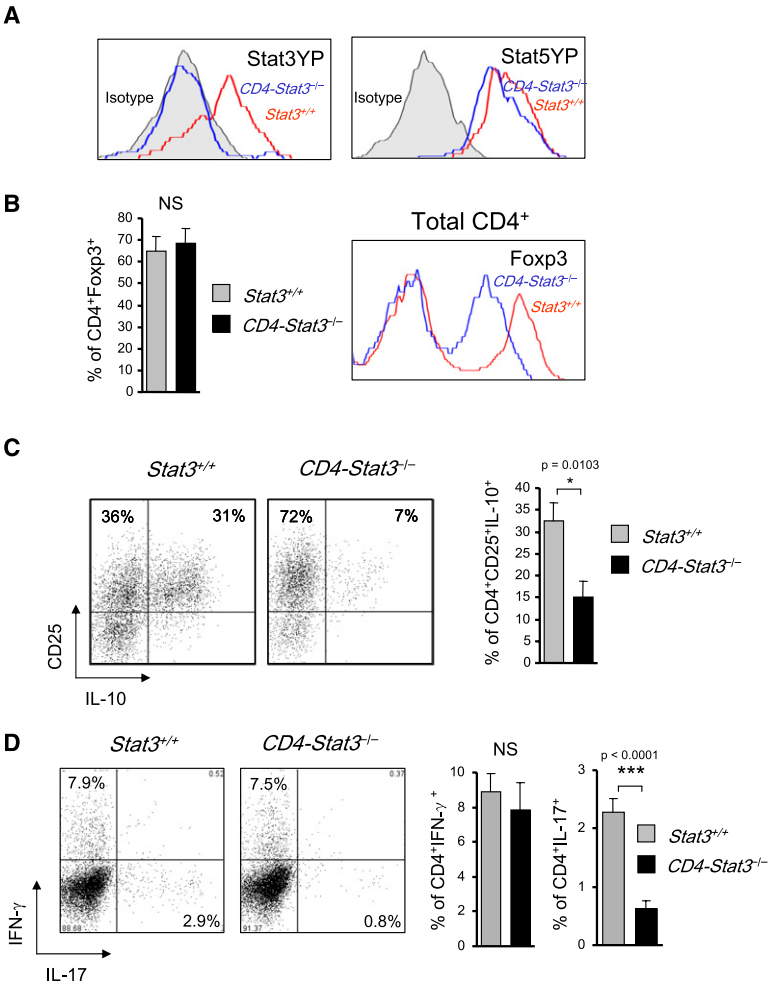

Figure 6. The Effects of *Stat3* Ablation on Tumor-Infiltrating Tregs

(A) *Stat3* ablation does not significantly affect Stat5 activity in tumor-infiltrating $CD4^+Foxp3^+$ Tregs as measured by intracellular staining with Stat3- and Stat5-phosphospecific antibodies and flow cytometry.

(B) The effect of Stat3 signaling in tumor $CD4^+$ T cells on the number of $Foxp3^+$ cells (left) and expression levels of Foxp3 (right), using $CD4^+$ T cells prepared from B16 tumors grown in $Stat3^{flox/flox}$ ($Stat3^{+/+}$) and *CD4-Cre*/$Stat3^{flox/flox}$ mice. Data shown at left are mean ± SEM; NS, not significant. The representative histogram at right shows one of three independent experiments with total n = 12.

(C) Lower expression of IL-10 by tumor-derived $CD4^+CD25^+$ Tregs from $Stat3^{-/-}$ mice. The two panels at the left show representative results for three independent experiments. The bar graph at right combines data from three independent experiments with total n = 12 and shows mean ± SEM; p = 0.0103.

(D) The two panels at the left show intracellular expression of IFN-γ and IL-17 cytokines by tumor-infiltrating $Stat3^{+/+}$ and $Stat3^{-/-}$ $CD4^+$ T cells as assessed by intracellular staining and flow cytometry using freshly isolated whole tumor cell preparations. The two bar graphs at the right represent three independent experiments with total n = 12. NS (not significant) and p < 0.0001, respectively. Data shown are means ± SEM.

evaluated directly whether IL-23R signaling impacts Tregs in the tumor microenvironment and whether this directly enhances B16 tumor growth in vivo. IL-23R blockade affected tumor-infiltrating Tregs in a manner similar but not identical to T cell-specific *Stat3* ablation (Figures 7A and 7B). Anti-IL-23R-treated mice displayed a modest but significant reduction in number of tumor-infiltrating Tregs compared with control antibody-treated mice. However, Foxp3 levels were diminished after anti-IL-23R treatment (Figure 7A, right), as was IL-10 production (Figure 7B). While these results are consistent with a role for IL-23-driven Stat3 activation in tumor-infiltrating Tregs, additional IL-23R-dependent signaling pathways (such as Stat5) are likely operative in tumor-infiltrating Tregs. In addition, other cytokines in the tumor microenvironment (such as IL-10) could additionally contribute to Stat3 activation in tumor-infiltrating Tregs. The effects of IL-23 within the tumor microenvironment indeed translate to an overall enhancement of tumor growth, as in vivo IL-23R blockade resulted in a significant reduction in tumor growth relative to isotype control antibody (Figure 7C).

DISCUSSION

The immune system acts as an extrinsic tumor suppressor (Dunn et al., 2005; Kaplan et al., 1998; Koebel et al., 2007). However, tumors acquire various mechanisms to facilitate immune escape (Dunn et al., 2002; Zou, 2005). Our results demonstrate that Stat3 signaling in the tumor microenvironment shifts inflammation from an antitumor IL-12 program to a tumor-enhancing IL-23 program via transcriptional activation of the IL-23-specific *p19* gene and concomitant transcriptional suppression of the IL-12-specific *p35* gene. Stat3 appears to coactivate the *p19* promoter with NF-κB/p65. In contrast, Stat3 also appears to suppress c-Rel in tumors, thereby reducing binding of c-Rel to the *IL-12/p35* promoter. This is in agreement with a recent report indicating that NF-κB subunits have unique roles in regulating gene expression in DCs (Wang et al., 2007). The p50 and c-Rel subunits were found to be critical for the induction of T cell stimulatory gene expression, whereas the NF-κB/p65 subunit regulated expression of a distinct group of inflammatory genes, some of which are procarcinogenic (Wang et al., 2007). While Stat3 transcriptionally activates the *IL-23/p19* gene in both tumor cells and myeloid cells within the tumor microenvironment, the major source of Stat3-driven IL-23 is tumor-associated macrophages. Stat3 restrains *IL-12* transcription in both tumors and infiltrating myeloid cells, though the majority of IL-12-producing cells in the absence of Stat3 activity are tumor-associated DCs. It is notable that, while these effects of Stat3 are not observed systemically, they appear to be operative in tumor-draining lymph nodes. We are currently testing the hypothesis that a preponderance of IL-23 production relative to IL-12 production by antigen-presenting cells in tumor-draining lymph nodes ultimately skews the differentiation of tumor-specific T cells, possibly leading to dominance of Treg and Th17 responses over antitumor Th1 responses.

IL-12 has been validated in numerous studies as a central cytokine in antitumor immunity and antiviral immunity due to its

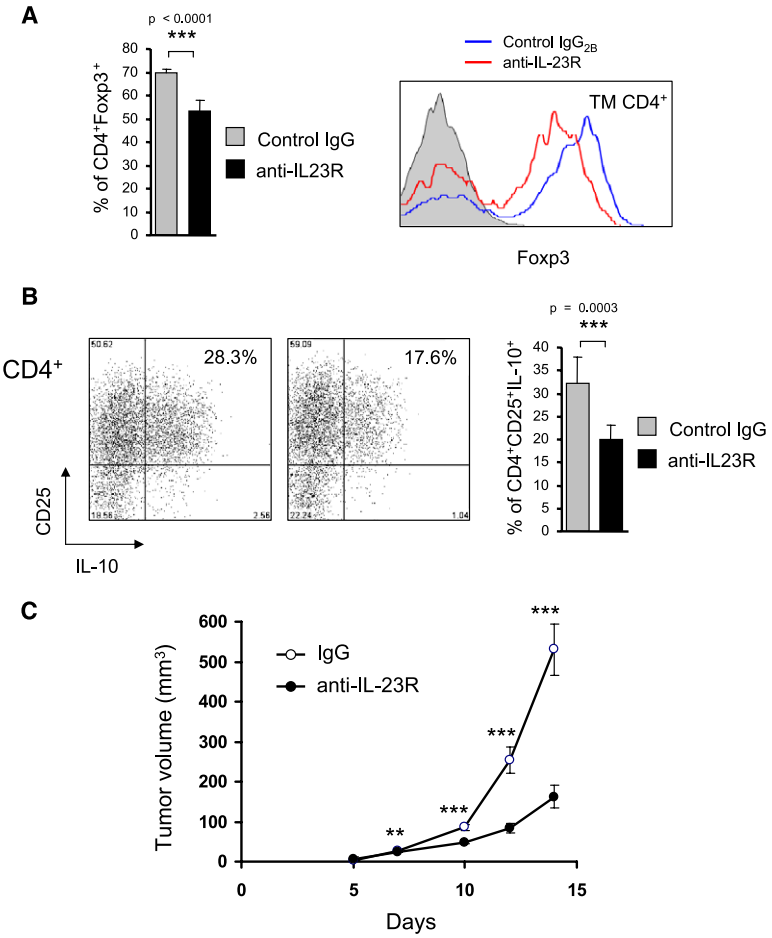

Figure 7. Blocking IL-23R Signaling In Vivo Affects Tumor Tregs and Tumor Growth

Mice were challenged with B16 tumors injected subcutaneously and treated with IL-23R-specific neutralizing antibodies or rat IgG2b control antibodies.

(A) Blocking IL-23 signaling reduces the number of tumor-infiltrating Tregs (left), and especially the expression level of Foxp3 (right).

(B) IL-23R neutralization reduces IL-10 production by $CD4^+CD25^+$ Tregs. Phenotypic analysis of tumor-infiltrating $CD4^+$ T cells was accomplished by cell surface/intracellular staining with specific antibodies and flow cytometry. For both (A) and (B), representative results of FACS analysis from one of two independent experiments are shown. Bar graphs show means ± SEM with total n = 7. $p < 0.0001$ (A), $p = 0.0003$ (B).

(C) Effects of IL-23R blockade on B16 tumor growth. Combined results of two independent experiments are shown as means ± SEM with total n = 10. ***$p < 0.001$, **$p < 0.01$.

role in activating NK cell, Th1 cell, and CTL responses (Trinchieri, 2003). The emergence of a second IL-12 family member, IL-23, which shares the p40 subunit with IL-12, has significantly altered our view of autoimmune disease since a number of murine autoimmune syndromes originally thought to be IL-12 dependent are instead IL-23 dependent. These more recent studies have suggested that IL-12-driven immunity is not only qualitatively distinct from IL-23-driven immunity but may also be mutually antagonistic. This notion also pertains to tumor immunity as emphasized by Langowski et al. (2006), who showed that tumorigenesis is diminished in *IL-23/p19* KO mice and increased in *IL-12/p35* KO mice. The studies presented here support the notion that IL-23 has a procarcinogenic role in contrast to the well-established anticarcinogenic role of IL-12. Importantly, our findings indicate that Stat3 signaling in both the tumor and the hematopoietic/immune microenvironment of the tumor is critical in shifting the balance from IL-12 to IL-23 production.

An additional unexpected finding of our study was the upregulation of IL-23R in tumor-associated Tregs. Although the underlying mechanism responsible for IL-23R upregulation in tumor-associated Tregs remains to be elucidated, our limited data indicate that the increase in IL-23R expression in tumor Tregs correlates with Stat3 activity. In addition to its central role in IL-23 upregulation, Stat3 appears to also play a role in IL-23R signaling within the tumor microenvironment. The role for Stat3 signaling in IL-10 production by Tregs has not been previously appreciated, although a recent report demonstrates that IL-27-driven IL-10 production by T cells is Stat3 dependent (Stumhofer et al., 2007). While T cell-specific *Stat3* ablation also diminished the number of tumor-infiltrating Th17 cells, this cell population is relatively minor in the B16 system. Recent studies have indicated that Th17 can contribute to tumor progression (Kryczek et al., 2007); however, it appears that, at least with B16 melanoma, the major role of T cell-specific Stat3 signaling (and also IL-23R signaling) lies within the Treg compartment. Indeed, ablation of Tregs has been shown to significantly enhance antitumor responses in B16 melanoma (Sutmuller et al., 2001; Turk et al., 2004). Whether IL-23R-dependent Stat3 signaling has a larger role in Th17 responses in other tumor systems remains to be determined. IL-23R signals through multiple other Stats, which also undoubtedly play a role in how IL-23 modulates the tumor microenvironment. For example, IL-23R also activates Stat5, which O'Shea and colleagues demonstrated to be very important in Treg expansion and possibly also differentiation (Yao et al., 2007). Indeed, tumor-infiltrating Tregs have high levels of tyrosine-phosphorylated Stat5. In this context, it was somewhat surprising that Stat3 signaling appears to enhance Foxp3 expression by tumor-infiltrating Tregs. This finding contrasts with studies suggesting that Stat3 activation (driven by IL-6 or IL-27) inhibits differentiation to the Treg lineage (Huber et al., 2008; Laurence et al., 2007). However, the role of Stat3 in *Foxp3* gene regulation may be quite distinct in tumor-infiltrating Tregs (where the cytokine milieu and ambient signals are quite distinct) versus naive T cells being induced into various differentiation pathways in vitro. Indeed, Pallandre et al. (2007) have demonstrated that, similar to our findings here, *Stat3* ablation decreases *Foxp3* expression by Tregs in a graft-versus-host disease model. Zorn et al. (2006) showed that both Stat3 and Stat5 can bind to a Stat consensus site in the *Foxp3* promoter. Thus, the role of Stat3 in regulating *Foxp3* expression by Tregs appears to be context dependent. In the context of the signals present within the tumor

microenvironment, Stat3 activity in Tregs appears to upregulate Foxp3 levels, which Flavell and colleagues have shown to be important in maintaining Tregs' inhibitory functions (Wan and Flavell, 2007).

It should also be emphasized that in addition to IL-23, at least two other cytokines, IL-6 and IL-10, appear to play an important role in enhancing cancer progression (Yu et al., 2007). These cytokines are somewhat interlinked in that they are all Stat3 inducible and their receptors activate Stat3 (O'Shea and Murray, 2008). While much has been written about the role of IL-6 and IL-10 in promoting tumorigenesis and inhibiting antitumor immunity, our data focus attention on IL-23 as an important mediator of procarcinogenesis by Stat3 signaling.

EXPERIMENTAL PROCEDURES

Cells

Mouse B16 melanoma cells were purchased from the American Type Culture Collection. Mouse C4 melanoma, MB49 bladder carcinoma, and MC38 colon carcinoma cells were generous gifts from J. Fidler (MD Anderson Cancer Center), T. Ratliff (University of Iowa), and M. Shurin (University of Pittsburgh), respectively. DC2.4 cells were originally obtained from K. Rock (University of Massachusetts Medical School). The generation of *v-Src*-transformed BALB/c 3T3 fibroblasts has been described previously (Wang et al., 2004).

In Vivo Experiments

Mice were maintained and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidelines and approved protocols from the Research Animal Care Committees of the City of Hope and Johns Hopkins University. We obtained *Mx1-Cre* mice from The Jackson Laboratory, *CD4-Cre* mice from Taconic, and *Stat3*$^{flox/flox}$ mice from S. Akira and K. Takeda (Osaka University). All transgenic mice were on a C57BL/6J background. Generation of mice with *Stat3*$^{-/-}$ hematopoietic cells by the inducible *Mx1-Cre* recombinase transgenic system has been described previously (Kortylewski et al., 2005). We generated *CD4-Cre/Stat3*$^{flox/flox}$ mice by standard interbreeding procedures. We verified specific *Stat3* deletion in T cells by PCR using primer sets that distinguish *Stat3*, *Stat3*^{loxP}, and *Stat3*-deleted alleles and by FACS analysis of Stat3 phosphorylation in IL-6-treated T cells. For tumor challenge, we injected 1×10^5 B16 or 5×10^5 MB49 or MC38 tumor cells subcutaneously into 7- to 8-week-old wild-type or transgenic mice, and tumor growth was monitored three times per week. For IL-23R neutralization experiments in vivo, mice were treated with 250 μg of anti-IL-23Rα antibodies (R&D Systems) injected intraperitoneally every third day during the course of the experiment, starting from the day of tumor inoculation. Mice were sacrificed 2 weeks after tumor inoculation. We prepared single-cell suspensions of spleen, lymph node, or tumor tissues by mechanic dispersion followed by collagenase D/DNase I treatment as described previously (Kortylewski et al., 2005).

Cytokine ELISA and Western Blotting

We enriched CD11b$^+$c$^+$ or CD11b$^+$c$^-$ immune cell subsets from spleens and tumors from *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ (*Mx1-Cre/Stat3*$^{flox/flox}$) mice using specific antibodies in combination with magnetic nanoparticles from StemCell Technologies as described previously (Kortylewski et al., 2005). For IL-12 and IL-23 measurement by ELISA (eBioscience), we cultured the enriched cell populations for 24 hr to collect supernatants. Equal protein amounts of lysates prepared from enzymatically digested tumors and/or tumor-draining lymph nodes were analyzed by western blotting using antibodies specific to IL-23/p19 (R&D Systems), phospho-S503-c-Rel (Thermo Scientific), and β-actin (Sigma).

Quantitative Real-Time PCR

We isolated total RNAs from various cell populations using the RNeasy System (QIAGEN) and then transcribed them into cDNAs using the iScript cDNA Synthesis Kit (Bio-Rad). PCR reactions were set up using specific primer pairs for mouse *IL-23/p19* (5′-TCCCTACTAGGACTCAGCCAACTC-3′ [forward] and 5′-ACTCAGGCTGGGCACTG-3′ [reverse]) or commercially available primers for mouse *IL-12/p35*, *18S* rRNA, or *GAPDH* (SuperArray). Sequence-specific amplification was assessed by measuring the fluorescent signal of SYBR green using a Chromo4 Real-Time PCR Detector (Bio-Rad).

Reporter Gene Assays

We inserted the mouse *IL-23/p19* promoter fragment (−1159 to +160) into the XhoI/HindIII site of the pGL3-Basic vector by PCR, generating the pGL3-*p19*-Pro-luciferase plasmid. For luciferase assays, we cotransfected 0.4 μg/ml pGL3-*p19*-Pro-luciferase plasmid containing 0.02 μg/ml pRL-*TK* normalization construct and 0.4 μg/ml of either the indicated pRC plasmid or siRNAs (80 pmol/ml) into BALB/c 3T3 fibroblasts and B16 and C4 mouse melanoma cells using Lipofectamine 2000 (Invitrogen). We analyzed luciferase activity in whole-cell lysates using the Dual-Luciferase Reporter Assay System (Promega) and quantified results using a Mikrotek Laborsysteme microplate reader. Data are presented as firefly luciferase activity normalized to Renilla luciferase activity in each triplicate sample.

Chromatin Immunoprecipitation Assays

We performed chromatin immunoprecipitations using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Briefly, cultured cells were fixed with 1% formaldehyde at 37°C for 10 min before lysis. For ChIP assays on freshly isolated whole tumors or tumor-draining or distant lymph nodes, prior to crosslinking with formaldehyde, tissues were frozen in liquid nitrogen and homogenized to enable nuclei isolation. We incubated the sonicated chromatin solutions with 2 μg of Stat3-, p65-, or c-Rel-specific antibodies (Santa Cruz) or with control rabbit IgG. Following immunoprecipitation and reversed crosslinking, DNA was extracted and analyzed by PCR using the following primer sets: mouse *IL-23/p19* promoter, 5′-GGATTCCCGTCCCTCGGTCTC-3′ (forward) and 5′-GGGCCAAGGCGCTTGGCACAG-3′ (reverse); mouse *IL-12/p35* promoter, 5′-GACAGTGGAGGCACCAGGCC-3′ (forward) and 5′-CAGACATCGCTGTCCCGGCG-3′ (reverse).

Flow Cytometry

For extracellular staining of immune markers, we prepared single-cell suspensions by mechanic dispersion and enzymatic digestion of spleen, lymph node, or tumor tissues. We preincubated 1×10^6 freshly prepared cells suspended in a mixture of PBS, 2% fetal calf serum, and 0.1% (w/v) sodium azide with FcγIII/IIR-specific antibody to block nonspecific binding and stained with different combinations of fluorochrome-coupled antibodies to CD3, CD4, CD25 (BD Biosciences), or IL-23R (Imgenex). Staining with the anti-IL-23R antibody required earlier labeling of the unconjugated antibody with a fluorescent dye, using the Zenon Labeling Kit (Invitrogen). Prior to intracellular staining with antibodies to phosphotyrosine-Stat3 or -Stat5 (BD Biosciences) and Foxp3 (eBioscience), we fixed cells in paraformaldehyde and permeated them in methanol. For intracellular staining of IFN-γ, IL-10, and IL-17 (BD Pharmingen), we followed the manufacturer's protocol after 4 hr incubation in the presence of Leukocyte Activation Cocktail (BD Pharmingen). Fluorescence data were collected on a FACSCalibur system (Beckton Dickinson) and analyzed using FlowJo software (Tree Star).

Statistical Analysis

An unpaired t test was used to calculate two-tailed p values to estimate statistical significance of differences between treatment groups. Statistically significant p values are indicated in figures as follows: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Data were analyzed using GraphPad Prism software.

SUPPLEMENTAL DATA

The Supplemental Data include two figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(08)00437-6.

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