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$CD4^+$ T Cell–Mediated Tumor Rejection Involves Inhibition of Angiogenesis that Is Dependent on IFNγ Receptor Expression by Nonhematopoietic Cells

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Summary

Immunity against MHC class II^- tumors can be mediated by $CD4^+$ T cells in the effector phase through an unknown mechanism. We show that this is IFNγ dependent but does not require IFNγ receptor (IFNγR) expression on tumor cells, T cells, or other hematopoietic cells and that IFNγR expression is not necessary in the priming phase. However, tumor immunity requires IFNγR expression on nonhematopoietic cells in the effector phase and involves inhibition of tumor-induced angiogenesis. This shows that an effective anti-tumor response involves communication between $CD4^+$ T cells and nonhematopoietic cells, most likely within the tumor stroma, and that tumor immunity must not entirely rely on direct tumor cell killing.

Introduction

Most tumor cells express MHC class I but not class II molecules and carry antigens that potentially can serve as rejection antigens. $CD8^+$ T cells usually recognize target antigens in an MHC class I–restricted fashion and have the ability to lyse tumor cells by direct cell-to-cell contact, whereas $CD4^+$ T cells usually recognize antigens presented by MHC class II molecules and only rarely have the potential to kill tumor cells. Therefore, it is reasonable to assume that $CD8^+$ cytolytic T lymphocytes (CTL) are important effector cells during tumor rejection. This view is supported by a number of experimental (Schreiber, 1999) and clinical data (Boon et al., 1994).

An unresolved question in tumor immunology is how $CD4^+$ T cells mediate tumor rejection. It is well known that $CD4^+$ T cells are often necessary for development of tumor immunity following immunization with tumor cells (Dranoff et al., 1993; Huang et al., 1994; Monach et al., 1995; Qin et al., 1998) or peptide antigens derived from the tumor (Ossendorp et al., 1998). Three possibilities of how $CD4^+$ T cells contribute to the development of tumor immunity have been postulated: (1) $CD4^+$ T cells are needed during the immunization (priming) phase and help CTL to develop (Qin et al., 1998). There is increasing evidence that CTL are often activated by antigen-presenting cells (APC), which take up, process, and present antigens of the tumor by MHC class I molecules (cross-priming) (Huang et al., 1994; Cayeux et al., 1997). For CTL priming, APC have to receive signals from $CD4^+$ T cells that have recognized tumor antigens presented by MHC class II molecules on the APC. This results in a reciprocal activation of APC and T cells by involvement of CD40/CD40-ligand interaction (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). (2) $CD4^+$ T cells are necessary for maintenance of CTL (Ossendorp et al., 1998), e.g., by providing cytokines such as IL-2. (3) In several tumor models, it has been shown that $CD4^+$ T cells are needed in the effector phase of an antitumor immune response against MHC class II^- tumors (Greenberg et al., 1981; Fujiwara et al., 1984; Greenberg et al., 1985; Hock et al., 1991; Dranoff et al., 1993; Hock et al., 1993; Monach et al., 1995; Hung et al., 1998). This was either shown by adoptive transfer of $CD4^+$ T cells to tumor bearing mice and demonstration of tumor rejection (Greenberg et al., 1985) or by depletion of $CD4^+$ T cells in immunized mice before challenge, showing that mice without $CD4^+$ T cells were unable to reject the challenge tumor (Dranoff et al., 1993; Hung et al., 1998). In these models, $CD8^+$ T cells were not needed at all for tumor rejection or at least part of the mice rejected the tumor in their absence. Because it is unlikely that $CD4^+$ T cells directly recognize MHC class II^- tumors, it has been proposed that they induce a delayed type hypersensitivity (DTH)-like reaction during which inflammatory cells like macrophages, granulocytes, or natural killer (NK) cells are attracted and activated by $CD4^+$ T cells and kill the tumor cells (Greenberg, 1991; Hung et al., 1998). For example, depletion of $CD4^+$ T cells resulted in abrogation of tumor rejection correlating with the absence of tumor infiltrating macrophages ($CR3^+$ cells) (Hock et al., 1991). However, direct tumor cell killing by $CD4^+$ T cell–activated innate effector cells in vivo has not been demonstrated.

It is likely that cytokines produced by effector $CD4^+$ T cells are important for tumor rejection. IFNγ is a key cytokine in cell-mediated immunity that is produced mainly by $CD4^+$ T helper 1 (Th1), $CD8^+$ T, and NK cells (Bach et al., 1997; Boehm et al., 1997). It has multiple biological activities on various cell types, consistent with the observation that the IFNγ-receptor (IFNγR) is expressed on almost all cell types (Valente et al., 1992; Farrar and Schreiber, 1993). A role of IFNγ for tumor rejection has been demonstrated (Dighe et al., 1994). IFNγ could have direct effects on tumor cells by (1) its cytotoxic activity on some tumor cells (e.g., together with TNF) (Williamson et al., 1983; Fransen et al., 1986), (2) upregulating MHC expression and thereby increasing tumor cell recognition and elimination (Dighe et al., 1994), or (3) inducing expression of angiogenesis inhibitors, like IP-10, by tumor cells (Coughlin et al., 1998). Alternatively, IFNγ may act in an immunoregulatory fashion and be important for Th1 development (Mosmann and Coffman, 1989) or activation of innate effector cells involved in tumor rejection (Murray et al., 1985). Yet another possibility is that IFNγ produced by T cells acts on nonhematopoietic cells within the tumor, which then indirectly contributes to tumor rejection. Here we demonstrate that an essential requirement for $CD4^+$ T cell–mediated tumor immunity is IFNγ production by T cells

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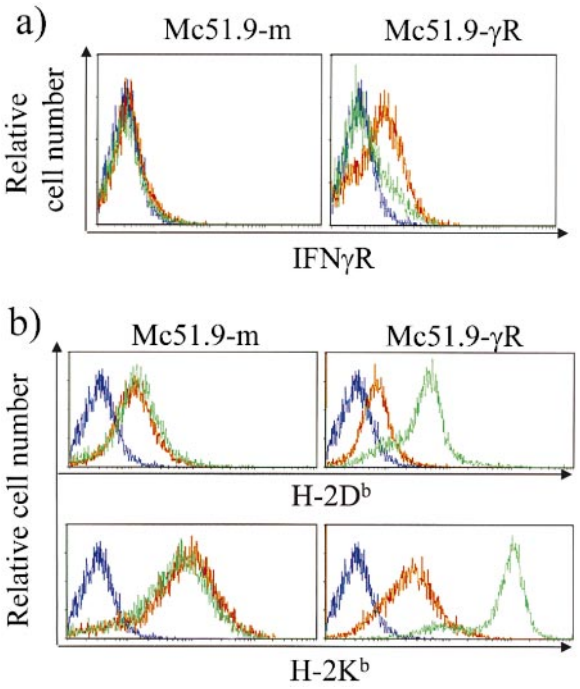


Figure 1. Functional Expression of IFNγR in Cell Line Mc51.9 Derived from an IFNγR$^{-/-}$ Mouse

(a) Cell surface IFNγR expression. Mock-transfected Mc51.9-m (left) and IFNγR plasmid-transfected Mc51.9-γR cells (right) were stained with an isotype-matched control mAb (blue) or the anti-IFNγR mAb GR-20 in the absence (red) or presence of 25 ng/ml IFNγ (green). PE-labeled rabbit anti-rat IgG2a was used as secondary mAb.

(b) IFNγ induced upregulation of MHC class I expression on Mc51.9-γR cells. Tumor cells were cultured for 24 hr in the absence (red) or presence of 25 ng/ml IFNγ (green) and stained with FITC-labeled mAb against H-2D^b (upper) or H-2K^b (lower). A FITC-labeled isotype-matched control mAb was used as control (blue).

and IFNγR expression by nonhematopoietic cells, which results in inhibition of tumor-induced angiogenesis.

Results

Tumorigenicity of IFNγR$^+$ and IFNγR$^-$ Tumor Cells

To analyze the mechanism by which CD4$^+$ T cells mediate tumor rejection and the relative contribution of IFNγR expression by tumor and different host cells, we established tumor models that allowed us to analyze the rejection of IFNγR$^+$ and IFNγR$^-$ tumor cells in IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice. Tumor cell lines (e.g., Mc51.9) induced by MCA in 129/Sv/Ev IFNγR$^{-/-}$ mice (H2^b) did not express IFNγR molecules. After transfection with an IFNγR expression plasmid (resulting in Mc51.9-γR cells) but not a control plasmid (resulting in Mc51.9-m cells), the cells expressed IFNγR on their surface (Figure 1a). Staining with anti-IFNγR mAb could be blocked by preincubation of the cells with IFNγ. Mc51.9 cells express MHC class I molecules (both H-2D^b and H-2K^b) (Figure 1b) and are negative for MHC class II (data not shown). The IFNγR was functionally expressed by Mc51.9-γR cells because exposure of these cells to IFNγ upregulated expression of MHC class I molecules (Figure 1b). IFNγ did not change MHC class I expression on Mc51.9-m cells. The expression of MHC class II molecules on Mc51.9-γR cells was not induced by IFNγ (data not shown). There was no difference in the in vitro proliferation rate between the IFNγ-responsive and -unresponsive cells, even when 0.5 to 500 ng/ml of IFNγ were added into the culture medium (data not shown).

To analyze whether IFNγR expression by tumor cells alters their tumorigenicity, 2×10^5 or 1×10^6 Mc51.9, Mc51.9-m, and Mc51.9-γR cells were injected into IFNγR$^{+/-}$ mice. Mc51.9 and Mc51.9-m cells grew with similar kinetics (Figures 2a and 2b). The growth of Mc51.9-γR cells was delayed in comparison to Mc51.9 or Mc51.9-m cells, reminiscent of results by Dighe et al. (1994). However, eventually all mice developed a tumor. In parallel, Mc51.9, Mc51.9-m, and Mc51.9-γR cells were injected into IFNγR$^{-/-}$ mice. As shown in Figures 2c and 2d, tumors grew with almost identical kinetics regardless of IFNγR expression by the tumor cells. Similar results were obtained when tumor lines Mc29.2 and Mc54.1 and their IFNγR-expressing variants were used (data not shown). This indicated that IFNγR expression by host cells was a prerequisite for the difference of tumor growth between IFNγ-responsive and -unresponsive tumor cells.

Generation of Tumor Immunity Requires IFNγR Expression by Host but Not Tumor Cells

To compare the relative importance of IFNγ responsiveness of tumor and host cells for generation of tumor immunity, IFNγR$^{+/-}$ or IFNγR$^{-/-}$ mice were immunized with irradiated Mc51.9-γR or Mc51.9-m cells or as control left untreated and 2 weeks later were contralaterally challenged with increasing numbers of parental tumor cells. IFNγR$^{+/-}$ mice immunized with Mc51.9-γR cells almost invariably rejected the Mc51.9 cell challenge (Figures 3a and 3b). Immunization of IFNγR$^{-/-}$ mice with Mc51.9-γR cells did not induce rejection of the challenge tumor, demonstrating that generation of tumor immunity required IFNγR expression on host cells. Furthermore, whereas IFNγR$^{-/-}$ mice immunized with Mc51.9-m cells were unable to reject Mc51.9 cells, IFNγR$^{+/-}$ mice immunized with Mc51.9-m cells rejected Mc51.9 cells as efficiently as those immunized with Mc51.9-γR cells, demonstrating that IFNγR expression by tumor cells used for immunization contributed little to tumor immunity in this model (Figures 3c and 3d). Challenge of immunized IFNγR$^{+/-}$ mice with an unrelated tumor did not lead to tumor rejection, indicating that immunity was tumor specific (data not shown).

To confirm the data with other tumors and exclude possible cell culturing artifacts, IFNγR$^{+/+}$ and IFNγR$^{-/-}$ mice were grafted with tumor fragments derived from hosts bearing primary MCA-induced tumors. Twelve to fourteen days later, tumors were surgically removed, and after another week a fragment of the same tumor passaged in nude mice was grafted onto the mice at a distant site. Altogether, five tumors (two from IFNγR$^{+/+}$ and three from IFNγR$^{-/-}$ mice) were analyzed. In IFNγR$^{+/+}$ mice, one of the two IFNγR$^+$ tumors and two of the three IFNγR$^-$ tumors were rejected (Figure 3e). In IFNγR$^{-/-}$ mice, all five tumors grew progressively regardless of whether their origin was from IFNγR$^{+/+}$ or IFNγR$^{-/-}$ mice. Thus, host but not tumor cells must express the IFNγR for development of tumor immunity. Furthermore, these results indicated that IFNγR expression was not necessary for tumor immunity on both the

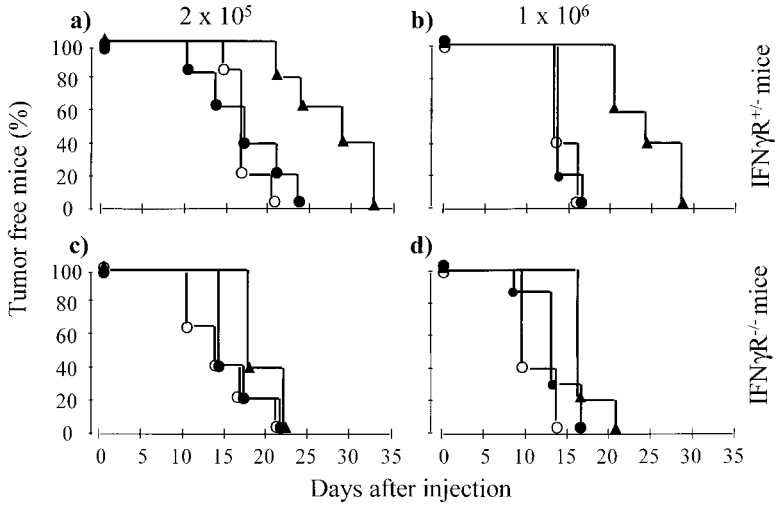


Figure 2. IFNγR Expression by Tumor Cells Reduces Tumorigenicity in IFNγR$^{+/-}$ but Not in IFNγR$^{-/-}$ Mice

IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice (5–6 mice/group) were s.c. injected with 2×10^5 (a and c) or 1×10^6 (b and d) Mc51.9 (open circles), Mc51.9-m (closed circles), or Mc51.9-γR cells (closed triangles). Tumor growth was monitored. Representative results from one out of four experiments with similar results are shown.

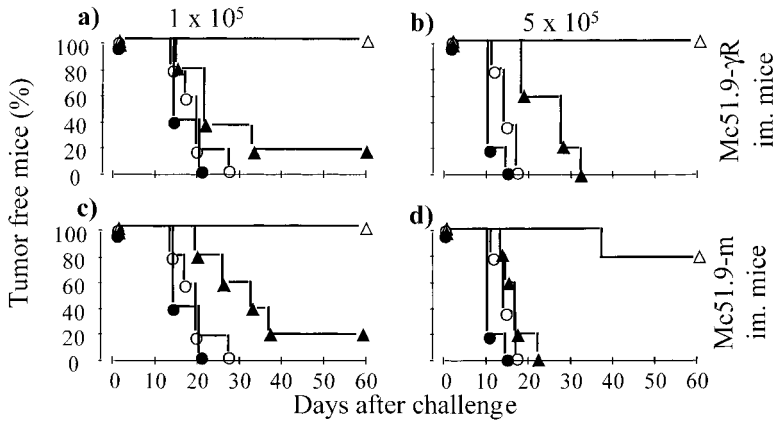
tumor cells used for immunization and those used for challenge.

Tumor Immunity Requires CD4$^+$ T Cells in the Effector Phase

Since the results above had shown that IFNγR expression by tumors contributes little to the generation of tumor immunity, subsequent experiments were performed with the IFNγR$^-$ tumor cell line Mc51.9. The contribution of T cell subsets in the effector phase of tumor immunity can variously be dominated by CD4$^+$ (Hung et al., 1998) or CD8$^+$ T cells (Hock et al., 1993; Qin et al., 1998). Therefore, CD4$^+$ or CD8$^+$ T cells were depleted in Mc51.9-immunized IFNγR$^{+/+}$ mice before challenge. All immunized control mice but none of the mice depleted of CD4$^+$ T cells rejected the challenge tumor, demonstrating that CD4$^+$ T cells were critically involved in the effector phase (Figure 4). Half of the mice depleted of CD8$^+$ T cells rejected the challenge tumor, half did not. Therefore, CD8$^+$ T cells appear to contribute to tumor rejection, but at least in part of the mice, tumor rejection occurred in their absence.

T Cell Responses to the Mc51.9 Tumor Are Not Impaired in IFNγR$^{-/-}$ Mice

To investigate whether the T cell response to the tumor was impaired in IFNγR$^{-/-}$ mice, spleen cells from IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice immunized twice with irradiated Mc51.9 cells were stimulated with tumor cell lysate and proliferation was measured. T cells of immunized IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice proliferated in response to the tumor cell lysate (Figure 5a). No significant proliferation was observed with spleen cells of naive mice. T cells from IFNγR$^{-/-}$ mice in fact proliferated



e)

Tumor	Tumor rejection in IFNγR$^{+/+}$ mice	Tumor rejection in IFNγR$^{-/-}$ mice
1	0/2 (13 ± 3 mm)	0/3 (18 ± 6 mm)
2	2/2*	0/2
3	2/2	0/2
4	1/3 (8 ± 4 mm)	0/3 (22 ± 4 mm)
5	2/2	0/2

Figure 3. Tumor Immunity Can Be Generated in IFNγR$^{+/-}$ but Not in IFNγR$^{-/-}$ Mice

(a–d) IFNγR$^{+/-}$ (open symbols) and IFNγR$^{-/-}$ mice (closed symbols) were left untreated (circles) or immunized (triangles) with 2×10^5 irradiated Mc51.9-γR (a and b) or Mc51.9-m cells (c and d) and 2 weeks later were contralaterally challenged with 1×10^5 (a and c) or 5×10^5 (b and d) Mc51.9 cells. Each group consisted of 5–6 mice. Similar results were obtained in three other experiments. For naive control mice, the same experiment is shown in (a) and (c) or (b) and (d), respectively. (e) Tumors were induced by MCA (1–2 in IFNγR$^{+/+}$ and 3–5 in IFNγR$^{-/-}$ mice). At a size of 11–12 mm, tumors were isolated and tumor fragments ($4 \times 4 \times 4$ mm) were grafted s.c. onto IFNγR$^{+/+}$ and IFNγR$^{-/-}$ mice (2–3/group) and onto a nude mouse. Tumors in IFNγR$^{+/+}$ and IFNγR$^{-/-}$ mice were subsequently removed at day 12–14, when they reached a size of 10 mm in diameter. One week later, the mice were challenged with a fragment of the same tumor ($4 \times 4 \times 4$ mm) passaged in nude mice. Shown are the numbers of mice that rejected the challenge tumor per total numbers of mice. For tumor 1 and 4, the mean tumor size and standard deviation at day 12 are also shown in parenthesis. Two tumor-free mice (asterisk) were challenged again with an unrelated tumor (tumor 5 from an IFNγR$^{-/-}$ mouse), which grew progressively in both cases.

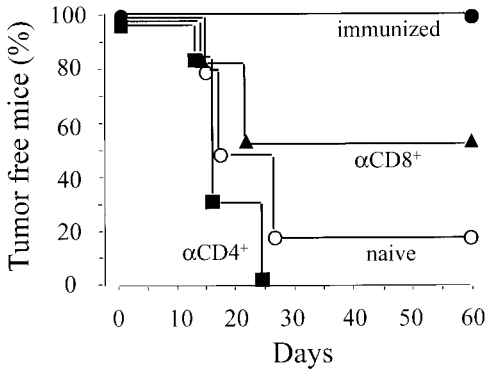


Figure 4. Tumor Immunity against Mc51.9 Tumor Cells Requires $CD4^+$ T Cells in the Effector Phase

$IFN\gamma R^{+/+}$ mice were immunized with 2×10^5 irradiated Mc51.9 cells and 14 days later were contralaterally challenged with 2×10^5 Mc51.9 cells. Depletion of $CD4^+$ (closed squares) or $CD8^+$ T cells (closed triangles) was started 3 days before the tumor challenge. Mice that were not T cell depleted (closed circles) and that were not immunized (open circles) served as control. Each group consisted of 8–10 mice.

more vigorously than those from $IFN\gamma R^{+/-}$ mice, indicating that their activation was not impaired. Because IFNγ production by host cells might be a prerequisite for tumor immunity in $IFN\gamma R^{+/-}$ mice, we analyzed the ability of $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice to produce IFNγ following immunization with Mc51.9 cells. Similar to the proliferation assay, spleen cells of immunized but not naive $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice secreted substantial amounts of IFNγ when stimulated with the tumor cell lysate (Figure 5b).

Because T cells in immunized $IFN\gamma R^{-/-}$ mice were activated and produced IFNγ, we analyzed whether T cells homed to the challenge site. Tumors in naive $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice contained few $CD4^+$ T cells 4 days after tumor cell injection (Figures 5c and 5d). At the same time, a clearly increased number of tumor-infiltrating $CD4^+$ T cells was detected in challenge tumors of immunized mice (Figures 5e and 5f). It appeared as if tumors in $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice were infiltrated by $CD4^+$ T cells to a similar extent, but subtle differences cannot be excluded. Similar results were obtained for $CD8^+$ T cells (data not shown). $Mac\text{-}1^+$ (data not shown) and $Gr\text{-}1^+$ cells (Figures 5g and 5h) massively infiltrated the tumor in immunized $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice 4 days after challenge, indicating that the inflammatory response to the challenge tumor was not dramatically impaired in $IFN\gamma R^{-/-}$ compared to $IFN\gamma R^{+/-}$ mice. In naive mice, clearly less inflammatory cells were observed in the tumor at that time point (data not shown).

Host Cells Other Than T Cells Must Express IFNγR for Inhibition of Tumor Growth

Because T cell activation and function appeared to be normal in immunized $IFN\gamma R^{-/-}$ mice and yet the mice were unable to reject a challenge tumor, we asked whether spleen cells from immunized $IFN\gamma R^{-/-}$ mice could inhibit tumor growth if transferred into $IFN\gamma R^{+/-}$ mice. $IFN\gamma R^{-/-}$ mice were immunized with irradiated Mc51.9 cells and spleen cells depleted of adherent cells were transferred into naive $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice. Three days later the mice were challenged with Mc51.9 cells and tumor growth was monitored. Untreated mice ($IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$) rapidly developed a tumor (Figure 6a). $IFN\gamma R^{-/-}$ mice that received spleen cells from immunized $IFN\gamma R^{-/-}$ mice developed a tumor with similar kinetics compared to control animals. Transfer of $IFN\gamma R^{-/-}$ spleen cells into $IFN\gamma R^{+/-}$ mice resulted in inhibition of tumor growth. In parallel, we transferred spleen cells from immunized $IFN\gamma R^{+/-}$ mice into $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice, which then were challenged with Mc51.9 cells. Transfer of $IFN\gamma R^{+/-}$ spleen cells into $IFN\gamma R^{+/-}$ mice resulted in inhibition of tumor growth (Figure 6b), a result similar to that found in the transfer of $IFN\gamma R^{-/-}$ spleen cells into $IFN\gamma R^{+/-}$ mice (Figure 6a). Transfer of $IFN\gamma R^{+/-}$ spleen cells into $IFN\gamma R^{-/-}$ mice had no inhibitory effect on tumor growth (Figure 6b). A similar result was obtained when spleen cells of naive or immunized $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice were transferred into SCID mice. Transfer of spleen cells from both immunized $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice inhibited tumor growth, whereas spleen cells from naive mice in either case had no effect (data not shown). Therefore, neither tumor nor spleen cells but other cells of the host must express IFNγR for inhibition of tumor growth. Because $CD4^+$ T cells are essential for tumor rejection in the effector phase, the results indicate that they must not express IFNγR to inhibit tumor growth, if other host cells are IFNγ responsive. Additionally, these experiments showed that IFNγR expression was necessary only in the effector but not in the priming phase.

Inhibition of Tumor Growth Requires IFNγR Expression on Nonhematopoietic Cells

In the following experiment, we asked whether hematopoietic cells or nonhematopoietic cells must express IFNγR. Lethally irradiated $IFN\gamma R^{-/-}$ (Figure 6c) and $IFN\gamma R^{+/-}$ mice (Figure 6d) were reconstituted with bone marrow cells from either $IFN\gamma R^{+/-}$ or $IFN\gamma R^{-/-}$ mice. Eleven weeks after bone marrow transplantation, the mice were immunized and challenged with Mc51.9 cells. Normal $IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-}$ mice served as control. Naive and immunized $IFN\gamma R^{-/-}$ mice rapidly developed a tumor (Figure 6c). Among the reconstituted animals, both $IFN\gamma R^{-/-} \rightarrow IFN\gamma R^{-/-}$ mice and $IFN\gamma R^{+/-} \rightarrow IFN\gamma R^{-/-}$ mice developed a tumor with similar kinetics, demonstrating that IFNγR expression on hematopoietic cells was not sufficient for tumor growth inhibition. In contrast, while all naive $IFN\gamma R^{+/-}$ mice developed a tumor at day 17, half of the immunized mice rejected the tumor (Figure 6d). Tumor growth in $IFN\gamma R^{+/-} \rightarrow IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-} \rightarrow IFN\gamma R^{+/-}$ mice was suppressed to a similar extent. There was no significant difference of tumor incidence between immunized $IFN\gamma R^{+/-}$ mice and chimeric $IFN\gamma R^{+/-} \rightarrow IFN\gamma R^{+/-}$ and $IFN\gamma R^{-/-} \rightarrow IFN\gamma R^{+/-}$ mice. In all three cases, tumor growth was delayed and at least part of the mice completely rejected the tumor. Thus, tumor growth inhibition required IFNγR expression by nonhematopoietic cells, whereas IFNγR expression by hematopoietic cells contributed little or not at all to tumor rejection.

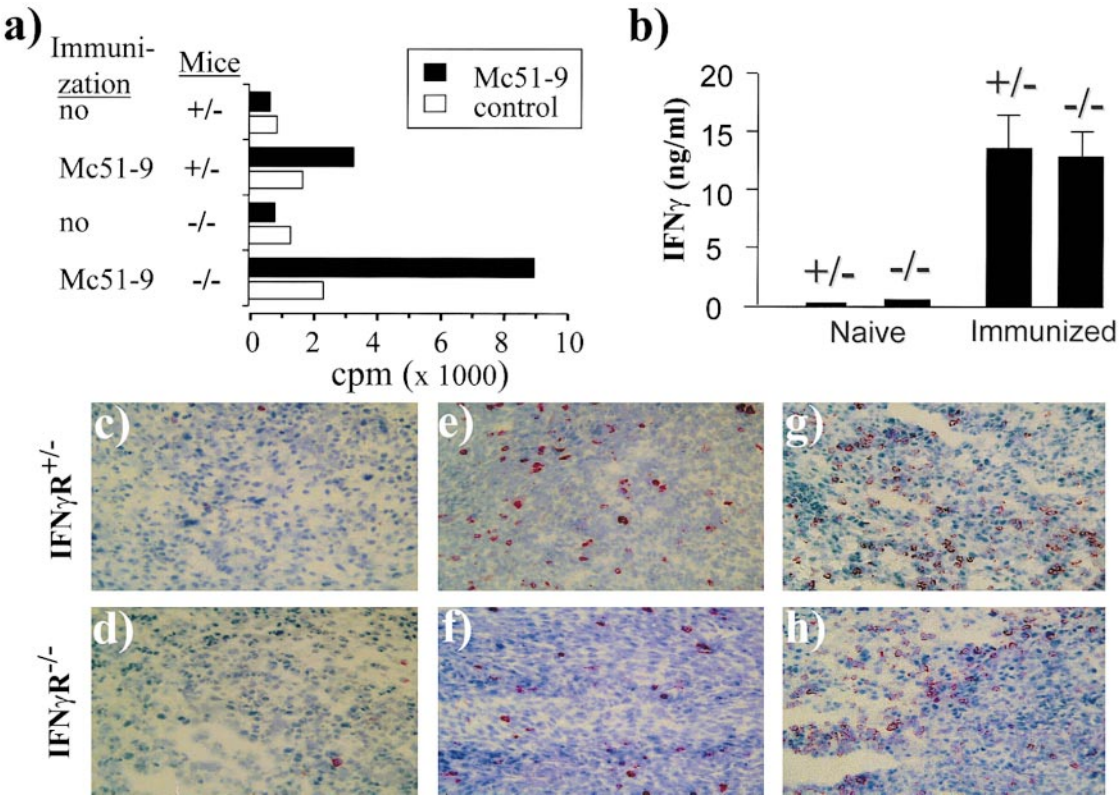


Figure 5. T Cell Responses to the Mc51.9 Tumor Are Normal in IFNγR$^{-/-}$ Mice

(a) T cell proliferation assay. IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice were left untreated or immunized twice with 2×10^5 irradiated Mc51.9 cells. Five days after the second immunization, spleen cells were isolated and stimulated with Mc51.9 tumor cell lysate at a spleen:tumor cell ratio of 40:1 for 5 days (closed bars). Spleen cell cultures without tumor cell lysate served as control (open bars). Proliferation was measured by a 12 hr ^{3}H-thymidine incorporation assay. One out of three experiments with similar results is shown.

(b) IFNγ production by immune spleen cells. Spleen cells from naive or Mc51.9-immunized mice were stimulated with tumor cell lysate, and IFNγ was determined by ELISA in culture supernatants. Shown are the representative results of three experiments.

(c–f) Infiltration of CD4$^+$ T cells into challenge tumors. IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice were left untreated (c and d) or immunized twice with 2×10^5 irradiated Mc51.9 cells (e and f). All mice were challenged with 1×10^6 Mc51.9 cells. Four days later tumor sections were prepared and stained for CD4$^+$ T cells. Magnification: 200×.

(g and h) Infiltration of Gr-1$^+$ cells into challenge tumors in IFNγR$^{+/-}$ (g) and IFNγR$^{-/-}$ (h) mice. Mice were treated as above and tumor sections were stained with anti-Gr-1 mAb. Magnification: 200×. Results of tissue sections are representative for 3–5 tumors per group.

Tumor Immunity Involves Inhibition of Angiogenesis

In both immunized IFNγ-R$^{+/-}$ and IFNγ-R$^{-/-}$ mice, T cells were activated, produced IFNγ, and homed to the challenge site (Figure 5), and IFNγR expression was necessary for tumor rejection only in the effector phase on nonhematopoietic cells (Figure 6). Therefore, we assumed that tumor immunity relied on IFNγR expression by cells within the stroma of MC51.9 tumors and analyzed tumor-induced blood vessel formation in naive and immunized IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice. Immunohistochemical analysis for the endothelial marker CD31 showed that tumors were already well vascularized in naive mice 4 days after inoculation. Angiogenesis typical for an established tumor was similarly seen in IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice (Figures 7a and 7b). In 4-day-old challenge tumors of immunized IFNγR$^{-/-}$ mice, tumor blood vessels similar to that in naive mice were observed (Figures 7d and 7f). In contrast, in challenge tumors of immunized IFNγR$^{+/-}$ mice, blood vessels within the tumor mass were completely absent (Figures 7c and 7e). At the border between tumor and adjacent tissue, they were visible; however, they were unable to grow into the tumor tissue. Correlating with the inability of blood vessels to grow into the tumor in immunized IFNγR$^{+/-}$ mice, large areas in the center of the tumor mass had already become necrotic at that time (Figure 7e). Thus, CD4$^+$ T cell–dependent tumor immunity involves tumor destruction indirectly by inhibition of angiogenesis.

Discussion

CD4$^+$ T cells play an important role not only in the priming but also in the effector phase of immune responses against MHC class II$^-$ tumors. It has been postulated that they induce a DTH-like reaction during which innate effector cells like macrophages (Greenberg, 1991) or NK cells (Levitsky et al., 1994) are attracted and activated by CD4$^+$ T cell–derived cytokines and subsequently kill the tumor cells. IFNγ is produced by CD4$^+$ Th1 cells (Mosmann and Coffman, 1989), is required for tumor

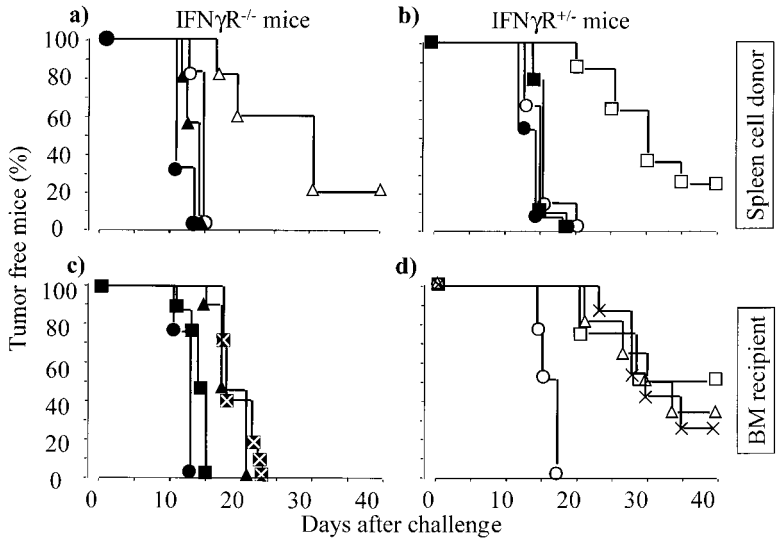


Figure 6. IFNγR Expression On Nonhematopoietic Cells Is Essential for Inhibition of Tumor Growth

(a) Adoptive transfer of tumor immunity with spleen cells of IFNγR$^{-/-}$ mice into IFNγR$^{+/-}$ recipients. Spleen cells (3×10^7) from immunized IFNγR$^{-/-}$ mice (twice with 2×10^5 irradiated Mc51.9 cells) were injected i.v. into IFNγR$^{+/-}$ or IFNγR$^{-/-}$ mice. Three days later, recipient IFNγR$^{+/-}$ (open triangles), IFNγR$^{-/-}$ (closed triangles), and as control, untreated IFNγR$^{+/-}$ (open circles) and IFNγR$^{-/-}$ (closed circles) mice were challenged with 1×10^6 Mc51.9 tumor cells. Numbers of mice per group were 5–6, and shown are results from 1 out of 2 experiments with similar results.

(b) Failure of adoptive transfer of tumor immunity with immune spleen cells from IFNγR$^{+/-}$ into IFNγR$^{-/-}$ mice. Spleen cells were prepared from immunized IFNγR$^{+/-}$ mice and adoptively transferred into naive mice as described above. Three days later, recipient IFNγR$^{+/-}$ (open squares), IFNγR$^{-/-}$ (closed squares), and as control, untreated IFNγR$^{+/-}$ (open circles) and IFNγR$^{-/-}$ (closed circles) mice were challenged with 1×10^6 Mc51.9 cells. Numbers of mice per group were 8–12, and shown are results from 1 out of 2 experiments with similar results.

(c) Failure to generate tumor immunity in IFNγR$^{+/-}$ bone marrow reconstituted IFNγR$^{-/-}$ mice. Lethally irradiated IFNγR$^{-/-}$ mice (8–10 mice/group) were reconstituted with 5×10^6 bone marrow cells from IFNγR$^{+/-}$ (closed squares with "×") or IFNγR$^{-/-}$ donors (closed triangles). Eleven weeks after reconstitution, mice were immunized with 2×10^5 irradiated Mc51.9 cells and challenged 2 weeks later with 1×10^6 Mc51.9 cells. Naive (closed circles) and immunized nonreconstituted IFNγR$^{-/-}$ (closed squares) mice served as control.

(d) Tumor immunity can be generated in IFNγR$^{-/-}$ bone marrow reconstituted IFNγR$^{+/-}$ mice. IFNγR$^{+/-}$ mice (8–12/group) were reconstituted with bone marrow cells from IFNγR$^{+/-}$ (open triangle) or IFNγR$^{-/-}$ donors ("×"), immunized and challenged as above. Naive (open circles) and immunized nonreconstituted IFNγR$^{+/-}$ (open squares) mice served as control.

rejection in some models (Dighe et al., 1994), and activates macrophages (Murray et al., 1985) and NK cells (Trinchieri and Perussia, 1985). Therefore, we analyzed the mechanism by which CD4$^+$ T cells mediate tumor rejection with specific emphasis on the role of IFNγ/IFNγR. To this end, we established tumor models allowing us to analyze the requirement of IFNγR expression on tumor and different host cells for tumor immunity mediated by CD4$^+$ T cells and the time point when the IFNγR must be expressed. We describe here that the IFNγR must be expressed in the effector but not the priming phase of the antitumor response, that nonhematopoietic cells within the tumor stroma were likely the target of IFNγ, and that CD4$^+$ T cell–mediated tumor immunity involved inhibition of angiogenesis.

Tumor cells lacking IFNγR were more tumorigenic in IFNγR$^{+/-}$ mice than transfected variants expressing the IFNγR (tumor growth did not differ in IFNγR$^{+/-}$ and IFNγR$^{+/+}$ mice). This was also observed by using tumor cells expressing a dominant-negative IFNγR mutant gene (Dighe et al., 1994; Coughlin et al., 1998). However, we observed in several tumor models (Mc29.2, Mc51.9, and Mc54.1) a slightly decreased tumorigenicity upon IFNγR expression, and finally all mice developed a tumor when IFNγR$^+$ tumor variants were injected into naive IFNγR$^{+/-}$ mice. We cannot exclude that decreased tumorigenicity would have been more obvious when less tumor cells were injected. Alternatively, the marginal effect of IFNγR expression on tumor cells for their tumorigenicity could be due to the experimental system (use of tumors from IFNγR$^{-/-}$ mice and moderate expression of IFNγR in the variants) or the effector mechanism during rejection. In our model, CD4$^+$ T cells played a dominant role in the effector phase of tumor rejection, and recently Mumberg et al. (1999) showed that CD4$^+$ T cell–mediated rejection did not require IFNγR responsiveness of the tumor cells. The decreased tumorigenicity of IFNγR$^+$ tumor cells in IFNγR$^{+/-}$ mice was abrogated for unknown reasons in IFNγR$^{-/-}$ mice, demonstrating that IFNγR expression by host cells was most important for an antitumor response. This was firmly established by demonstrating that IFNγR$^{+/-}$ mice could be immunized with IFNγR$^+$ or IFNγR$^-$ tumor cells. Conversely, IFNγR$^{-/-}$ mice were severely impaired in development of tumor immunity regardless whether they were immunized with IFNγR$^+$ or IFNγR$^-$ tumor cells. The fact that IFNγR expression and IFNγ-induced MHC upregulation was not necessary on cells used for immunization is compatible with the observation that T cells appear to be activated by "cross-priming" (Huang et al., 1994; Cayeux et al., 1997). The observation that IFNγR expression was also not necessary on challenge tumor cells indicates that IFNγ-induced upregulation of MHC molecules in the effector phase did not play a significant role for better T cell recognition/elimination in our tumor model.

The inability of IFNγR$^{-/-}$ mice to develop tumor immunity most likely did not reside in the T cell compartment. It is known already from the initial description of IFNγR$^{-/-}$ mice that T cell development and activation appear to be normal in these mice (Huang et al., 1993). Additionally, IFNγ$^{-/-}$ mice, which might have a phenotype similar to IFNγR$^{-/-}$ mice, can develop immunity to some infectious pathogens such as *Listeria monocytogenes* (Harty and Bevan, 1995). We showed that immunization with tumor cells activated T cells in IFNγR$^{-/-}$ as well as in IFNγR$^{+/-}$ mice as judged by T cell proliferation, IFNγ production, and homing to the challenge site. Moreover, transfer of spleen cells from immunized IFNγR$^{-/-}$ mice

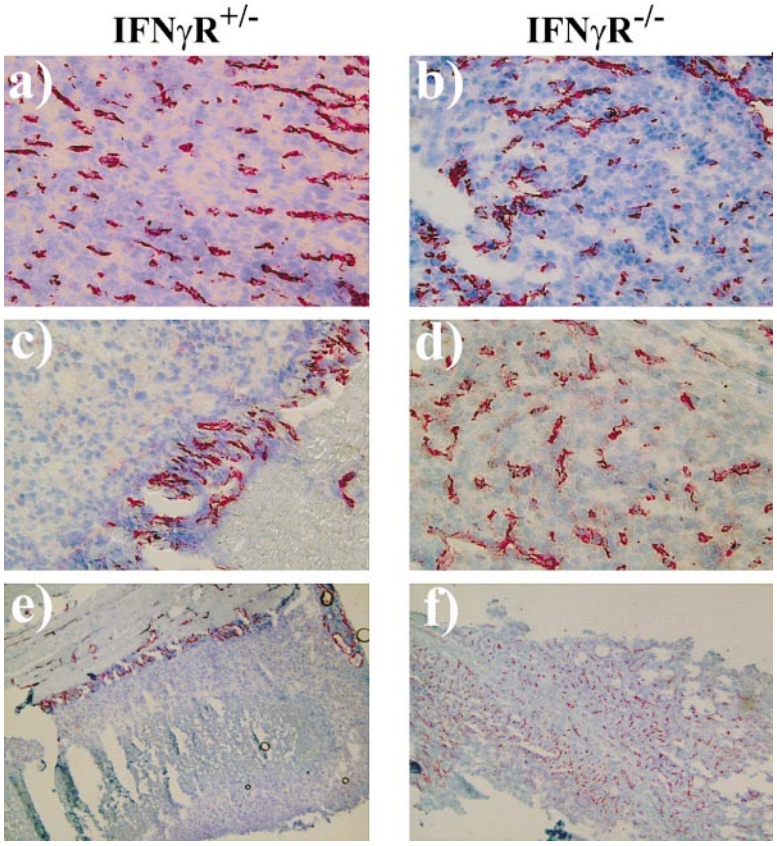


Figure 7. Inhibition of Tumor-Induced Angiogenesis in Immunized IFNγR$^{+/-}$ but not IFNγR$^{-/-}$ Mice

Mice (3–5/group) were left untreated or immunized twice with 2×10^5 irradiated Mc51.9 tumor cells and challenged one week later by an s.c. injection of 1×10^6 Mc51.9 cells. Four days after challenge, cryostat tumor sections were prepared and stained with anti-CD31 mAb for endothelial cells. Shown in (a) and (b) are tumor sections of naive IFNγR$^{+/-}$ (a) and IFNγR$^{-/-}$ (b) mice. Shown in (c)–(f) are tumor sections of immunized IFNγR$^{+/-}$ (c and e) and IFNγR$^{-/-}$ (d and f) mice. Note the necrotic area in the center of the tumor in immunized IFNγR$^{+/-}$ mice in (e). Magnification: 200× (a–d) and 40× (e and f).

inhibited tumor growth in IFNγR$^{+/-}$ mice to a similar extent as transfer of IFNγR$^{+/-}$ spleen cells. This experiment also showed that IFNγR expression was not necessary in the priming but only in the effector phase of the antitumor response.

While the adoptive transfer experiments indicated that neither tumor nor T cells must express IFNγR for tumor immunity, it left open the question of whether other hematopoietic cells needed to express IFNγR. For instance, IFNγ produced by CD4$^+$ T cells could be needed for a DTH-like reaction and activate other cells such as NK cells or macrophages for tumoricidal activity (Greenberg et al., 1985) or induce production of other cytokines (Boehm et al., 1997). Surprisingly, bone marrow chimeric mice showed that IFNγR expression was not necessary on hematopoietic cells for tumor immunity. This does not argue against innate effector cells involved in the antitumor response, which then, however, would act in an IFNγR-independent fashion. In fact, challenge tumors in IFNγR$^{-/-}$ mice were infiltrated by large numbers of Mac1$^+$ and Gr1$^+$ cells.

IFNγR expression by nonhematopoietic cells was of critical importance for inhibition of tumor growth. Since its expression was required only in the effector phase, it is likely that these cells were within the tumor stroma. It is well known that tumors above a critical size require blood supply and induce the formation of blood vessels in the tumor (Folkman, 1998). Only 4 days after injection of tumor cells in naive mice, tumors were well vascularized, both in IFNγR$^{-/-}$ and IFNγR$^{+/-}$ mice. In challenge tumors of immunized IFNγR$^{+/-}$ mice, angiogenesis was strongly inhibited, which correlated with a rapid infiltration of T cells. At least in vitro T cells produced IFNγ in response to Mc51.9 cells (Figure 5b). Whether tumor-infiltrating CD4$^+$ T cells also produce IFNγ has to be analyzed. In IFNγR$^{-/-}$ mice, nonhematopoietic cells within the tumor stroma could not respond to IFNγ, and therefore angiogenesis was not impaired and the tumor was not rejected. It is possible that IFNγ directly acted on endothelial cells that express the IFNγR (Valente et al., 1992). Alternatively, IFNγ acted on other cells in the tumor stroma, e.g., fibroblasts, and induced secondary cytokines, which contributed to inhibition of angiogenesis. For example, IFNγ can induce production of IP-10 and Mig by fibroblasts (Luster et al., 1985; Farber, 1990), two chemokines known to inhibit angiogenesis (Sgadari et al., 1996, 1997). We cannot yet exclude the possibility that impaired tumor angiogenesis results from tumor cell killing and reduced tumor cell–derived angiogenic factors. However, because at the rim of the tumor, sufficient viable cells and blood vessel formation were visible, we think that IFNγ-producing CD4$^+$ T cells inhibited invasion of blood vessels into the tumor.

The finding that CD4$^+$ T cell–mediated tumor immunity involves inhibition of angiogenesis has several implications. (1) It shows that tumor immunity, at least in models in which CD4$^+$ T cells are essential effector cells, does not exclusively rely on direct tumor cell killing. Inhibition of angiogenesis is an effective way to prevent rapid tumor growth but is unlikely to be sufficient to lead to complete tumor rejection. Therefore, innate effector cells, which alone have rarely the ability to completely eradicate a tumor (Hock et al., 1993), may be necessary

to kill residual tumor cells, e.g., those growing at the periphery of the tumor. This is supported by the finding that mice with a defective nitric oxide synthase and diminished macrophage tumoricidal activity have a reduced ability to reject tumors (Hung et al., 1998). The requirement of residual tumor cell killing in addition to antiangiogenesis is furthermore supported by the observation that half of the mice depleted of $CD8^+$ T cells could not reject the challenge tumor. However, because the potential of $CD8^+$ T cells to eliminate a tumor can correlate with their ability to produce IFNγ and TNF rather than with their cytotoxic activity (Barth et al., 1991), we can not exclude the possibility that inhibition of angiogenesis contributed to the antitumor effect mediated by $CD8^+$ T cells. (2) The inhibition of angiogenesis leads to a rapid necrosis in the center of the tumor (Figure 7e), which should result in the release of high amounts of tumor-derived antigens in a short time to be taken up by APC. This might be important for activation of $CD4^+$ T cells and an enhanced antitumor response. (3) A well-known dilemma in immunotherapy is that cancer vaccines can be quite effective to prevent the growth of a challenge tumor, but they usually fail to induce tumor rejection when tumor-bearing animals are treated (Lollini and Forni, 1999). This must not be due to the failure of T cell activation in tumor-bearing mice, as the phenomenon of "concomitant immunity" suggested. Rather, T cells activated in tumor-bearing mice are confronted with a well-vascularized tumor, and the effect of antiangiogenesis may be less pronounced. The effect of IFNγ, alone or in combination with other factors, must not be restricted to inhibiting the growth of new blood vessels. Recently, we found that rejection of large vascularized tumors by cyclophosphamide is mediated by the destruction of tumor blood vessels, which also required IFNγR expression on host cells (unpublished data).

Experimental Procedures

Mice

The IFNγR-deficient ($IFN\gamma R^{-/-}$) mice congeneic to 129/Sv/Ev and control wild type mice ($IFN\gamma R^{+/+}$) were kindly provided by M. Aguet (Huang et al., 1993). Sex and age (6 to 12 weeks) matched control litter mates of the breeding between $IFN\gamma R^{-/-}$ and $IFN\gamma R^{+/-}$ mice were used unless otherwise indicated. In some experiments, tumor rejection between $IFN\gamma R^{+/+}$ and $IFN\gamma R^{+/-}$ mice was compared, which showed no phenotypical difference. The mutant IFNγR gene was confirmed by polymerase chain reaction (PCR) using tail DNA as described elsewhere. The primers used were located on exon V of the IFNγR gene: (sense) 5′-CCC ATT TAG ATC CTA CAT ACG AAA CAT ACG G-3′ and (antisense) 5′-TTT CTG TCA TGG AAA GGA GGG ATA CAG-3′. The PCR-amplified DNA fragment for the normal IFNγR gene, including expression plasmid pBabe-γR, was 190 bp, and for the mutated allele, 1300 bp. A primer specific for the neomycin phosphotransferase gene was also used to distinguish the $IFN\gamma R^{+/+}$ and $IFN\gamma R^{+/-}$ mice: (*neo*) 5′-CCT GCG TGC AAT CCA TCT TG-3′. The amplified DNA fragment using the *neo* primer and the IFNγR sense primer was about 460 bp for the mutant IFNγR allele.

Generation of Tumor Cell Lines from $IFN\gamma R^{-/-}$ Mice

Tumors were induced by intramuscular injection of 0.8 mg MCA in $IFN\gamma R^{-/-}$ mice and excised 21–23 weeks later at a size of 1.1–1.2 cm in diameter. Fragments of the primary tumors were grafted twice onto nude, twice onto $IFN\gamma R^{-/-}$, and twice onto $IFN\gamma R^{+/+}$ mice. The in vivo passaged tumors were then isolated, minced, and incubated in trypsin-EDTA solution (Life Technologies, Karlsruhe, Germany) containing 1 mg/ml collagenase (Life Technologies) overnight at 0°C and then 5 min at 37°C in the presence of 0.1 mg/ml DNase I (Boehringer Mannheim, Germany). Single-cell suspensions were prepared and subsequently tumor cells were cloned by limiting dilution. The tumor cell lines and the mock- and IFNγR-transfected variants were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Three cell lines, Mc29.2, Mc51.9, and Mc54.1, were used in this study.

Plasmids and Cell Transfections

The full-length mouse IFNγR cDNA was excised from the expression plasmid pHMGmGIFR (Hemmi et al., 1989) as an SmaI/SalI fragment, blunt-ended, and then cloned into the SnaBI site of the retrovirus vector pBabe (Morgenstern and Land, 1990). The resulting plasmid pBabe-γR contains the IFNγR gene under the control of the viral long terminal repeat promoter and a puromycin resistance gene under the control of the SV40 promoter. Mc29.2, Mc51.9 and Mc54.1 cells were transfected with the plasmid pBabe-γR using a mammalian transfection kit (Stratagene GmbH, Heidelberg, Germany) and selected in RPMI 1640 culture medium containing 4 μg/ml puromycin, resulting in Mc29.2-γR, Mc51.9-γR, and Mc54.1-γR cell lines. As controls, cells were also transfected with the empty plasmid pBabe and selected for puromycin resistance, resulting in the mock-transfected Mc29.2-m, Mc51.9-m, and Mc54.1-m cell lines.

Flow Cytometry

Cell surface IFNγR expression was determined by staining of cells with the rat anti-mouse IFNγR mAb (GR20, PharMingen, Hamburg, Germany) or an isotype-matched control mAb (R35–95, PharMingen). PE-conjugated rabbit-anti-rat IgG2a (Jackson Immunoresearch Laboratories, West Grove, PA) was used as secondary antibody. To verify the specificity of the GR20 binding, tumor cells were preincubated with 25 ng/ml murine IFNγ (PharMingen) overnight and then stained for IFNγR. For MHC class I expression, tumor cells cultured without or with 25 ng/ml IFNγ for 24 hr were incubated with FITC-conjugated mAb anti-H-$2K^b$ (AF6–88.5), anti-H-$2D^b$ (HK95), isotype-matched control mAb, mouse IgG_{2a} (G155–178), or mouse IgG_{2b} (49.2) (PharMingen). All samples were analyzed with an Epics-XL flow cytometer (Coulter Electronics, Hamburg, Germany).

Tumor Growth In Vivo

The parental, mock-, or IFNγR-transfected tumor cells were washed in D-PBS and subcutaneously (s.c.) injected into $IFN\gamma R^{+/-}$ or $IFN\gamma R^{-/-}$ mice at the indicated numbers in 0.2 ml D-PBS. Tumor growth was monitored every 2 to 3 days and mice bearing a tumor of >10 mm in diameter were scored as tumor positive. For immunization of mice, 2×10^5 tumor cells were irradiated with 100 Gy and inoculated s.c. into mice. Two weeks later, mice were challenged contralaterally by s.c. injection of parental tumor cells in numbers as indicated.

Tumor Fragment Transplantation

Tumor fragments were removed from mice bearing primary MCA-induced tumors and passaged in nude mice as previously described (Prehn and Main, 1957). Altogether, five primary tumors (two from $IFN\gamma R^{+/+}$ and three from $IFN\gamma R^{-/-}$ mice) were analyzed. For immunization, tumor fragments of $4 \times 4 \times 4$ mm were s.c. transplanted onto $IFN\gamma R^{+/+}$ and $IFN\gamma R^{-/-}$ mice (2–3/group). Tumors were cut out by a surgical operation when they reached a size of 10 mm in diameter at day 12–14. One week later, the mice were challenged contralaterally with a fragment ($4 \times 4 \times 4$ mm) of the same tumor passaged in nude mice. The growth of the challenge tumor was monitored every 2–3 days.

T Cell Depletion

Groups of $IFN\gamma R^{+/+}$ mice were immunized with 2×10^5 irradiated (100 Gy) Mc51.9 cells and challenged 14 days later contralaterally with the same number of viable Mc51.9 cells. T cell subset depletion was done by intraperitoneal (i.p.) injection of 2 mg rat mAb GK1.5 (anti-CD4) or 2.43 (anti-CD8) in 0.5 ml D-PBS at day −3 and +4 relative to challenge. Specific depletion was confirmed by flow cytometric analysis of PBL at day 7, 14, and 21 by flow cytometric

analysis using FITC-conjugated mAbs anti-CD4 (RM4–4) and anti-CD8 (53–6.7, PharMingen).

Analysis of T Cell Proliferation and Cytokine Production

Three to five mice per group were immunized twice with a 2 week interval by i.p. injection of 2×10^5 irradiated Mc51.9 cells. Five days later, spleen cells from immunized and, as control, naive IFNγR$^{+/-}$ or IFNγR$^{-/-}$ mice were cultured at a concentration of 2×10^6/ml in RPMI medium in 96-well plates (0.1ml/well) for 5 days. For specific T cell stimulation, Mc51.9 tumor cell lysate (made three times by freeze/thaw) was added to spleen cells at a ratio of 1:40. T cell proliferation was determined by a 12 hr ^{3}H-thymidine incorporation assay. To determine IFNγ production by immune spleen cells, supernatants of the cell culture as described above were collected 5 days after in vitro restimulation with tumor cell lysates. A commercially available kit (OptEIA Mouse IFNγ Set, PharMingen) was used for determination of IFNγ concentration.

Adoptive Transfer of Tumor Immunity

Donor mice were immunized twice with a 2 week interval by i.p. injection of 2×10^5 irradiated Mc51.9 cells, and 5 days later spleen cells were isolated. After the depletion of red blood cells by NH_4Cl treatment, cells were cultured for 60 min at 37°C to remove adherent cells. After washing in D-PBS, 3×10^7 cells (CD4$^+$, 30%–45%; CD8$^+$, 10%–15%; and B220$^+$, 40%–60%) were resuspended in 0.2 ml D-PBS and intravenously (i.v.) injected into the tail vein of IFNγR$^{+/-}$, IFNγR$^{-/-}$, or SCID mice. Three days after the cell transfer, mice were challenged with Mc51.9 cells and tumor growth was monitored.

Bone Marrow Chimeric Mice

Freshly prepared bone marrow cells of 6- to 8-week-old female IFNγR$^{+/-}$ and IFNγR$^{-/-}$ mice were injected i.v. into lethally irradiated (10 Gy) recipient mice (5×10^6 cells/age- and sex-matched mice). The following groups of mice were included: IFNγR$^{+/-}$ → IFNγR$^{+/-}$, IFNγR$^{+/-}$ → IFNγR$^{-/-}$, IFN-γR$^{-/-}$ → IFN-γR$^{+/-}$, and IFN-γR$^{-/-}$ → IFN-γR$^{-/-}$. Successful reconstitution of the hematopoietic system was determined by flow cytometric analysis of peripheral blood mononuclear cells for IFNγR expression. Additionally, chimerism of mice was confirmed by PCR analysis of the IFNγR gene using tail DNA that contained genomic DNA from both nonhematopoietic and hematopoietic cells. Eleven weeks after bone marrow transplantation, mice were immunized with 2×10^5 irradiated Mc51.9 cells and challenged 2 weeks later with 1×10^6 Mc51.9 cells.

Immunohistochemistry

Isolation of tumor tissues, preparation of cryostat sections, and alkaline phosphatase immunostaining were done as described (Blankenstein et al., 1991). Here, 1×10^6 Mc51.9 cells were injected s.c. into naive or twice (with 2×10^5 irradiated Mc51.9 cells) immunized IFNγR$^{+/-}$ or IFNγR$^{-/-}$ mice at a shaved belly region, and tumor tissue was obtained after 4 and 6 days. The mAbs used for staining were anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD31 (MEC13.3), anti-Gr-1 (RB6–8C5), anti-Mac-1 (M1/70), and isotype-matched control mAbs (PharMingen). Alkaline phosphatase-conjugated goat anti-rat IgG and rabbit anti-goat IgG were purchased from Jackson Immunoresearch Laboratories.

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