Decreased accumulation of immune regulatory cells is correlated to the antitumor effect of IFN-γ overexpression in the tumor

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Abstract. During mammary tumorigenesis, there is a profound tumor-induced immunosuppression and a progressive thymic atrophy associated with tumor development. IFN-γ has been shown to be effective in enhancing antitumor responses in several tumor models, however, how IFN-γ exerts its anti-tumor effect is largely controversial. In the present study we have used a mammary tumor model to investigate whether the levels of IFN-γ have an important role in the tumor-induced immunosuppression as well as in the pathogenesis of the thymic atrophy. We evaluated this possibility using DA-3 cells transfected to express IFN-γ (DA-3/IFN-γ), a system that provides constant, local production of IFN-γ within the tumor microenvironment. Overexpression of IFN-γ in the mammary tumor results in a marked delay of tumor growth, a reduction in regulatory T cells and myeloid-derived suppressor cells accumulation mostly due to down-regulation of chemokines implicated in the recruitment of immune regulatory cells, and a blockage in the tumor-associated thymus atrophy. Collectively, our data suggest that the replacement of the faulty levels of IFN-γ in the tumor results in a diminution of the tumor-induced immune suppression caused by the mammary tumor development.

Introduction

Interferon-gamma (IFN-γ) is a crucial cytokine primarily produced by T cells, NK cells, and NKT cells that have diverse roles in the innate and adaptive immune responses (1). Among its different immunologically related activities, IFN-γ induces the expression of MHC II antigens on macrophages, T and B cells, as well as many tumor cells (2). IFN-γ has been used clinically in the therapy of malignancies and viral diseases (3). Moreover, several studies have shown that IFN-γ is vital to tumor surveillance by the immune system and a high correlation between IFN-γ production and tumor regression has been seen in immunotherapy (4). IFN-γ is also responsible, together with LPS, for the induction of the enzyme nitric oxide synthase (iNOS) on macrophages (5); iNOS participates in the production of nitric oxide, a highly reactive gaseous molecule that plays a role in the antiviral, antimicrobial, antiparasitic and antitumor activities of IFN-γ. Because of the variety of relevant roles that this cytokine exhibits, its expression is tightly regulated, and is considered critical in the assurance of the immunological defenses of the host, in particular during tumor development. For that reason, it is not surprising to discover tumor-induced diminished expression of IFN-γ in tumor bearers, as a mechanism developed by the neoplasia that contributes to tumor tolerance. Indeed, it has been reported that IFN-γ production is greatly depressed in cancer patients (6).

Using a murine mammary adenocarcinoma originally induced in BALB/c mice by dimethylbenzanthracene (D1-DMBA-3) (7), and an *in vitro* cell line derived from it (DA-3) (8) we have previously described a profound immunosuppression and a progressive thymic atrophy associated with tumor development (9). We have demonstrated that the tumor-induced immunosuppression observed in these mammary tumor bearers is associated, among other, with an accumulation of myeloid derived suppressor cells in several peripheral organs (10). In addition, the mammary tumor cells used in these studies secrete several molecules which have effects in the immune system (11-13); for example, there is a decrease in the IFN-γ in the peripheral circulation and its production by T cells mainly due to a downregulation of IL-12 (14). Moreover, we have also shown that the tumor-induced thymic involution is accompanied by a severe depletion of the most abundant subset of thymocytes; $CD4^+CD8^+$ double positive (DP) immature cells and an increase in the percentages of $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive populations and $CD4^-CD8^-$ double negative population (9). We have investigated several possible mechanisms leading to this thymic atrophy. In this regard, previous results from our laboratory have shown that

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Abbreviations: NM, normal mice; TBM, tumor-bearing mice; IFN-γ, interferon gamma; Tregs, regulatory T cells; MDSC, myeloid-derived suppressor cells

Key words: tumor-bearing mice, IFN-γ, regulatory T cells, myeloid-derived suppressor cells

the severe thymic atrophy and the impaired T cell development seen in mammary tumor bearers are associated with an arrest in at least two steps of T cell differentiation (15,16); changes in the levels of crucial cytokines expressed in the thymus microenvironment (17); a progressive increase in apoptosis during the tumor development, mainly due to downregulation of important molecules that control programmed cell death (16); as well as an alteration in interferons and Jak/Stats signaling pathways (18).

In the present study we have used our DA-3 mammary tumor model to further investigate whether the overexpression of IFN-γ in the tumor microenvironment has an important role in antitumor activity, in the tumor-induced immunosuppression as well as in the thymic atrophy in these tumor-bearing mice. Our results suggest that the overexpression of IFN-γ in the tumor could help the host to escape from the tumor-induced immune suppression caused by the mammary tumor development.

Materials and methods

Mice and cell lines. BALB/c female mice used in these studies were 10-12 weeks of age and were bred and housed under barrier conditions in the Division of Veterinary Resources at the University of Miami, Miller School of Medicine. Animal care and use was according to the guidelines of the National Institutes of Health. The DA-3 mammary tumor cell line was derived in our laboratory from the D1-DMBA-3 tumor and maintained in DMEM/high glucose, 10% characterized heat-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin with OPI medium supplement (Sigma-Aldrich, St. Louis, MO). Tumor cells ($1x10^6$ DA-3, and $1x10^6$ IFN-γ-transfected DA-3 cells) were injected s.c., and 4-5-week-old tumor-implanted animals were used for the indicated studies.

Transfection of DA-3 tumor cells. Stable DA-3/IFN-γ transfectants were generated by isolating the gene-encoding IFN-γ from pORF5-mIFNγ Vector (Invivogen, San Diego, CA) and inserting it into the expression plasmid pcDNA3.1/Hygro (Invitrogen). DA-3 cells were then plated and transfected using Lipofectamine 2000 as per manufacturer's protocol (Invitrogen). Cells were cloned under selection with 400 μg/ml hygromycin and tested for constitutive IFN-γ expression by ELISA using the Mouse IFN-γ ELISA set (BD Biosciences, San Diego, CA). The IFN-γ-transfected cells with the highest specific IFN-γ production were selected for cloning by limiting dilution.

Proliferation assays. Cell proliferation rate was determined using CyQuant® NF Cell proliferation Assay kit (Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated at density of 1000 per well in a 96-well plate. Numbers of cells in wells were counted every 24 h. Growth medium was removed, 50 μl of green-fluorescent CyQuant GR dye was added to the well and incubated for 30 min at 37˚C. The fluorescence intensity of each sample was measured using a fluorescence microplate reader with excitation at 485 nm and emission detection at 530.

Measurement of tumor volume and survival curve. Tumor cells were implanted by s.c. injection of $1x10^6$ tumor cells in 0.9% saline. Tumor volumes were calculated by measuring two diameters of the tumor (x, small diameter; y, large diameter) using digital calipers and entering measurements into the equation tumor volume = $x^2y(0.52)$. To determine the effect of IFN-γ overexpression in the survival of tumor bearers, mice were injected with $1x10^6$ DA-3 or IFN-γ-transfected DA-3 cells and the percent of survival was determined over time.

Preparation of T cells and culture. Spleens were removed from normal mice and tumor bearers and mashed through 70 μmol/l cell strainers (BD Biosciences) to obtain single-cell suspensions. Cells were washed in RPMI-1640 and centrifuged at 1,500 rpm for 10 min. RBCs were lysed by hypotonic shock. Cells were quickly resuspended in HBSS and centrifuged. Cells from the different mice were resuspended in RPMI, counted and placed in 24-well tissue culture plates (Costar, Cambridge, MA) at a concentration of 10^6 cell/ml and cultured in RPMI-1640 containing 10% FCS, glutamine (30 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-ME ($5x10^{-5}$ M) (CM) and stimulated with α-CD3 (1 μg/ml) or Con A (5 μg/ml). Supernatants were collected at 48 h.

Plasma collection. Blood from the different mice was collected into a Microvette CB 300 (Braintree Scientific, Inc.) containing heparin. After centrifugation, plasma was removed and stored at -80˚C until assay by ELISA.

Macrophage collection. Normal and 4-week tumor-bearing mice were injected i.p. with 1.5 ml of 3% thioglycolate (Difco Laboratories). On day 4, peritoneal macrophages were obtained as described (19).

Thymocyte collection. Mice were sacrificed and both lobes of the thymus were carefully dissected from the chest cavity and placed in a Petri dish containing 1X Hanks' balanced salt solution, 1% calf serum, 10 mM HEPES, pH 7.2, to prevent drying. The thymic lobes were weighed and placed in a cell strainer in a Petri dish with a drop of medium on the top, and gently compressed with the base of a 3-ml syringe followed by a wash with cold media and transfer to polypropylene tubes. Cell were counted and analyzed by flow cytometry.

Flow cytometry. The following antibodies were used for flow cytometry: APC anti-CD4 (RM4-5), Per-CP anti-CD8 (53-6.7), FITC anti-Ly-6C (Gr-1) and PE anti-CD11b (M1/70) purchased from BD PharMingen (San Diego, CA) as well as FITC anti-CD4 (L3T4) and APC anti-CD25 (PC61.5) purchased from eBioscience (San Diego, CA). Splenic cells were also immunostained for CD4, CD25, and Foxp3, using eBioscience mouse regulatory T cell staining kit according to the manufacturer's instructions. The cells were analyzed using a BD Biosciences LSRII Cytometer (BD Biosciences, San Jose, CA) and Diva software (BD Biosciences). Percentages of Tregs are detected by the percentages of $CD25^+Foxp3^+$ cells within the gate of $CD4^+$ cells. The total number of events collected for analysis was between 100,000-500,000 cells.

Cytokine ELISA. The amounts of IFN-γ, CCL2/MCP-1 and CCL-5/RANTES present in the supernatants from stimulated T cells were measured by ELISA according to the manufacturer's instructions. IFN-γ was analyzed using standard ELISA kit (BD Biosciences, San Diego, CA) as well as CCL2/MCP-1

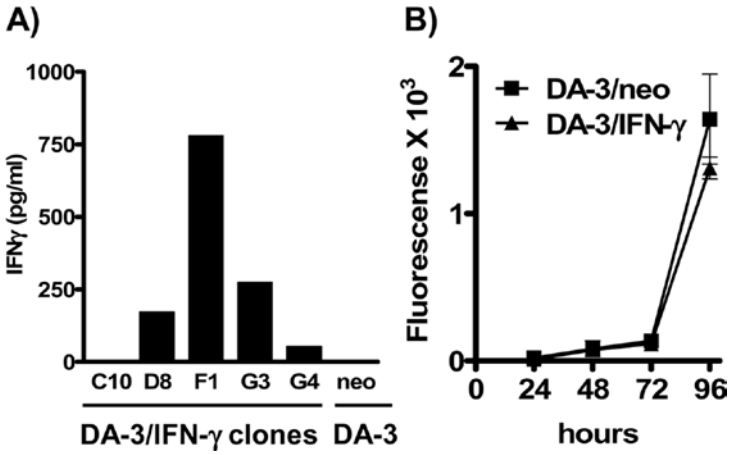

Figure 1. Functional properties of IFN-γ-transfected DA-3 mammary tumor cell line. DA-3 tumor cell line was transfected as described in Materials and methods. (A) IFN-γ expression from the different clones of DA-3 cells, and the DA-3 cells transfected with an empty vector as control were quantified by ELISA. (B) The proliferation rate from IFN-γ-transfected DA-3 cell line and empty vector-transfected DA-3 cells was analyzed to compare the effect of IFN-γ overexpression in cell proliferation. Data are representative (A) or the mean ± SD (B) of three independent experiments.

and CCL-5/RANTES (R&D Systems, Minneapolis, MN) following the manufacturer's protocol. Plasma VEGF levels were measured by Quantikine Murine VEGF ELISA (R&D Systems) according to the manufacturer's instructions. The amounts of cytokine present in each well were quantitated by measuring absorbance at 450-550 nm using a Tecan SLT Rainbow Reader (Lab Instruments, Research Triangle Park, NC). OD values were converted to pg/ml by including dilutions of known amounts of recombinant murine cytokines in the ELISA. A standard curve was generated by plotting the OD of the standards vs their known cytokine concentration.

Results

Characterization and functionality of IFN-γ-transfected mammary tumor cell line. Interferon-gamma (IFN-γ) is a cytokine that acts on cell-surface receptors, activating transcription of genes that offer treatment potential by increasing tumor immunogenicity, disrupting proliferative mechanisms, and inhibiting tumor angiogenesis. However, abnormally low levels of IFN-γ are produced by tumor bearing hosts. In this study, we examined the ability of IFN-γ to control tumor cell growth in an *in vivo* model as well its effects in the tumor microenvironment by IFN-γ overexpression directly on the tumor cells by gene transfection. DA-3 mammary tumor cell lines were transfected with pCDNA3.1-IFN-γ using Lipofectamine 2000 as described in Materials and methods and later cloned by limiting dilution in order to select the IFN-γ-transfected cells with the highest IFN-γ production by ELISA. As control, empty vector was also cloned in DA-3 cells. As shown in Fig. 1A, several clones were obtained and clone F1 was selected for this study by its elevated expression of IFN-γ. Furthermore, before implantation of IFN-γ transfected DA-3 mammary tumor cell line *in vivo*, we evaluated whether this IFN-γ overexpression could have any affect on the proliferative properties of the tumor cell line. As shown in Fig. 1B, IFN-γ stable-transfectants did not influence the *in vitro* growth characteristics of DA-3 cells when compared to empty vector-transfected DA-3 cells.

Antitumor effect of IFN-γ overexpression in the tumor cells. To investigate the *in vivo* contribution of IFN-γ overexpression in the tumor cells during tumor development, mice were injected with $1x10^6$ IFN-γ transfected DA-3 cells or $1x10^6$ empty vector-transfected DA-3 cells as controls. Tumor development was observed during 4 weeks. Although both groups of mice showed initial tumor growth, mice injected with IFN-γ transfected DA-3 cells had a significantly reduced primary tumor size when compared to control mice (Fig. 2A). Moreover, mice injected with IFN-γ transfected DA-3 cells had significantly prolonged survival as compared to DA-3 tumor bearers with >75% of the mice remaining alive for >60 days (Fig. 2B).

Since the mammary tumor model used forms spontaneous tumor metastasis in the lung, we next examined the effect of IFN-γ overexpression in the tumor on the development of lung metastasis. As shown in Fig. 2C and D, mice injected with empty vector-transfected DA-3 cells showed a higher number of metastasis as well as an augmented lung volume when compared to mice injected with DA-3 cells expressing high levels of IFN-γ, or normal mice.

Decreased VEGF expression on the plasma of IFN-γ-transfected DA-3 tumor-bearing mice. In previous studies we have shown that DA-3 tumor bearers produce several factors that affect the hosts' immunity. In this regard, the tumor used in our studies, as well as T lymphocytes from tumor bearers produce VEGF which is detected at high levels in their sera (20). Thus, we investigated whether the overexpression of IFN-γ in the tumor induces changes in the levels of VEGF in the blood of tumor bearers. Plasma from normal, DA-3 tumor bearers and IFN-γ-transfected DA-3 tumor-bearing mice were collected as described in Materials and methods and the VEGF levels were evaluated by ELISA. As can be seen in Fig. 3, VEGF is expressed at high levels in DA-3 tumor bearers when compared to normal mice as previously described (17). However, when VEGF levels were analyzed in the plasma of IFN-γ-transfected DA-3 tumor bearers, its expression decreased towards normal levels. These data suggest that the antitumor effect of IFN-γ

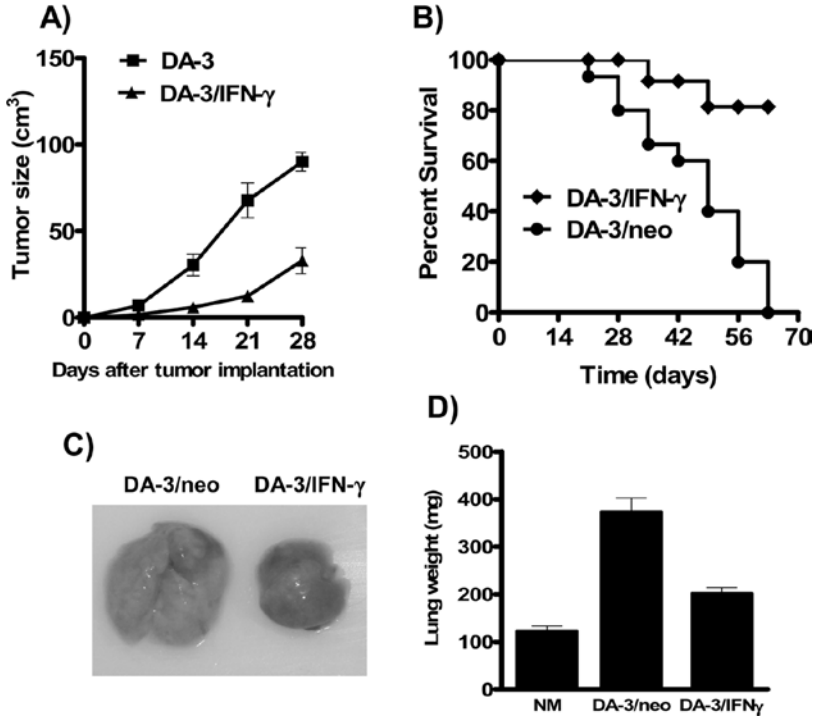

Figure 2. Effect of IFN-γ overexpression during the tumor development. BALB/c mice were injected with DA-3/neo or DA-3/IFN-γ and tumor growth (A) and percent of survival (B) were determined at different days after tumor implantation. (B). Lungs from these two groups of mice were collected on the same day. The presence of lung metastases in IFN-γ transfected DA-3 tumor bearers was determined after lung morphology evaluation (C) as well as by lung volume when compared to mice injected with DA-3/neo cells, or normal mice (D). For all tumor studies, the results from one experiment consisting of 10 mice/treatment group are shown and results are representative of three similar experiments.

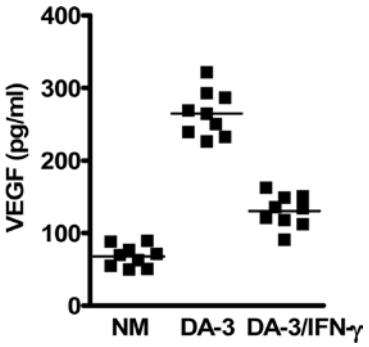

Figure 3. VEGF expression from tumor-bearing mice following IFN-γ overexpression in the tumor. Mice were injected with IFN-γ transfected DA-3 and DA-3/neo cells as described in Materials and methods. Normal untreated mice were used as controls. Four weeks after tumor injection, plasma from the different mice was collected as described in Materials and methods. Plasma levels of VEGF were assessed by ELISA. Data shown are the mean ± SD of three independent experiments.

could be related to a diminution and/or blockage on tumor-derived factors secretion.

Antitumor effect of IFN-γ overexpression is associated with normal levels of leukocyte recruitment in the tumor microenvironment of IFN-γ-transfected DA-3 tumor-bearing mice. Tumor-induced immune suppression involves the accumulation of suppressive infiltrates of regulatory T-cells (Tregs) and myeloid derived suppressor cells (MDSC) in the spleen of tumor bearers (21-23). Since the studies above demonstrated an overall significantly reduced tumor development, we hypothesized that part of the anti-tumor effect could be also due to a reduction in the regulatory immune cell accumulation. To determine the possible contribution of IFN-γ to the leukocyte recruitment during the tumor-induced immune suppression, we analyzed the profile of leukocytes in normal, DA-3 tumor bearers and IFN-γ-transfected DA-3 tumor-bearing mice (Fig. 4). Macrophages and spleen cells of the different groups of mice were collected as described in Materials and methods. As shown in Fig. 4A, analysis of the leukocyte subsets in normal and DA-3 tumor-bearing mice showed a decrease in total numbers of peritoneal macrophages as well as an augmentation in total numbers of T cells in the spleens when compared to normal mice. Interestingly, when IFN-γ-transfected DA-3 tumor-bearing mice were analyzed, these populations were very similar to those observed in normal mice. Next, we characterized the profile of the T cell subpopulations in response to the IFN-γ overexpression by flow cytometry. As can be seen in Fig. 4B and C, the percentages of $CD4^+$ and $CD8^+$ populations were diminished in DA-3 tumor bearers when compared to normal mice. However, when T cells from mice injected with IFN-γ-transfected DA-3 cells were analyzed, only $CD8^+$ cells reached levels similar to those observed in normal mice. In contrast, the percentage of $CD4^+$ T cells did not increase, maintaining levels similar to those observed in DA-3 tumor bearers (Fig. 4B and D).

T regulatory (Tregs) cells and myeloid-derived suppressor cells (MDSC) have been identified as functional suppressor cells within the tumor microenvironment (24-26). Therefore, we further examined these populations in the different mice by flow cytometry. As shown in Fig. 4C, consistent with other investigators, the spleens of tumor bearers have an augmented

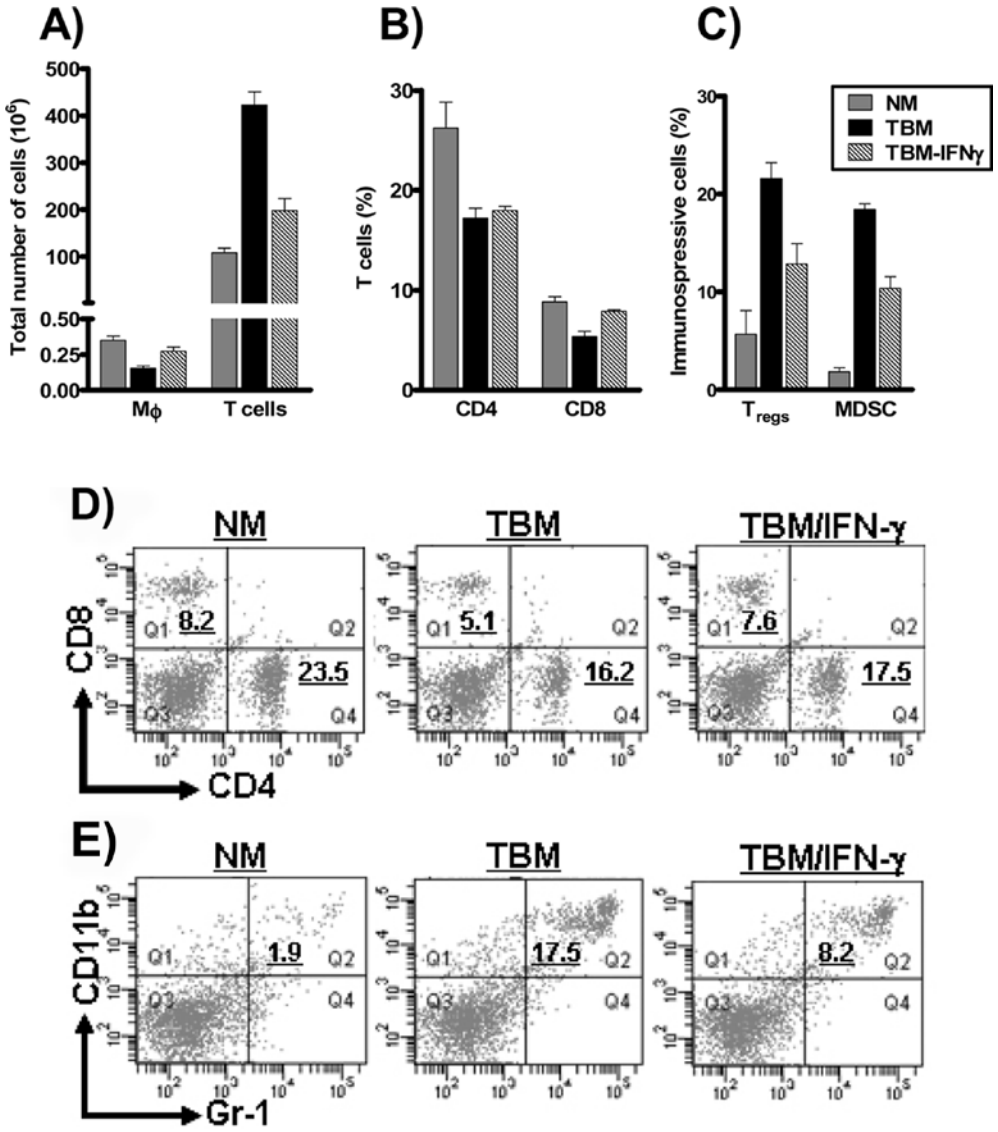

Figure 4. Antitumor effect of IFN-γ overexpression is associated with normal levels of leukocyte recruitment in the tumor microenvironment. Mice were injected with IFN-γ transfected DA-3 and DA-3/neo cells as described in Materials and methods. Four weeks after tumor injection, the total number of peritoneal macrophages as well as the total T cells in the spleens of tumor bearers was evaluated in the different tumor bearers and compared to normal mice (A). Splenocytes from the different mice were stained with antibodies anti-CD4 and anti-CD8 to calculate the percent of each T cell subsets by flow cytometry (B and D). Splenic cells were immunostained with anti-CD4, -CD25, and Foxp3, for the detection of mouse regulatory T cells or anti-Ly-6C and -CD11b for myeloid derived suppressor cells (C and E). Data represent the mean ± SD (A, B and C) or are representative (D and E) of 4-5 mice/group in three separate experiments.

percentage of Tregs in comparison to the levels observed in the spleens from normal mice (21.6±2.8 vs 5.6±2.4). Interestingly, when the spleens from mice injected with IFN-γ-transfected DA-3 cells were analyzed, the percentages of Tregs were diminished (12.8±3.6) when compared to those from DA-3 tumor bearers, although it never reached to levels observed in the spleen of normal mice (Fig. 4C). In addition, when the MDSC populations were analyzed, the spleens of DA-3 tumor-bearing mice showed a greatly increased percentage of these cells (18.4±1.2), when compared to the spleens of normal mice (1.8±0.5). However, when the spleens from mice implanted with IFN-γ-transfected DA-3 cells were analyzed, the percentages of myeloid derived suppressor cells were diminished (10.3±2.1) when compared to DA-3 tumor bearers, although as was the case with Tregs, never reached to the low levels observed in the spleens of normal mice (Fig. 4C and E). These data suggest that an effect of IFN-γ overexpression on the tumor cells could be to limit the recruitment of regulatory cells to the tumor microenvironment.

Reduction in Tregs and MDSC accumulation is associated with a down-regulation of chemokines implicated in the recruitment of immune regulatory cells. In previous investigations, we have documented that mammary tumor development is accompanied by a significant down-regulation of IFN-γ in splenic T cells from mammary tumor-bearing mice (14,27). Thus, we first evaluated whether the overexpression of IFN-γ in the tumor affects the expression of IFN-γ in the splenocytes of IFN-γ-transfected DA-3 tumor-bearing mice. To this end, splenocytes from normal, DA-3 tumor bearers and IFN-γ-transfected DA-3 tumor-bearing mice 4 weeks after tumor inoculation were isolated as described in Materials and methods. Splenocytes from the different mice

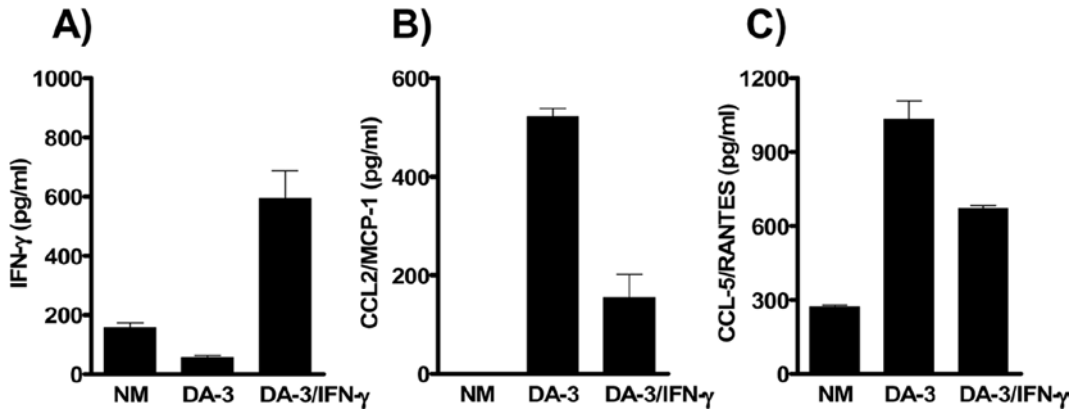

Figure 5. IFN-γ overexpression in the tumor is associated with a down-regulation of chemokines implicated in the recruitment of immune regulatory cells. Mice were injected with IFN-γ transfected DA-3 and DA-3/neo cells as described in Materials and methods. Four weeks after tumor injection, total splenic T cells from DA-3/neo or IFN-γ transfected tumor-bearing and normal mice, as control, were activated for 48 h with anti-CD3 antibody (A) or ConA (B and C). (A) IFN-γ, (B) CCL2/MCP-1 and (C) CCL5/RANTES production was quantified by ELISA. Data represent the mean ± SD of 4-5 mice/group in three separate experiments.

were activated with α-CD3 and IL-2 during 48 h, and their level of IFN-γ was assessed by ELISA. As shown in Fig. 5A, consistent with our previous findings (27), the expression of IFN-γ in DA-3 tumor bearer splenocytes is diminished when compared to the expression of this cytokine in the spleens from normal mice. However, when splenocytes from IFN-γ-transfected DA-3 tumor-bearing mice were analyzed, the expression of IFN-γ was significantly elevated when compared to DA-3 tumor bearers, and even higher to those observed in normal mice.

The studies described above suggest that the prevention of immunosuppression in mammary tumor-bearing mice by IFN-γ overexpression could be closely related, to a decrease of immune regulatory cell accumulation in the tumor bearers. We hypothesized that the reduced numbers of Tregs and MDSC could be due in part to a reduction in chemoattractant signals for these cells. Therefore, we analyzed the splenocytes from the different types of mice for the expression of CCL2/MCP-1 and CCL5/RANTES, two chemokines implicated in the recruitment of immune regulatory cells (28,29). As shown in Fig. 5B and C, the expression of CCL2/MCP-1 and CCL5/RANTES were significantly elevated in splenocytes from tumor-bearing mice when compared to those from normal mice. Interestingly, when splenocytes from IFN-γ-transfected DA-3 tumor-bearing mice were analyzed, the levels of CCL2/MCP-1 and CCL5/RANTES were greatly diminished when compared to those from DA-3 tumor bearers, although never reached the low levels observed in normal mice (Fig. 5B and C). These data suggest that the reduced number of regulatory immune cells in the spleen of DA-3-transfected tumor bearers could be related to an IFN-γ-dependent downregulation of chemokines implicated in the recruitment of immune regulatory cells.

Antitumor effect of IFN-γ prevents the thymic involution and impaired T cell development of mammary tumor-bearing mice. In previous studies we have shown that during mammary tumorigenesis, there is a profound thymus involution associated with a severe depletion of the most abundant subset of thymocytes, $CD4^+CD8^+$ double positive (DP) immature cells (30). To investigate whether the IFN-γ overexpression-caused anti-tumor effect also prevents the thymic involution associated with the tumor development, thymuses from normal mice, DA-3 tumor bearers, and mice injected with IFN-γ-transfected DA-3 cells were analyzed. Mice were sacrificed 4 weeks after inoculation with $1x10^6$ DA-3 or IFN-γ-transfected DA-3 cells. Normal mice were used as controls. Thymuses from the different mice were dissected as described in Materials and methods and the thymic lobes were weighed and their cell numbers were assessed. As shown in Fig. 6A and B, thymuses from DA-3 tumor bearers were dramatically decreased in size and cell number when compared to those of normal mice, as previously described (16). However, thymuses from tumor-bearing mice that were injected with IFN-γ-transfected DA-3 cells did not demonstrate the characteristic thymic atrophy of tumor bearers. Their thymuses resembled the size and weight of those of normal mice (Fig. 6A). Moreover, when cell number was determined, the thymic hypocellularity in DA-3 tumor-bearing mice was not apparent in the thymuses from tumor bearers injected with IFN-γ-transfected DA-3 cells (Fig. 6B).

Further analysis of the thymic subsets in normal and tumor-bearing mice as well as in those of tumor bearers injected with IFN-γ-transfected DA-3 cells was performed by flow cytometry. Fig. 6C shows that within the total thymus population of DA-3 tumor-bearing mice there is a diminished percentage of double positive ($CD4^+CD8^+$) cells and an increase in the percentages of ($CD4^+CD8^-$) and ($CD4^-CD8^+$) single populations and double negative ($CD4^-CD8^-$) subsets, as previously described (16). However, it should be emphasized that the absolute numbers of all these populations were diminished in DA-3 tumor bearer thymuses (data not shown). Interestingly, when the thymuses from mice injected with IFN-γ-transfected DA-3 cells were analyzed, the percentages of double positive ($CD4^+CD8^+$), single positive ($CD4^-CD8^+$) and ($CD4^+CD8^-$) and double negative ($CD4^-CD8^-$) populations were very similar to those observed in the thymuses of normal mice (Fig. 6C). Moreover, the absolute numbers of all these thymic populations were found to be similar to those of thymuses from normal mice (data not shown). Collectively, these data suggest that in addition to a decreased immune regulatory cell accumulation, the antitumor effect caused by the IFN-γ overexpression also prevents the thymic involution and the impaired T cell development present in the thymuses of DA-3 mammary tumor-bearing mice.

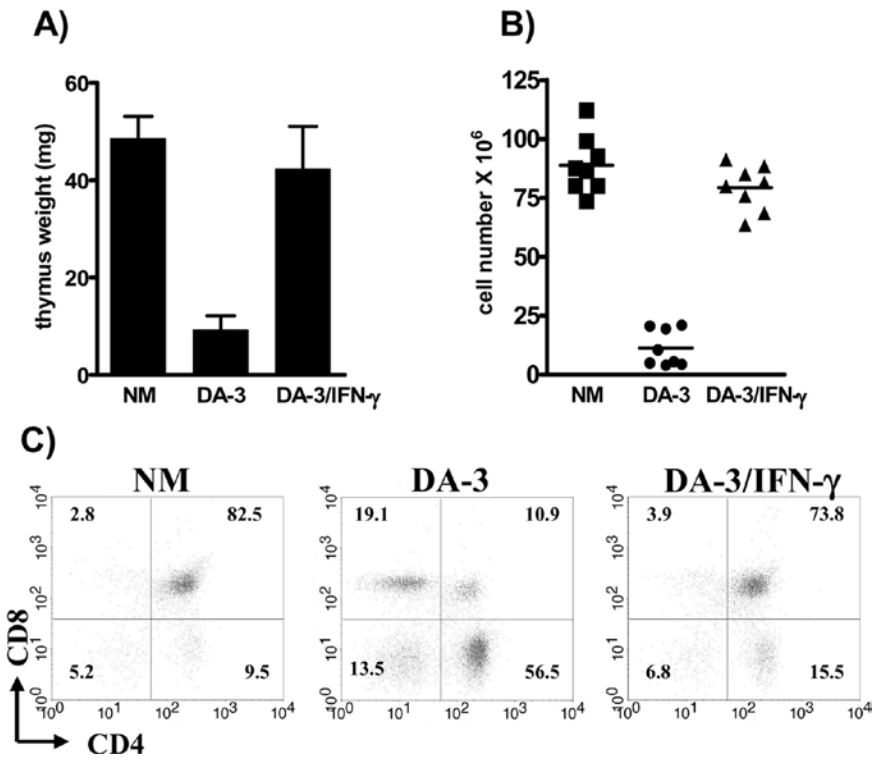

Figure 6. Thymic involution in tumor-bearing mice is mostly prevented by IFN-γ overexpression in the tumor. Mice were injected with IFN-γ transfected DA-3 and DA-3/neo cells as described in Materials and methods and were analyzed 3.5 weeks after tumor injection. Thymus weight (A) and thymic cell numbers (B) of DA-3/neo tumor bearers and mice injected with IFN-γ-transfected DA-3 cells were compared to normal mice. (C) Thymocytes from the different mice were stained with antibodies anti-CD4 and anti-CD8 to compare their phenotypes by flow cytometry. Data represent the mean ± SD (A and B) of three independent experiments each with 4-5 animals per group or representative (C) of three different experiments.

Discussion

We have previously reported a profound immunosuppression and a progressive thymic atrophy associated with the development of a murine mammary adenocarcinoma (D1-DMBA-3), and an *in vitro* cell line derived from it (DA-3), implanted in BALB/c mice (9,13). We have also shown that the tumor-induced immuno-suppression observed in these mammary tumor bearers is associated with an accumulation of myeloid-derived suppressor cells in several peripheral organs (10) as well as a decrease in the IFN-γ peripheral circulation and its production by T cells (27). A number of studies have revealed an important role for IFN-γ in tumor immunity (31-33). It has also been shown that IFN-γ has direct anti-proliferative, apoptotic, and anti-angiogenic effects that could complement its indirect effects on anti-tumor immunity (4). From these reports it is apparent that IFN-γ has a multiplicity of actions, one or more of which may be operating in host defense against a particular tumor.

Cancer-induced immunosuppression is a major problem as it reduces the anti-tumor effects of immunotherapies. In cancer tissues, cancer cells, immune cells, and other stromal cells interact and create an immunosuppressive microenvironment through a variety of mechanisms. Cancer immunotherapy is a form of treatment that aims to improve the ability of a cancer-bearing individual to reject the tumor immunologically. However, anti-tumor immunity elicited by the host or by immunotherapeutic strategies, can be actively attenuated by mechanisms that limit the strength and/or duration of immune responses, including the presence of immunoregulatory cell types or the production of immunosuppressive factors. In the present study we have used our DA-3 mammary tumor model to explore whether the overexpression of IFN-γ in the tumor has any role in anti-tumor activity, and tumor-induced immunosuppression observed in these mice. To this aim, we performed implantation of IFN-γ transfected DA-3 mammary tumor cell line *in vivo*, to provide constant, local production of IFN-γ during the tumor development.

Our results demonstrate clear differences in the antitumor responses in mice implanted with IFN-γ transfected DA-3 cells, as compared to those implanted with DA-3 cells. Moreover, as expected, the antitumor activity observed in IFN-γ transfected DA-3 tumor bearers, is also related to a significant reduction in lung metastasis as well in the mortality of these mice. Our results agree with a significant amount of evidence showing that endogenously produced IFN-γ plays a major role in regulating tumor development, not only by promoting protective host defenses to tumors but also orchestrating responses in hosts that facilitates their escape from immune attack (34-36).

Previous work from our laboratory reported that the tumor cells used in our studies secrete several molecules that we have shown to have effects in various compartments of the immune system. Among these factors are prostaglandin E_2 (PGE_2) (11,12), angiogenesis factor(s) (12), phosphatidyl serine (PS) (37), and vascular endothelial growth factor (VEGF) (20). The possibility that the IFN-γ overexpression may be down-regulating the expression of tumor-derived factors that could be preventing the tumor-induced immune suppression, cannot be excluded at present. In this regard, our results demonstrated a decreased expression of VEGF in the plasma of IFN-γ transfected DA-3 tumor bearers when compared to DA-3 tumor-bearing mice. Using a rheumatoid arthritis model,

Mathieu *et al* (38) showed that IFN-γ also inhibits the release of PGE2 and its receptors in whole blood and in normal and inflammatory cell populations. Moreover, in a previous study, Sorensen *et al* (39) showed that the IFN-γ produced within or by the tumor suppresses the expression of the VEGFR3, one of the three receptors that bind members of the proangiogenic endothelial growth factor (VEGF) family.

Another important finding from our study was to demonstrate a central role for IFN-γ in regulating the peritoneal and spleen cellular composition after therapy. When compared to DA-3 tumor bearers, IFN-γ-transfected DA-3 tumor-bearing mice showed a population of total T cells and peritoneal macrophages comparable to that observed in normal mice. Second, and perhaps more interesting, was the effects of IFN-γ overexpression in the reduction of Tregs and MDSC accumulation within the spleen of tumor bearer's. At the present we do not know the mechanisms by which IFN-γ provokes a reduction in regulatory T cells and myeloid-derived suppressor cell accumulation in tumor bearers. The possibility exists that IFN-γ is acting via reduction in chemoattractant signals for these cells. Therefore, we analyzed the splenocytes from the different types of mice for the expression of CCL2/MCP-1 and CCL5/RANTES, two chemokines implicated in the recruitment of immune regulatory cells (28,29). To this aim, splenocytes from the different mice were activated with α-CD3 and IL-2 or LPS during 48 h and further analyzed. In previous studies, we have shown that splenocytes from DA-3 tumor bearers are characterized with diminished or inexistent expression of IFN-γ when compared to splenocytes from normal mice. In this report we revealed that T cells from the spleen of IFN-γ-transfected DA-3 tumor-bearing mice are characterized by an elevated expression of IFN-γ when compared to normal mice and thus, to DA-3 tumor bearers. Moreover, when the levels of CCL2/MCP-1 and CCL5/RANTES were determined, these levels were extensively diminished in IFN-γ-transfected DA-3 tumor-bearing mice when compared to those from DA-3 tumor bearers, although never reached the low levels observed in normal mice. One hypothesis for this diminished expression in CCL2/MCP-1 and CCL5/RANTES could be that the down-regulated expression on VEGF observed in IFN-γ-transfected DA-3 tumor-bearing mice affects their expression. This argument is supported by Sow and colleagues who reported that VEGF induced the expression of these chemokines (40). Moreover, the fact that these chemokines never reached the low levels observed in normal mice could be explained by other reports which demonstrated that CCL2/MCP-1 and CCL5/RANTES are strongly induced *in vitro* by IFN-γ (41). Overall, these results suggest that the reduced numbers of immune regulatory cells in the IFN-γ-transfected DA-3 tumor-bearing mice could be in part due to a reduction in chemoattractant signals for these cells.

We have reported that the thymic involution and impaired T cell development observed in our mammary tumor-bearing mice are associated with an arrest in at least two steps of T cell differentiation (15,16); changes in the levels of crucial cytokines expressed in the thymus microenvironment (17); as well as a progressive increase in apoptosis during the tumor development (16); and an alteration in the interferons and Jak/Stats signaling pathways (18). Moreover, our previous studies suggest that thymic stromal cells from tumor bearers may not produce the necessary cytokines for appropriate T cell development possible due to the action of tumor-derived factors. The studies presented herein suggest that the marked delay in tumor growth of IFN-γ transfected tumor bearers may be closely related to the thymus involution and impaired T cell development observed in mammary tumor bearers. To gain insight into this possibility, we performed thymus analysis of these mice. In this regard, in IFN-γ-transfected DA-3 tumor-bearing mice, we observed a lack of involution and hypocellularity in the thymuses of these mice when compared to DA-3 tumor bearers. Moreover, the phenotypes of the various thymic subsets, and the absolute numbers of all these thymic populations, were similar to those observed in the thymuses of normal mice. Although the mechanisms for the thymic involution and impaired T cell development present in our model are not completely elucidated, based on our studies some possibilities can be discussed. One cause for these phenomena may be a decline in tumor derived factors expressed during the tumor development. This argument is supported by our observations showing a diminished VEGF expression in IFN-γ-transfected tumor-bearing mice. In agreement with our results, Lipnik and colleagues have suggested that the overexpression of IFN-γ reduces the expression of VEGF-A protein in mammary tumors *in vivo* (42). Moreover, we have recently showed that injection of hepatocyte growth factor (HGF) into mice implanted with mammary tumors resulted in normalization of the levels of VEGF and thymic volume (17). Based on the data presented herein we suggest that the overexpression of IFN-γ in the tumor cells could inhibit the tumor growth and the secretion of tumor-derived factors. Since in mammary tumor-bearing mice there is an up-regulated production of several factors that affect the hosts' immunity, a concomitant breakdown of this process may be resulting in the normal expression of chemokines implicated in the recruitment of immune regulatory cells and also prevents the thymic involution associated with the tumor development.

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