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Stat3 Activity in Melanoma Cells Affects Migration of Immune Effector Cells and Nitric Oxide-Mediated Antitumor Effects

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Infiltration of immune effector cells in tumors is critical for antitumor immune responses. However, what regulates immune cell infiltration of tumors remains to be identified. Stat3 is constitutively activated with high frequency in diverse cancers, promoting tumor cell growth and survival. Blocking Stat3 signaling in tumors in vivo results in tumor growth inhibition that involves killing of nontransfected tumor cells and infiltration of immune effector cells, suggesting that Stat3 activity in tumor cells might affect immune cell recruitment. However, dying tumor cells can also attract immune cells. In this study, we show in isogenic murine melanomas that natural Stat3 activity is associated with tumor growth and reduction of T cell infiltration. Blocking Stat3 signaling in the melanoma cells containing high Stat3 activity results in expression of multiple chemotactants, leading to increased migration of lymphocytes, NK cells, neutrophils, and macrophages. In addition, blocking Stat3 triggers tumor cells to produce soluble factors capable of activating macrophage production of NO in vitro and in vivo. TNF-α and IFN-β, which are secreted by Stat3-inhibited tumor cells, are able to activate macrophage NO production, whereas neutralizing TNF-α in the tumor supernatant from Stat3-blocked tumor cells abrogates nitrite production. Moreover, interrupting Stat3 signaling in tumor cells leads to macrophage-mediated, nitrite-dependent cytostatic activity against nontransduced tumor cells. These results suggest that tumor Stat3 activity affects recruitment of diverse immune effectors and it can be manipulated to activate the effector phase of innate immune responses. The Journal of Immunology, 2005, 174: 3925–3931.

It is well documented that the presence of immune cells inside tumors is necessary for cellular immune responses against tumors and that immune effector cells are often physically excluded from progressing tumors (for review, see Ref. 1). However, the mechanism(s) within tumors that might affect immune cell infiltration is largely unknown (for review, see Ref. 1). As a point of convergence for multiple prevalent oncogenic signaling pathways, Stat3 is constitutively activated with very high frequency in a wide range of cancers (for reviews, see Refs. 2–4). Blocking Stat3 signaling in tumor cells has been shown to down-regulate expression of genes that are critical for cell proliferation and survival. They include Bcl-xL (5), Mcl-1 (6), cyclin D1 (7), and c-Myc (8). Using a dominant-negative variant of Stat3, designated Stat3Δ, we recently showed that inhibiting Stat3 signaling in tumors caused tumor growth inhibition and regression in vivo (9). Interestingly, in vivo tumor transfection efficiency was only 10–15%, suggesting that targeting Stat3 signaling in tumors involves a substantial “bystander” antitumor effect (9). The bystander effect was associated with tumor infiltration of immune cells (10). Our recent study also showed that blocking Stat3 signaling in tumor cells led to elevated expression of chemokines (10), suggesting that Stat3 activity might influence tumor-immune cell infiltration. However, it remains to be demonstrated that infiltration of the immune cells is not due to apoptotic/necrotic tumor cells associated with the gene therapy and that the elevated expression of chemokines has biological functions.

Neutrophils and macrophages are considered as the first effector cells in the chain of innate immune response (11, 12). Activated by cytokines such as TNF-α, IL-6, IL-1, and IFNs, these effector cells can exert direct cytotoxic antitumor effects mediated by reactive forms of oxygen and/or nitrogen (13–15). NO production by activated macrophages has been shown to inhibit tumor cell growth and tumor metastasis (for review, see Ref. 16). Production of NO by tumor cells as a result of the transfecting inducible NO synthase (iNOS)5 gene, which is primarily expressed in activated macrophages or up-regulating endogenous iNOS expression, can induce tumor regression and prevent metastasis (17–19). Although our previous study indicated that Stat3 blockade in B16 tumor cells can lead to production of NO, whether macrophage activation by Stat3 blockade in tumor cells is a general phenomenon is unknown. Moreover, since NO could either promote or inhibit tumor growth depending on the concentration available at the tumor site (for review, see Ref. 16), whether NO production induced by Stat3 blockade in tumor cells could lead to antitumor effects remains to be assessed.

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5 Abbreviations used in this paper: iNOS, inducible NO synthase; CM, conditioned medium; EGFP, enhanced green fluorescent protein; NMDA, N-monomethyl-L-argi- nine; IP-10, IFN-γ-inducible protein 10; siRNA, small interfering RNA; EGFP, enhanced GFP.
In this study, we show that growth of K1735 murine melanoma sublines in vivo depends on natural Stat3 activity. Although tumor cells containing constitutively activated Stat3 are able to form tumors in immunocompetent mice, those sublines with low/no Stat3 activity either fail to form tumors or are spontaneously rejected. Moreover, before they were rejected the tumors with low/no Stat3 activity had heavy infiltration of T lymphocytes. Our data further indicated that the sublines with low/no Stat3 activity were able to form tumors in SCID mice that lack T cells. These results indicate for the first time that endogenous Stat3 activity correlates with tumor growth and reduced T cell infiltration and response. We further demonstrate that blocking Stat3 in a K1735 melanoma subline displaying high Stat3 activity leads to the production of multiple chemotaxtractants, including RANTES, IFN-γ-inducible protein 10 (IP-10), MCP-1, MIP-2, and TCA-3, inducing migration of lymphocytes, NK cells, neutrophils, and macrophages. In addition, inhibiting Stat3 either genetically or pharmacologically in diverse tumor cells results in activation of macrophage NO production. Blocking Stat3 signaling in tumor cells in vivo also leads to activation of distal peritoneal macrophages. Moreover, we identify TNF-α as one of the factors produced by Stat3-blocked B16 tumor cells required for activating macrophage nitrite production. We further demonstrate that release of nitrite by activated macrophages as a consequence of Stat3-blockade in tumor cells in turn inhibits the growth of nontransduced tumor cells. A critical role of Stat3 in tumor progression and cancer therapy has gained considerable recognition and momentum (2–4). Our current results demonstrate that Stat3 activity in melanoma negatively regulates infiltration of diverse immune effector cells and that targeting Stat3 in tumor cells leads to production of chemokines and cytokines capable of recruiting immune cells and eliciting innate immune cell-mediated antitumor effects.

Materials and Methods

Mice and cell lines

Female C57Bl/6, BALB/c, C3H, and SCID mice (National Cancer Institute, Frederick, MD) as well as iNOS knockout mice (6– to 8-wk old; The Jackson Laboratory) were used for the experiments. Two days after transfection of B16 cells (1 × 105) with either pIREs-Stat3β or pIREs-enhanced GFP (EGFP), cells were gamma irradiated and injected s.c. Five days later, peritoneal macrophages were obtained, followed by 24-h incubation in vitro before NO production was determined with Griess reagent (Sigma-Aldrich). All mice were maintained under pathogen-free conditions and experiments involving mice were performed in accordance with established institutional guidance and approved protocols. The B16 and A2058 tumor cell lines were obtained from American Type Culture Collection. JW human melanoma cells have been described elsewhere (20). A2058 tumor cell lines were obtained from American Type Culture Collection. JW human melanoma cells have been described elsewhere (20). K1735 melanoma sublines were kindly provided by Dr. J. Fidler (M. D. Anderson Cancer Center, Houston, TX). All of the cell lines were maintained in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

Immunostaining and photomicrograph analysis

Tumor tissues were formalin fixed and paraffin embedded. Three-micrometer sections were deparaffinized and stained with CD3E Ab (1/500; BD Pharmingen). For each K1735 melanoma clone (CL-19, CL-4, M-2, and X-21), two tumors were analyzed and four typical photomicrographs (magnification, ×20) per sample were included for data analysis. Within these photomicrographs (visible area, 2.5 × 105 μm²), positively stained T cells were counted using Image Pro-Plus version 5.0 software (Media Cybernetics).

Cell transfection and treating tumor cells with Stat3 small molecule inhibitor

Transient transfection of tumor cells with pIREs-Stat3β or control empty vector pIREs-EGFP were conducted using LipofectAMINE plus reagent (Invitrogen Life Technologies). Where indicated, a Stat3 small interfering RNA (siRNA) oligonucleotide was used to suppress Stat3 expression in tumor cells. The sequences for Stat3 siRNA and negative control (scrambled sequence) were 5'-GATCCCGTGAGTTGCTGTCGAAATTTCAGAGAATTGTGACACGACACCTGACTTTTTTGGAAA-3' and 5'-GATCCACCTACCGTTGATTAAGGTGTCTAAGAGACACTTATACCAACGGTATGTTTTTGGAAA-3', respectively. Forty-eight hours after transfection, the cells or their cell-free supernatants were used for migration and/or NO assays in vitro and in vivo. Tumor cells were treated with a small-molecule Stat3 inhibitor, CPA-7 (21) at 20 μM for 24 h before supernatants were collected.

EMSA

EMSA to detect Stat3 DNA binding were performed essentially as previously described (9). Briefly, nuclear extracts (1–8 μg of total protein), normalized for protein concentration, were incubated with the 32P-radiolabeled high-affinity Sis-inducible element oligonucleotide probe. Protein-DNA complexes were resolved by 5% non-denatured PAGE and specific Stat-DNA complexes were detected by autoradiography.

Preparation of peritoneal macrophages and neutrophils

To prepare macrophages for NO production, peritoneal cells were obtained by washing the peritoneal cavity with serum-free RPMI 1640 culture medium. The macrophage population was enriched by adhesion on plastic plates for 2 h, followed by washing off nonadherent cells. Adherent cells were identified as macrophages (>95%) based on morphological criteria using Giemsa staining and CD11b expression using Mac-1 Abs. For migration assays, peritoneal macrophages and neutrophils were collected from the peritoneal cavity after thioglycolate i.p. injection (3%, 1.5 ml, 72 h and overnight, respectively). Peritoneal cells contained >75% neutrophils based on morphological criteria.

NO production

Peritoneal macrophages (2 × 10^6/ml) were cultured with various conditioned media (CM) for 48 h. In some experiments, CM was pretreated with anti-TNF-α or anti-IL-1 Abs for 1 h at room temperature (Santa Cruz Biotechnology). Nitrite accumulation in macrophage supernatants was determined using Griess reagent (Sigma-Aldrich).

Macrophage cytotoxic activity

NO-mediated macrophage cytotoxic activity against nontransfected B16 cells was determined by DNA synthesis inhibition of target tumor cells as previously described (22). Briefly, B16 tumor cells (2 × 10^5/ml) were cocultured for 48 h with and without macrophages in the presence of CM collected from pIRES-Stat3β- or control empty vector pIREs-EGFP-transfected B16 cells. The tumor supernatants were then used as they were or were diluted at 1:1 or 1:5 ratios with CM. The iNOS inhibitor, N-mono-methyl-L-arginine (10^-4 M), was added to macrophage cultures before addition of tumor cell supernatants. To estimate DNA synthesis, cells were pulsed with [H]-Thymidine (0.25 μCi/well) during the last 6 h of incubation. [H]-Thymidine incorporation was determined using a liquid scintillation beta counter (Pharmacia Wallac).

RNase protection assay

The RNase protection assay was performed as previously described (9).

Neutrophil, splenocyte, and macrophage migration assays

Migration assays for various subsets of immune cells were performed using a 48-well microchemotaxis chamber (NeuroProbe) following the manufacturer’s protocol. Neutrophil, splenocyte, and macrophage migration rates were determined by counting respective cells that have migrated into the filter of the chemotaxis chamber. The filter was stained with modified Wright-Giemsa.

Results

Endogenous Stat3 activity vs tumor growth and T cell infiltration

Tumor infiltration by immune effector cells, especially T cells, is indicative of prognosis and response to immunotherapy (1, 23). In our previous study, we found that blocking Stat3 signaling in B16 mouse melanoma tumors by gene therapy using a plasmid vector encoding a dominant-negative Stat3 protein, Stat3β, caused tumor growth inhibition/regression and heavy infiltration of T cells (9, 10). We further showed that blocking Stat3 in tumor cells led to...
expression of T cell chemoattractants, RANTES and IP-10 (10). These results suggest that Stat3 activity in tumor cells may influence T cell infiltration and growth. However, dying tumor cells, which were abundantly present in Stat3β-treated B16 tumors, are also known to attract infiltration of diverse immune cells. Furthermore, the biological consequences of the elevated expression of chemoattractants remained unclear. To assess whether natural Stat3 activity within a tumor can influence T cell infiltration, we tested six subclones of the murine melanoma K1735 for growth and T cell infiltration. Three of these clones had high Stat3 activity and three displayed low or no Stat3 activity. Although all of the subclones of K1735 melanoma with high Stat3 activity were able to form tumors, only one of the subclones, CL-19, with low/no Stat3 activity formed tumors in C3H immunocompetent mice (Fig. 1A). However, CL-19 tumors grew no larger than 2 mm in diameter before they regressed (Fig. 1A). Previous reports indicated that CL-19 represented a nonmetastatic clone, whereas CL-4, M-2, and X-21 were highly aggressive and metastatic clones of K1735 melanoma (24). As shown by EMSA, Stat3 activity is low in CL-19 tumor cells, but is elevated in CL-4, M-2, and X-21 tumor cells (Fig. 1B). In addition, natural, endogenous Stat-3 activity is associated with reduced T cell infiltration as shown in both photomicrographs (Fig. 1A, right columns) and actual T cell numbers (Fig. 1C). Our results further indicated that all three K1735 sublines with low/no Stat3 activity were able to form tumors in SCID (C3H background) mice, which do not have T cells (data not shown).

Lowering Stat3 activity in K1735 subclone results in lymphocyte migration and expression of multiple chemoattractants

To directly test whether Stat3 activity would affect lymphocyte migration, we used a Stat3 small-molecule inhibitor, CPA-7 (21), to block Stat3 signaling in CL-4 tumor cells, which display high levels of endogenous Stat3 activity (Fig. 1B). Inhibition of Stat3 activity in the CL-4 cell line leads to secretion of soluble factors that stimulated splenocyte migration (Fig. 2A). Based on the morphology, both lymphocyte and NK cell migration rates were induced by CM from CPA-7-treated CL-4 cells (Fig. 2B). Along with the in vivo data shown in Fig. 1, these findings suggest that tumor’s Stat3 activity can influence tumor lymphocyte infiltration.

We next determined whether blocking Stat3 with CPA-7 might induce the expression of chemoattractants that could account for the increased lymphocyte migration. An RNase protection assay to detect expression levels of multiple chemokines was performed. CL-4 tumor cells treated with CPA-7 displayed increased expression of RANTES, IP-10, TCA-3, MIP-2, and MCP-1 chemokines (Fig. 2C). RANTES, IP-10, and TCA-3 are best known to attract T cells and MIP-2 and MCP-1 macrophage chemoattractants. Many of these chemoattractants are capable of stimulating migration of multiple subsets of immune cells (25, 26). We further examined chemoattractant expression in A2058 human melanoma cells. Blocking Stat3 could also stimulate the expression of multiple chemoattractants, including RANTES, IP-10, and IL-8 (data not shown).

Migration of neutrophils and macrophages induced by Stat3-interrupted tumor cells

Our previous study showed that blocking Stat3 signaling in B16 tumors by Stat3β gene therapy in vivo led to infiltration of neutrophils and macrophages (10). To assess whether expression of the chemoattractants by B16 tumor cells after blocking Stat3 could attract/recruit neutrophils, we performed migration assays. Stat3β is a dominant-negative variant of Stat3 capable of displacing wild-type Stat3 (Stat3α) DNA binding (5). B16 tumor cells display constitutively activated Stat3 and transient transfection of B16 cells with pIRES-Stat3β led to high levels of Stat3β DNA-binding as detected by EMSA (Fig. 3A). Exposure of freshly isolated neutrophils to CM collected from B16 cells expressing pIRES-Stat3β elicited a robust migration response comparable to IL-8, known to induce neutrophil migration (27), whereas little neutrophil migration was detected using control medium or CM from control vector, pIRES-EGFP, transfected B16 cells. Neutrophil migration rate-induced supernatants from Stat3-blocked tumor cells is dose dependent (Fig. 3, B and C). To make sure neutrophil migration due to Stat3 blockade in tumor cells is not a unique feature of Stat3β, we also inhibited Stat3 in CL-4 melanoma cells with CPA-7. Inhibiting Stat3 activity in tumor cells stimulates expression of a variety of chemoattractants, such as MIP-2, IP-10, MCP-1, and TCA-3, that are known to recruit neutrophils and
macrophages (Fig. 2C). Data in Fig. 3D indicate that CPA-7 treatment of CL-4 tumor cells leads to secretion of a soluble factor that increased neutrophil migration. These results indicate that induction of neutrophil migration can be achieved by Stat3 blockade in diverse melanoma cells. Because blocking Stat3 in melanoma cells induces expression of several chemokines, such as MIP-2 and MCP-1, that are known to attract macrophages, we tested whether blocking Stat3 in CL-4 melanoma cells will lead to macrophage migration. As shown in Fig. 3E, the macrophage migration rate was increased when the macrophages were exposed to supernatant of CPA-7-treated tumor cells.

**Tumor cell Stat3 activity and macrophage nitrite production**

Activated nitrite production by macrophages has been shown to play a role in inhibiting tumor growth and metastasis (18, 19, 28–30), and our previous study showed that inhibiting Stat3 signaling in B16 tumor cells by Stat3 DNA-binding activity as determined by EMSA. Although B16 tumor cells display Stat3 DNA-binding, Stat3 DNA binding is expected to displace the binding of Stat3α. NIH3T3 cells stimulated with epidermal growth factor serve as a positive control for endogenous Stat3 DNA-binding activity. B. Neutrophil migration induced by CM from Stat3β-transfected tumor cells: mean ± SD of three experiments. IL-8 = 100 ng/ml recombinant human IL-8 (positive control); CM-EGFP = CM from EGFP-transfected B16 cells (vector control); CM-Stat3β = CM from Stat3β-transfected B16 cells; 50% = 1/1 and 20% = 1/4 dilutions of CM from Stat3β-transfected B16 cells. *, p < 0.05. C. Neutrophil migration induced by CM from Stat3β-transfected B16 tumor cells shown in photomicrographs (magnification, ×100). D. Neutrophil migration induced by CM from CL-4 tumor cells treated with CPA-7. Tumor supernatants collected from CL-4 tumor cell cultures after treating with either DMSO-containing medium or 20 μM CPA-7. Data shown represent mean ± SD; n = 3. E, Macrophage migration induced by CM from CL-4 cell line treated with CPA-7. Tumor supernatants collected from indicated tumor cell cultures after treating with either DMSO-containing medium or 20 μM CPA-7. Data shown represent mean ± SD; n = 3. *, p < 0.05.

**FIGURE 3.** Migration of neutrophils and macrophages in response to Stat3 blockade in melanoma cells. A. Stat3 is activated in B16 tumor cells and transfecting the tumor cells with Stat3β expression vector leads to high Stat3β DNA-binding activity as determined by EMSA. Although B16 tumor cells display Stat3α DNA-binding, Stat3α DNA binding is expected to displace the binding of Stat3α. NIH3T3 cells stimulated with epidermal growth factor serve as a positive control for endogenous Stat3α DNA-binding activity. B. Neutrophil migration induced by CM from Stat3β-transfected tumor cells: mean ± SD of three experiments. IL-8 = 100 ng/ml recombinant human IL-8 (positive control); CM-EGFP = CM from EGFP-transfected B16 cells (vector control); CM-Stat3β = CM from Stat3β-transfected B16 cells; 50% = 1/1 and 20% = 1/4 dilutions of CM from Stat3β-transfected B16 cells. *, p < 0.05. C. Neutrophil migration induced by CM from Stat3β-transfected B16 tumor cells shown in photomicrographs (magnification, ×100). D. Neutrophil migration induced by CM from CL-4 tumor cells treated with CPA-7. Tumor supernatants collected from CL-4 tumor cell cultures after treating with either DMSO-containing medium or 20 μM CPA-7. Data shown represent mean ± SD; n = 3. E, Macrophage migration induced by CM from CL-4 cell line treated with CPA-7. Tumor supernatants collected from indicated tumor cell cultures after treating with either DMSO-containing medium or 20 μM CPA-7. Data shown represent mean ± SD; n = 3. *, p < 0.05.
secretion of factors that activate macrophages is a general phenomenon in response to disruption of Stat3 signaling, we next evaluated whether targeting Stat3 in a variety of human and mouse melanoma cell lines and primary melanoma cells would lead to increased production of NO by macrophages. Blocking Stat3 signaling in a number of mouse and human melanoma cells, both established cell lines and primary tumor cells, with either Stat3B, a small-molecule Stat3 inhibitor, or a Stat3 siRNA led to production of soluble factors that activated NO synthesis in peritoneal macrophages (Fig. 4A).

To determine whether blocking Stat3 signaling in tumor cells could activate macrophages in vivo, mice were injected s.c. with B16 cells transiently expressing either the control empty vector, pIRES-EGFP, or pIRES-Stat3 expression vector. Five days later, resident peritoneal macrophages were harvested and tested for NO production. Although only a subset of B16 cells express Stat3B (~30–40% based on the percentage of fluorescent cells), injection of Stat3B-transfected B16 cells resulted in significantly higher level of NO production by peritoneal macrophages than GFP-transfected B16 cells ex vivo (Fig. 4C).

**TNF-α is required but not sufficient for stimulating macrophage nitrite production**

Results shown above indicate that blocking Stat3 signaling in tumor cells leads to secretion of factors that stimulate macrophages to produce NO. However, what factors might be involved in this process remained to be determined. TNF-α and IFN-β are among the proinflammatory cytokines secreted by the tumor cells after Stat3 inhibition (10). To test whether they could stimulate macrophages to produce NO, recombinant TNF-α and IFN-β proteins were added to cultured peritoneal macrophages, both individually and simultaneously before assayying for NO production (Fig. 5A). Peritoneal macrophages were prepared from both C57BL and BALB/c mice. LPS (1 μg/ml) and IFN-γ (50 U/ml) treatment was included as positive control. When added individually, TNF-α and IFN-β did not induce significant NO production after 48 h of incubation. However, when TNF-α and IFN-β were added simultaneously, a dramatic induction of NO production by macrophages was observed. Moreover, neutralizing experiments to block TNF-α function in CM showed that preincubation with a TNF-α Ab reduced nitrite induction (Fig. 5B). In contrast, preincubation of CM from B16 tumor cells with IL-1 Ab had no effect on stimulation of nitrite production by macrophages. These results suggest that activation of macrophages likely involve multiple proinflammatory mediators. In addition, at least in the B16 tumor cell system, TNF-α is required for the observed macrophage activation.

**Nitrite produced by Stat3 blockade can exert cytostatic activity against tumor cells**

Although NO is the key mediator of tumoricidal activity by macrophages (18, 19), NO when present at low levels at the tumor site is also known to promote tumor growth and metastasis (for review, see Ref. 16). Whether nitrite produced in response to Stat3 blockade in tumor cells could lead to cytotoxic activity against tumor cells remains to be determined. To address this question, macrophages preincubated in CM collected from either Stat3-transfected or control vector-transfected B16 cells were tested for their capability to induce cytotoxic effects on nontransfected B16 cells.

**FIGURE 4.** Peritoneal macrophage nitrite secretion in response to Stat3 blockade. A, Blocking Stat3 signaling in both established melanoma cell lines and primary melanoma cells induced nitrite secretion by peritoneal macrophages. Left panel, CM collected from untransfected (WT), empty vector-transfected (EGFP), or Stat3β-transfected (Stat3β) B16 cells were added to peritoneal macrophages at the indicated concentrations. NO production was measured 48 h later. Data shown represent mean ± SD; n = 3. Right panel, Blocking Stat3 signaling in diverse melanoma cells with different approaches also leads to macrophage production of NO. Data shown represent mean ± SD, with n = 3 for each set of experiments. *, p < 0.05. B, Stat3 inhibition in indicated melanoma cells by CPA-7 or siRNA, as determined by EMSA. C, Activation of distal macrophages (peritoneal) in vivo by B16 tumor cells transfected with Stat3B. B16 tumor cells transfected with either pEGFP-Stat3B or control pEGFP vector were irradiated and injected s.c. into C57BL/6 mice. Peritoneal macrophages were harvested 5 days postinjection and NO production was measured. Nitrite production by peritoneal macrophages from PBS-injected mice was used as a normal control. Data shown represent mean ± SD; n = 3, *, p < 0.01.

**FIGURE 5.** Activation of macrophage nitrite production by Stat3-blocked B16 tumor cells might be TNF-α and IFN-β dependent. A, TNF-α and IFN-β contribute to macrophage activation triggered by Stat3 disruption in tumor cells. Peritoneal macrophages were individually or simultaneously incubated with recombinant proteins (TNF-α, 100 ng/ml, and IFN-β, 1000 U/ml). Data shown represent two independent experiments for both strains of mice. LPS (1 μg/ml) and IFN-γ (50 U/ml) treatment was included as a positive control, *, p < 0.01. B, Blocking TNF-α reduces nitrite production. CM from indicated B16 tumor cells were pretreated with neutralizing anti-TNF-α and IL-1 Abs before adding to macrophage cultures.
As shown in Fig. 6, those macrophages that were exposed to supernatant derived from Stat3β-transfected B16 tumor cells were able to inhibit growth of nontransfected B16 tumor cells. It is well established that NO is produced as a consequence of enzymatic oxidation of the amino acid L-arginine by NO synthase, which consists of both constitutive and inducible (iNOS) forms (16). The expression of iNOS can be induced by various immunological and proinflammatory stimuli. We next determined whether the observed cytotoxic effect was mediated by NO as a result of iNOS gene induction in macrophages. N-monomethyl-L-arginine (NMMA), an analogue of arginine that blocks NO production, was added to tumor CM. As shown in Fig. 6, NMMA abrogated macrophage cytotoxicity induced by soluble factors secreted by Stat3-interrupted tumor cells. Furthermore, CM from Stat3β-B16 tumor cells failed to activate \( \text{iNOS}^{-/-} \) macrophage cytotoxicity, confirming that the observed cytotoxic effect is nitrite mediated and iNOS dependent (Fig. 6).

**Discussion**

The present study shows for the first time that endogenous Stat3 activity is associated with tumor growth and reduced T cell infiltration. Results presented here also demonstrate that blocking Stat3 signaling in melanoma cells with high levels of Stat3 activity triggers production of multiple chemokine attractants, stimulating migration of diverse subsets of immune cells critical for induction of antitumor immune responses. Furthermore, we show that blocking Stat3 signaling in a variety of melanoma cells by either Stat3 dominant-negative protein or a pharmacological Stat3 inhibitor leads to expression of soluble factors capable of activating macrophages, which in turn exert iNOS-dependent and NO-mediated tumor cell growth inhibition. We also identify TNF-\( \alpha \) as one of the factors secreted by Stat3-interrupted B16 melanoma cells involved in stimulating macrophage nitrite production.

Progressing and advanced tumors are often associated with lack of infiltration of immune effector cells (for review, see Ref. 1). However, the mechanism(s) regulating recruitment of immune effector cells at the tumor site remains to be further defined. Our results provide evidence that activation of Stat3, which is a point of convergence for numerous commonly activated oncogenic signaling pathways, negatively regulates expression of chemokines and infiltration of diverse immune effector cells. At the same time, many immunotherapeutic strategies have been attempted to recruit immune effector cells with the goal to enhance immune responses to cancer (for review, see Ref. 1). These approaches usually aim to increase one chemokine expression. Our results show that targeting Stat3 in tumor cells can lead to up-regulation of multiple chemokines and thereby recruit diverse immune effectors to tumors.

Bystander tumor growth inhibition has been observed in several current cancer gene therapies. A number of independent studies have shown that p53 gene overexpression in tumor cells in vivo is associated with a significant reduction in tumor angiogenesis attributable to down-regulation of vascular endothelial growth factor expression (31, 32). In addition to antiangiogenic effects, the efficacies of some gene therapies have been shown to benefit greatly from antitumor immune responses (33). However, the antitumor immune responses associated with a majority of the current gene therapies are nonspecific. For example, HSVtk-expressing B16 tumor cells die predominantly by necrosis upon exposure to ganciclovir. Dying tumor cells, especially necrotic tumor cells, attract/activate macrophages as well as T cells, leading to antitumor immunity (33). Although a role for activated macrophages in mediating antitumor immunity is implicated in HSVtk/ganciclovir-based gene therapy, activation was attributed to tumor cell necrosis rather than a specific mechanism (33). In contrast, our present study demonstrates that targeting Stat3 signaling in tumor cells triggers secretion of soluble factors, including TNF-\( \alpha \) and IFN-\( \beta \), that activate production of nitrite by macrophages, causing cytostatic activity against nontransfected tumor cells. Although low levels of NO production by tumor cells have been associated with tumor progression (16, 19, 34), high levels of NO produced by tumor cells and macrophages are known to inhibit tumor growth and metastasis (17). Our findings suggest that macrophage nitrite production, induced by disrupting Stat3 signaling in B16 tumor cells, participates in growth inhibition of “bystander” B16 tumor cells. The fact that local injection of Stat3-interrupted tumor cells activates distal macrophages (peritoneal) to produce relatively high NO also implies that targeting Stat3 in tumor cells may reduce metastasis.

Numerous independent studies have demonstrated that Stat3 is constitutively activated in diverse cancers and that blocking Stat3 leads to tumor cell apoptosis and growth inhibition in vitro and in vivo (for reviews, see Refs. 2–4). Importantly, tumor cells’ dependence on Stat3 for survival is significantly greater than that of normal cells (for review, see Ref. 4). It has been demonstrated that Stat3 blockade by either genetic approaches or small-molecule drugs causes the death of tumor cells but not normal cells (8, 35). Moreover, recent studies have also shown that Stat3\(^{–/–}\) normal cells can survive and grow well in vitro and in vivo (36–38). With the emergence of Stat3-specific inhibitors (4, 21, 35), Stat3 as one of the most promising molecular targets for cancer therapy is gaining momentum (4, 39). In addition to causing tumor cell death, blocking Stat3 in tumor cells also allows activation of dendritic cells, which in turn activates Ag-specific T cells in vitro and in vivo (10). A critical role of Stat3 in both macrophages and dendritic cells in mediating tumor-induced immune suppression has also been documented recently (10, 40). The findings from our present study suggest that tumor Stat3 activity negatively impacts the immune cell effector phase, including infiltration and direct tumor growth inhibition. Targeting Stat3 in tumor cells may lead to recruitment of diverse immune effector cells and innate immune cell-mediated direct antitumor effects.

**FIGURE 6.** Soluble factors secreted by Stat3-interrupted B16 tumor cells induce iNOS-dependent cytostatic effects in vitro. Macrophages (Mph) treated with CM from Stat3-interrupted B16 tumor cells have cytostatic activity against nontransfected B16 tumor cells. This cytostatic activity is NO and iNOS dependent since inhibition of nitrite production using NMMA or absence of the iNOS gene (iNOS\(^{–/–}\)) in the macrophages abrogates the antitumor effects. *, \( p < 0.01 \).
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