Spontaneous Vitiligo in an Animal Model for Human Melanoma: Role of Tumor-specific CD8\(^+\) T Cells


Department of Immunology, Institut Cochon, INSERM U567, CNRS UMR 8104, Laboratoire membre de l’IFR 116, Université R. Descartes, Paris, France; Laboratory of Tumor Immunology, Division of Oncology, Geneva, Switzerland; Unité de Recherche et d’Expertise en Histotechnologie et Pathologie, Institut Pasteur, Paris, France; Department of Immunology, Nagoya University School of Medicine, Nagoya, Japan; UPR 9021 CNRS, Strasbourg, France; and INSERM CRI/IFR69, Hôpital P. Brousse, Villejuif, France

ABSTRACT

Tumor antigen-reactive T cells can be detected in a large proportion of melanoma patients, but their efficacy on tumor control in vivo remains unclear. On the other hand, vitiligo, a skin disorder characterized by patchy depigmented macules, may occur spontaneously or after antitumor therapies. Moreover, vitiligo is significantly associated with positive clinical response, but the mechanism is not understood. Therefore, the establishment of a relevant animal model in which melanoma and vitiligo spontaneously develop stepwise may be useful for better understanding of the parameters involved in the destruction of both benign and malignant melanocytes. In a previous work, we established a mouse model for melanoma in which MT/ret transgenic mice express the ret oncogene fused to the metallothionein promoter. Here we report that melanoma leads to spontaneous vitiligo. We further investigate, for the first time in this model, the natural antitumor T-cell response and evaluate the role of cellular immunity in the development of the disease. Interestingly, the occurrence of spontaneous tumor nodules in MT/ret mice with melanoma-associated vitiligo is significantly delayed when compared in melanoma mice without vitiligo. Moreover, a significant proportion of mice with melanoma-associated vitiligo resisted a challenge with syngeneic melanoma cells in contrast to animals without vitiligo. Our results confirm that vitiligo is associated with clinical benefit and further demonstrate the crucial role of CD8\(^+\) T cells for tumor control in melanoma-associated vitiligo.

INTRODUCTION

A large proportion of melanoma patients spontaneously develop a strong response to melanocyte differentiation antigens (MDAs), providing direct evidence for induction of antitumor immunity during cancer progression. However, the efficiency of tumor-reactive T cells in controlling tumor progression in vivo remains unclear. The onset of vitiligo, a skin disorder characterized by patchy depigmented macules, has been thought to be the result of immune-mediated destruction of melanocytes, although other mechanisms may lead to their destruction (1). Recent studies support the involvement of cellular immunity in vitiligo development. Ogg et al. (2) were the first to identify, in patients with non-melanoma-associated vitiligo, skin-homing autoreactive circulating T cells that specifically recognize melanoma cells in vitro. Moreover, we recently studied T cells from vitiliginous skin margins, and our data clearly support the role of CD8\(^+\) T cells specific for MDAs in vitiligo occurring naturally in melanoma patients (3). Antimelanoma therapies sometimes lead to the development of vitiligo, and this permanent loss of skin pigment is significantly associated with positive clinical response. Indeed, it has been observed specifically in patients with metastatic melanoma who respond favorably to interleukin (IL)-2 (4). Vitiligo also occurs in melanoma patients after infusion of tumor-specific CD8\(^+\) T cells (5, 6). The prevalence of antibodies and T cells that are MDA specific in both melanoma and vitiligo patients highlights the similarities between autoimmune observed in vitiligo and antitumor immunity observed in melanoma immune surveillance (1). However, the exact role of the immune response specific for MDA in vitiligo development and melanoma control remains unclear.

Progressive skin depigmentation occurs spontaneously in several animal models of nonmelanoma-associated vitiligo, including swine, Smyth chickens, and the C57BL6/Ler-vit/vit mouse strain (7). It has been suggested that the vitiligo pathogenesis is similar in humans and animals. Indeed, antibodies against the pigmented cell surface antigens have been found in dogs, cats, and horses with vitiligo (8), and T cells infiltrating feather tissue are abundant before visible signs of vitiligo and in tissues undergoing active loss of pigment in Smyth chickens (9). Frequent combination of melanoma and vitiligo has been observed in Arabian horses and Sinclair swine (10), heralding regression or slow progression of the melanoma, but no relevant mouse model is currently available. Therefore, the establishment of a suitable animal model in which melanoma and vitiligo spontaneously develop stepwise would contribute to a better understanding of parameters involved in the destruction of both benign and malignant melanocytes. By introducing the human ret oncogene fused to the murine metallothionein (MT) promoter-enhancer, Iwamoto et al. (11) produced MT/ret transgenic mouse lines with a mixed strain background (C57BL/6 × BALB/c). In these mice, skin melanosis and benign melanocytic tumors developed slowly. More recently, a new line was established by back-crossing one MT/ret line with C57BL/6 mice. In this line, the process of tumor development and malignant transformation bears multiple resemblances with that of the human giant congenital melanocytic nevus that gives rise to cutaneous melanoma during aging (12).

In the present report, we show that a large proportion of MT/ret animals spontaneously develop melanoma-associated vitiligo. We further used this model to evaluate the role of cellular immunity in the disease development and to address the following questions: Can we detect melanoma-specific T cells in peripheral blood lymphocytes? What are their antigen specificities? Does T-cell frequency correlate with disease severity? Does the occurrence of spontaneous vitiligo correlate with a higher frequency of melanoma-specific circulating T cells? Does vitiligo correlate with regression of the spontaneous melanoma or protect mice against challenge with syngeneic melanoma cells? Are CD8\(^+\) T cells crucial for melanoma control in mice with melanoma-associated vitiligo?

MATERIALS AND METHODS

Mice. MT/ret transgenic 304/B6 mice (12) were established by crossing line 304 MT/ret mice (originating from a BCF, mouse, H-2\(^b\)) five times with...
Following the cross between C57BL/6 and BALB/c, all of the mice used in the present report have been back-crossed six times with C57BL/6 mice and contain <15% of BALB/c genes. Mice were kept under standard conditions except that zinc (115 μg/l) was present in their drinking water. Clinical signs were assessed twice a month, and development of exophthalmus, facial or dorsal tumor nodules, and vitiligo was recorded. Mice were used for experiments at different time points in the course of malignancy. Control mice were nontransgenic littermates.

**Histology.** Skin samples were taken from the periphery of vitiligo patches. Skin tissues were fixed in a solution containing zinc acetate (0.5%), zinc chloride (0.5%), and calcium acetate (0.05%) in Tris buffer at pH 7 for 72 h. They were then embedded in low-melting-point paraffin (37°C; polyethylene glycol distearate; Sigma-Aldrich, St. Louis, MO). Sections (5 μm) were deparaffinized in absolute ethanol, air dried, and stained with Fontana-Masson for melanin pigments.

**Peptides and Cell Lines.** Peptides corresponding to murine TRP2 180–188 (VYDFFVWL), TRP2 180–185 (SVYDFFVW), and GP100 25–33 (EGRSRQDWL) melanocyte lineage normal differentiation proteins were synthesized and purified by high-performance liquid chromatography to >90% purity. Lyophilized peptides were diluted to 2 mg/ml in 10% DMSO and 90% water and were aliquoted and stored at -20°C.

A Melanoma cell line (Melan-ret) was established from a facial nodule that developed in a Melan-ret mouse back-crossed six times with the C57BL/6 strain. This line was grown in RPMI 1640 with 10% FCS, 2 mm L-glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). For ELISPOT assays, this line was cultured for 24 h in complete medium containing 100 units/ml mouse recombinant IFN-γ (Boehringer Mannheim, Mannheim, Germany). IFN-γ treatment of Melan-ret cells significantly enhanced MHC expression (data not shown), as already reported.

**Results.**

Treatment with Heavy Metal Leads to Vitiligo Development in MT/ret Mice Spontaneously Developing Melanoma. In the present study, we used transgenic mice expressing the human ret oncogene back-crossed five times with C57BL/6 mice. After 3 months, all MT/ret mice developed benign melanocytic tumors that slowly progressed to cutaneous melanoma in 65% of the mice within 10 months (12). Tumor cells metastasized mostly in lymph nodes, lung, and brain. These are also privileged metastatic sites in melanoma patients. Because the transgene construct contained a MT promoter, we thought we could promote melanoma development by adding zinc to the water given to mice from birth. In a first series of 88 mice, the tumor incidence increased, and melanoma progressed more rapidly in mice bred under these conditions compared with those described previously (12). Indeed, 80% of our MT/ret mice displayed exophthalmus or cutaneous nodules 6 months after birth. Exophthalmus was in most cases the first sign of tumor development. It was followed by the development of cutaneous melanocytic nodules on the face (cheek, nose, ear, and neck) and on the back of the head (back, tail, leg muscles, and genitals). Sixty-eight % of mice 6 months of age displayed exophthalmus, in contrast to the 35 and 26% of mice that displayed facial and dorsal nodules, respectively. Exophthalmus was diagnosed earlier than facial (P = 0.004) and dorsal (P = 0.001) nodules. No significant difference was observed between the onset of facial and dorsal nodules (P = 0.656).

A clear-cut correlation between mortality and exophthalmus or nodules was observed. The development of dorsal nodules was a worse prognostic factor (Pearson coefficient, r = 0.841; P < 0.001; n = 36). The correlation coefficient with mortality decreased to 0.73 (P < 0.001) for exophthalmus and facial nodules. These observations allowed us to define a severity grade of melanoma, where exophthal-mus and facial nodules corresponded to a less aggressive disease compared with dorsal nodules. Skin of MT/ret mice exhibited a black color attributable to melanosis as illustrated on newborn mice (Fig. 1A) and on depilated adult mice (Fig. 1B, left mouse) in contrast to the pink color of control nontransgenic mice (data not shown). Unexpectedly, vitiligo was observed in nearly one-half of MT/ret animals (45 of 88), and this depigmentation was permanent. The site and pattern of pigment loss differed from mouse to mouse [face, back (right mouse in Fig. 1B) or tail (Fig. 1, C and D)]. Vitiligo occurred as depigmentation surrounding tumors (Fig. 1D) or distant from tumor nodules (Figs. 1, B and C). Histopathological analysis of skin samples taken from the margin of vitiligo patches confirmed the loss of melanocytes in the epidermis and dermis. Fontana-Masson staining of skin sections (Fig. 1, E, middle part and F, right part) revealed the absence of pigments in the depigmented area of the tail (Fig. 1C). Only few pigments were still detectable in the deep dermis (Fig. 1E). Interestingly, in sections of some cutaneous nodules, pigments were absent in a large proportion of the dermis above the tumor nodule.
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1H). To assess such a hypothesis, we monitored the tumor-specific IFN-γ response in MT/ret melanoma mice and compared it with the response in mice with concurrent vitiligo. Many reports have indicated that IFN-γ plays a critical role in the development of tumor immunity, and recent reports including ours using IFN-γ knock-out mice demonstrated a crucial role for IFN-γ in the control of B16 melanoma growth in vivo (15–17). To evaluate natural tumor-specific response, ex vivo IFN-γ-ELISPOT assays were performed by using PBLs isolated from MT/ret mice as effector cells and a Melan-ret cell line as melanoma antigen-presenting cells. This Melan-ret cell line was established from a nodule of a MT/ret mouse. IFN-γ secretion by PBLs in response to Melan-ret cells revealed that naturally processed melanoma-associated antigens were presented to and recognized by T cells (Fig. 2A). IFN-γ was not secreted when EL-4 thymoma cells were used as antigen-presenting cells (not shown). The number of melanoma-specific PBLs was significantly different from mouse to mouse and may be as high as 1 PBL of 1000 PBLs in mice without vitiligo (Fig. 2A, left part) and as high as 1 PBL of 680 in mice with melanoma-associated vitiligo (Fig. 2A, right part). Reactivity was found in 61% (28 of 46) of melanoma mice. Thirty-nine % (18 of 46) of them displaying <1 IFNγ-secreting PBL of 10⁴ in response to Melan-ret cells were classified as nonresponders. Interestingly, the number of responders decreased as the severity of symptoms increased. Indeed, 71% (10 of 14) and 69% (11 of 16) of melanoma mice with exophthalmus and facial nodules, respectively, had IFN-γ-secreting cells in response to melanoma cells, whereas only 44% (7 of 16) of animals with dorsal nodules showed this response (Fig. 2B). Antitumor reactivity was found in 93% (25 of 27) of mice with melanoma-associated vitiligo, and in contrast to mice without vitiligo, this reactivity did not significantly depend on disease severity (Fig. 2B). To further determine the immune effector cells responsible for melanocyte destruction, biopsy samples taken from the margin of a vitiligo patch were minced and cultured as we reported recently in humans (3). Interestingly, vitiligo-infiltrating lymphocytes that proliferated expressed CD8 antigen and specifically recognized Melan-ret cells (Fig. 2C).

One of our recent studies in human has highlighted the natural induction of CD8⁺ T cell-mediated cellular immunity in the course of vitiligo and melanoma progression (3). Therefore, we focused our investigations on CD8⁺ T cell-mediated antitumor immunity. As a prelude to studying the fine antigen specificities of tumor-specific CD8⁺ T cells, the expression of MDA transcripts was studied in the tumors by reverse transcription-PCR. TRP2 and GP100 transcripts were expressed in most organs (skin, lymph nodes, and lungs) macroscopically invaded by tumor cells (data not shown). TRP2₁₈₁–₁₈₈ (18) and TRP₂₁₈₀–₁₈₈ (19) epitopes are presented by the K⁺ MHC class I molecule, whereas GP100₂₅–₃₃, epitope (20) is presented by D⁵. Interestingly, pooled PBLs from mice responding with a high frequency to the Melan-ret cell line also responded with a high frequency to a pool of these TRP2 and GP100 epitopes (Fig. 2D). To ensure that both epitopes were recognized, pools of PBLs isolated from MT/ret mice were restimulated with TRP2- or GP100-derived peptides separately. T cells specific for each epitope were detected in PBLs from responder mice (Fig. 2D). PBLs from C57BL/6 mice immunized with human GP100₂₅–₃₃ or murine TRP2₁₈₀–₁₈₈ peptides secreted IFN-γ both in response to these peptides and to the Melan-ret cells, confirming that these peptides are naturally presented by the Melan-ret cell line (not shown). As expected, no significant response against GP100₂₅–₃₃ and TRP₂₁₈₀–₁₈₈ peptides was found when pooled PBLs isolated from MT/ret mice that exhibited no antitumor response were used (data not shown).

![Fig. 1. Spontaneous vitiligo in MT/ret melanoma mice. Features of vitiligo and correlation with tumor growth suppression. A–D, photographs of MT/ret transgenic mice. Pigmentation of the whole skin after birth (A, 1–5-day-old transgenic mice). The melanosis is homogeneous in newborn (A) and in adult (B, left mouse). In B–D, depigmentation occurs on the back (B, right mouse) and on the tail (C and D). Note a small tumor nodule on the depigmented tail (D). E and F, skin biopsy specimens at the margin between the normal and vitiliginous tail skin of the 30-week-old mouse in C were stained with Fontana-Masson (×125). Melanin-containing cells are abundant in the dermis and epidermis of the pigmented skin, and only a few such cells are detectable in the deep dermis of the depigmented zone. G, biopsy of a cutaneous nodule consisting of melanoma cells stained with Fontana-Masson (×125). The upper dermis nodule shows melanin-producing cells on the right, in contrast with absence of pigments on the left. H, comparison of the percentage of 6-month-old mice with melanoma alone (n = 43) or with melanoma-associated vitiligo (n = 45) that presented defined clinical signs (exophthalmus, facial and dorsal nodules). No significant difference in exophthalmus incidence was found when melanoma mice (n = 31), and mice with melanoma-associated vitiligo (n = 26) were compared (χ² test, P = 0.995). In contrast, facial nodules were significantly more frequent in melanoma mice (n = 20) than in mice with melanoma-associated vitiligo (n = 10, P = 0.012). Similar data were obtained with dorsal nodules that were also significantly more frequent in melanoma mice (n = 15) than in mice with melanoma-associated vitiligo (n = 10, P = 0.03).](image-url)
Spontaneous Vitiligo Development Is Associated with Melanoma Protection. To assess whether self-reactivity can be useful for melanoma control, MT/ret mice were challenged s.c. with Melan-ret cells. The outgrowth of the s.c. tumor (referred as s.c. tumor) was significantly suppressed in mice with melanoma-associated vitiligo. Indeed, 5 of 12 MT/ret mice in this group (42%) were protected 40 days after transplantation (Fig. 3A). In contrast, s.c. tumor developed in most control littermates (82%). Tumor incidence was even higher in MT/ret mice that had already spontaneously developed a melanoma (88%). The difference in s.c. tumor development was more significant 140 days after tumor challenge, when 25% of MT/ret mice with vitiligo were still protected, in contrast to 100% of MT/ret mice with melanoma alone that developed a s.c. tumor (data not shown, Fisher’s exact test, $P = 0.0261$). Interestingly, 6 of 6 mice protected against tumor at day 40 displayed a significantly higher level of melanoma-specific PBLs, in contrast to mice that developed a s.c. tumor (Fig. 3B).

To further assess the role of the CD8$^+$ T-cell subpopulation in controlling melanoma in MT/ret mice with melanoma-associated vitiligo, depletion experiments were conducted starting 1 day before the challenge with Melan-ret cells. Two of 6 mice (33%) injected with the rat immunoglobulin non-depleting antibody controlled the s.c. tumor (Fig. 4A), consistent with the number of mice resistant in the absence of antibody treatment (Fig. 3A). In contrast, CD8$^+$ T-cell depletion favored the transplanted tumor outgrowth. In this latter group, tumor incidence was increased and tumor development was more rapid. Thirty-three % of MT/ret mice treated with control antibody were still free of tumor at 100 after tumor challenge in contrast to CD8$^+$ T-cell depleted mice, who were all dead by day 65 (Fig. 4B).

DISCUSSION

In this study, we used MT/ret transgenic mice, an animal model for human cutaneous melanoma recently established by some of us. We investigated for the first time in this model the natural antitumor T-cell response and further evaluated the role of cellular immunity in the course of disease development. Interestingly, our data show that a large proportion of MT/ret animals spontaneously developed melanoma-associated vitiligo. To our knowledge, this is the first mouse model in which melanoma leads to vitiligo. The pattern we observed bears strong resemblance to the vitiligo that occurs spontaneously in some melanoma patients. In humans, melanoma therapy also sometimes leads to the development of vitiligo that is significantly associated with a positive clinical response (4–6, 21, 22). Similarly, we obtained a good correlation between vitiligo development and melanoma control in our MT/ret mouse model. Indeed, mice with vitiligo and melanoma displayed significantly fewer tumor nodules than mice of the same age with melanoma alone (Fig. 1H). Vitiligo was rarely observed without signs of melanoma. However, we cannot exclude the possibility that some nodules may be too small to be detectable by gross clinical examination. Therefore, it remains difficult to determine whether vitiligo delays melanoma outgrowth or whether melanoma precedes vitiligo in this model.

Autoimmune T cells have been identified in the blood of patients with progressive autoimmune vitiligo (2) and in the depigmented skin of patients with spontaneous melanoma-associated vitiligo (3). Vitiligo also occurs in melanoma patients after infusion of melanoma-specific PBLs, in contrast to mice that developed a s.c. tumor (Fig. 3B).
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specific CD8⁺ T cells (5, 6). In existing animal models, immunotherapy against melanoma may favor the appearance of vitiligo. Indeed, vaccine consisting of granulocyte/macrophage-colony stimulating factor-producing irradiated B16 cells and CTLA-4 blockade successfully treated mice carrying a small load of B16 melanoma cells. In the surviving mice, a melanoma-specific CD8⁺ T cell-dependent mechanism produces depigmented lesions (19). Similarly, adoptive transfer of T cells specific for MDA-derived peptides favors induction of vitiligo (17, 23). CTL-mediated depigmentation has also been reported in mice immunized with DNA encoding human TRP-2 (24).

Moreover, the antitumor effect of locally secreted IL-12 on B16 melanoma cells mediated by CD8⁺ T cells also leads to vitiligo-like coat color alteration (25). Finally, the tissue damage observed after melanoma treatment may result from an immune response independent of CD8⁺ T cells. Indeed, mice vaccinated with recombinant vaccinia virus encoding murine TRP1 display extensive skin depigmentation, and this melanocyte-directed autoimmunity depends on both TRP1-specific antibodies and CD4⁺ T cells (26).

Our results show a significant frequency of PBLs derived from MTRtet mice that secrete IFN-γ ex vivo on recognition of Melan-ret melanoma cells, in marked contrast to the absence of IFN-γ secretion by PBLs from nontransgenic littermates. T cells that secrete IFN-γ in response to Melan-ret cells were statistically more frequent in melanoma mice that developed vitiligo (mean, 0.058% of PBLs in 27 mice) than in mice that did not (mean, 0.029% of PBLs in 46 mice). This result suggests a crucial role for IFN-γ-secreting T cells in tumor control.

The antitumor reactivity is mediated by CD8⁺ T cells that are specific for TRP2- and GP100-derived epitopes, the only B16 tumor rejection antigens that have been characterized to date. The number of IFN-γ-secreting PBLs was as high as 1 PBL of 1686 in response to Melan-ret cells and 1 of 2673 in response to cells pulsed with TRP2- and GP100-derived peptides (Fig. 2D). Given an average of 10% CD8⁺ T cells in PBLs, the mean number of circulating CD8⁺ T lymphocytes specific for melanoma-derived peptides from responder mice corresponded to 1 of 270 (0.37%). Interestingly, this is relatively close to the proportion found in humans. Indeed, the circulating CD8⁺ T-cell population specific for Mart127–35 and for tyrosinase368–376, may represent 0.014–0.5% and 0.19–2% of total CD8⁺ T cells, respectively, in HLA-A*0201-positive metastatic melanoma patients (27–29). Tumor-specific circulating lymphocytes in MTRtet mice are more frequent than cells specific for TRP2181–188, TRP2180–188, and GP10025–33, suggesting recognition of other CD8 T-cell epitopes presented at the melan-net cell surface and which remain to be identified. Melanoma-specific T cells in freshly isolated PBLs of MTRtet mice are currently used to identify naturally processed and MHC class I-presented peptides derived from the Melan-ret line. In this study, we focused our investigations on T-cell-mediated antitumor immunity and more particularly on CD8⁺ T cells.

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However, cells that secrete IFN-γ in response to Melan-ret cells were also found in PBLs depleted of CD8+ lymphocytes (data not shown), suggesting the recognition of tumor-derived CD4+ T-cell epitopes. Such naturally processed and MHC class II-presented peptides derived from melanoma cells have yet to be identified.

Thirty % of mice with melanoma-associated vitiligo were totally resistant to challenge with syngeneic Melan-ret cells (Fig. 3A), and this effect depended on CD8+ T cells (Fig. 4A). Interestingly, 4 of 4 vitiligo mice that were protected had a higher frequency of melanoma-specific T cells than the vitiligo mouse that developed a s.c. tumor as assessed by IFN-γ ELISPOT. Our data indicate that CD8+ T cells mediate, at least in part, spontaneous development of vitiligo, which can be seen as an early sign of protective immunity against melanoma.

This model enables antitumor response and tumor escape to be studied in parallel. The reduced number of responders in mice with dorsal nodules may be attributable to poor expansion of tumor-specific cells or to the loss of their capacity to secrete IFN-γ. It is therefore crucial to characterize in longitudinal in vivo studies the different functional stages (homing and effector capacities) of T lymphocytes and their frequency. In melanoma patients, tumor-specific CD8+ T cells may display an antigen-experienced phenotype both in the periphery and in metastatic lymph nodes (27–31). Alternatively, melanoma-specific CD8+ T cells may become functionally unresponsive as shown for tyrosinase368-376-specific T cells in one patient (28). The MT/ret model will be essential for in vivo study of the functional status of antimielanoma effector cells at different stages of tumor development, because such longitudinal studies are not possible in humans. The MT/ret model will also be helpful for further assessment of the role of autoantibodies and CD4+ or CD8+ T cells in the development of spontaneous melanoma-associated vitiligo compared with treatment-induced vitiligo.

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Renée Lengagne, Frédérique-Anne Le Gal, Marylène Garcette, et al.


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