

## **Implicating a Role for Immune Recognition of Self in Tumor Rejection: Passive Immunization against the Brown Locus Protein**

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### **Summary**

The immune system can recognize differentiation antigens that are selectively expressed on malignant cells and their normal cell counterparts. However, it is uncertain whether immunity to differentiation antigens can effectively lead to tumor rejection. The mouse *brown* locus protein, gp75 or tyrosinase-related protein 1, is a melanocyte differentiation antigen expressed by melanomas and normal melanocytes. The gp75 antigen is recognized by autoantibodies and autoreactive T cells in persons with melanoma. To model autoimmunity against a melanocyte differentiation antigen, mouse antibodies against gp75 were passively transferred into tumor-bearing mice. Passive immunization with a mouse monoclonal antibody against gp75 induced protection and rejection of both subcutaneous tumors and lung metastases in syngeneic C57BL/6 mice, including established tumors. Passive immunity produced coat color alterations but only in regenerating hairs. This system provides a model for autoimmune vitiligo and shows that immune responses to melanocyte differentiation antigens can influence mouse coat color. Immune recognition of a melanocyte differentiation antigen can reject tumors, providing a basis for targeting tissue autoantigens expressed on cancer.

Experimental evidence shows that the immune system can recognize antigens expressed by cancer cells. Recently, the isolation of genes encoding cancer antigens has led to the identification of these molecules (1–3). From these studies, a paradigm emerges in which the immune repertoire recognizes a set of self-antigens, termed differentiation antigens, expressed by malignant cells but also by their normal cell counterparts (3–5).

The immune response to melanoma has been studied more extensively than that to any other human cancer. Dissection of the immune response to melanoma has shown that differentiation antigens expressed by normal melanocytes and related neuroectoderm-derived cells are dominant antigens recognized by the immune system (3, 5–16). Among the best-characterized melanoma autoantigens are proteins of the tyrosinase family. Tyrosinase (the product of the *albino* locus) is the prototype molecule, and other members include gp75 or tyrosinase-related protein 1 (the product of the *brown* locus) and gp100 (the presumed product of the *silver* locus) (17–19). These molecules, plus the MelanA/MART-1 antigen, are all melanocyte-specific molecules recognized by the immune system of melanoma patients (8, 9, 11–16). The question of whether immune recognition of self (differentiation antigens) can lead to tumor rejection arises. In particular, we considered whether immunity to melanocytes can actually lead to rejection of melanoma, and whether there are sequelae in normal tissues that contain melanocytes.

### **Materials and Methods**

*Mice and Tumors.* C57BL/6 (6–8 wk-old females) were obtained from The Jackson Laboratory (Bar Harbor, ME). B16F10 is a mouse melanoma cell line of C57BL/6 origin kindly provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX) (20). B78H.1 is a variant of B16 melanoma that does not express the gp75 antigen. JBRH is a melanoma from C57BL/6 provided by Dr. P. Livingston (Memorial Sloan-Kettering, NY). Other tumors derived from C57BL/6 mice include the RMA and EL-4 lymphomas, Lewis lung carcinoma, and MC58 sarcoma (from Dr. J. Nicolic-Zugic, Memorial Sloan-Kettering, NY). All animal experiments and care were in accordance with institutional guidelines.

For subcutaneous tumors, tumor cells were injected subcutaneously in the mouse flank. B16F10 melanoma cells ( $5 \times 10^4$  cells), B78H.1, RMA, EL-4, JBRH, and Lewis lung carcinoma ( $10^4$ – $10^6$  cells) were injected subcutaneously in 0.1 ml normal saline into the flank of syngeneic C57BL/6 mice. Tumors were checked at least four times per week by palpation and inspection. For each tumor type, palpable tumors formed in 10–21 d. For B16F10 melanoma lung metastases, C57BL/6 mice were injected intravenously through the tail vein with  $1 \times 10^5$  B16F10 melanoma cells in sterile saline. For depilation experiments, C57BL/6 mice were depilated on the day before starting treatment over the posterior flank and observed for coat color. The same results were observed if the coat was plucked manually under anesthesia or depilated with hair remover (JMC, Inc., Tokyo).

Mice were treated intraperitoneally with mAb TA99 or control mouse IgG2a mAb UPC10 diluted in 0.3–0.4 ml of normal saline. TA99 mAb was purified from mouse ascites by protein A

affinity column. F(ab')<sub>2</sub> fragments of mAb TA99 were produced by digestion with pepsin and purification over protein A Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ). Control IgG2a mAb UPC10 was from Sigma Chemical Co. (St. Louis, MO). Injection with control mAb UPC10 did not induce any difference in growth of B16F10 melanoma compared with untreated control mice. For subcutaneous tumor measurements, the longest surface length (*a*) and its perpendicular width (*b*) were measured, and tumor size reported as *a* × *b*. For lung metastases, at 16–20 d after tumor challenge, mice were killed and surface lung metastases were scored and counted as black nodules under a dissecting microscope. Surface lung metastases were detected by day 4–7 under a dissecting microscope and by day 7–10 by eye. For histologic evaluation, tissues and tumors were fixed in formalin solution, blocked in paraffin, sectioned every 4 μm, and stained with hematoxylin and eosin. Statistical analysis of tumor growth was performed using the Student's *t* test or Bonferroni two-sided *t* test, a conservative analysis to allow for multiple comparisons.

**Antibody Treatments In Vivo.** Depletion of T cells in vivo was accomplished by intraperitoneal administration of rat mAb GK1.5 (anti-CD4; IgG2b) and mAb 2.43 (anti-CD8; IgG2b), and Thy1.2<sup>+</sup> cells by mAb 30-H12 (produced by hybridomas from the American Type Culture Collection, Rockville, MD). These mAbs were used as ascites fluids (titer >1:10,000 by staining of mouse thymocytes by flow cytometry). mAb preparations (0.2 ml) were injected intraperitoneally at day -3 and every 7 d thereafter. Throughout experiments, these treatments depleted respective T cell subpopulations and Thy-1<sup>+</sup> populations >97% as determined by indirect immunofluorescence staining and cytofluorometric analysis of lymph nodes and thymocytes with mAbs GK1.5 (CD4), 2.43 (CD8), or 30-H12 (Thy1.2). Natural killer (NK) cell depletion was performed using mAb PK136 (anti-NK-1.1) (American Type Culture Collection). Antibody (0.2 ml) was injected intraperitoneally at day -3 and every 7 d thereafter. Depletion of NK cells was assessed by 4-h <sup>51</sup>Cr-release assays with 5,000 YAC cells as targets and spleen cells as effector cells at effector-to-target ratios of 100:1, 50:1, and 25:1, and depletion was shown to abrogate completely detectable NK activity. For depletion of complement, mice were injected intraperitoneally with 10 U cobra venom factor (Diamedix Corp., Miami, FL) on day -1 and every 4 d. Depletion of complement in sera of treated mice was verified using lysis of sensitized rabbit RBC (21).

**Expression of gp75 Antigen on B16F10 Melanoma.** Intracellular expression of gp75 was determined using mAb TA99 in indirect immunofluorescence assays against B16F10 melanoma cells fixed and permeabilized in methanol/acetone (1:1) at 4°C for 15 min. Intracellular expression was confirmed by immunoelectron microscopy using protein A-labeled colloidal gold particles as described (22). Cell-surface expression was shown by binding of mAb TA99 to intact, live B16F10 cells by enzyme-linked immunoassay (titer >1/10,000) and by protein A-mixed hemadsorption assay (titer >1/5,000) (5, 22).

## Results and Discussion

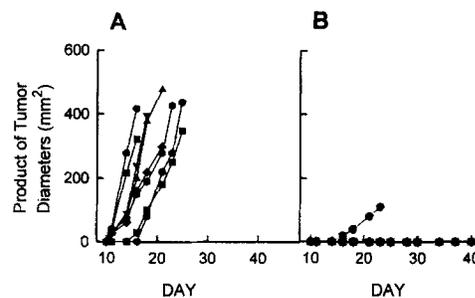
Syngeneic mouse tumor models were established to examine whether immunity against antigens expressed on normal melanocytes and melanoma can lead to tumor rejection. The *brown* locus product, gp75, was the target antigen in these models. The gp75 autoantigen is relevant because it is potentially immunogenic in persons with melanoma,

recognized on melanomas by both autoantibodies and autoreactive T cells (8, 9). The mouse mAb TA99 binds to both human and mouse gp75 and reacts with normal melanocytes and melanoma but does not react with other tissues (22). TA99 mAb has been shown to localize efficiently to melanoma xenografts in mice (tumor/normal tissue ratios >100:1 to 10<sup>5</sup>:1), showing that systemic administration of antibody against gp75 can specifically localize to tumor sites (23).

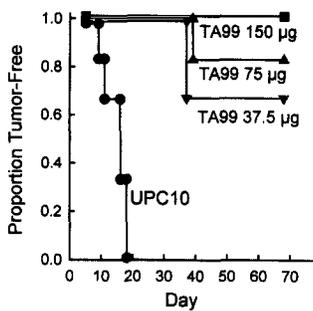
The gp75<sup>+</sup> B16F10 melanoma is a spontaneously arising tumor that is very weakly immunogenic in syngeneic C57BL/6 mice. B16F10 cells from fresh tumors express gp75 in melanosomes within the cell and on the cell surface (see Materials and Methods). Incubation of mAb TA99 (up to 600 μg/ml for 7 d) with B16F10 melanoma cells in vitro did not affect the cell growth, morphology, or pigmentation. Mice were challenged subcutaneously with B16F10 melanoma and treated with either mAb TA99 or isotype-matched control mAb UPC10. With this tumor challenge, B16F10 uniformly forms palpable tumors in ~2 wk. Tumors were rejected in mice treated with mAb TA99 but not in control mice (Fig. 1). Protection against tumor growth was observed with doses of mAb TA99 as low as 37.5 μg, but optimal protection was seen with doses of ≥150 μg mAb TA99 (Fig. 2). Tumor protection was observed beyond 50–70 d.

This antitumor effect was specific for tumors that expressed gp75 antigen. Tumor protection was seen for the gp75<sup>+</sup> JBRH melanoma after subcutaneous challenge in syngeneic C57BL/6 mice (time to median appearance of tumors delayed by >31 d). However, no antitumor effects were observed in syngeneic C57BL/6 mice with a gp75<sup>-</sup> variant of the parental B16 melanoma (B78H.1 melanoma), nor with other subcutaneous gp75<sup>-</sup> tumors, including EL4 lymphoma, RMA lymphoma, Lewis lung carcinoma, or MC58 sarcoma.

Intravenous injection of B16F10 leads reproducibly to lung metastases (20, 24). Treatment with mAb TA99 mark-



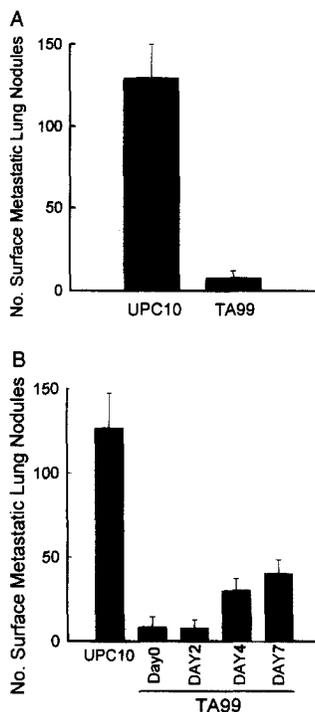
**Figure 1.** Protective immunity induced by mAb TA99 against B16F10 melanoma cells. Melanoma cells ( $5 \times 10^4$ ) were injected subcutaneously into the flank of C57BL/6 mice. Mice were treated intraperitoneally with (A) control mouse IgG2a mAb UPC10 or (B) mAb TA99, injected at a dose of 150 μg (days 0, 2, 4, 7, 9, and 11). There were eight mice in each group. Treatment with mAb TA99 produced a significant increase ( $P < 0.0001$ , Bonferroni two-sided *t* test) in proportion of tumor-free mice. Each symbol in A represents an individual mouse. In group B, eight mice were treated with mAb TA99, and only one mouse developed palpable tumor (●).



**Figure 2.** Protective immunity by different doses of mAb TA99 against B16F10 melanoma. Mice were treated with 150 µg of control mAb UPC10 (●) or mAb TA99 at 150 µg (■), 75 µg (▲), or 37.5 µg (▼) per dose as described in the legend of Fig. 1. There were six to eight mice in each group. Mice were scored as tumor free or tumor bearing, and the proportion of tumor-bearing mice (ratio of tumor-bearing/total number of mice per group) was calculated for each time point.

edly reduced the number of lung surface metastases even at individual doses as low as 50 µg (Fig. 3 A and data not shown). Injection of F(ab')<sub>2</sub> fragments of mAb TA99 did not have any effect on the number of B16F10 metastases, supporting a crucial role for the Fc portion of mAb TA99 in antitumor effects (data not shown).

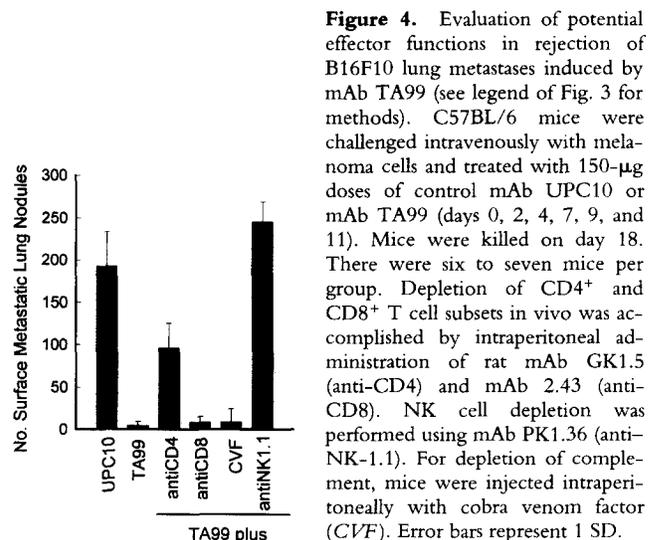
We asked whether treatment with mAb TA99 would have antitumor effects in established metastases. Delaying treatment with mAb TA99 for 2–7 d after challenge with B16F10 still induced substantial protection against metastases but required higher doses of mAb TA99 ( $P < 0.001$ , paired Student's *t* test at 7 d) (Fig. 3 B). Effects of mAb TA99 were also observed on growth of subcutaneous B16F10 melanoma when treatment was delayed 4 d after tumor challenge (median time to appearance of tumor increased by 14 d), and slightly but reproducibly when treatment was delayed 7 d (median time to appearance of tumor increased 6 d).



**Figure 3.** Antitumor effects of mAb TA99 on B16F10 lung metastases, including established metastases. Syngeneic C57BL/6 mice were injected intravenously through the tail vein with B16F10 melanoma cells. Mice were treated intraperitoneally with control mAb UPC10 or mAb TA99. 18 d after tumor challenge, mice were killed, and surface lung metastases were scored. There were eight to nine mice in each group. (A) mAb TA99 and control mAb UPC10 were administered at a 150-µg dose (days 0, 2, 4, 7, 9, and 11). (B) 600-µg doses of mAb TA99 were given three times per week for 2 wk starting on day 0 (6 h after tumor challenge), day 2, day 4, or day 7; a 600-µg dose of mAb UPC10 was given on days 0, 2, 4, 7, 9, and 11. Error bars represent 1 SD.

Histologic examination of residual B16F10 subcutaneous lesions and metastatic lung lesions in mice treated with mAb TA99 showed occasional infiltration of lymphocytes and macrophages, compared with no mononuclear or inflammatory infiltrates observed in control mice. To assess what components of the immune or inflammatory system might be involved in tumor rejection, T lymphocyte subsets, complement, or NK cell populations were depleted before challenge with tumor cells. Depletion of CD8<sup>+</sup> T cells and complement did not alter tumor rejection mediated by mAb TA99 (Fig. 4 shows results for lung metastases; data not shown for subcutaneous tumors). Depletion of CD4<sup>+</sup> cells partially decreased rejection of B16F10 lung metastases by mAb TA99 (Fig. 4) (depletion of CD4<sup>+</sup> cells without mAb TA99 treatment did not affect the number of B16F10 metastases; data not shown) but did not affect growth of subcutaneous B16F10 tumors.

Depletion of an NK1.1<sup>+</sup> cell population appeared to abrogate the protective effect of mAb TA99 in both metastatic B16F10 in the lung (Fig. 4) and subcutaneous B16F10 tumors (data not shown), supporting a role for NK cells in tumor rejection mediated by mAb TA99. NK1.1<sup>+</sup> cells appear to provide natural immunity against B16F10 lung metastases (24). We further examined the role of NK1.1<sup>+</sup> cells in the lung metastases model (using the same experimental design described in Fig. 5 with six to eight mice per group): (a) Depletion of NK1.1<sup>+</sup> cells from C57BL/6 mice led to a significant increase in the number of metastases (mean  $314 \pm 58$  metastases) compared with control undepleted mice ( $136 \pm 25$  metastases); (b) treatment with mAb TA99 markedly decreased metastases ( $12 \pm 11$  metastases); and (c) the number of lung metastases in mice treated with mAb TA99 and also depleted of NK1.1<sup>+</sup> cells was significantly greater (mean  $186 \pm 42$ ) than in mice treated with TA99 alone ( $12 \pm 11$ ), but not as great as the number of metastases in untreated NK1.1-depleted mice ( $314 \pm 58$ ). These results suggested that other components of the host in addition to NK cells, such as CD4<sup>+</sup> cells (Fig. 4), partic-



**Figure 4.** Evaluation of potential effector functions in rejection of B16F10 lung metastases induced by mAb TA99 (see legend of Fig. 3 for methods). C57BL/6 mice were challenged intravenously with melanoma cells and treated with 150-µg doses of control mAb UPC10 or mAb TA99 (days 0, 2, 4, 7, 9, and 11). Mice were killed on day 18. There were six to seven mice per group. Depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in vivo was accomplished by intraperitoneal administration of rat mAb GK1.5 (anti-CD4) and mAb 2.43 (anti-CD8). NK cell depletion was performed using mAb PK1.36 (anti-NK-1.1). For depletion of complement, mice were injected intraperitoneally with cobra venom factor (C/F). Error bars represent 1 SD.

ipated in rejection of lung metastases mediated by mAb TA99. It is possible that NK1.1<sup>+</sup> cells binding mAb TA99 through Fc receptors secondarily activated CD4<sup>+</sup> cells to participate in tumor rejection. Alternatively, CD4<sup>+</sup> T cells might be activated by APC through internalization and presentation of antigen-antibody complexes.

Mice treated with mAb TA99 were examined for changes in pigmentation and other autoimmune-type manifestations. The coat of animals remained black unless animals were depilated to prepare skin sites for tumor injection. Pigmented melanocytes on the trunk of adult mice are found in hair bulbs. Regenerating hairs on the trunk were depigmented in 13 of 13 tumor-bearing mice treated with mAb TA99 at 300–900  $\mu\text{g}$  per dose (three times per week for 2 wk) but not in mice treated with control mAb UPC10 (0 of 36 mice) or untreated control mice (0 of 30 mice). Depigmentation was not related to injection of tumor cells; treatment of depilated C57BL/6 mice without injection of tumor cells still led to depigmentation in 12 of 12 mice treated with 300–1,000- $\mu\text{g}$  dose of mAb TA99 (Fig. 5). At TA99 doses  $\leq 150$   $\mu\text{g}$ , depigmentation was not observed (0 of 54 mice). Thus, the threshold dose of mAb TA99 required for coat color changes was fivefold greater than the threshold required for antitumor effects in tumor protection experiments. Histologic sections of skin from mice treated with mAb TA99 showed depigmented hair follicles and regenerating hairs in previously depilated areas. The bulbs of white hairs did not contain pigment, and follicles lacked pigmented melanocytes. There were no signs of decrease in pigmentation or pigmented granules, inflammation, or changes in cellular morphologies or tissue architecture in the eyes (choroid and retina) of mice treated with mAb TA99. There were no detectable alterations in behavior or weights of mice during or after treatment. In vivo depletion with an mAb against Thy1.2 showed that Thy1<sup>+</sup> cells were necessary for depigmentation, but NK1.1<sup>+</sup> cells, complement, and the individual CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets were not necessary.

These findings suggest that autoimmunity directed against tyrosinase-related proteins and other antigens expressed by melanocytes can influence coat color in mice. This implies that coat color can be regulated at the level of a host response in addition to previously defined genetic controls at the cellular level. The induction of concomitant tumor rejection and autoimmunity recalls a relevant clinical observation in persons with metastatic melanoma who develop vitiligo. The spontaneous appearance of vitiligo has been associated with an improved prognosis in persons with metastatic melanoma (25, 26). A related observation is that the immune system of patients with vitiligo can recognize tyrosinase and other specific antigens expressed by melanocytes (27, 28). This mouse model has recapitulated an association between melanoma and vitiligo, showing that a



**Figure 5.** Alteration of coat color in C57BL/6 mice treated with mAb TA99. Mice were depilated on day  $-1$  over the left posterior flank and abdomen. On day 0, mice started treatment with either mAb TA99 (*left mouse*) or mAb UPC10 (*right mouse*) three times per week for 2 wk (see schedule in Fig. 1) at individual daily doses of 300  $\mu\text{g}$ . The line demarcating the upper border of the shaved coat (*dorsal trunk of the animal*) can be seen in the control animal (*right mouse*). The coat of mice treated with mAb TA99 showed depigmentation in areas of regenerating hairs but not in the nondepilated coat, while no alteration in coat color was observed in mice treated with isotype control mAb UPC10.

biologic response to the tyrosinase family of proteins in melanocytes of the skin can mediate melanoma rejection.

In summary, an increasing body of data suggests that immune recognition of melanoma in humans is directed most frequently against molecules expressed by melanocytes or other neural crest-derived cell types (it should be pointed out that this perceived “immune repertoire” against melanoma can be biased by the use of selected in vitro assays and may not be a random sampling). These experiments suggest that the threshold for depigmentation, a potential autoimmune manifestation, can be greater than that for tumor protection, and that the biologic state of melanocytes may be important in the development of autoimmune signs. Passive transfer of mAb against gp75 was able to lead to rejection even of established B16F10 tumors in the lung (similar effects have been observed with mAb treatment against other antigens expressed by B16F10) (29). We have shown that passive immunity against melanocytes can lead to effective rejection of even established tumors. Melanoma is not the only tumor type where differentiation antigens are recognized by the immune system. Immune recognition of differentiation antigens has also been detected in patients with breast, colon, and pancreas carcinomas (30–32).

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