Tumor-Derived Cytokines Dysregulate Macrophage Interferon-γ Responsiveness and Interferon Regulatory Factor-8 Expression

DAVID W. MULLINS,[1,2] RYAN S. MARTINS, AND KLAUS D. ELGERT

Department of Biology, Microbiology and Immunology Section, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061–0406

Tumors can evade immune responses through suppressor signals that dysregulate host effector cell function. In this study we demonstrate that tumor-derived suppressor molecules impede host antitumor immune activity through dysregulation of multiple macrophage (Mϕ) pathways, including suppressed production of cytotoxic and immunostimulatory agents and impaired expression of the interferon regulatory factor-8 (IRF-8) protein, a critical transducer of interferon-γ-mediated activation pathways. The tumor-derived immunosuppressive cytokines interleukin-10 and transforming growth factor-β_1 constrain IRF-8 production by normal Mϕs, regardless of priming, and IRF-8 is also dysregulated in primary Mϕs from tumor-burdened hosts. Collectively, these data describe a new mechanism by which tumors disrupt immune function and suggest that abrogation of tumor-derived immunoregulatory factors *in situ* can restore immune function and enhance antitumor efficacy. Exp Biol Med 228:270–277, 2003

Key words: immunosuppression; IFN-γ; IRF-8; macrophage; tumors

A functional immune system is a potential barrier to tumor development and progression. To evade immune mechanisms, many tumors release immunosuppressive factors that attain high concentration *in situ* and inhibit cell-mediated effector function. Suppression of immune responses, coupled with superior growth kinetics of tumor cells, enable the neoplasm to surpass the control capacity of the host, leading to progressive decreases in cell-mediated antitumor responses and accelerated disease (1). We and others have shown that tumor cell-derived factors, such as interleukin (IL)-10 and transforming growth factor (TGF)-β_1, induce both local (2) and systemic (3) immunosuppression and selectively alter macrophage (Mϕ) expression of pro-inflammatory cytokines and other potential mediators of immunosuppression. Thus, tumors subvert Mϕ function to suppress T cell function and simultaneously support tumor growth (4, 5).

A mechanism by which tumors may modulate Mϕ activation and effector function involves the control of interferon (IFN)-γ production and responsiveness. IFN-γ plays a central role in Mϕ-T cell interactions. T cells produce IFN-γ in response to antigen stimulation and the antigen-presenting cell-derived cytokines IL-12 and IL-18 (6, 7); in turn, T cell-derived IFN-γ primes Mϕs for enhanced production of cytotoxic effector molecules and proinflammatory cytokines, including IL-12 and IL-18. IFN-γ also exerts direct anti-tumor effector functions and mediates immune surveillance (8). Thus, tumors may subvert the induction of innate and adaptive immunity by modulating IFN-γ production or dysregulating IFN-γ-responsive signal pathways. To evaluate this possibility, we used our well-established murine fibrosarcoma model (3, 9) to determine whether tumor-derived factors interfere with Mϕ responsiveness to, or T cell production of, IFN-γ. In this study, we describe tumor-induced dysregulation of Mϕ effector, pro-inflammatory, and signaling molecule production. Collectively, these data suggest that therapies aimed at disrupting tumor cell suppressor activity or restoring tumor-dysregulated Mϕ responsiveness to IFN-γ-mediated signaling may have significant therapeutic value in the treatment of neoplasia.

Materials and Methods

Murine Tumor Model. Eight to twelve-week-old Balb/c (H-2^d) male mice (Harlan Sprague-Dawley, Madison, WI) were used. A Balb/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma, designated Meth-KDE, was used as described (9). The Meth-KDE fibrosarcoma induces significant immunosuppression through the production of IL-10, TGF-β_1, and prostaglandin E_2 (PGE_2;

This work was supported by Horsley Cancer Research Fund (K.D.E.), Virginia Academy of Sciences (R.S.M. and K.D.E), *Sigma Xi* (R.S.M.), and the American Cancer Society (D.W.M.).

[1] Current Address: Carter Immunology Center and Department of Microbiology, Box 801386, University of Virginia, Charlottesville, VA 22908–1386

[2] To whom requests for reprints should be addressed at Carter Immunology Center, Box 801386, University of Virginia, Charlottesville, VA 22908–1386. E-mail: dmullins@virginia.edu

Received July 3, 2002.
Accepted October 30, 2002.

1535-3702/03/2283-0270$15.00

Ref. 3). Meth-KDE tumors were induced by intramuscular injection of 4×10^5 transplanted cells and palpable tumors developed within 10 days. Tumor-bearing host (TBH) Balb/c mice were used 21 days post-tumor induction because tumor-induced Mϕ suppressor activity is maximal at this time, without cachexia or necrosis. All protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee and conformed to accepted National Institutes of Health guidelines for animal use.

Media and Reagents. Mϕs were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma, St. Louis, MO). All media contained 50 mg/l gentamicin sulfate (Tri-Bio Laboratories, State College, PA), 25 mM sodium bicarbonate ($NaHCO_3$), and 25 mM HEPES buffer (Sigma). RPMI-1640 medium was endotoxin-free (<10 pg/ml) as assessed by the *Limulus* amebocyte lysate assay (Sigma). T cell cultures were supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). Bacterial lipopolysaccharide (LPS; *Escherichia coli* serotype 026:B6) was purchased from Sigma. Recombinant murine IFN-γ (specific activity 2.98×10^6 U/ml; endotoxin content <10 pg/ml) was purchased from Genzyme, Inc. (Cambridge, MA). Rabbit anti-TGF-β_1 polyclonal Ab (6.5 mg/ml; endotoxin content <10 pg/ml) was a generous gift from Genentech, Inc. (South San Francisco, CA). Anti-murine IL-10 mAbs were generated using SXC-1 (clone HB 10739, American Type Culture Collection [ATTC], Manassas, VA) hybridoma supernatants. PGE_2 and indomethacin were obtained from Sigma.

Tumor Cell Culture and Supernatant Preparation. Purified Meth-KDE cells were used to generate tumor-derived supernatants. Meth-KDE cultures were established as previously described (10) and maintained by diluting 1:10 in fresh medium every fifth day. Tumor cell supernatant was obtained by culturing the purified Meth-KDE (4×10^6 cells/ml at 37°C, 5% CO_2) in 24-well culture plates (Corning, Corning, NY) for 72 hr in a total volume of 1.0 ml of complete RPMI medium. Cell-free Meth-KDE supernatants were prepared by centrifugation (400 × g, 5 min) and filtration through a 0.4-micron filter.

Fresh Meth-KDE supernatants were depleted of IL-10 or TGF-β_1 using plate-bound specific Abs, as described (3). PGE_2-depleted tumor supernatants were prepared by incubating Meth-KDE cells with 10^{-7} M indomethacin (Sigma), an arachidonic acid pathway inhibitor. Resulting supernatants did not contain detectable IL-10, TGF-β_1, or PGE_2, as measured by specific enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, and Advanced Magnetics, Boston, MA).

Immune Cell Collection and Culture. Primary normal host and TBH peritoneal exudate Mϕs were collected by lavage with cold RPMI-1640 medium 4 days after intraperitoneal injection with 2 ml of 3% thioglycollate (Difco, Detroit, MI). Mϕs were purified by plating peritoneal exudate cells for 2 hr (37°C, 5% CO_2) in glass plates, washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium using a rubber cell scraper. The final Mϕ preparations contained cells that were >95% viable, and flow cytometric analysis with M1/70 and F4/80 mAb (ATCC) showed them to be >80% Mac-1^+ and F4/80^+ respectively (not shown).

T cells were collected by plating pooled normal host or TBH-derived whole spleen cells for 2 hr in glass plates and collecting the nonadherent cell fraction. Red blood cells were lysed with 0.83% ammonium chloride (Sigma). To obtain purified $CD4^+$ T cells, the nonadherent cell fraction was treated with anti-CD8 (ATCC clone 3.155), anti-IA^d (ATCC clone MK-D6), and anti-B cell and immature T-cell (ATCC clone J11d) monoclonal antibodies and complement (Accurate). The resulting cell population was >90% $CD4^+$ by flow cytometric analysis using GK1.5 mAb (ATCC, not shown). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Mϕ Nitrite Production. Either 4×10^6 normal host or TBH peritoneal Mϕs were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells) without or with 72-hr Meth-KDE supernatants (1:2 dilution). 50 U/ml IFN-γ, or a combination of 50 U/ml IFN-γ and 1 μg/ml LPS was added at the start of incubation. Supernatants were collected after 48 hr (optimal incubation time) for nitrite assessment following centrifugation (400 × g, 5 min). Mϕ viability remained >95% throughout the culture period as determined by the MTT assay. Nitrite level in culture supernatants was measured using Griess reagent, as described (9).

Mϕ-Mediated Cytotoxicity Assays. To assess direct cytotoxic activity of Mϕs against Meth-KDE tumor cells, Mϕs (2×10^5 cells) were added to 1×10^5 tumor cells. Cytotoxicity was measured using a modification of the Alamar blue™ (Biosource International, Camarillo, CA) colorimetric viability assay, as described (7).

Quantification of Cytokine Production. IL-12, IL-18, and tumor necrosis factor (TNF)-α production was induced by culturing Mϕs as described for nitrite production. IFN-γ production was measured by culturing 4×10^6 purified $CD4^+$ T cells from either normal host or TBH Balb/c mice with 8 μg/ml Con A or a combination of PMA + Cal; after 12 h, cell-free supernatants were collected for analysis. For all cytokines, supernatants were assayed by specific ELISA (R&D Systems) per the manufacturer's recommended protocol.

Western Blot Analysis of IL-18 and Interferon Regulatory Factor-8 (IRF-8). For Western blot analysis of Mϕ IRF-8 and IL-18, normal host and TBH peritoneal Mϕs were cultured as described. Cells were lysed with 50 mM Tris-Cl, pH 7.6, containing 10 μg/ml leupeptin and aprotinin (Sigma) and 300 mM NaCl. Membranes were pelleted by centrifugation (12,000 × g), and protein concentration was determined using the Lowry method. Protein (15 μg) was denatured by boiling in β-mercaptoethanol (2-ME, 5%) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% ProtoGel (National Diagnostics, Atlanta, GA) vertical gel, and transferred to

nitrocellulose. The membrane was blocked using 5% nonfat milk (for IRF-8 visualization) or PBS supplemented with 0.5% Tween-20 (for IL-18 visualization). IRF-8 was specifically detected using polyclonal rabbit anti-mouse IRF-8 Ab (Zymed Laboratories, Inc., South San Francisco, CA) at 2 μg/ml and HRP-conjugated goat-anti-rabbit IgG (Transduction Laboratories) at 1:2,000 dilution. IL-18 was detected using polyclonal goat anti-mouse IL-18 (R&D Systems) at 0.2 mg/ml and horseradish peroxidase-conjugated rabbit-anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used at 1:20,000 dilution. Bound IRF-8 and IL-18 were visualized using Luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and documented on Kodak Biomax film after 30 sec of exposure.

Statistics and Calculations. Cells from four to six normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested in the specific ELISA, Griess reagent test, and cytotoxicity assays. Data are presented as means ± SEM of triplicate independent determinations. All experiments were repeated at least three times; representative experiments are shown. All comparisons were tested for significance by Student's *t* test, and all comparisons are significant at the $P < 0.05$ level, unless otherwise stated.

Results

Tumor Cell-Derived Supernatants Downregulate Mϕ Cytotoxic Effector Functions. IFN-γ priming of normal host Mϕs leads to enhanced cytotoxic and effector function following LPS activation; however, tumors induce functional and phenotypic changes that alter Mϕ responsiveness to activating agents in tumor-associated (2, 11) or tumor-distal (12) compartments. Furthermore, tumor-derived supernatants inhibit *in vitro* activation of RAW 264.7 Mϕs (10) and primary ex vivo Mϕs (13). To further define the negative impact of tumor-derived cytokines on Mϕ activation and effector function, normal host-derived peritoneal Mϕs were cultured either without or with 72-hr Meth-KDE-derived cell-free supernatants (normal host Mϕs were used to avoid the interference of *in vivo* activation from systemically-acting tumor-derived cytokines; Ref. 5). Although tumor supernatant did not decrease nitric oxide (NO) production by naïve Mϕs, tumor factors suppressed ($P < 0.05$) LPS-triggered NO production by IFN-γ-primed Mϕs (Fig. 1A), almost to basal levels. Regulation of TNF-α is fundamentally coupled to NO production (13), and tumor supernatant also suppressed ($P < 0.05$) LPS-triggered TNF-α production by IFN-γ-primed Mϕs (Fig. 1B), although the level did remain greater than basal. Priming and activation with higher doses of IFN-γ (up to 100 U/ml) and LPS (up to 10 μg/ml) further enhanced normal host Mϕ NO and TNF-α production, but tumor supernatant-induced suppression of NO or TNF-α production could not be reversed, even with optimal priming and activation (not shown).

To evaluate whether tumor-derived factors dysregulate direct Mϕ-mediated antitumor cytotoxicity, primed and activated primary Mϕs were added to growing Meth-KDE tumor cells and cytotoxicity measured (14). Mϕs cultured with 72-hr Meth-KDE-derived cell-free supernatants killed significantly ($P < 0.05$) fewer tumor cells than untreated Mϕs (51.4% and 47.1% decrease in cytotoxic activity of naive and activated Mϕs, respectively, Fig. 1C). Meth-KDE cells were sensitive to exogenous TNF-α and an NO source (sodium nitroprusside) added to cultures (not shown), suggesting that Mϕ production of these factors could account for cytotoxic effector activity. Interestingly, in this system, unactivated Mϕs demonstrate cytotoxic activity, which is diminished by tumor supernatant. Likely, tumor cell coculture with Mϕs leads to Mϕ activation and effector func-

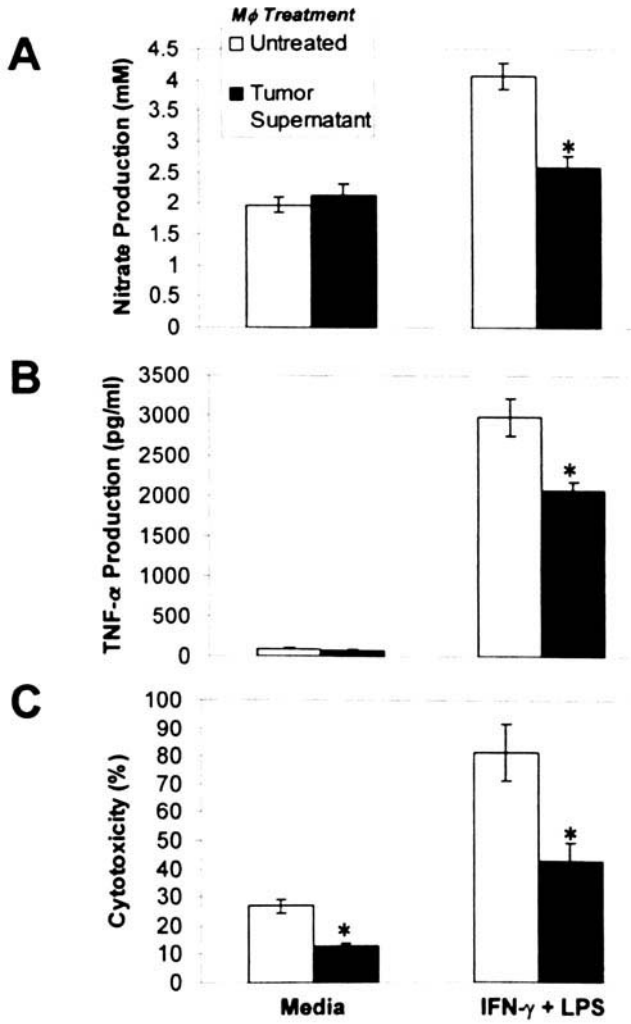

Figure 1. Tumor cell-derived supernatants inhibit Mϕ activation and effector function. (A) Four × 10^6 thioglycollate-elicited Balb/c normal host peritoneal Mϕs were cultured in 200 μl of serum-free medium without (□) or with (■) 72-hr Meth-KDE supernatants (1:2 dilution). IFN-γ (50 U/ml) and 1 μg/ml LPS were added to some cultures at the start of incubation, and supernatants were collected after 48 hr (optimal incubation time) for nitrite assessment using the Griess reagent test. (B) Four × 10^6 peritoneal Mϕs were cultured and activated, as described, and TNF-α levels assessed after 12 hr of incubation. (C) Mϕs (2 × 10^5 cells) were cultured and activated, as described, then added to 1 × 10^5 Meth-KDE tumor cells and cultured for 24 hr in the presence of 10 nM actinomycin-D. Cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay. Percent cytotoxicity was calculated using the formula [1 – [(control – test) / test]] × 100. *$P < 0.05$ compared with untreated.

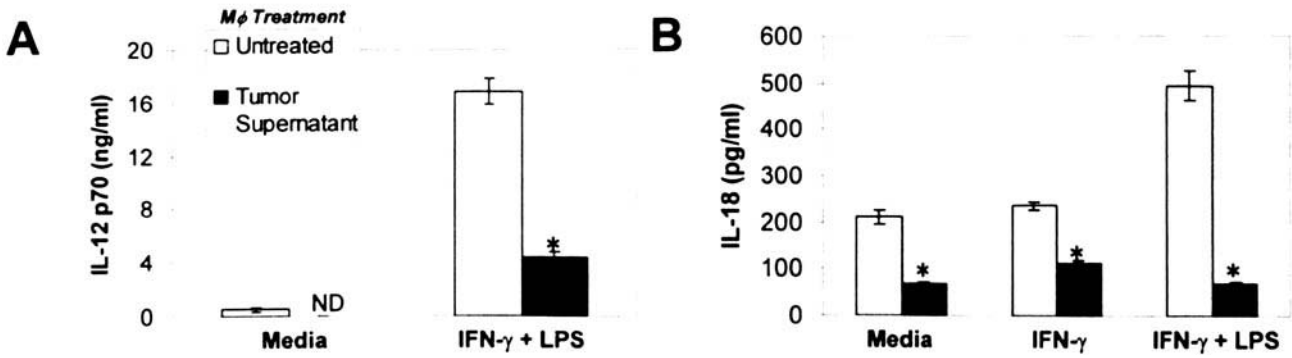

Figure 2. Mϕ IL-12p70 and IL-18 production are impaired by tumor cell-derived factors. (A) Four × 10^6 normal host peritoneal Mϕs were cultured in 200 μl of serum-free medium without (□) or with (■) 72-hr Meth-KDE supernatants (1:2 dilution). IFN-γ (50 U/ml) and 1 μg/ml LPS were added to some cultures at the start of incubation; supernatants were collected after 12 hr and assayed for IL-12 heterodimer by p70-specific ELISA. ND, not detected. (B) 4 × 10^6 normal host peritoneal Mϕs were cultured, as described for IL-12. Supernatants were collected at 12 hr, and IL-18 protein levels were determined by specific ELISA. In both panels, data are averages ± SEM of triplicate independent determinations from one of three similar experiments. *$P < 0.05$ compared with untreated.

tion, and tumor-derived suppressor factors are not sufficiently concentrated in the culture to mediate suppression; however, addition of tumor supernatant prevents Mϕ activation and cytolytic function. Collectively, these data suggest that Mϕs possess the potential to mediate anti-tumor cytotoxic function, but tumor-derived factors substantially reduce Mϕ cytotoxic activity regardless of priming and activation.

Tumor-Derived Factors Downregulate Mϕ Production Of Proinflammatory Effector Cytokines. In addition to direct cytotoxic activity, Mϕs perform a critical support role for induction of active T cell-mediated helper and anti-tumor functions. To determine whether tumor-derived factors also compromise primary Mϕ production of proinflammatory effector cytokines capable of promoting T cell function, normal host Mϕs were cultured, as described, in the absence or presence of 72-hr Meth-KDE supernatants, and Mϕ IL-12 (p70) and IL-18 production was assessed. Tumor-derived factors strongly inhibited IL-12 production ($P < 0.05$) by primary normal host Mϕ, regardless of activation (Fig. 2A), in agreement with observations using the RAW264.7 Mϕ cell line (15). Tumor-derived factors also strongly inhibited normal host Mϕ IL-18 production (Fig. 2B), and activation in the presence of tumor supernatant failed to increase IL-18 levels. Thus, IL-12 and IL-18 are suppressed by tumor-derived factors in a manner that cannot be reversed or compensated by activation. Similar inhibition of IL-12 and IL-18 were observed in tumor supernatant-treated splenic Mϕ populations (not shown).

To determine whether tumor growth impairs the capacity of primary Mϕ to generate IL-12 and IL-18 *in situ*, peritoneal Mϕs from normal host and TBH were treated as described, and evaluated for cytokine production. The presence of tumor significantly inhibited Mϕ IL-12 production capacity *ex vivo*, regardless of *in vitro* activation. The level of IL-12 production was significantly ($P < 0.05$) less than similarly treated normal host Mϕs (Fig. 3A). Both priming with IFN-γ alone or priming and activation with LPS enhanced Mϕ IL-18 protein production (Fig. 3B), but tumor

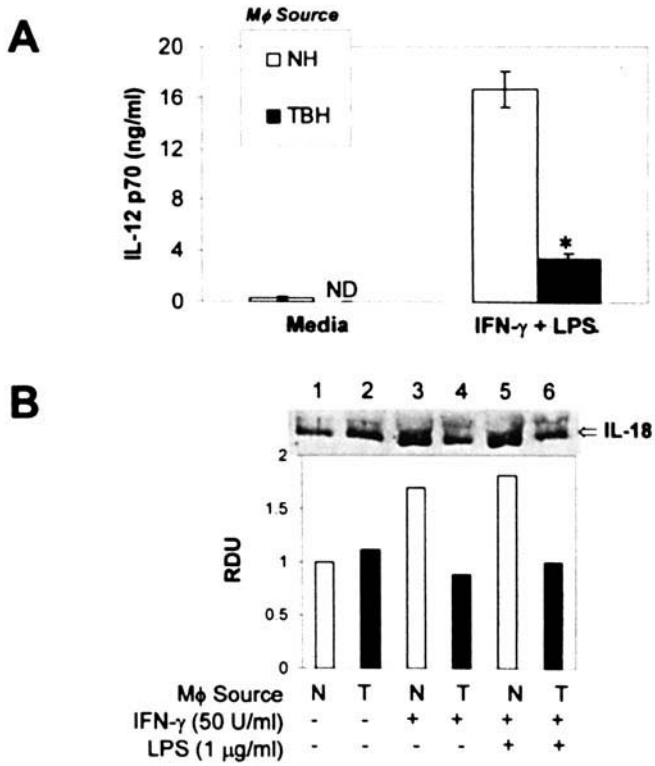

Figure 3. Mϕ IL-12p70 and IL-18 production are impaired by tumor growth. (A) Four × 10^6 normal host (□) or TBH (■) peritoneal Mϕs were harvested, then cultured and activated as described. After 12 hr, supernatants were collected and assayed for IL-12 heterodimer by p70-specific ELISA. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments. *$P <$ 0.05 compared with normal host (NH). (B). Four × 10^6 normal host (N, □, lanes 1, 3, 5) or TBH (T, ■, lanes 2, 4, 6) peritoneal Mϕs were cultured in fresh medium (lanes 1 and 2), IFN-γ (50 U/ml, lanes 3 and 4), or LPS (1 μg/ml) + IFN-γ (50 U/ml, lanes 5 and 6). Cell lysates were obtained after 12 hr, and IL-18 was visualized by Western blotting with specific antibody. Each lane was loaded with 15 μg of total protein, as determined by Lowry microassay. Densitometry, expressed as relative densitometic units, was calculated using NIH Image software and normalized to untreated normal host responses (relative densitometic unit value of 1). Data are from one of three experiments with similar results.

growth inhibited activation-induced increases in IL-18 levels as compared to similarly treated normal host Mϕs. IL-18-specific ELISA confirmed tumor-induced inhibition of activation-induced IL-18 production from TBH Mϕs (not shown). These data demonstrate a lesion in both IL-12 and IL-18 production during tumor outgrowth, even by Mϕs derived from distinct immunologic compartments distal from the tumor.

Tumor Growth Limits Activation-Induced Mϕ IRF-8 Expression. Because tumor growth can inhibit Mϕ production of IFN-γ-inducible factors, such as IL-12 and IL-18, we asked whether tumor growth dysregulates the crucial signal transduction molecule IRF-8. Primary normal host or TBH peritoneal Mϕs (4×10^6 cells) were treated with IFN-γ, LPS, or IFN-γ and LPS, and then assayed for IRF-8 (Fig. 4A). Although the presence of tumor did not diminish the basal level of IRF-8 in unactivated cells, IFN-γ-induced IRF-8 expression was impaired by tumor growth, in a manner consistent with tumor-induced modulation of NO production. Further, the IRF-8 dampening effect of tumor was not overcome by activation of IFN-γ-primed Mϕs with LPS. These data demonstrate a lesion in Mϕ IRF-8 production in response to immuno-stimulatory signals, and soluble effectors mediate this phenomenon, as IRF-8 dysfunction is observed in Mϕs from tumor-distal immunologic compartments. The Meth-KDE fibrosarcoma produces several immunomodulatory factors, including TGF-β_1 and IL-10 (5, 16), and these factors could potentially modulate Mϕ production of IRF-8.

To determine the role of TGF-β_1 and IL-10 in the tumor-induced modulation of activation-induced IRF-8 production, Meth-KDE supernatants were depleted of these cytokines (as described in Ref. 10) and used to treat normal host-derived Mϕs. Tumor cell-derived supernatant downregulated Mϕ IRF-8 protein expression, even after priming and activation with IFN-γ and LPS (Fig. 4B). Recombinant TGF-β_1 (10.0 ng/ml; Ref. 13) or recombinant IL-10 (3.0 U/ml; Ref. 13) suppressed activated Mϕ IRF-8 expression. Unlike TGF-β_1 and IL-10, exogenous PGE_2 (also produced by Meth-KDE fibrosarcoma cells) failed to suppress IRF-8. We have observed, in separate experiments, that a combination of TGF-β_1 and IL-10 yields IRF-8 levels that are approximately equivalent to the level achieved in the presence of unabsorbed tumor supernatant (not shown). To our surprise, depletion of either TGF-β_1 or IL-10 from tumor supernatant reversed suppression and almost fully restored IRF-8 levels to full expression; these data suggest that the doses of recombinant cytokines used in these experiments can mask a synergistic suppressive activity of TGF-β_1 and IL-10 in tumor supernatant on IRF-8 expression. Collectively, these data demonstrate that TGF-β_1 and IL-10—but not PGE_2—are significant mediators of tumor-induced suppression of primary Mϕ responsiveness to IFN-γ priming and subsequent activation.

Tumor Growth Downregulates T Cell IFN-γ Production and Costimulatory Molecule Expression. *In situ*, Mϕ antitumor activity and cytokine expression are reliant upon priming by IFN-γ. Mϕ-derived IL-12 and IL-18 both promote cell-mediated immune responses and synergize to induce or enhance T cell IFN-γ production (17, 18), which in turn is crucial for antitumor function. Because IFN-γ is a pivotal mediator of immune responses to tumor, we evaluated the impact of tumor growth on T cell function in our model. Normal host or TBH $CD4^+$ T cells were

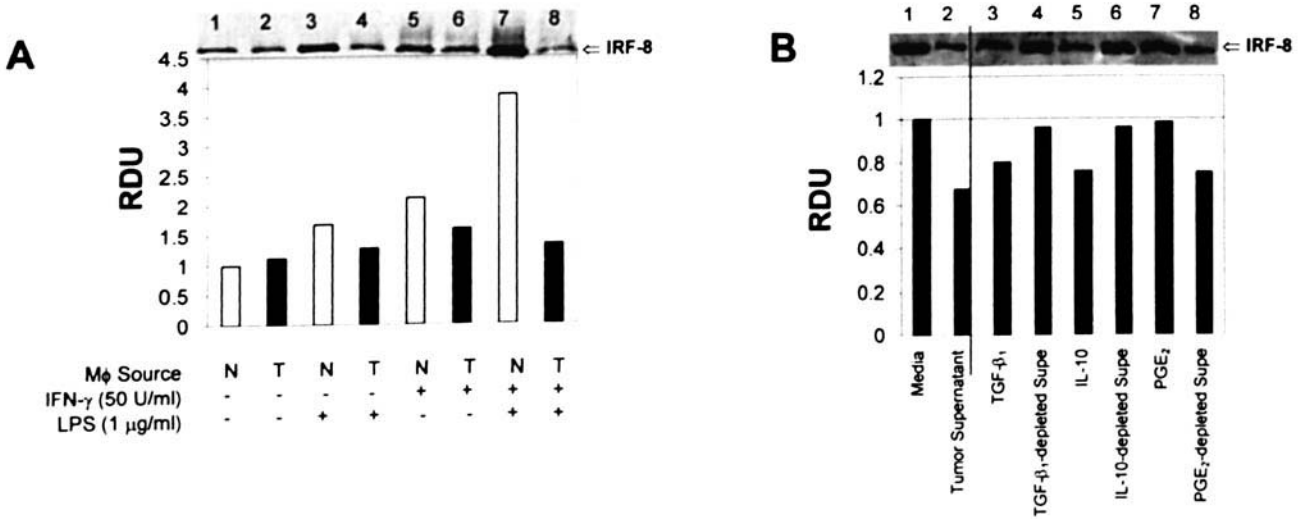

Figure 4. Tumor-derived TGF-β_1 and IL-10 impair Mϕ IRF-8 production. (A) Either 4×10^6 normal host (□) or TBH (■) peritoneal Mϕs were cultured in fresh medium (lanes 1 and 2), LPS (10 μg/ml, lanes 3 and 4), IFN-γ (50 U/ml, lanes 5 and 6), or LPS (10 μg/ml) + IFN-γ (50 U/ml, lanes 7 and 8). After 12 hr of incubation, lysates were obtained and IRF-8 was detected by Western analysis. Each lane was loaded with 15 μg of total protein, as determined by Lowry microassay. Representative data from one of three similar experiments are shown. (B) 4×10^6 primary normal host peritoneal Mϕs were primed and activated with 10 μg/ml LPS + 50 U/ml IFN γ, either without (lane 1) or with (lane 2) 72-hr Meth-KDE supernatants (1:2 dilution). Additional cultures were supplemented with TGF-β_1 (10.0 ng/ml, lane 3), IL-10 (3.0 U/ml, lane 5), and PGE_2 (25.0 ng/ml, lane 7), and parallel cultures were treated with Meth-KDE supernatants depleted of TGF-β_1 (lane 4), IL-10 (lane 6), or PGE_2 (lane 8). After 12-hr lysates were prepared and assayed for IRF-8 production. Each lane was loaded with 15 μg of total protein, as determined by Lowry microassay. Representative data from one of three similar experiments are shown.

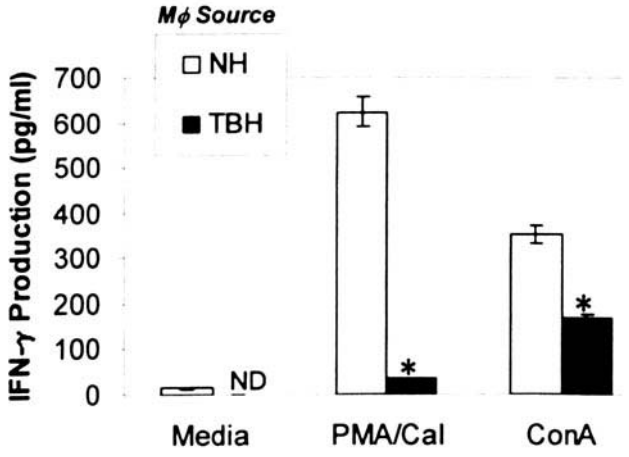

Figure 5. Tumor growth compromises T cell IFN-γ production. Four × 10^6 purified $CD4^+$ T cells from normal host (□) or TBH (■) Balb/c mice were cultured with 8 μg/ml Con A or a combination of PMA + Cal. After 12-hr supernatants were collected and assayed for IFN-γ production by specific ELISA. ND, not detected. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments. *$P < 0.05$ compared with normal host (NH).

stimulated with Con A or a combination of PMA + CaI, and IFN-γ production was measured using an IFN-γ-specific ELISA. Tumor growth significantly impaired T cell IFN-γ production, regardless of activation (Fig. 5), suggesting that $CD4^+$ T cell-mediated helper activities may be significantly compromised during tumor outgrowth. This paucity of T cell-derived IFN-γ may contribute to the apparent lack of activation and effector function by TBH-derived Mϕs.

Discussion

The cytokine IFN-γ is a central and vital mediator of anti-tumor immune responses and tumor surveillance (Fig. 6A). Here, we demonstrate that a nonmetastatic fibrosarcoma, through the production and release of immunomodulatory cytokines, adversely alters IFN-γ-associated functions of Mϕs and T cells (Fig. 6B). The resultant failure of the anti-tumor response may allow for unchecked growth of cancerous lesions.

In this study, we have investigated several mechanisms of immune dysfunction related to the response to, or production of, IFN-γ by immune cells functioning in the context of a tumor-burdened immune system. First, tumor-derived cytokines suppress priming- and activation-induced TNF-α and NO production from Mϕs, with a corresponding decrease in direct Mϕ-mediated cytotoxicity. TNF-α promotes the generation of $CD8^+$ T cell-mediated antitumor cytotoxicity through reversal of TGF-β_1-induced inhibition (19), and TNF-α-dependent production of reactive nitrogen intermediates can mediate Mϕ tumoricidal activity through IFN-γ and IL-2 (20). Additionally, TNF-α may enhance tumor cell sensitivity to NO, rendering tumors susceptible to even moderate levels of NO. Thus, abrogation of these factors significantly impedes anti-tumor responses, as tumors likely escape Mϕ-mediated cytotoxicity by overproducing Mϕ-deactivating cytokines that inhibit local NO and TNF-α production.

Another mechanism by which tumors evade immune response involves limiting or abrogating the production of T cell-stimulating agents by Mϕs. Mϕs may well provide both chemotactic signals to guide T cell migration into tumors, as well as immunostimulatory factors that promote T cell IFN-γ production, activation, and effector function. Tumor cell-derived factors downregulate IL-12 and IL-18 production by both normal Mϕs exposed to supernatant *in vitro*, as well as TBH Mϕs examined *ex vivo*. Downregulation of IL-12 production may contribute to lowered T cell IFN-γ production and subsequent loss of a variety of antitumor effects. Likewise, IL-18 induces IFN-γ production by activated murine and human T cells, in synergy with IL-12 (6, 21); upregulation of IL-18Rα gene expression by IL-12 may explain its capacity to increase IL-18-induced IFN-γ production by T cells (22). When administered to mice as an adjuvant for increased tumor immunogenicity, IL-18 enhanced natural killer (NK) cell activation, induced IL-2 release, and decreased IL-10 levels. Against Meth-A sarcoma, IL-18 stimulated NK-mediated antitumor effects, induced cytotoxic $CD4^+$ T cells, and evoked immunologic memory (1). Abrogation of IL-18 production will likely have profound detrimental effects on tumor control. Furthermore, NO and TNF-α induce or enhance the production of other cytokines that mediate antitumor immune responses (including IL-12), both directly (10) and indirectly through

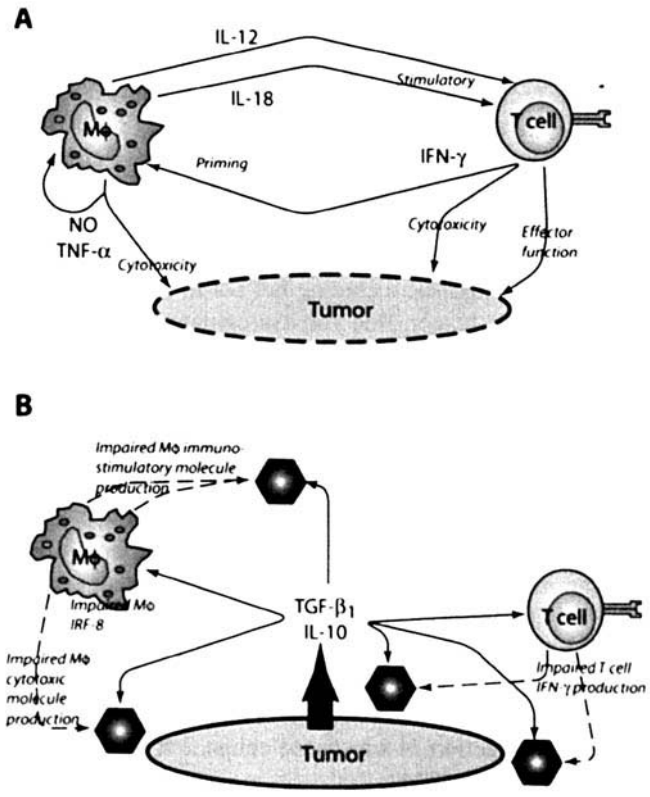

Figure 6. Model of tumor-induced immune suppression. (A) Immune responses to tumors. (B) Tumor-induced dysfunction (dysregulated functions indicated by dashed lines). Through TGF-β_1 and IL10, tumors inhibit Mϕ responsiveness through downregulation of IRF-8, leading to a loss of effector function and diminished support of T cell activity.

increased T cell production of IFN-γ (23). Thus, loss of Mϕ-derived cytotoxic effector molecules may also contribute to decreased immunostimulatory function.

Compounding the tumor-induced defects in Mϕ function, the suppressive effect of the tumor cell-derived factors on T cells provides yet another mechanism of immune evasion. T cell-derived IFN-γ promotes immune recognition of tumor cells by enhancing MHC expression, and IFN-γ stimulates cytotoxicity mediated by NK cells, T lymphocytes, and Mϕs (24). IFN-γ promotes tumor regression through the inhibition of cell proliferation, direct toxic effect on cells in combination with TNF-α, and induction of Mϕ inducible NO synthase expression (24) and NO production. We showed a deficit in IFN-γ production in TBH T cells, potentially compromising Mϕ activation *in situ*.

To further understand the mechanisms of tumor-induced dysregulation of immune responses, we evaluated Mϕ expression of IRF-8, a molecule in the IFN-γ-induced signal cascade. The transcription factors that control IFN-γ activity form an integral part of a host's immune response. IRF-8 is unique among these factors in that it is expressed exclusively in cells of the immune system (25). IFN-γ induces IRF-8 expression in cells of lymphocyte or macrophage lineage through the GAS sequence in its promoter (26). In turn, IRF-8 regulates IL-12 (25, 27) and IL-18 (28) gene expression through the interferon-stimulated response element in the promoter of these genes. IRF-8 regulates IL-12p40 gene activation in Mϕs and, consequently, IFN-γ-dependent host resistance (25). IRF-8 is a critical factor for IL-18 promoter activity, performing a dominant regulatory role in the inducible expression of IL-18 (28). We observed that tumor growth disrupted the IFN-γ signaling pathway through downregulation of IRF-8. Activation-induced expression of IRF-8 was diminished in TBH Mϕs, and tumor supernatants inhibited IRF-8 expression in primary Mϕs. Cytokines, including but not limited to TGF-β_1 and IL-10, are responsible for dysregulated activation of IRF-8 expression. Thus, tumors disrupt immune responses by altering Mϕ responsiveness to IFN-γ through downregulation of an integral signaling molecule.

Collectively, tumor-induced downregulation of IFN-γ production by T cells, and disruption of Mϕ cytotoxic, stimulatory, and signaling pathways may effectively break the Mϕ-T cell interactive cycle, leading to profound loss of immune-mediated tumor control. These data further emphasize the importance of IFN-γ in antitumor activity, suggesting that abrogation of tumor-derived TGF-β_1 and IL-10 may provide a therapeutic approach to restoration of effective anti-tumor responses. Likewise, therapeutic restoration of IRF-8 production *in situ* could enhance the tumoricidal activity of primary Mϕs.

1. Micallef MJ, Yoshida K, Kawai S, Hanaya T, Kohno K, Arai S, Tanimoto T, Torigoe K, Fujii M, Ikeda M, Kurimoto M. In vivo antitumor effects of murine interferon-gamma-inducing factor/interleukin-18 in mice bearing syngeneic Meth A sarcoma malignant ascites. Cancer Immunol Immunother **43**:361–367, 1997.
2. Lopez DM, Handel-Fernandez ME, Cheng X, Charyulu V, Herbert LM, Dinapoli MR, Calderon CL. Cytokine production by lymphoreticular cells from mammary tumor bearing mice: The role of tumor-derived factors. Anticancer Res **16**:3923–3929, 1996.
3. Alleva DG, Burger CJ, Elgert KD. Tumor-induced regulation of suppressor macrophage nitric oxide and TNF-α production: Role of tumor-derived IL-10, TGF-β, and prostaglandin E_2. J Immunol **153**:1674–1686, 1994.
4. Eisenstein TK. Suppressor macrophages. Immunol Ser **60**:203–224, 1994.
5. Elgert KD, Alleva DG, Mullins DW. Tumor-induced immune dysfunction: The macrophage connection. J Leukoc Biol **64**:275–290, 1998.
6. Ahn H-J, Maruo S, Tomura M, Mu J, Hamaoka T, Nakanishi K, Clark S, Kurimoto M, Okamura H, Fujiwara H. A mechanism underlying synergy between IL-12 and IFN-γ-inducing factor in enhanced production of IFN-γ. J Immunol **159**:2125–2131, 1997.
7. Mullins DW, Walker TM, Burger CJ, Elgert KD. Taxol-mediated changes in fibrosarcoma-induced immune cell function: Modulation of antitumor activities. Cancer Immunol Immunother **45**:20–28, 1997.
8. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci USA **95**:7556–7561, 1998.
9. Mullins DW, Burger CJ, Elgert KD. Paclitaxel enhances macrophage IL-12 production in tumor-bearing hosts through nitric oxide. J Immunol **162**:6811–6818, 1999.
10. Mullins DW, Martins RS, Burger CJ, Elgert KD. Tumor cell-derived TGF-β and IL-10 dysregulate paclitaxel-induced macrophage activation. J Leukoc Biol **69**:129–137, 2001.
11. Dinapoli MR, Calderon CL, Lopez DM. The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduced expression of the inducible nitric oxide synthase gene. J Exp Med **183**:1323–1329, 1996.
12. Alleva DG, Burger CJ, Elgert KD. Tumor growth causes suppression of autoreactive T-cell proliferation by disrupting macrophage responsiveness to interferon-γ. Scand J Immunol **39**:31–38, 1994.
13. Mullins DW, Alleva DG, Burger CJ, Elgert KD. Taxol, a microtubule-stabilizing antineoplastic agent, differentially regulates normal and tumor-bearing host macrophage nitric oxide production. Immunopharmacology **37**:63–73, 1997.
14. Ahmed SA, Gogal RM Jr., Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assays. J Immunol Meth **171**:211–224, 1994.
15. Mullins DW, Askew D, Elgert KD. Tumor growth dysregulates macrophage production of bioactive interleukin-12. J Allergy Clin Immunol. **99**:449, 1997.
16. Walker TM, Yurochko AD, Burger CJ, Elgert KD. Ia$^-$ macrophages and cytokine networks contribute to tumor-induced suppression of CD4$^+$ autoreactive T cells. Immunol Invest **22**:169–187, 1993.
17. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol **13**:251–276, 1995.
18. Dinarello CA. Interleukin-18. Methods **19**:121–132, 1999.
19. Gorelik L, Bar-Dagan Y, Mokyr MB. Insight into the mechanism(s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearer splenic cells. J Immunol **156**:4298–4308, 1996.
20. Cox GW, Melillo G, Chattopadhyay U, Mullet D, Fertel RH, Varesio L. Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-γ plus IL-2-induced murine macrophage tumorcidal activity. J Immunol **149**:3290–3296, 1992.
21. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin

(IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. J Exp Med **187:**2103–2108, 1998.
22. Shibata Y, Foster LA, Kurimoto M, Okamura H, Nakamura RM, Kawajiri K, Justice JP, Van Scott MR, Myrvik QN, Metzger WJ. Immunoregulatory roles of IL-10 in innate immunity: IL-10 inhibits macrophage production of IFN-γ-inducing factors but enhances NK cell production of IFN-γ. J Immunol **161:**4289–4300, 1998.
23. Liu W, Kurlander RJ. Analysis of the interrelationship between IL-12, TNF-alpha, and IFN-gamma production during murine listeriosis. Cell Immunol. **163:**260–267, 1995.
24. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol **15:**749–795, 1997.
25. Scharton-Kersten T, Contursi C, Masumi A, Sher A, Ozato K. Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. J Exp Med **186:**1523–1534, 1997.
26. Contursi C, Wang IM, Gabriele L, Gadina M, O'Shea J, Morse HC, III, Ozato K. IFN consensus sequence binding protein potentiates STAT1-dependent activation of IFNgamma-responsive promoters in macrophages. Proc Natl Acad Sci USA **97:**91–96, 2000.
27. Giese NA, Gabriele L, Doherty TM, Klinman DM, Tadesse-Heath L, Contursi C, Epstein SL, Morse HC. Interferon (IFN) consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses In vivo through control of interleukin 12 expression. J Exp Med **186:**1535–1546, 1997.
28. Kim YM, Kang HS, Paik SG, Pyun KH, Anderson KL, Torbett BE, Choi I. Roles of IFN consensus sequence binding protein and PU.1 in regulating IL-18 gene expression. J Immunol **163:**2000–2007, 1999.