Cutaneous Tumors Cease CXCL9/Mig Production as a Result of IFN-γ–Mediated Immunoediting

Marianne Petro,* Danielle Kish,* Olga A. Guryanova,† Galina Ilyinskaya,† Anna Kondratova,† Robert L. Fairchild,*,‡ and Anton V. Gorbachev*

During growth in the host, tumor cells are subjected to the stresses of innate and adaptive immunity (immunoediting), which provoke epigenetic changes in the tumor and increase tumor resistance to these immune responses. Our recent studies in methylcholanthrene-induced fibrosarcomas have indicated the appearance and rapid growth of tumor variants deficient in producing the T cell chemoattractant chemokine CXCL9/Mig, an important component of antitumor immunity. In the current report, we demonstrate that highly tumorigenic Mig-deficient tumor variants arise in both cutaneous fibrosarcoma and melanoma as a result of immune stress imposed by IFN-γ and T cells. The consequence of the loss of tumor-derived Mig expression is the increased resistance of Mig-deficient tumors to T cell-mediated immunity, which promotes the accelerated growth of these tumor variants. Remarkably, the ability of Mig-deficient tumor cells to express another CXCR3 ligand, CXCL10/IFN-γ–inducible protein, does not compensate for the absent antitumor functions of Mig, suggesting a nonredundant role for this chemokine in the suppression of tumor growth. To our knowledge, these studies report for the first time that IFN-γ–mediated stress leads to the loss of specific chemokine expression by tumor cells, which in turn promotes tumor growth and evasion of the immune response. ***The Journal of Immunology*****, 2013, 190: 832–841.**

Malignant tumors grow under host immune pressure and constantly evolve to acquire resistance to antitumor immune responses in otherwise immunocompetent tumor hosts. This cancer immunoediting was first demonstrated in methylcholanthrene (MCA)–induced murine fibrosarcomas and has been confirmed in other experimental cancer models, as well as in clinical studies (1–9). IFN-γ, cytolytic T cells, and NK and NKT cells are critical components of antitumor immunity and constitute host components potentially promoting immunoediting of tumors (10, 11). Because immunoediting clearly enhances tumor resistance to host immune responses and to immunotherapeutic intervention, understanding mechanisms underlying the development of this resistance is required to design more effective therapeutic approaches. Recent studies have identified several mechanisms of tumor immune evasion, including decreased expression of tumor Ags and/or MHC class I molecules, production of immunosuppressive cytokines, and the recruitment of immunoregulatory cells into the tumor microenvironment (1, 7–9). However, specific tumor-derived genes and/or molecules that are edited under the pressure of antitumor immunity remain poorly identified.

*Department of Immunology, Cleveland Clinic Foundation, Cleveland, OH 44195; †Department of Molecular Genetics, Cleveland Clinic Foundation, Cleveland, OH 44195; and ‡Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

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Address correspondence and reprint requests to Dr. Anton V. Gorbachev, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195-0001. E-mail address: gorbaca@ccf.org

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Abbreviations used in this article: IP-10, IFN-γ–inducible protein; MCA, methylcholanthrene; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA.

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Our recent studies indicated that the parental murine fibrosarcoma cell line MCA205 produces CXCL9/Mig in response to IFN-γ stimulation in vitro, but the majority of tumor cells lose the ability to produce Mig during growth as cutaneous tumors in syngeneic wild-type mice (12). Mig-deficient tumor variants isolated from these tumors are highly tumorigenic with accelerated growth in recipient mice, when compared with Mig-producing tumor cells. Restoration of Mig expression in a Mig-deficient tumor variant reverses its high tumorigenicity and results in T cell–dependent suppression of tumor growth, indicating a critical role of tumor-derived Mig in the immune-mediated inhibition of tumor growth. In the current report, we addressed the hypothesis that tumor cells cease Mig expression as a result of immunoediting mediated by IFN-γ and/or T cells. We demonstrate that Mig-producing clones of MCA205 fibrosarcoma and B16 melanoma that are stable in Mig expression when growing in vitro convert into Mig-deficient variants when growing in vivo as cutaneous tumors. Mig-deficient tumor variants of MCA205 are generated and expand in immunocompetent mice, but not in hosts lacking critical components of antitumor immunity, including IFN-γ, T cells, and NK cells. Similarly, prolonged exposure of MCA205 and B16 cells to IFN-γ in vitro results in decreased Mig expression by the tumor cells. Overall, the results implicate IFN-γ–mediated downregulation of Mig expression in tumor cells as an important mechanism of tumor evasion triggered by immune stress.

Materials and Methods

Mice and reagents

C57BL/6 mice and RAG1$^{-/-}$ and IFN-γ$^{-/-}$ mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice and RAG2$^{-/-}$ and RAG2/γcR$^{-/-}$ mice on the C57BL/6 background were purchased from Taconic Farms (Hudson, NY). Female mice 8–10 wk old were used throughout these studies. All animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Mab to murine H-2K^{b}, CD11b, NK1.1, CD19, CD45, CD3, CD4, and CD8 molecules were purchased from BD Pharmingen (San Diego, CA). MAb to murine CXCL9/Mig and CXCL10/IFN-γ–inducible protein (IP-10)

were purchased from R&D Systems (Minneapolis, MN). Recombinant murine IFN-γ was purchased from PeproTech (Rocky Hill, NJ).

Induction of cutaneous tumors

MCA205-10 and B16-F10 cells were grown in complete RPMI 1640 culture medium and were injected intradermally into syngeneic C57BL6 mice (3×10^5 cells per mouse). Tumor sizes were measured in two dimensions, beginning on day +7 post implant. Tumor sizes were calculated as previously described: $(\text{tumor width})^2 \times (\text{tumor length})$ (12, 13).

Generation of Mig-deficient tumor variants

MCA205-10 tumors were retrieved on day 20 post implant. Cell suspensions were prepared by tumor digestion with 0.1% collagenase, 0.01% DNase and 2.5 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO). The tumor cells were seeded in 96-well culture plates at 0.2 cells per well in complete RPMI 1640 culture medium. Growing single colonies of tumor cells were stimulated with 10 ng/ml IFN-γ, and supernatants were collected 72 h later and tested in Mig-specific ELISA. Selected tumor clones that were low or negative in Mig production were harvested and expanded for secondary screening for IFN-γ–induced Mig production.

Analysis of Mig and IP-10 protein production

Tumor homogenates were prepared in the presence of protease inhibitors: 10 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml chymostatin (Sigma-Aldrich). After centrifugation at $12{,}000 \times g$ for 10 min, the supernatants were collected and the total protein concentration was quantified using a Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL). All samples were diluted to an equivalent total protein concentration and tested for concentrations of CXCL9 or CXCL10 by ELISA. Levels of chemokines were expressed as a ratio of protein concentration to the weight of the tumor sample.

Cytokine-specific ELISPOT assays

ELISPOT assays to enumerate IFN-γ–producing T cells were performed as previously described (12). Briefly, ELISPOT plates (Unifilter 350; Polifiltronics, Rockland, MA) were coated with anti–IFN-γ mAb R26A2 overnight at 4°C. Then the plates were blocked with 1% BSA in PBS for 90 min at 37°C. Lymph node cells were prepared from naive mice or from tumor-challenged mice on day 14 after tumor implant and used as responder cells. MCA205 tumor cells were treated with 50 μg/ml mitomycin C and used as stimulator cells. Responder lymph node cells were resuspended in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) and cultured at 5×10^5 cells per well with 5×10^5 stimulator cells per well for 24 h at 37°C in 5% CO_2. Responder cells from tumor recipients cultured with syngeneic splenocytes and responder cells from naive mice cultured with MCA205 tumor cells were used as negative controls. After 24 h, cells were removed from the culture wells by extensive washing with PBS and then PBS–0.2% Tween 20. Biotinylated anti–IFN-γ mAb XMG1.2 was added, and the plate was incubated overnight at 4°C. The wells were washed three times with PBS–0.2% Tween 20 and incubated with anti-biotin alkaline phosphatase conjugate for 2 h at room temperature. The wells were washed with PBS, and nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. The resulting spots were counted on an Immuno-Spot Series 1 Analyzer (Cellular Technology, Cleveland, OH) that was designed to detect spots with predetermined criteria for size, shape, and colorimetric density. The results are expressed as the mean number of spots detected in triplicate wells after subtraction of spots from negative control wells containing T cells from the same experimental group cultured with syngeneic spleen cells (typically fewer than five spots per well).

Flow cytometry

To test IFN-γ–induced upregulation of MHC class I expression by tumor cells, cells were cultured for 48 h with or without 10 ng/ml IFN-γ and then were stained with FITC-labeled anti-mouse $H\text{-}2K^b$ mAb.

To assess the recruitment of T and NK cells into tumors, tumors were excised and cell suspensions prepared. The suspensions were filtered through a 40-μm nylon cell strainer (BD Falcon), incubated with Mouse F_c Block, and then stained with FITC-labeled anti-CD45 mAb and PE-labeled anti-CD4 mAb, anti-CD8 mAb, or anti-NK1.1 mAb.

Analysis of gene expression by quantitative RT-PCR

Total cell RNA was extracted from tumor cells 18 h after culture with or without IFN-γ by dissolving the cells in TRIzol Reagent (Life Technologies BRL, Gaithersburg, MD) and subsequent chloroform extraction. The expression of Mig or IP-10 mRNA was tested by quantitative RT-PCR (qRT-PCR). Briefly, cDNA was synthesized from 2 μg total mRNA using the TaqMan Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using custom primers and FAM dye–labeled probes (Applied Biosystems, Foster City, CA) for mouse CXCL9/Mig, CXCL10/IP-10, and Mrpl 32 as a housekeeping gene (assay no. Mm00434946_m1, Mm00445235_m1, and Mm00777741_sH, respectively). The expression level of RNA isolated from Mig-deficient tumor clone 1B-10 not stimulated with IFN-γ was arbitrarily set at 1.0 and used to determine the relative expression levels of the remaining samples.

Knocking down Mig gene expression in tumor cells

Lentiviral constructs expressing short hairpin RNA (shRNA) specific to CXCL9/Mig (no. TRCN0000067295) and control nontargeting shRNA (no. SHC002) were purchased from Sigma-Aldrich. Lentiviral particles were packaged in 293T cells using a pGag1 and pRev2 set of helper plasmids as described (14). Virus-containing supernatants were collected and incubated with target cells in growth media supplemented with 4 μg/ml polybrene for 8 h at 37°C, 5% CO_2. Then the cells were washed and cultured in RPMI 1640 containing 5 μg/ml puromycin to select transduced puromycin-resistant cells expressing shRNA constructs. To test inhibition of Mig expression, cells were stimulated with IFN-γ and tested for Mig mRNA expression by qRT-PCR and for Mig protein production by ELISA.

T cell transfer

Lymph node cells from 10–12 mice were pooled and incubated with PE-labeled anti-CD11b, anti-CD19, and anti-NK1.1 mAb. Then cells were incubated with magnetic beads coated with anti-PE mAb and loaded onto a MACS separation column (Miltenyi Biotec, Auburn, CA) in a magnetic field to remove PE-labeled monocytes, B cells, and NK cells. The resulting cell population contained >95% $CD3^+$ cells. Aliquots of 2×10^7 cells were transferred by i.v. injection into tumor-bearing mice.

In vitro proliferation assay

Tumor cells were seeded in 96-well plates (2×10^4 cells per well) in complete RPMI 1640 culture medium. After 48 h of culture, plates were washed and adherent cells were fixed with 70% methanol and stained with 1% methylene blue for 10 min at room temperature. After extensive washing, 1% SDS was added to lyse cells and methylene blue uptake was measured at 595 nm.

Statistical analysis

Student *t* test was used to assess differences between experimental groups. At least three tumors from three individual mice were used from each experimental group to assess chemokine production, T cell recruitment, and generation of Mig-deficient tumor variants. At least five mice per group were used to evaluate tumor growth.

Results

Mig-expressing tumor cells convert into highly tumorigenic Mig-deficient variants during growth in immunocompetent mice

Our previous studies indicated that cutaneous tumors induced by intradermal implants of MCA205 fibrosarcoma cells contain highly tumorigenic variants deficient in Mig production (12). It was unclear if these Mig-deficient tumor variants preexisted in the parental MCA205 cells and then expanded during growth in vivo or if parental Mig-expressing cells converted into Mig-deficient variants during their growth in vivo. To directly address these possibilities, we used tumor cell clone MCA205-10, generated in previous studies by subcloning tumor cells retrieved from a growing MCA205 tumor (12). This tumor clone is stable in its ability to produce Mig after multiple passages and rounds of stimulation with IFN-γ in vitro. To test the potential conversion of Mig-expressing tumor cells into Mig-deficient variants during in vitro versus in vivo growth, clone MCA205-10 was grown either as a cell culture or as a cutaneous tumor in wild-type mice. Production of Mig was readily detectable in tumors at early stages of growth but decreased >2-fold at later times (Fig. 1A, day 10 versus 20 postimplant). To test whether loss of Mig expression by tumor cells contributed to this decrease in intratumor Mig pro-

duction, growing tumors were retrieved on day 20 post implant and were digested to prepare single-cell suspensions, and the tumor cells were subcloned by limiting dilution. Panels of individual clones generated were stimulated with IFN-γ, and culture supernatants were tested for Mig by ELISA. A considerable proportion of tumor clones generated from Mig-expressing MCA205-10 tumors excised from wild-type mice produced little or no Mig in response to IFN-γ stimulation (Fig. 1B). In contrast, tumor cell suspensions retrieved from cultures did not contain Mig-negative clones. These results indicated that Mig-expressing tumor cells lose their ability to express Mig when growing in vivo as cutaneous tumors.

It was important to test whether the Mig-deficient tumor variants do not produce Mig because they become unable to respond to IFN-γ signaling. We tested mRNA expression and protein production of Mig and another IFN-inducible chemokine, CXCL10/IP-10, after IFN-γ stimulation of the parental Mig-expressing clone MCA205-10 and seven Mig-deficient tumor variants derived from five individual tumors generated by implantation of MCA205-10 cells into wild-type mice. Although expression of Mig mRNA was low to undetectable in all Mig-deficient clones tested, most of these tumor clones expressed IP-10 mRNA when stimulated with IFN-γ. These results were confirmed by testing culture supernatants for Mig and IP-10 production by ELISA (Fig. 1C, 1D). Furthermore, Mig-deficient tumor cells upregulated class I MHC expression when stimulated with IFN-γ, further indicating that these variants did not lose responsiveness to IFN-γ stimulation (Fig, 2A).

Next, we tested whether the transition to Mig deficiency had an impact on tumor cell growth in vitro and/or in vivo. We cultured cell aliquots of Mig-expressing and Mig-deficient clones for 48 h and then assessed their proliferation. In vitro growth of Mig-deficient clones derived from MCA205-10 tumors was similar to that of the parental MCA205-10 cells (Fig. 2B). In vivo growth of Mig-deficient and Mig-expressing tumor cells as intradermal implants was similar for the first 2 wk post implant; however, growth of Mig-deficient tumor variants accelerated >2-fold beginning at days 14–16 post implant, whereas the growth of the parental Mig-producing clone MCA205-10 remained relatively slow (Fig. 2C). It was noteworthy that the growth of tumor variants deficient in Mig expression only versus tumor variants deficient in both Mig and IP-10 expression was similar (Fig. 2C, clones 1B-10 and G-9 versus clones 8G-6 and 9F-6, respectively), indicating that a deficiency in IP-10 expression did not further increase growth of Mig-deficient tumor cells. Furthermore, growth of Mig-deficient tumor variants was significantly accelerated in immunocompetent (wild-type) mice when compared with a Mig-expressing clone derived from the same tumor, indicating the increased tumorigenicity of Mig-deficient tumor variants when compared with the tumor-derived Mig-expressing variants (Fig. 2D). In striking contrast,

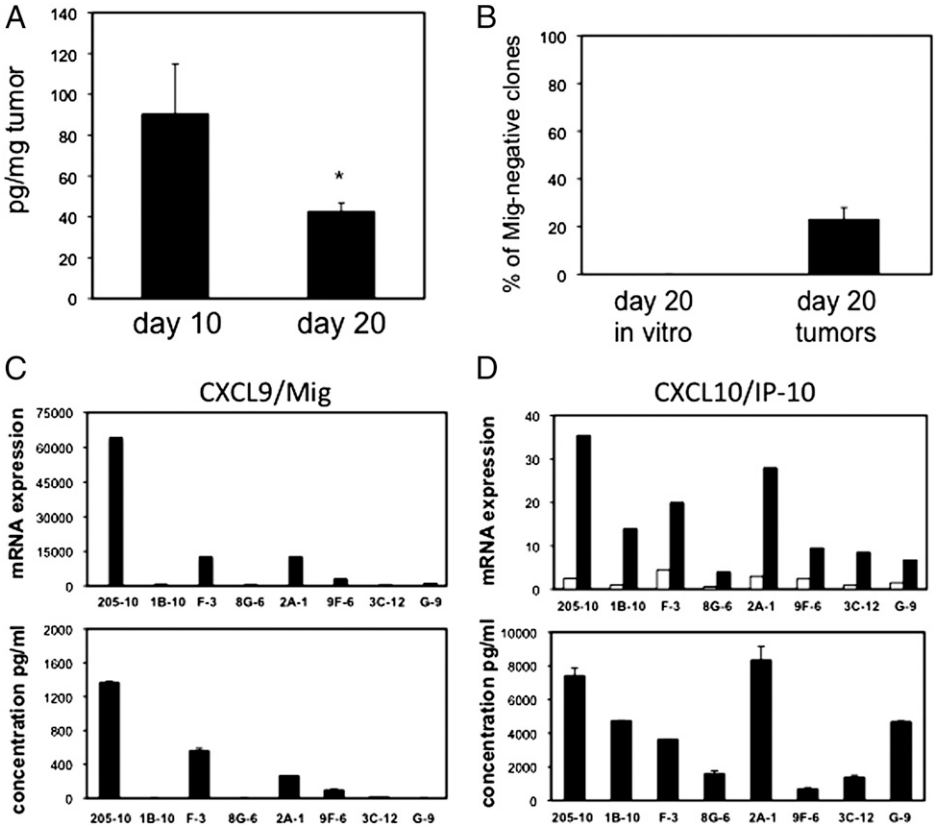

FIGURE 1. Highly tumorigenic Mig-deficient variants arise from Mig-expressing MCA205 cells during growth in wild-type mice. Mig-expressing tumor clone MCA205-10 was grown in vitro or in vivo as intradermal implants in syngeneic wild-type mice. (**A**) Intratumor production of Mig was tested by ELISA of tumor protein extracts prepared on days 10 and 20 post implant. Tumors from three recipient mice were analyzed in each group. $*p < 0.05$. (**B**) Tumor cells were retrieved from in vitro cultures of the tumors and subcloned by limiting dilution. Panels of tumor clones were tested for Mig production after stimulation with IFN-γ. Bars indicate the percentage of tumor clones that were low or negative in Mig production (50–90% decrease of Mig production when compared with parental cell line). Tumors from four recipient mice were analyzed. (**C** and **D**) *Upper panels*, The parental tumor clone 205-10 and seven tumor-derived Mig-negative variants were stimulated with IFN-γ; total cell RNA was prepared and tested for mRNA expression of Mig and IP-10 by qRT-PCR. The expression of Mig and IP-10 mRNA in IFN-γ−stimulated cells (black column) versus nonstimulated cells (white column) is shown. *Lower panels*, The parental tumor clone 205-10 and tumor-derived Mig-deficient clones were stimulated with IFN-γ, and then Mig and IP-10 protein production was assessed by ELISA. All results are representative of two individual experiments, with similar results observed each time.

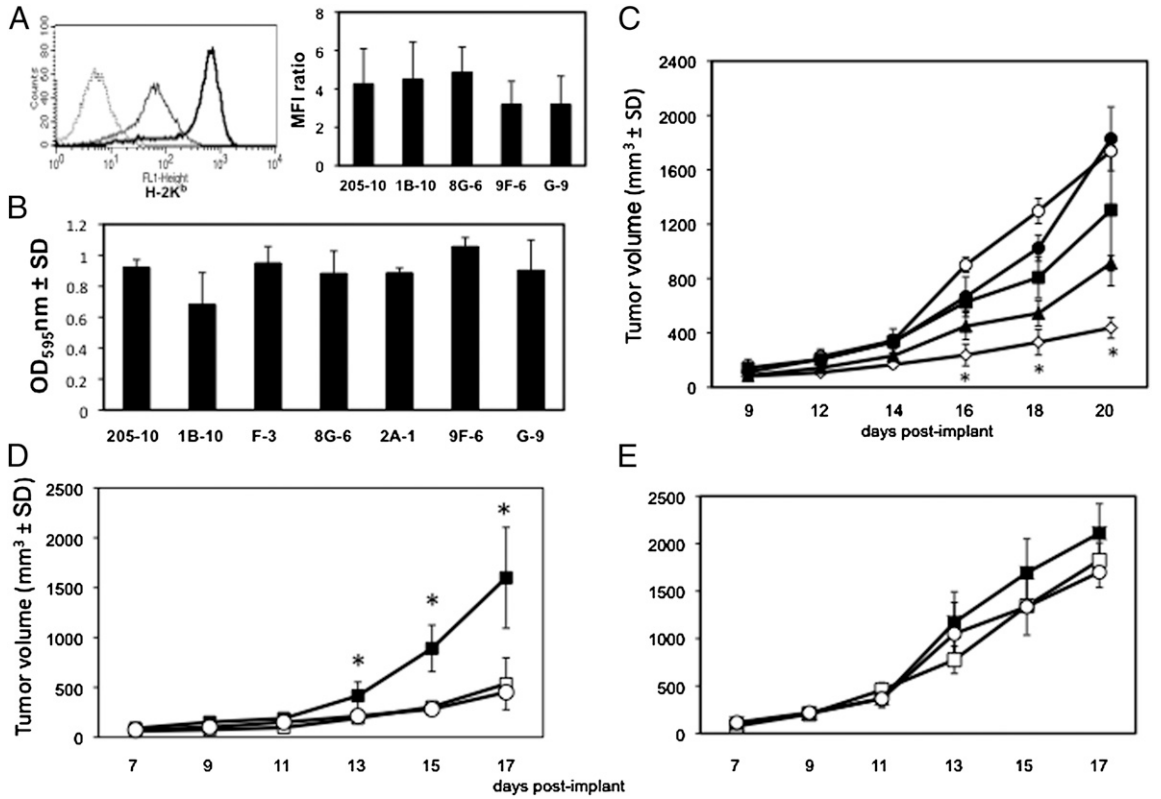

FIGURE 2. Tumor variants deficient in Mig expression are highly tumorigenic. (**A**) Tumor cell aliquots were cultured in triplicate with or without IFN-γ for 48 h and then were stained with FITC-labeled anti-mouse H-2K^b mAb. Histograms represent staining with isotype control Ab (…), or anti–H-2K^b mAb staining of nonstimulated tumor cells (- - -) or IFN-γ–stimulated tumor cells (**- - -**). Bar graph represents the IFN-γ−mediated upregulation of H-2K^b by showing the mean fluorescence intensity (MFI) ratios of IFN-γ–stimulated cells to nonstimulated cells. (**B**) Tumor cell aliquots were seeded in 96-well plates and cultured for 48 h. Then cell proliferation was assessed using methylene blue dye uptake. (**C**) Wild-type mice were implanted with 3×10^5 cell aliquots of parental Mig–expressing MCA205-10 cells (◇), Mig-deficent clones 1B-10 (●) and G-9 (○), or Mig- and IP-10–deficient clones 8G-6 (■) and 9F-6 (▲). Growth of tumors induced by these implants was compared (*p* values show differences between growth of Mig-expressing and Mig-deficient tumor implants). *$p < 0.05$. Results are representative of two individual experiments. (**D** and **E**) Equal numbers of parental 205-10 (○) or tumor-derived (□) Mig-expressing tumor cells or Mig-deficient (■) tumor cells were implanted into wild-type mice (D) or into RAG1$^{-/-}$ mice (E). Results are representative of two individual experiments. *$p < 0.05$.

growth of Mig-deficient and Mig-expressing tumor variants was similar in immunodeficient RAG1$^{-/-}$ mice, indicating similar abilities of these tumor variants to grow in vivo in the absence of T cells (Fig. 2E). These results suggested that the growth of Mig-expressing tumor cells is suppressed by host antitumor immunity activated within the first 2 wk of tumor growth, whereas Mig-deficient tumor variants are more resistant to this immune surveillance.

To directly test this idea, we evaluated the therapeutic effect of T cells transferred into RAG1$^{-/-}$ mice on the growth of Mig-expressing tumors versus Mig-deficient tumors. RAG1$^{-/-}$ mice were implanted with MCA205-10 tumor cells and were left untreated (control) or received aliquots of T cells purified from naive wild-type mice. This T cell transfer resulted in inhibition of tumor growth beginning at days 11–12 after T cell transfer for at least 3 consecutive days when compared with the control RAG1$^{-/-}$ group (Fig. 3A, *upper panel*). In contrast, transfer of T cells had no detectable effect on the growth of Mig-deficient tumors in the same experimental setting (Fig. 3A, *lower panel*). Although transferred T cells were activated in the tumor-draining lymph nodes of RAG1$^{-/-}$ recipients of Mig-expressing or Mig-deficient tumors, the numbers of tumor-specific IFN-γ–producing T cells in the lymph nodes draining Mig-deficient tumors were significantly decreased when compared with the numbers of these cells in lymph nodes draining Mig-expressing tumors (Fig. 3B). The absent therapeutic effect of adoptive T cell transfer on Mig-deficient tumors also correlated with decreased recruitment of transferred CD4 and CD8 T cells and host NK cells into these tumors when compared with recruitment into Mig-expressing tumors (Fig. 3C). These results indicate that Mig-deficient tumor variants are more resistant to T cell–mediated immunity than are Mig-expressing tumors and that resistance is mediated by the decreased activation and recruitment of tumor-reactive T cells.

Knocking down Mig gene expression in tumor cells increases their tumorigenicity

To further address the importance of ceasing Mig expression for cutaneous tumor growth, we transduced parental Mig-expressing MCA205 tumor cells with specific shRNA that target Mig mRNA to knock down expression of the Mig gene. Control tumor cells were transduced with shRNA that does not affect expression of murine genes (scramble control vector). Generated tumor variants were stimulated with IFN-γ and were tested by qRT-PCR for mRNA expression and by ELISA for protein production. IFN-γ−stimulated expression of Mig mRNA (not shown) and protein (Fig. 4A, black column) was inhibited in the tumor cells transduced with Mig-specific shRNA TRCN0000067295, whereas the expression of IP-10 was not (Fig. 4A, white column). Likewise, Mig production in tumors induced by Mig-knockdown tumor cell implants was completely inhibited when compared with control tumors induced by cells transduced with control shRNA (Fig. 4B). Inhibition of intratumor Mig production correlated with the accelerated growth of Mig-knockdown tumors in wild-type mice

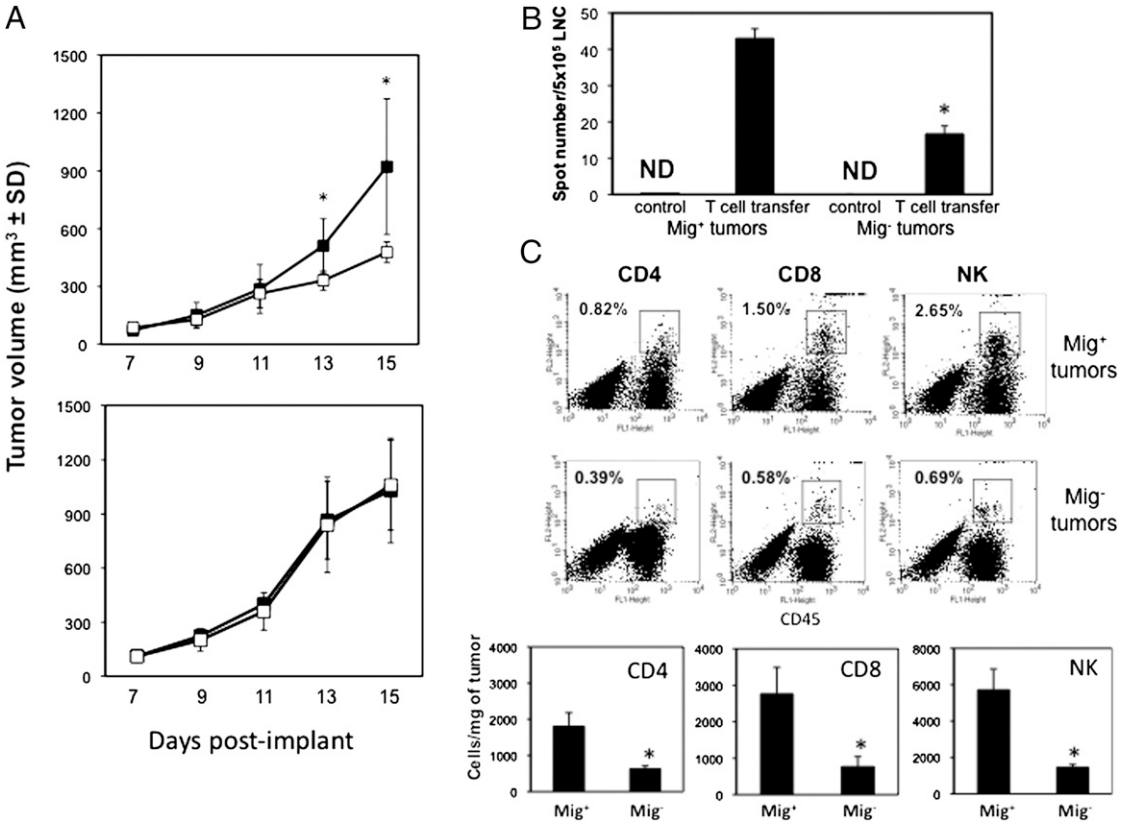

FIGURE 3. Mig-deficient tumor clones are resistant to T cell–mediated immunity. (**A**) Mig-expressing tumor cells MCA205-10 (*upper panel*) or Mig-deficient clone 1B-10 (*lower panel*) was implanted into RAG1$^{-/-}$ mice. On day 1 post implant, these tumor recipients were transferred with 2×10^7 T cells purified from spleens and lymph nodes of naive mice. Tumor growth was compared in mice receiving T cells (□) and in control RAG1$^{-/-}$ mice that did not receive T cells (■). Results are representative of two individual experiments. $*p < 0.05$. (**B**) Tumor-reactive T cell activation in T cell–transferred RAG1$^{-/-}$ mice: Tumor-draining lymph nodes were removed on day 14 after tumor implant from control RAG1$^{-/-}$ recipients or from T cell–transferred RAG1$^{-/-}$ recipients of Mig-expressing or Mig-deficient tumors (designated as Mig$^+$ and Mig$^-$ tumors, respectively). Aliquots of 5×10^5 cells were cultured with mitomycin-treated tumor cells, and numbers of IFN-γ producing cells were assessed by ELISPOT, as previously described (12). $*p < 0.05$; ND, Not detectable. (**C**) Intratumor infiltration of tumor-reactive cells: Cell suspensions were prepared from Mig-expressing tumors (Mig$^+$ tumors) or Mig-deficient tumors (Mig$^-$ tumors) and were stained with FITC-labeled mAb to CD45 and PE-labeled mAb to CD4, CD8, or NK1.1 to analyze intratumor infiltration of T and NK cells by flow cytometry. Numbers in dotplots represent the percentages of the indicated cell populations in total cell population of tumor. Bar graphs indicate mean numbers of indicated cells in Mig-expressing (Mig$^+$) versus Mig-deficient (Mig$^-$) tumors growing in T cell–transferred RAG1$^{-/-}$ mice. Three tumors per each group were analyzed. All results are representative of two individual experiments, with similar results observed each time. $*p < 0.05$.

when compared with control tumors having intact Mig expression (Fig. 4C). This accelerated growth of Mig-knockdown tumors, in comparison with control tumors, was associated with very low infiltration of CD8 T cells and NK cells (Fig. 4D). These results indicated an important role for tumor-derived Mig in the immune-mediated suppression of cutaneous tumors growth.

Conversion of Mig-expressing tumor cells into Mig-deficient variants is promoted by antitumor immunity

Our results indicated that tumor cells cease Mig expression when growing in vivo and become more resistant to T cell–mediated inhibition of tumor growth. Next we tested whether conversion of Mig-expressing tumor cells into Mig-deficient variants occurs as a result of immune pressure. Tumor cell suspensions retrieved from tumors growing in immunocompetent wild-type mice or in mice deficient in critical components of antitumor immunity were subcloned, and panels of the clones generated from these tumors were tested for IFN-γ–induced Mig production. Because IFN-γ is a critical factor of antitumor immunity and tumor immunoediting in the MCA205 model (2), we first determined the presence of Mig-deficient tumor clones within Mig-expressing tumors growing in IFN-γ$^{-/-}$hosts. Very few tumor clones low in Mig production were detected during initial screening of a large panel of clones generated from tumors retrieved from eight tumor-bearing IFN-γ$^{-/-}$ mice (a total of 552 clones from eight tumors were tested). When these Mig-low tumor clones from IFN-γ$^{-/-}$ mice were expanded, repeatedly stimulated with IFN-γ, and then tested for Mig production, most clones regained the ability to produce Mig. In contrast, the frequencies of Mig-low clones were high in tumors retrieved from wild-type mice (Fig. 5A; 517 clones from nine tumors were tested). Although some of these Mig-low clones also regained their ability to produce Mig after subsequent expansion in vitro and stimulation with IFN-γ, at least 29 tumor clones remained Mig negative after second and third rounds of stimulation with IFN-γ. Because T cells and NK and NKT cells are sources of IFN-γ and are important components of tumor immunity as well, we compared the presence of Mig-deficient tumor variants in T cell–deficient RAG1$^{-/-}$ mice and in RAG2/γcR$^{-/-}$ mice, deficient in both T cells and NK cells. Frequencies of Mig-negative tumor variants in tumors retrieved from these immunodeficient mice, compared with tumors growing in wild-type mice, were significantly decreased, indicating the role of T cells in the generation and expansion of Mig-deficient tumor variants in vivo (Fig. 5A).

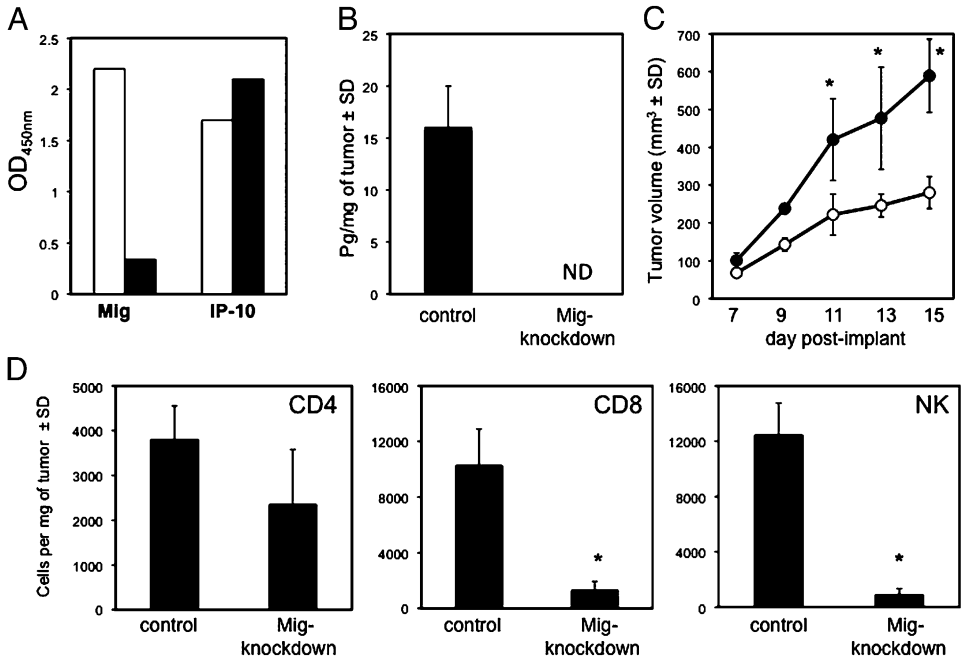

FIGURE 4. Knocking down Mig transcription in tumor cells results in accelerated tumor growth. (**A**) Mig-expressing tumor cells were transduced with control shRNA or with shRNA that specifically targets Mig mRNA. Supernatants were collected from control shRNA-transduced (white column) or from Mig-specific shRNA-transduced cells (black column) after stimulation with IFN-γ and tested for Mig and IP-10 production by ELISA. (**B**) Protein extracts were prepared from tumors induced in wild-type mice by implants of control or Mig-knockdown tumor cells and were tested for Mig production by ELISA. ND, detectable. (**C**) Control (○) or Mig-knockdown (●) tumor variants were implanted into wild-type mice, and their growth was compared. $*p < 0.05$. (**D**) Cell suspensions were prepared from Mig-expressing tumors (control) or Mig-knockdown tumors and were stained with FITC-labeled mAb to CD45, and PE-labeled mAb to CD4, CD8, or NK1.1, to analyze intratumor infiltration of T and NK cells by flow cytometry. Bar graphs indicate the numbers of indicated cells per milligram of tumor in control versus Mig-knockdown tumors. Four tumors per each group were analyzed. Results are representative of two individual experiments, with similar results observed each time. $*p < 0.05$.

Because the results suggested that Mig-deficient tumor variants expand in tumors as a result of immune pressure mediated by T cells, we tested whether delivery of T cells into $RAG1^{-/-}$ recipients of Mig-expressing tumors would result in the expansion of Mig-deficient tumor variants. $RAG1^{-/-}$ mice were implanted with Mig-expressing tumor cells and were left untreated (control) or received aliquots of purified T cells on day 1 post implant. Transfer of T cells into $RAG1^{-/-}$ mice resulted in increased frequencies of Mig-deficient tumor clones in the tumors excised on day 20 post implant when compared with tumors retrieved from control $RAG1^{-/-}$ mice at the same time point (Fig. 5B).

B16 melanoma cells cease Mig expression during growth in vivo

Next we tested whether loss of Mig expression by tumor cells occurs in other types of cancer. We chose murine melanoma B16-

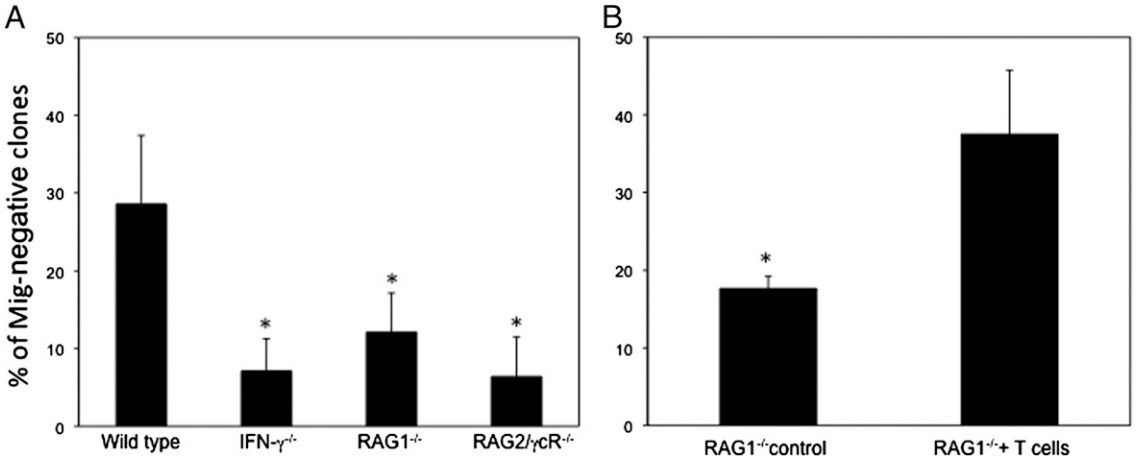

FIGURE 5. Mig-deficient tumor variants expand in immunocompetent, but not in immunodeficient, mice. (**A**) Mig-expressing MCA205-10 cells were implanted into wild-type mice, IFN-$\gamma^{-/-}$ mice, $RAG1^{-/-}$ mice, or RAG2/$\gamma cR^{-/-}$ mice. Tumors were excised at 3 wk post implant, and tumor cell suspensions were subcloned. Tumor clones were tested for Mig production induced by IFN-γ stimulation. The mean percentages of Mig-deficient tumor clones derived from each group are shown. $*p < 0.05$ when compared with wild-type control group. Results are representative of three individual experiments. (**B**) Mig-expressing MCA205-10 cells were implanted into $RAG1^{-/-}$ mice. On day 1 post implant, these mice received 2×10^7 T cells purified from naive wild-type mice. Cell suspensions were prepared from tumors retrieved from control or T cell-recipient $RAG1^{-/-}$ mice on day 20 post implant and were subcloned and tested for IFN-γ–induced Mig production. $*p < 0.05$.

F10 and breast carcinoma 4T-1 cell lines, because both produced Mig and IP-10 when stimulated with IFN-γ in vitro (not shown). Cutaneous B16 and 4T-1 tumors were induced in wild-type mice by intradermal implants, tumors were grown for 15 d and then retrieved, and tumor cell suspensions were subcloned to generate panels of tumor clones. Whereas the frequencies of Mig-negative tumor variants were low in 4T-1 tumors (not shown), we observed high frequencies of Mig-deficient variants in B16 tumors retrieved on day 15 post implant, but not in parental B16 cultures (Fig. 6A). Similar to our findings in MCA205 tumors, growth of Mig-deficient B16 tumor–derived variants was significantly accelerated when compared with that of Mig-expressing tumor-derived variants of B16 implanted into immunocompetent wild-type mice (Fig. 6B). Next, we tested whether a stable Mig-expressing clone derived from B16 tumor would lose the expression of Mig when growing in vivo, as was demonstrated in the MCA205 sarcoma model. B16 clone 1D-7 was grown in vitro or in vivo as a tumor for 20 d. When tumor cell suspensions from in vitro culture or from tumors were subcloned and tested for Mig production, high frequencies of Mig-negative tumor clones were observed in panels of clones derived from growing tumors (Fig. 6C). All of the Mig-deficient clones of B16 remained responsive to IFN-γ stimulation by producing IP-10 (Fig. 6D). These results indicated that loss of Mig expression and expansion of highly tumorigenic Mig-deficient tumor variants occur during growth of both cutaneous MCA205 sarcomas and B16 melanomas.

IFN-γ directly mediates loss of Mig expression by tumor cells

Our studies using IFN-$\gamma^{-/-}$ mice in the MCA205 model suggested that IFN-γ–induced stress provokes loss of Mig expression by tumor cells during their growth in vivo. We further tested this potential mechanism of IFN-γ–mediated tumor immunoediting by exposing parental Mig-expressing MCA205 or B16 cells to IFN-γ during growth in vitro. Our previous studies indicated that short-term exposure of tumor cells to IFN-γ (24–72 h) did not result in loss of their ability to express Mig. However, when tumor cell cultures were treated with IFN-γ for extended periods (7 consecutive days, beginning on the first day of culture), a significant loss of Mig expression by tumor cells was observed. Because this exposure of tumor cells to IFN-γ resulted in decreased growth of tumor cells when compared with control nontreated cultures (most likely owing to proapoptotic and antiproliferative effects of IFN-γ on tumor cells), it was important to test equal numbers of IFN-γ–treated and control tumor cells for the ability to produce Mig. After the culture with or without IFN-γ, tumor cells were washed and counted, and then aliquots of viable IFN-γ–treated or control

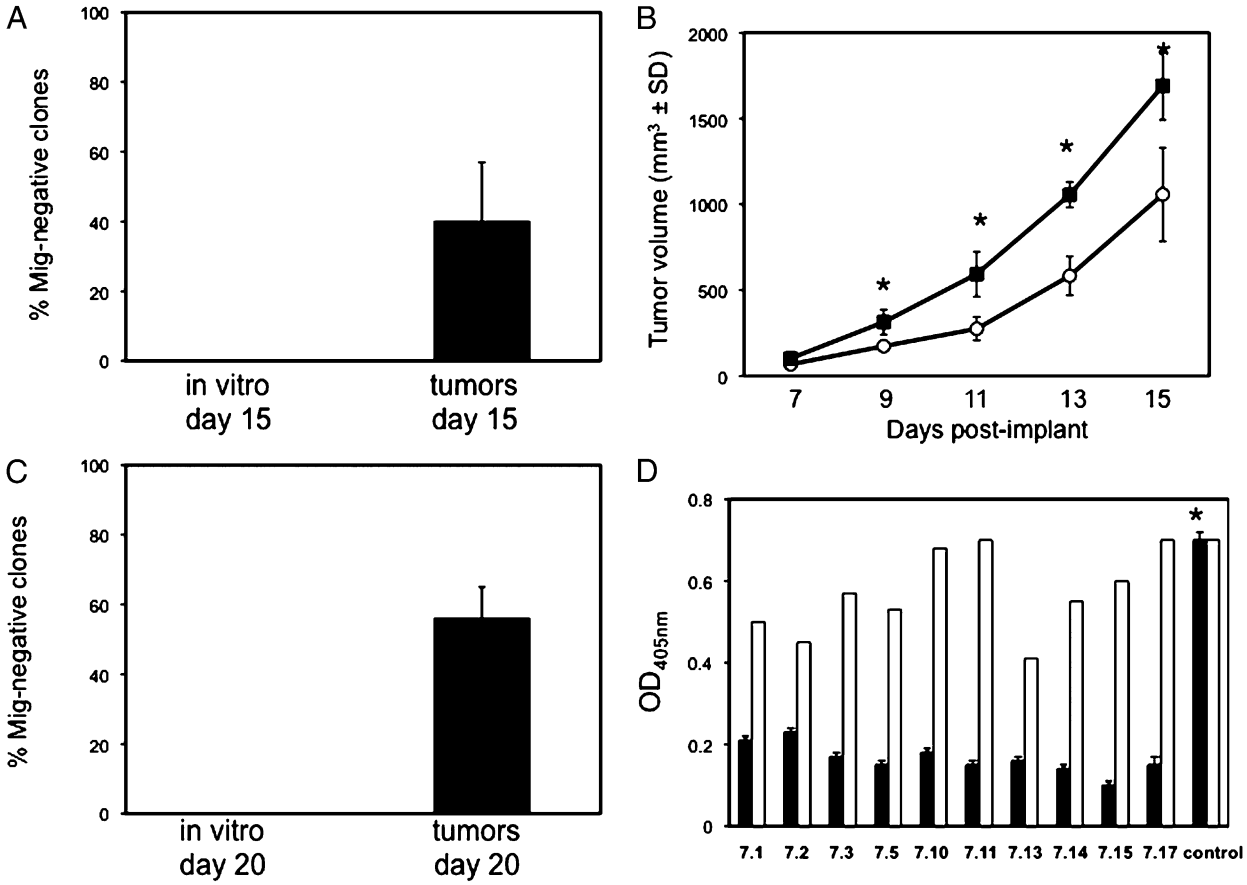

FIGURE 6. Highly tumorigenic Mig-deficient variants arise from Mig-expressing B16 melanoma cells during growth in wild-type mice. (**A**) Mig-expressing B16-F10 cells were grown in vitro or in vivo as intradermal implants in wild-type mice. Tumor cells were retrieved from in vitro cultures of the tumors on day 15 and subcloned by limiting dilution. Panels of tumor clones were tested for Mig production after stimulation with IFN-γ. Tumors from seven recipient mice were analyzed. (**B**) Wild-type mice were implanted with 3×10^5 cell aliquots of tumor-derived Mig-expressing clone 1D-7 (○) or with Mig-deficient clone 7A-7 (■). Growth of tumors induced by these implants was compared. *$p < 0.05$. (**C**) Mig-expressing B16 tumor clone 1D-7 was grown in vitro or in vivo as intradermal implants in wild-type mice. Tumor cells were retrieved from in vitro cultures of the tumors on day 20 and subcloned by limiting dilution. Panels of tumor clones were tested for Mig production after stimulation with IFN-γ. Tumors from three recipient mice were analyzed. (**D**) The parental Mig-expressing tumor clone 1D-7 (control) and ten clones generated from growing 1D-7 tumors were stimulated with IFN-γ, and then protein production of Mig (■) and IP-10 (□) was assessed by ELISA. Results are representative of two individual experiments, with similar results observed each time. *$p < 0.05$ when compared with tumor-derived clones.

tumor cells were stimulated with IFN-γ for 48 h and tested for Mig production. The ability of B16-F10 melanoma cells to produce Mig in response to IFN-γ was completely lost after the extended growth of these cells in the presence of IFN-γ, whereas the IFN-γ–induced production of IP-10 and upregulation of class I MHC molecule by tumor cells remained intact, indicating that the responsiveness of tumor cells to IFN-γ is not lost (Fig. 7A, 7B). Growth of these in vitro–generated Mig-deficient variants in wild-type mice was significantly increased when compared with growth of parental B16 cells that were able to produce Mig, indicating that IFN-γ–induced loss of Mig expression in these tumor variants increased their tumorigenicity (Fig. 7C).

Although loss of Mig production by MCA205-10 cells by extended culture with IFN-γ was less profound than loss of Mig by B16 cells, at least 27% of tumor clones derived from IFN-γ–treated MCA205 cultures were Mig negative, whereas clones from control MCA205-10 cultures remained Mig positive (Supplemental Fig. 1). These results indicate that Mig-expressing MCA205 tumor cells can also convert into Mig-deficient tumor variants in response to constitutive stimulation with IFN-γ. Overall, our findings in both MCA205 and B16 models of skin cancer support the hypothesis that aggressive cutaneous tumors cease Mig expression when growing under the stress mediated by IFN-γ.

Discussion

The role of Mig and IP-10 in the recruitment of activated NK and Th1 cells to inflammatory tissue sites and in the inhibition of angiogenesis is well established (15–18). Studies in murine cancer models indicate that the antitumor effects of many immunotherapeutic agents are mediated through angiostatic and chemoattractant properties of Mig and/or IP-10 (13, 19–23). Clinical studies have documented expression of both Mig and IP-10 in tumors and the correlation between decreased intratumor expression of these chemokines and more aggressive tumor growth (24–28). What is not clear is whether the expression of these T cell chemoattractants in the tumor microenvironment is suppressed in all tumor cells during tumor growth or is selectively lost in a clonal manner.

Our recent studies indicated high frequencies of Mig-deficient tumor variants in advanced MCA205 fibrosarcomas that accounted for decreased intratumor production of Mig and for decreased activation and recruitment of tumor-reactive T cells (12). In the current report, we extended these studies and demonstrated that highly tumorigenic Mig-deficient variants arise from Mig-expressing MCA205 sarcomas and B16 melanomas. Importantly, these Mig-deficient tumor variants were derived from in vivo growing Mig-expressing tumor clones that were stable regarding their ability to produce Mig during growth in vitro. Therefore, the conversion of Mig-expressing tumor cells into Mig-deficient variants occurs during their growth in the host. Consistent with our previous findings, these Mig-deficient tumor variants were more aggressive than the parental Mig-expressing tumor cells when growing in immunocompetent mice. Accelerated growth of Mig-deficient tumors was not mediated by their increased ability to proliferate, but rather by resistance to antitumor immunity. In vitro growth of Mig-deficient tumor cells, as well as their growth in vivo in immunodeficient RAG1$^{-/-}$ mice, was similar to the growth of Mig-expressing tumor cells. Unlike growth of Mig-expressing tumors, that of Mig-deficient tumor variants in RAG1$^{-/-}$ mice was not suppressed by T cell transfer, indicating that these tumor variants are more resistant to T cell immunity than are parental Mig-expressing tumor cells. Accelerated growth of Mig-deficient tumors was associated with poor recruitment of T and NK cells into these tumors when compared with recipients of Mig-expressing tumors, suggesting a critical role for tumor-derived Mig in promoting antitumor immune responses. Furthermore, we observed that shRNA-mediated knockdown of Mig expression in parental tumor cells resulted in similar increases in tumor growth and decreases in T and NK cell recruitment into tumors, despite the ability of these tumor variants to express IP-10. These findings are consistent with our previous studies indicating a nonredundant role of Mig in promoting antitumor immune responses to MCA205 tumors (12). It was noteworthy that in addition to inhibited T cell recruitment into Mig-deficient tumors, we also observed decreased numbers of tumor-specific IFN-γ–producing T cells in the lymph nodes draining these tumors when compared with lymph nodes draining Mig-expressing tumors growing in RAG1$^{-/-}$ mice populated with T cells. These results are in line with our earlier findings indicating enhanced activation of tumor-specific IFN-γ–producing T cells in lymph nodes draining Mig-expressing tumors and suggesting that besides the recruitment of tumor-reactive T cells, tumor-derived Mig has

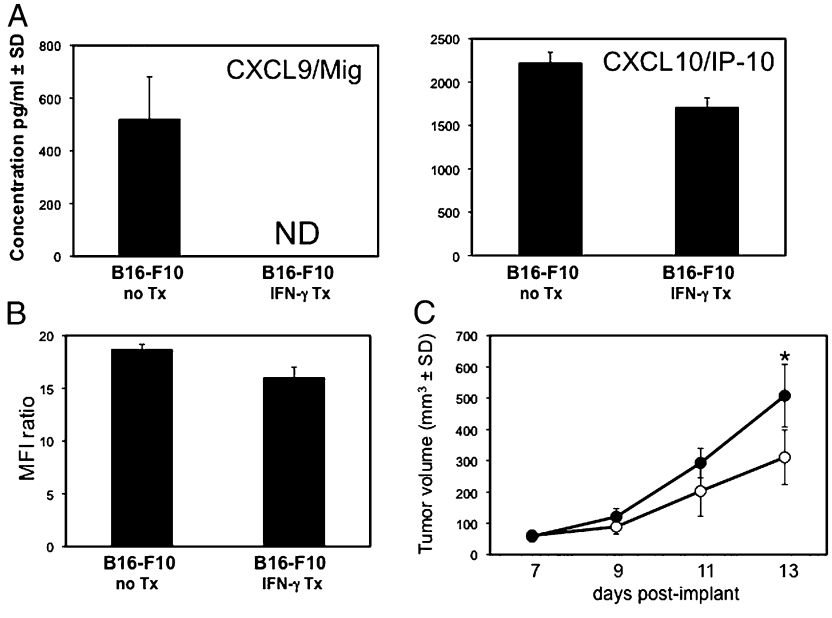

FIGURE 7. Treatment of B16 cells with IFN-γ results in loss of Mig production by tumor cells. (**A**) Tumor cells were seeded on six-well plates and were left untreated (no Tx) or treated (Tx) with 50 ng/ml of IFN-γ for 7 consecutive days. Then cells were washed and counted, and 3×10^5 cell aliquots of untreated or IFN-γ–treated tumor cells were stimulated with 10 ng/ml of IFN-γ for 48 h. Supernatants were tested by ELISA for production of Mig and IP-10. Results are representative of two individual experiments. (**B**) Tumor cell aliquots were cultured with or without IFN-γ for 48 h and then were stained with FITC-labeled anti-mouse H-2K^b mAb. Bar graph represents the IFN-γ−mediated upregulation of H-2K^b by showing the MFI ratios of IFN-γ–stimulated cells versus nonstimulated cells. (**C**) Wild-type mice were implanted with 3×10^5 cell aliquots of parental Mig-expressing B16-F10 cells (○) or with Mig-deficient variant generated in vitro by IFN-γ treatment (●). Growth of tumors induced by these implants was compared. *$p < 0.05$.

an impact on T cell activation, possibly by affecting tumor-infiltrating dendritic cells that acquire tumor Ags and then migrate into draining lymph nodes to prime tumor-specific T cells.

Remarkably, Mig-deficient tumor variants were rare in immunogenic MCA205-10 tumors growing in immunodeficient mice lacking critical components of antitumor immunity (IFN-γ, T cells, or NK and NKT cells). These results suggest that Mig-deficient tumor variants expand as a result of immune selection mediated by IFN-γ produced by tumor-infiltrating effector cells. Consistent with this hypothesis, we observed an increase in the frequencies of Mig-deficient variants when tumor-bearing RAG1$^{-/-}$ mice were populated with T cells, suggesting a role for T cells in immune selection of these tumor variants. Frequencies of Mig-negative tumor variants in tumors retrieved from RAG2/γcR$^{-/-}$ mice were moderately decreased when compared with tumors growing in RAG1$^{-/-}$ mice, indicating a possible function for NK cells in the selection of Mig-deficient tumor variants. It is known that NK cells are more abundant in RAG1$^{-/-}$ and RAG2$^{-/-}$ mice than in wild-type hosts and may partially compensate for the absent T cell–mediated tumor immunosurveillance in these mice (29). We were unable to completely deplete NK cells in RAG1$^{-/-}$ mice, using the standard anti-NK1.1 mAb depletion protocol that always resulted in nearly 100% depletion of NK cells in wild-type hosts (A. Gorbachev, unpublished observations). Therefore, the contribution of NK cells to immune selection of Mig-deficient tumor variants remains unclear at this point.

To gain further insight into IFN-γ–mediated editing of tumor cells, we demonstrated that prolonged exposure of MCA205 or B16 cells to IFN-γ decreased Mig production by these cells after restimulation with IFN-γ and resulted in the appearance of Mig-deficient tumor variants in IFN-γ−treated tumor cell cultures. This IFN-γ–mediated downregulation of Mig production was more profound in B16 melanoma than in MCA205. Consistent with this observation, the frequencies of Mig-deficient tumor variants were higher in growing B16 melanomas than in MCA205 fibrosarcomas (40–60% versus 20–30%, respectively). Melanoma is known for its increased ability to mutate and rapidly change in response to stress, and this ability contributes to high tumorigenicity and resistance of this cancer to all therapies currently used (30). Rapid loss of Mig expression by melanoma cells in response to immune stress may be part of a mechanism that mediates the notorious resistance of melanoma to antitumor immunity and to its aggressive growth and dissemination.

It is noteworthy that the majority of Mig-deficient tumor variants generated in these studies retained their ability to respond to IFN-γ stimulation by production of IP-10 and by upregulation of class I MHC. Previous studies have indicated loss of IFN-γ responsiveness by various cancers (including melanoma) as an important mechanism of tumor evasion (31, 32). More recent studies in human melanoma demonstrated that IFN-γ−edited tumor cells could also decrease their sensitivity to cytotoxic T cell–mediated lysis without losing responsiveness to IFN-γ (33). In this article, we demonstrated for the first time, to our knowledge, the IFN-γ–mediated loss of chemokine expression by tumor cells as another potent mechanism of tumor resistance to immune responses. Apparently, the expression of IP-10 by tumor cells does not compensate for loss of Mig expression and does not suppress tumor growth in the absence of Mig in the tumor microenvironment. We generated several Mig-deficient MCA205 clones that express low levels of both Mig and IP-10; however, growth of these tumor variants was not increased when compared with tumor variants deficient in Mig expression only. Consistent with our findings, recent studies in murine breast carcinoma demonstrated generation of immune-resistant tumor variants that downregulated the expression of Mig, but not IP-10 (5). Although these two chemokines are similar in their structure and angiostatic and chemoattractant functions, evidence exists that they exert some nonredundant or even antagonistic functions as well. Although Mig expression is induced specifically by IFN-γ, the expression of IP-10 can be also induced by type 1 IFNs, TNF-α, and LPS (34, 35). Moreover, the expression pattern of these chemokines in tissues during inflammation is different (36–38). Recent studies in a murine cardiac allograft model demonstrated antagonistic effects of Mig and IP-10 on the costimulation of alloreactive IFN-γ–producing CD8 T cells (39), and we recently observed differential effects of these two chemokines on the activation of tumor-specific T cells as well (A. Gorbachev, manuscript in preparation).

Overall, these studies demonstrate the IFN-γ–mediated loss of Mig expression in cutaneous tumors as a potent mechanism of immunoediting that results in increased tumor resistance to T cell–mediated immunity. The molecular mechanism contributing to loss of Mig expression in tumor cells is currently under investigation in our laboratory. Defining this mechanism is likely to result in the identification of new molecular targets to counteract tumor resistance to T cell–based immunotherapies.

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Disclosures

The authors have no financial conflicts of interest.

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