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IFN- γ Can Promote Tumor Evasion of the Immune System In Vivo by Down-Regulating Cellular Levels of an Endogenous Tumor Antigen

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IFN- γ -Dependent Inhibition of Tumor Angiogenesis by Tumor-Infiltrating CD4⁺ T Cells Requires Tumor Responsiveness to IFN- γ ¹

Gregory L. Beatty and Yvonne Paterson²

The importance of CD4⁺ T cells in the induction of an optimal antitumor immune response has largely been attributed to their ability to provide costimulatory signals for the priming of MHC class I-restricted CD8⁺ CTL. However, many reports have demonstrated a requirement for CD4⁺ T cells in the effector phase of tumor rejection indicating a greater responsibility for CD4⁺ T cells in controlling tumor outgrowth. We demonstrate here a critical role for CD4⁺ T cells in restraining initial tumor development through the inhibition of tumor angiogenesis. Using a tumor variant that is unresponsive to IFN- γ , we show that tumor responsiveness to IFN- γ is necessary for IFN- γ -dependent inhibition of tumor angiogenesis by CD4⁺ T cells. These studies reveal a pivotal role for CD4⁺ T cells in controlling early tumor development through inhibition of tumor angiogenesis. *The Journal of Immunology*, 2001, 166: 2276–2282.

The foundation of immunotherapy is based on the ability of the immune system to specifically identify tumor cells through the recognition of tumor-specific Ags. The T cell arm of the immune system defines this tumor specificity. Tumor Ags expressed by tumor cells are recognized by both CD4⁺ and CD8⁺ T cells. Although in many models CD8⁺ T cells in the absence of CD4⁺ T cells are effective in eliminating tumors (1–3), the role of CD4⁺ T cells is recognized to be more than provision of help for the induction of CD8⁺ CTL. Indeed, in some models CD4⁺ T cells in the absence of CD8⁺ T cells are effective in eliminating tumor cells (4–7). Although CD4⁺ cytolytic T cells that lyse MHC class II⁺ tumors have been identified in both rodents and humans (8–11), the ability of CD4⁺ T cells to promote rejection of MHC class II-negative tumors suggests that CD4⁺ T cells may use additional effector mechanisms in promoting tumor rejection (4, 12, 13). Consequently, because most tumors of non-hemopoietic origin do not express MHC class II molecules, a major contribution of CD4⁺ T cells in tumor immunity has been attributed to their secretion of cytokines (13, 14).

CD4⁺ T cells can be divided into two subpopulations, Th1 and Th2, based on the profile of secreted cytokines. Th1 cells predominantly produce IL-2 and IFN- γ , whereas Th2 cells produce IL-4 and IL-10 (15, 16). Although both Th1 and Th2 cells can be effective in promoting tumor rejection, Th1 cells are more effective in the generation and maintenance of strong immunological memory (17). Indeed, IFN- γ secretion by CD4⁺ T cells has been proposed to be important in vivo for the anti-tumor effects of CD4⁺ T cells (18, 19). IFN- γ can enhance MHC class I and II expression (19, 20), inhibit tumor cell proliferation (21, 22), activate macro-

phages (23–25), inhibit the production of immunosuppressive molecules (26, 27), and enhance the secretion of antiangiogenic chemokines (28–30). However, recent studies by us (31) and others have also shown that IFN- γ has the potential to modulate tumor cells to prevent the presentation of tumor Ags either through the down-regulation of tumor Ag protein expression or by less efficient processing of tumor Ags upon IFN- γ -dependent induction of the immunoproteasome (32).

Given the apparent opposing effects that IFN- γ can display, we sought to further investigate the role of IFN- γ in a tumor system in which we previously described the ability of IFN- γ to down-regulate the protein expression of an immunodominant tumor Ag (31). In this system, IFN- γ down-regulation of tumor Ag expression promotes tumor escape through loss of tumor recognition by CD8⁺ CTL. Using this model, we demonstrate here that IFN- γ secretion by CD4⁺ T cells controls early tumor outgrowth by inhibiting tumor angiogenesis before the involvement of CD8⁺ CTL.

Materials and Methods

Mice and cell lines

Female BALB/c mice, 6–8 wk of age, were obtained from Charles River Breeding Laboratories (Wilmington, MA). BALB/cByJ SCID mice, 6–8 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). CT26 is an *N*-nitroso-*N*-methylurethane-induced colon carcinoma that is syngeneic to BALB/c (33). CT26.IFN was constructed as previously described (31) by transducing CT26 cells with a retroviral vector bearing a mutant IFN- γ gene that has been modified to include coding sequences for the carboxyl-terminal endoplasmic reticulum retention signal Lys-Asp-Glu-Leu (KDEL) to obtain CT26.IFN (34). CT26.mugR cells were constructed by transfecting CT26 with a plasmid encoding a truncated form of the IFN- γ R α -chain as previously described (22).

Vaccinations and tumor growth in vivo

Irradiated (15,000 rad) CT26 (2×10^6) cells were inoculated s.c. in the right flank of BALB/c mice. After 21 days, naive or immune mice were challenged with 1×10^5 CT26 cells s.c. in the left flank (day 0 in all experiments). Where indicated, 0.2 mg anti-CD4 (GK1.5), 0.2 mg anti-CD8 (2.43), or 0.5 mg anti-IFN- γ (XMG1.2) mAb were injected i.p. on days -1, 0, 1, 3, 7, and 14 post tumor challenge. We have shown using a control Ab (GL117.41) specific for *Escherichia coli* β -galactosidase that this depletion protocol does not influence the growth of a similar CT26-derived tumor (35). Mice were monitored three times per week for the development of tumor nodules. In each experiment, eight mice were used

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per group. Tumorigenicity studies using CT26, CT26.IFN, and CT26.mugR were conducted by injecting 5×10^5 cells s.c. in the left flank. Mice were monitored three times per week, and the mean tumor diameter was recorded from two perpendicular diameters of the tumor mass.

In vitro tumor cell proliferation assay

To measure cell proliferation in vitro, 3×10^6 /ml cells were first labeled with $5 \mu\text{M}$ CFSE (Molecular Probes, Eugene, OR) in PBS for 10 min at room temperature (36). Staining was stopped with an equal volume of FCS, and the cells were washed two times with RPMI 1640 containing 10% FCS and penicillin/streptomycin (complete medium). Cells were cultured in complete medium for 24, 48, 72, 96, and 120 h. At the appropriate time point, cells were harvested and fixed in 2% paraformaldehyde (pH 7.4) before analysis by flow cytometry.

Matrigel angiogenesis assay

Angiogenesis assays were conducted by injecting BALB/c or BALB/cByJ SCID mice s.c. with 0.5 ml Matrigel (Collaborative Biomedical Products, Bedford, MA) (37) mixed with CT26, CT26.IFN, or CT26.mugR cells in the right flank (day 0 in all experiments). Immune mice were generated by immunizing BALB/c mice with 2×10^6 irradiated (15,000 rad) CT26 cells 14 days before implantation with Matrigel mixed with tumor cells. Where indicated, 0.2 mg anti-CD4 (GK1.5), 0.2 mg anti-CD8 (2.43), or 0.5 mg anti-IFN- γ (XMG1.2) mAb was injected i.p. on days -1, 0, 1, and 3. Where indicated, 30 μl anti-asialo GM1 (Wako Chemicals, Richmond, VA) or 30 μl normal rabbit serum was injected i.v. on days -1, 0, 1, 3, and 5. Depletion of CD4, CD8, and NK cells was confirmed on day 7 after Matrigel implantation by flow cytometric analysis of spleens, tumor draining lymph nodes, and Matrigel plugs for CD4 $^+$, CD8 $^+$, and DX5 $^+$ (a pan NK cell marker) cells. Seven days after implantation, Matrigel pellets were harvested, surrounding tissue was dissected away, and the pellets were liquefied by incubation at 4°C overnight in 300 μl PBS. To quantify angiogenesis, hemoglobin content of the liquefied pellets was assayed by the Drabkin method (Sigma Diagnostics, St. Louis, MO) as described (38).

Flow cytometry

Infiltration of cells into Matrigel pellets was analyzed by double staining with FITC-anti-CD45 and PE-anti-CD8 β or PE-anti-CD4 and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA). For detection of cytoplasmic cytokine expression by infiltrating cells, 2×10^6 cells were cultured per well in a 96-well round-bottom plate for 7 h in the presence of Brefeldin A. Cells were stained with PE-anti-CD4 mAb and Cy5-anti-CD45, fixed and permeabilized with Cytotfix/CytoPerm Solution (PharMingen, San Diego, CA), and stained with FITC-anti-IFN- γ mAb. The percentage of cells expressing cytoplasmic IFN- γ was determined by flow cytometry.

Results

An early role for IFN- γ in the effector phase of tumor immunity

We undertook this study to further investigate the role of IFN- γ in a tumor model in which we had previously shown that IFN- γ decreases tumor immunogenicity (31). To identify whether IFN- γ can play a positive role in this tumor model, BALB/c mice that were immunized with 2×10^6 irradiated CT26 cells s.c. in the right flank were treated with mAbs against IFN- γ and challenged with 1×10^5 live CT26 cells. As shown in Fig. 1, IFN- γ plays an important role early in the effector phase of tumor immunity as demonstrated by the early onset of tumor growth in ~90% of mice. In 50% of mice receiving treatment with mAbs against IFN- γ , tumor growth is similar to that observed in naive mice. However, as some tumors are observed to regress, it is clear that the role of IFN- γ is not essential later in the effector phase of tumor immunity. This finding is consistent with our previous work demonstrating that IFN- γ decreases CT26 tumor immunogenicity allowing for escape from CD8 $^+$ CTL recognition (31), although other explanations for this observation are possible.

Tumor responsiveness to IFN- γ reduces tumorigenicity

Because the observation of early tumor outgrowth in the absence of IFN- γ could be the result of a direct effect of IFN- γ on tumor cellular proliferation, we assessed the role of IFN- γ in slowing

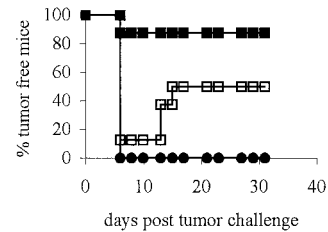


FIGURE 1. Tumor immunity against CT26 tumor cells requires IFN- γ during the early stage of effector phase. BALB/c mice were immunized with saline (●) or 2×10^6 irradiated CT26 (squares) s.c. on day -14. On day -1, immunized mice were treated with i.p. injection of either saline (■) or 500 μg of anti-IFN- γ XMG1.2 (□). Mice were then s.c. injected with 1×10^5 CT26 on day 0 with CT26 tumor cells. Saline or Ab treatment was given on days -1, 0, 1, 3, 7, and 14. The differences in tumor incidence between immune mice treated with saline and anti-IFN- γ Abs at day 10 is statistically significant by Fisher's Exact test ($p < 0.05$).

tumor growth both in vitro and in vivo using two previously described variants of CT26 called CT26.IFN and CT26.mugR. CT26.IFN overexpresses a mutant IFN- γ that is targeted to the endoplasmic reticulum allowing for signaling within the tumor cell but no secretion (34), whereas CT26.mugR expresses a dominant negative form of the IFN- γ R α -chain rendering the cell unresponsive to IFN- γ (22). Using these two variants of CT26, we show in Fig. 2A that CT26 responsiveness to IFN- γ is associated with slowed in vivo tumor growth in naive mice. The significant difference in tumor growth observed between CT26.mugR and CT26 suggests the in vivo presence of IFN- γ in the tumor microenvironment. In other tumor models, IFN- γ has been reported to slow tumor growth by inhibiting tumor cellular proliferation (21, 22) or

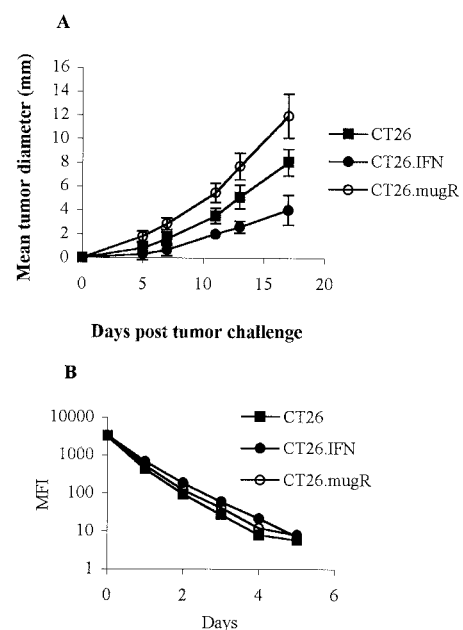


FIGURE 2. Tumor responsiveness to IFN- γ reduces tumorigenicity. A, Naive BALB/c mice were s.c. injected with 5×10^4 CT26 (■), CT26.IFN (●), or CT26.mugR (○). Tumor growth was monitored every 2–3 days. Data are represented as mean tumor diameter \pm SEM of six mice per group. B, CT26 (■), CT26.IFN (●), and CT26.mugR (○) were labeled with CFSE and subsequently cultured in vitro for the indicated time periods. At each time point, cells were assessed by flow cytometry for loss of CFSE, an indication of cell division. The mean fluorescence intensity is presented. No difference in the rates of proliferation between cell lines was observed.

by inducing apoptosis (39). To examine whether IFN- γ may have a direct effect on CT26 growth, we compared the in vitro growth rates of CT26, CT26.IFN, and CT26.mugR. We found the growth rate of each cell line to be similar in vitro. In addition, no effect on CT26 in vitro growth was observed in the presence of exogenous IFN- γ (data not shown). Therefore, CT26 responsiveness to IFN- γ reduces CT26 tumorigenicity.

IFN- γ slows tumor growth by inhibiting tumor angiogenesis

Because previous work has demonstrated the importance of tumor responsiveness to IFN- γ for the inhibition of tumor angiogenesis (22, 39), we hypothesized that IFN- γ in the CT26 tumor model may be controlling tumor outgrowth by inhibiting tumor angiogenesis. To investigate this possibility, we used in vivo Matrigel assays to measure the extent of angiogenesis stimulated by CT26, CT26.IFN, and CT26.mugR in both naive and immune mice. Using hemoglobin content as an index of Matrigel vascularization, tumor cells were mixed with Matrigel matrix and implanted s.c. into naive or immune BALB/c mice. This assay demonstrated no effect on the structure or survival aspects of the tumor cells as determined by analysis of histological sections of Matrigel-tumor plugs (data not shown). In naive mice CT26.mugR induces a higher degree of vascularization compared with CT26 (Fig. 3A),

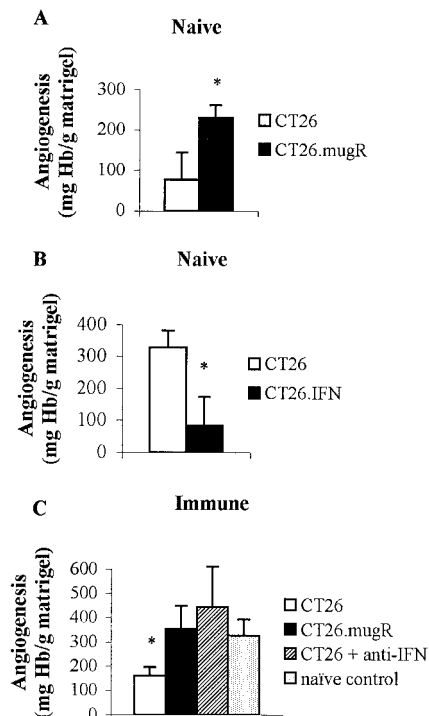


FIGURE 3. IFN- γ -dependent inhibition of tumor angiogenesis requires tumor responsiveness to IFN- γ . *A*, Naive mice were s.c. injected with Matrigel containing 10^5 CT26 or CT26.mugR cells. On day 7 the Matrigel plugs were harvested, and angiogenesis was quantitated by determining the hemoglobin content of individual pellets. *, $p = 0.03$. *B*, Naive mice were s.c. injected with Matrigel containing 5×10^5 CT26 or CT26.IFN cells. Matrigel plugs were analyzed on day 7 as described above. *, $p = 0.015$. *C*, Mice were immunized with 2×10^6 irradiated CT26 s.c. on day -14. On day 0 immune mice were s.c. injected with Matrigel containing 5×10^5 CT26 or CT26.mugR cells. Where indicated, anti-IFN- γ XMG1.2 was administered i.p. on days -1, 0, 1, 3, and 5. Naive mice s.c. injected with Matrigel containing 5×10^5 CT26 cells served as control. Matrigel plugs were harvested on day 7 and analyzed as described above. Asterisks indicate groups significantly different ($p < 0.05$) by Student's *t* test from naive control.

whereas CT26 induces a higher degree of vascularization compared with CT26.IFN (Fig. 3B). In addition, immune mice are more efficient than naive mice in inhibiting tumor angiogenesis stimulated by CT26. This inhibition of angiogenesis requires tumor responsiveness to IFN- γ and can be abrogated by treatment with mAbs against IFN- γ (Fig. 3C). These results demonstrate the potential for IFN- γ to slow CT26 outgrowth by inhibiting tumor angiogenesis.

CD4⁺ T cell secretion of IFN- γ inhibits tumor angiogenesis

The main cellular producers of IFN- γ are NK cells, NKT cells, Th1 CD4⁺ T cells, and TC1 CD8⁺ T cells (24). We first assessed the roles of NK cells and T cells as cellular producers of IFN- γ responsible for inhibiting tumor angiogenesis by implanting CT26 cells mixed with Matrigel s.c. into naive BALB/c or BALB/cByJ SCID mice. A higher degree of vascularization was observed in SCID mice compared with naive BALB/c mice indicating that T cells are essential for inhibiting tumor angiogenesis (Fig. 4A). Furthermore, NK cells were not required for inhibiting tumor angiogenesis because no effect on Matrigel vascularization was apparent with anti-asialo GM1 depletion. However, by depleting CD4⁺ and CD8⁺ cells we found that CD4⁺ T cells were essential for inhibiting tumor angiogenesis (Fig. 4A). We next addressed whether

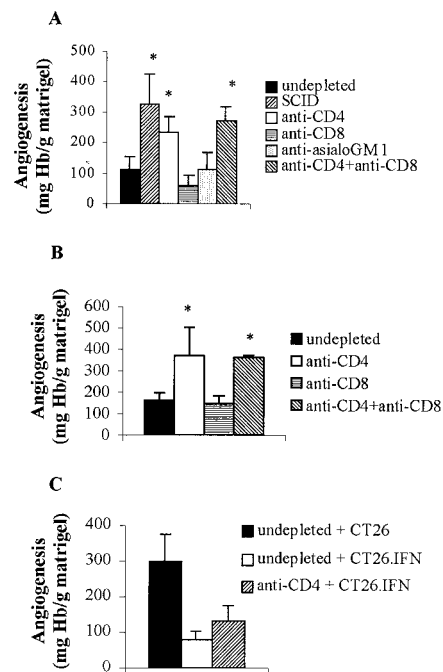


FIGURE 4. IFN- γ -dependent inhibition of angiogenesis requires CD4⁺ T cells. *A*, On day 0 naive BALB/c mice or SCID mice were s.c. injected with Matrigel containing 10^5 CT26 cells. *B*, Immune BALB/c mice were s.c. injected with Matrigel containing 5×10^5 CT26 cells. *C*, Naive BALB/c mice were s.c. injected with Matrigel containing 5×10^5 CT26.IFN cells. Where indicated, Abs against CD4, CD8, or asialo GM1 were administered i.p. on days -1, 0, 1, 3, and 5. Immune mice were obtained by immunizing with 2×10^6 irradiated CT26 s.c. on day -14. On day 7 Matrigel plugs were harvested and assessed for hemoglobin content as an index of angiogenesis. *A*, Undepleted naive BALB/c mice receiving no Ab served as control. Asterisks indicate groups significantly different ($p < 0.05$) by Student's *t* test from undepleted naive BALB/c mice. *B*, Undepleted immune mice receiving no Ab served as control. Asterisks indicate groups significantly different ($p < 0.05$) by Student's *t* test from undepleted immune mice. *C*, Naive BALB/c mice s.c. injected with Matrigel containing 5×10^5 CT26 served as control. No significant difference ($p > 0.05$) by Student's *t* test was observed between mice injected with Matrigel containing CT26.IFN and treated with or without Abs against CD4.

CD4⁺ T cells were required for the enhanced inhibition of tumor angiogenesis observed in immune mice. In the absence of CD4⁺ T cells, CD8⁺ T cells were still observed to infiltrate the tumor at comparable levels to mice with an intact immune system (data not shown). However, depletion of CD4⁺ T cells abrogated the ability of immune mice to inhibit tumor angiogenesis. Depletion of CD8⁺ cells either alone or in combination with depletion of CD4⁺ cells had no effect on tumor angiogenesis, indicating that CD4⁺ T cells were solely responsible for the enhanced inhibition of tumor angiogenesis observed in immune mice (Fig. 4B).

To address whether the role of CD4⁺ T cells in the inhibition of tumor angiogenesis was simply to provide a source of IFN- γ for signaling in the tumor cell, we compared the requirement for CD4⁺ T cells for the decreased ability of CT26.IFN cells to stimulate angiogenesis compared with CT26. Although CD4⁺ cells are clearly important for decreasing the ability of CT26 to stimulate angiogenesis (Fig. 4A), the decreased ability of CT26.IFN to stimulate angiogenesis does not require CD4⁺ T cells (Fig. 4C). This result emphasizes the role for CD4⁺ T cell secretion of IFN- γ for inhibition of tumor angiogenesis.

CD4⁺ T cell secretion of IFN- γ slows early tumor outgrowth by inhibiting tumor angiogenesis

As the aforementioned results suggest that CD4⁺ T cells are the source of IFN- γ responsible for inhibiting angiogenesis, we sought to confirm this hypothesis by analyzing tumor-infiltrating CD4⁺ T cells for their ability to secrete IFN- γ . As shown in Fig. 5, 7 days after tumor implantation into immune mice tumor-infiltrating CD4⁺ T cells are observed to secrete IFN- γ . However, at 7 days post implantation we also observed CD8⁺ T cells to be secreting IFN- γ in some but not all of the tumors analyzed. Therefore, we hypothesized that CD4⁺ T cells may infiltrate the tumor before CD8⁺ T cells and provide an early source of IFN- γ necessary for inhibiting tumor angiogenesis. To test this hypothesis, CT26 cells in Matrigel were implanted s.c. in immune mice and analyzed 3, 5, and 7 days later for the presence of infiltrating CD4⁺ and CD8⁺ T cells. We found that CD4⁺ T cells infiltrate the tumor earlier than CD8⁺ T cells in both naive (Fig. 6A) and immune mice (Fig. 6B). In addition, we observed no difference in the levels of CD4⁺ and CD8⁺ T cells infiltrating CT26 compared with CT26.mugR tumors (data not shown). Tumor infiltration by CD4⁺ T cells commences as early as day 3 and peaks by day 5, whereas infiltration by CD8⁺ T cells is not appreciated until day 7. Finally, the increased levels of CD4⁺ T cells infiltrating tumors in immune mice

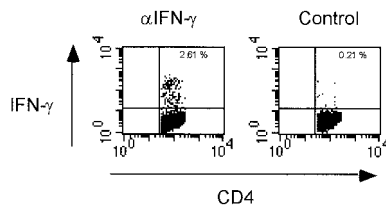


FIGURE 5. Secretion of IFN- γ by tumor-infiltrating CD4⁺ T cells inhibits tumor angiogenesis. BALB/c mice were immunized with 2×10^6 irradiated CT26 cells s.c. on day -14. On day 0 immune mice were s.c. injected with Matrigel containing 10^5 CT26 cells. Seven days later, Matrigel plugs were harvested, and single cell suspensions were prepared. Tumor-infiltrating cells were cultured in the presence of Brefeldin A for 7 h and subsequently analyzed by flow cytometry. Cells were first stained for surface expression of CD4 and then for intracellular expression of IFN- γ using either an anti-IFN- γ Ab (*left*) or control isotype-matched Ab (*right*). The percentage of CD4 cells secreting IFN- γ is shown in the top right corner of each plot.

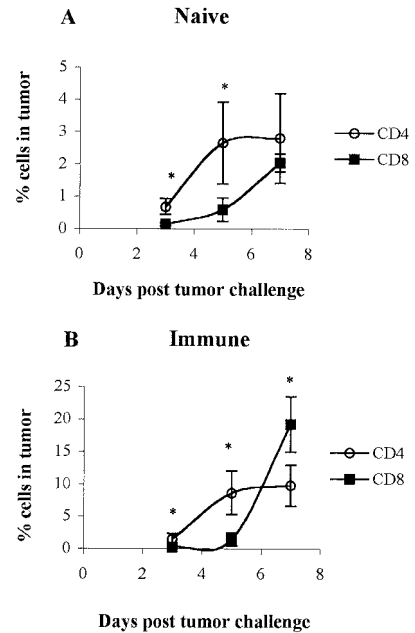


FIGURE 6. CD4⁺ T cells infiltrate tumor before CD8⁺ T cells. Naive (A) or immune (B) mice were s.c. injected with Matrigel containing 10^5 CT26 cells on day 0. Immune mice were obtained by immunizing with 2×10^6 irradiated CT26 cells s.c. on day -14. On days 3, 5, and 7 Matrigel plugs were harvested, and single cell suspensions were prepared. Tumor-infiltrating cells were analyzed by flow cytometry for CD4⁺ and CD8⁺ cells. Data are presented as the mean percentage of live cells \pm SEM. For each time point, three tumors from three individual mice were analyzed. Asterisks indicate a significant difference ($p < 0.05$) by Student's t test between the percentages of CD4⁺ and CD8⁺ tumor-infiltrating cells.

likely explains the enhanced inhibition of tumor angiogenesis observed compared with naive mice.

We have shown that CD4⁺ T cell secretion of IFN- γ is required for the early inhibition of angiogenesis in the CT26 model. Our results would predict that in the absence of CD4⁺ T cells, immune mice challenged with CT26 would display an early onset of tumor growth potentially followed by regression due to the infiltration of tumor-specific CD8⁺ CTL (Fig. 6B). To test this hypothesis, BALB/c mice immunized with 2×10^6 irradiated CT26 cells were depleted of CD4⁺ cells and challenged with 1×10^5 CT26. As shown in Fig. 7, ~90% of mice display an early onset of tumor outgrowth followed by complete regression of tumors in all mice. These results establish a role for CD4⁺ T cells beyond providing

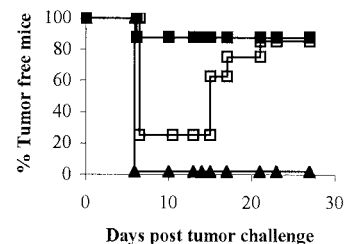


FIGURE 7. CD4⁺ T cells are required for early inhibition of tumor growth. BALB/c mice were immunized with 2×10^6 irradiated CT26 s.c. on day -14. On day 0 mice were challenged s.c. with 10^5 CT26 cells. Immune mice were treated i.p. on days -1, 0, 1, 3, 7, and 14 with either saline (■) or an Ab against CD4 (□). Naive mice (▲) challenged s.c. with 10^5 CT26 cells served as control. The difference in tumor incidence between immune mice treated with saline and anti-CD4 Abs at day 10 ($p < 0.05$) but not day 25 is statistically significant by Fisher's Exact test.

help for the induction of CD8⁺ CTL and emphasize the importance of CD4⁺ T cells in controlling early tumor outgrowth before the infiltration of CD8⁺ CTL.

Discussion

An expanding volume of evidence exists defining a critical role for CD4⁺ T cells in antitumor immunity. Although many studies have focused on the role of CD4⁺ T cells in the provision of help for the induction of CTLs (40–43), it is clear that tumor-specific CD4⁺ T cells can orchestrate several effector mechanisms in an antitumor immune response (13, 14). Evidence provided from cell-based vaccine models demonstrates that CD4⁺ T cells can coordinate several antitumor effector pathways independent of CTLs (14), whereas adoptive transfer studies illustrate the critical importance of CD4⁺ T cells in antitumor immunity (4–6). Although positive evidence for the involvement of both Th1 and Th2 CD4⁺ cells is apparent, the critical importance of CD4⁺ T cell-derived IFN- γ , a Th1 cytokine, is suggested from several studies (4, 14, 18). However, as CD8⁺ T cells and NK cells are capable of secreting IFN- γ , the significance of CD4⁺ T cell-derived IFN- γ has remained ill defined. In this study, we provide evidence that tumor infiltration of CD4⁺ T cells precedes CD8⁺ T cells and provides an early source of IFN- γ critical for the control of tumor growth by inhibiting tumor angiogenesis.

This report demonstrates the essential role for CD4⁺ T cells in the inhibition of tumor angiogenesis. Although it is understood that NK cells can harbor a critical role in anti-tumor immunity (44, 45) and may be important in the inhibition of tumor angiogenesis induced by IL-12 therapy (44), we have found that NK cells are not required for inhibition of tumor angiogenesis using a cell-based vaccine strategy. This finding may be a consequence of the high level of MHC class I expression present on CT26, because MHC class I molecules are capable of negatively regulating NK cell activity (46–48). Nonetheless, the finding that CD4⁺ T cells can participate during the effector phase of an antitumor immune response against an MHC class II-negative tumor is consistent with previous adoptive transfer studies and underscores the importance of APCs in processing and presenting tumor Ags (49, 50).

The importance of CD4⁺ T cells during the effector phase of an immune response to tumors has previously been attributed to the local release of cytokines that recruit and activate tumoricidal macrophages, eosinophils, and other cellular populations (14). The role of CD4⁺ T cell secretion of IFN- γ has thus been linked to the activation of macrophages to secrete TNF- α and NO (23–25). However, IFN- γ is also known to induce monocytes, macrophages, fibroblasts, and even some tumor cells to produce two CXC chemokines, monokine-induced by γ -IFN (MIG) and IFN-inducible protein 10 (IP-10), that are known to exert potent anti-angiogenic activity (29, 30, 51). Our findings that tumor cell responsiveness to IFN- γ is essential for CD4⁺ T cell-dependent inhibition of tumor angiogenesis is consistent with the possibility that CD4⁺ T cell-derived IFN- γ induces tumor cells to secrete angiogenesis inhibitors. Indeed, we have also found that immune mice are effective in inhibiting tumor angiogenesis stimulated by a mixture of IFN- γ -unresponsive (CT26.mugR) and -responsive (CT26) tumor cells, which is consistent with this scenario (G.L.B and Y.P., unpublished data).

Unlike many tumor models, where neutralization of IFN- γ is followed by complete loss of therapeutic efficacy (39, 52, 53), in the CT26 tumor model a significant level of protection against tumor challenge is maintained in the absence of IFN- γ . Although several interpretations are imaginable, we ascribe this phenomenon to our previously reported finding that IFN- γ can promote tumor escape through the down-regulation of an immunodominant en-

dogenous tumor Ag (31). However, the importance of IFN- γ in this model clearly extends beyond just regulation of tumor angiogenesis and tumor Ag expression, otherwise treatment of immune mice with mAbs against CD4 and IFN- γ should yield similar results. This observation implies a significant yet nonessential role for CD8⁺ T cell secretion of IFN- γ . Indeed, CD8⁺ T cells infiltrating the CT26 tumor are observed to secrete IFN- γ . It is possible that production of IFN- γ by CD8⁺ T cells, which infiltrate after CD4⁺ T cells (Fig. 6), is important for the maintenance of macrophage activation and the production of other molecules such as TNF- α and NO that are tumoricidal (23–25). Alternatively, IFN- γ may inhibit the secretion of immunosuppressive molecules such as TGF- β (54), which is constitutively secreted by CT26 and down-regulated by IFN- γ (G.L.B. and Y.P., unpublished results). Nonetheless, our findings demonstrate a delicate balance of positive and negative effects associated with IFN- γ in providing efficient tumor immunity.

Several tumor models have demonstrated the importance of tumor responsiveness to IFN- γ in defining therapeutic efficacy (21, 22, 31, 39). However, Mumberg et al. have shown that although elimination of MHC class II-negative tumor cells by adoptive transfer of tumor-specific CD4⁺ T cells requires IFN- γ for efficacy, tumor rejection is not dependent on tumor sensitivity to IFN- γ (4). This observation implies that host cells are the targets of IFN- γ rather than tumor cells, which is the finding of this paper. Indeed, while preparing this manuscript, another study was published that defined a role for IFN- γ secreted potentially by CD4⁺ T cells in the rejection of an MCA-induced tumor (55). Similar to the results observed by Mumberg et al., effective tumor immunity in this model did not require tumor responsiveness to IFN- γ . In addition, tumor angiogenesis did not require tumor responsiveness to IFN- γ , in contrast to our findings where the major impact on tumor angiogenesis was directly attributable to the ability of tumor cells to respond to IFN- γ . Qin et al. demonstrated that IFN- γ responsiveness by nonhemopoietic cells during the effector phase of tumor immunity was essential for inhibition of tumor-induced angiogenesis (55). Although both CD4⁺ and CD8⁺ T cells were found to infiltrate the tumor, effective tumor immunity required CD4⁺ T cells and, to a lesser degree, CD8⁺ T cells. Based on these results, the authors inferred that CD4⁺ T cell secretion of IFN- γ might be required for inhibition of tumor angiogenesis, although the contribution of IFN- γ secreted by CD8⁺ T cells or even NK cells could not be eliminated. In our study, we provide the first report to definitively identify CD4⁺ T cells as essential for inhibition of tumor-induced angiogenesis that is likely dependent on the kinetics of tumor infiltration. As tumor responsiveness to IFN- γ alone (CT26.IFN) was sufficient for promoting inhibition of tumor angiogenesis (Fig. 4C) similar to immune mice, IFN- γ responsiveness by nonhemopoietic cells does not appear to be important in this model. Indeed, even immune mice were unable to inhibit angiogenesis of IFN- γ -unresponsive tumors, further decreasing the likelihood that IFN- γ responsiveness by nonhemopoietic cells provides an alternative mechanism for IFN- γ -dependent inhibition of tumor angiogenesis. It is not clear at this point what differences in the tumor models studied by Qin et al. and presented here account for the disparate cell populations required for inhibition of tumor angiogenesis. Nonetheless, we find that IFN- γ secreted by CD4⁺ T cells is required to promote inhibition of tumor angiogenesis likely through the induction of angiogenesis inhibitors by IFN- γ -responding tumor cells.

Inhibition of tumor-induced angiogenesis is a well-accepted mechanism for limiting tumor growth (56). Therefore, the finding that CD4⁺ T cells can inhibit tumor angiogenesis has significant implications in the design of current immunotherapeutic strategies.

Although CD4⁺ T cell responses are central to the regulation of virtually all Ag-specific immune responses, the incorporation of CD4⁺ T cells into the design of cancer vaccine strategies has been limited. This limitation has primarily been fueled by a lack of techniques for efficiently isolating and characterizing MHC class II-restricted tumor Ags. However, recent efforts in the development of new strategies to identify MHC class II-restricted tumor Ags is now facilitating the identification of several tumor-specific CD4⁺ T cell Ags (57). Although inhibition of angiogenesis alone by CD4⁺ T cells is not likely to result in tumor rejection, it offers an attractive mechanism for the immune system to acquire time to prime, activate, and recruit tumor-specific effector cells required for elimination of tumor cells. Indeed, CD4⁺ T cells have been implicated in the recruitment and activation of the tumoricidal activities of eosinophils and macrophages (14). Nonetheless, inhibition of tumor angiogenesis also discourages tumor progression and the development of metastatic potential (58–60). It is likely that a combination of strategies that limit tumor outgrowth by inhibiting angiogenesis while specifically targeting the killing of tumor cells will be required for the regression of many rapidly growing tumors. Therefore, the ability of CD4⁺ T cells to orchestrate an immune response by recruiting effector cells as well as by inhibiting tumor angiogenesis encourages the incorporation of CD4⁺ T cell-specific tumor Ags into current vaccine strategies.

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