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Distinct Role for CD8 T Cells toward Cutaneous Tumors and Visceral Metastases

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The growth of immunogenic tumors in immunocompetent individuals is one of the oldest conundrums in tumor immunology. Although the ability of mouse CD8+ T cells to control transplanted tumors is well documented, little is known about their impact on autochthonous tumors. To gain insight into the role of CD8+ T cells during the course of cancer development, we produced a novel model of spontaneous melanoma. The metallothionein (MT)-ret/AAD mouse is transgenic for the RET oncogene and the chimeric MHC molecule AAD (α1-α2 domains of HLA-A2 linked to α3 domain of H2-Dd). This model recapitulates the natural history of human melanoma, and expression of the AAD molecule makes it suitable for analyzing CD8+ T cell responses directed against peptide Ags that have been previously identified in HLA-A2+ melanoma patients. We found that, as tumors grow, mice develop a broad melanoma-specific CD8+ T cell response. Occurrence of cutaneous nodules is not affected by CD8+ T cell depletion, showing that although CD8+ T cells are functional, they have no effect on established cutaneous tumors. However, depleted mice die from visceral disease much earlier than controls, showing that CD8+ T cells control metastasis spreading and disease progression. Antigenic modulation is observed in visceral metastases, suggesting that visceral nodules may be subject to immunoediting. Our data demonstrate that growth of melanoma in the MT-ret/AAD model involves several tolerance mechanisms sequentially. They also reveal a different role for CD8+ T cells toward early stage of cutaneous tumors and late visceral metastatic stage of the disease. The Journal of Immunology, 2008, 180: 130–137.

Although a large number of melanoma-associated Ags recognized by T lymphocytes have been identified, activation of tumor-specific lymphocytes is rarely associated with clinical response in patients (1, 2). On one hand, in a few experimental models, tumor-specific CD8+ T lymphocytes can prevent or eradicate tumors. Moreover, a handful of clinical studies have reported expansion of tumor-specific CD8+ T cells in regressing melanomas (3, 4) or correlation between clinical response to immunotherapy and presence of tumor-specific CD8+ T cells (5–8), strongly supporting a role for these cells in disease control. But in contrast, in most patients, CD8+ T cells are detected and fail to control tumor growth (9), a situation not unique to melanoma. Indeed, attempts to harness the immune system against cancer have met with little success so far (10). Three classes of mechanisms have been proposed to explain the paradoxical growth of immunogenic tumors in the presence of an active immune response. First, in the absence of lymphatic drainage or “danger” signal, tumors could simply be ignored by the immune system (11). A second mechanism, tumor immunoediting, has recently received experimental support. Tumors, which are genetically and epigenetically unstable, can easily adapt to the immune attack, either by down-regulating tumor Ags or MHC molecules, or by shutting down death-signaling pathways leading to resistance to death-inducing cytokines and immune effector cells (12, 13). Finally, a third group of mechanisms involves systemic or local alterations of the immune system, especially in advanced cancer patients: impaired T cell function (defective cytokine secretion and proliferation) (14, 15), lack of perforin expression (16–18), down-modulation of the CD3ζ chain of the TCR (19), inhibition by ligands such as programmed death ligand 1 (20), extrinsic suppression by regulatory T cells (21–23), T cell propensity to apoptosis (24), accumulation of immature dendritic cells (25), or myeloid suppressor cells (26). All these mechanisms recently reviewed (27) could contribute to the lack of efficacy of the immune system against tumors. However, their respective contribution has been difficult to analyze hitherto due to the lack of relevant experimental models and the significance of active ongoing antitumor immune responses in cancer patients remains largely unexplained.

Mechanisms by which tumors escape the immune system have been mostly studied with transplanted tumors expressing surrogate tumor Ags. Tumor cell lines used in such studies had been previously selected for their ability to grow in vitro and therefore...
strongly differ from primary cancer cells isolated from spontaneous tumors, which usually have a poor clonogenic potential. It is now recognized that such tumor models fail to recapitulate the complex network of cellular interactions that is established during the course of disease progression in human cancers. Spontaneous tumor models are more likely to circumvent some of these drawbacks because gradual oncogenesis allows for prolonged interaction between growing tumors and the immune system.

In a recent study (28), we characterized the immune response specific for melanoma in the single-transgenic metallothionein (MT)6-ret mouse expressing the human RET oncogene (29), one of the fewmurine models of genetically driven melanoma (30). Our data indicated that naturally occurring CD8+ T cells were functional and protected MT-ret mice against an ectopic challenge with syngeneic melanoma cells. In the present study, we investigate the role of these CD8+ T cells against autochthonous tumors. The immune response to melanoma-associated Ags has been mostly studied in HLA-A2 patients, and many HLA-A2-restricted epitopes have been characterized; we therefore produced a double-transgenic mouse, referred to as MT-ret/AAD, that expresses both the human RET oncogene and the chimeric MHC class I molecule AAD (α3 and α5 domains of HLA-A2 linked to the α3 domain of H2-Dd3), which has been shown to present HLA-A2-restricted peptides. In this new model, we evaluated the role of CD8+ T cells during the course of melanoma progression. The MT-ret/AAD model provides a unique opportunity for analyzing the mechanisms by which tumors eventually escape immune control.

Materials and Methods

Mice

MT-ret mice (29) were crossed with AAD mice expressing a chimeric MHC class I composed of the α3 and α5 domains of HLA-A*0201 and the α3 domain of H2-Dd3 (31) to produce MT-ret/+ AAD+/− (MT-ret/AAD) mice. Both MT-ret and AAD mice were on the C57BL/6 background. Clinical signs of MT-ret/AAD mice were assessed twice a month. All experiments were performed in compliance with French Ministry of Agriculture regulations for animal experimentation.

Tumor cell lines and flow cytometry

The B16AAD cell line (32) was derived by gene transfection using the B16.F10 melanoma cell line provided by Christiane Biever (University of California, Los Angeles) and Selection of the B16AAD cell line was performed using the Graphpad software.

Real-time quantitative RT-PCR

RNA was prepared from fresh tumors isolated from MT-ret/AAD mice with the Qiagen RNA extraction kit. RNA (1 μg) was reverse transcribed using reverse transcriptase (Invitrogen Life Technologies). Real-time quantitative PCR was performed using Light Cycler and SYBR Green (Roche). Primers are available upon request. Expression of GAPDH was used as endogenous reference, and the target gene expression in each sample was normalized to B16.F10. The relative gene expression level is presented as the following equation: R = ΔCP_target (B16.F10 − sample)/ΔCP_GAPDH (B16.F10 − sample), where ΔCP represents target expression and GAPDH expression as indicated. The fold increase or decrease expression for one particular Ag corresponds to the ratio between the value of R for one tumor and the value of R for the primary ocular tumor.

Abbreviations used in this paper: MT, metallothionein; MDA, melanocyte differentiation Ag; FI, fluorescence index; TRP, tyrosinase-related protein.

Peptides

Peptides corresponding to human HLA-A2-restricted epitopes gp100134 (KTVWWYQW), gp100166 (VLYRYSQFSV), Ty360 (Y5MNTM5QVY), Mart127 (AAAGILTV), Mart132 (ILTVIL0VLV), Mart136 (ALMDDK3LIV) derived from melanocyte differentiation Ags (MDA), and to the murine K* -restricted tyrosinase-related protein 2 (TRP2132) (SVYDFVWVL) and D*-restricted gp10056 (EGRSNQDWL) were synthesized and purified by HPLC.

IFN-γ ELISPOT

Single-cell suspensions from spleens of MT-ret/AAD mice were prepared. After erythrocyte lysis, splenocytes were either used directly in ELISPOT assays to assess their capacity to secrete IFN-γ in response to tumor cells, or restimulated for 5 days with a pool of murine (gp100134 and TRP2132), or human (Mart127, 132, Mart136, gp100134, gp100166, Ty360) peptides in the presence of human recombinant IL-2 (10 IU/ml) (Boehringer Mannheim) and then tested either against the same pool of peptides or against individual peptides. ELISPOT assay was performed as previously described (28). Splenocytes (106 cells/well) were incubated with tumor cells (105 cells/well) or with peptides (10−6 M). When indicated CD8+ or CD4+ T cells (105 cells/well) were purified from pooled PBLs of MT-ret/AAD tumor bearing mice by using magnetic beads conjugated to anti-mouse CD8 mAb and anti-CD4 mAb (Miltenyi Biotech) according to the manufacturer’s recommendation. Purified T cells were kept overnight at 37°C in complete medium (RPMI 1640 with 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin) before the incubation with a cutaneous Melan-ret/AAD cell line. To assess the HLA-A2 restriction, B16AAD cells were preincubated for 1 h with B7.2 before the assay.

Western blot analysis

Western blot was performed as recommended by Santa Cruz Biotechnology. Briefly, proteins from the whole tumor lysates (5 μg per lane) were separated by polyacrylamide 4−20% linear gradient gel electrophoresis and transferred to nitrocellulose membranes. They were incubated in Blotto B, then in primary goat Ab (specific for tyrosinase (sc-7834) or TRP2 (sc-10452) at 1 μg/ml) for 1 h at room temperature and then with HRP-conjugated secondary donkey anti-goat Ab (sc-2020, 1:1000) in Blotto B for 45 min at room temperature. Chemiluminescence ECL+ reagent (Amer sham) was used for Western blot visualization.

CD8 T cell depletion in vivo

The purified rat IgG2b 2.43 anti-mouse CD8 Ab (TIB 210; American Type Culture Collection) was injected i.p. (100 μg) in MT-ret/AAD mice at day 8−11 after birth and every week for at least 14 wk. Onset of melanoma in CD8-depleted MT-ret/AAD mice was recorded once a week and compared with a control group of animals treated with a purified rat IgG control Ab (see Fig. 6) or a group of untreated animals (Figs. 6 and 7). Statistical analysis was performed using the Graphpad software.

Results

Vitiligo occurrence in MT-ret/AAD mice is associated with delayed melanoma onset

MT-ret mice were crossed with animals expressing the chimeric AAD molecule. More than 85% of the MT-ret/AAD mice (n = 101) displayed tumors within 4 mo after birth, and 50% of them had several evident cutaneous nodules at day 64 (Fig. 1A). In all mice, the eye was the first site of tumor occurrence as revealed by the presence of marked exophthalmus. Nodules appeared significantly earlier on the face than in the posterior part of the body (trunk, tail, leg muscles, or genitals, hereafter referred as to back) (p < 0.001 by log-rank test) (Fig. 1B). Similar to data obtained in single-transgenic MT-ret animals (28), vitiligo was observed in 39% of MT-ret/AAD animals (Fig. 1B), and was associated with delayed appearance of cutaneous nodules (p < 0.0001) (Fig. 1C). Indeed, 50% of mice without vitiligo had visible tumors at day 50, whereas the median time for tumor detection in animals with vitiligo was day 88. However at later time points, there was not significant difference in the number and size of the tumors between animals with and without vitiligo and eventually, 95% of the mice developed melanomas.
MT-ret/AAD mice spontaneously develop an HLA-A2-restricted, antitumor T cell response

Next, we evaluated the spontaneous antitumor T cell response in individual tumor-bearing mice. Ex vivo IFN-γ ELISPOT assays were performed using fresh spleen cells isolated from tumor-bearing MT-ret/AAD mice as effector cells and B16.F10 melanoma cells expressing AAD (B16AAD) as APC. Jurkat or EL4-HHD cells were used as HLA-A2+ non-melanoma APC. B16AAD was recognized by splenocytes from eight representative mice (3- to 6-mo-old), even in the absence of in vitro restimulation (Fig. 2A). The frequency of B16AAD-reactive T cells ranged from 1:1.400 to 1:2.500, and was not significantly different in MT-ret/AAD mice with or without vitiligo. No response to Jurkat (Fig. 2A) or EL4-HHD (data not shown) was detected. To determine which T cells recognize the tumors, CD4+ and CD8+ T cells were purified from pooled PBLs derived from tumor-bearing mice. As shown in Fig. 2B, only CD8+ T cells produce IFN-γ in response to cutaneous Melan-ret/AAD syngeneic tumors. T cells secreting IFN-γ in response to B16.F10, which does not express AAD, were significantly less frequent than those responding to B16AAD, showing that tumor recognition was frequently HLA-A2 restricted (Fig. 2C). Consistent with this result, preincubation of B16AAD with the BB7.2 mAb specific for HLA-A2 reduced the number of IFN-γ-secreting cells by 80%, to a level equivalent to B16.F10. Similarly, in a chromium release assay, preincubation of B16AAD cells with BB7.2 reduced their lysis by splenocytes isolated from MT-ret/AAD mice (our unpublished data). All together, our data show that all tumor-bearing mice develop a strong spontaneous CD8+ T cell response against Ags expressed on B16AAD melanoma and this response is in part HLA-A2-restricted.

The fine specificity of the spontaneous antitumor response was analyzed by testing splenocytes against defined MDA-derived epitopes. Spleen cells from tumor-bearing MT-ret/AAD mice were restimulated with a pool of MDA-derived epitopes of murine (Kb-restricted TRP2180 and Dγ-restricted gp10023) or human (HLA-A2-restricted Mart127, Tyr369, gp100154, gp100476) origin and then tested against the same pool of peptides. Results shown in Fig. 3A established that splenocytes from tumor-bearing MT-ret/AAD mice react against MDA of both origins. This response was Kβ-, Dγ-, or AAD-restricted. As expected, splenocytes from single-transgenic tumor-bearing MT-ret mice did not recognize HLA-A2-restricted MDA-derived peptides. To further refine their specificity, splenocytes isolated from MT-ret/AAD mice displaying facial and dorsal cutaneous nodules were restimulated with a pool of MDA-derived epitopes (Tyr369, Mart127, Tyr369, gp100154, gp100476, TRP2180) and then tested against individual peptides (Fig. 3B). Different recognition profiles were observed despite the similar tumor location and tumor burden. In all analyzed mice, the response was directed against several immunodominant peptides (multispecificity). In particular, T cells specific for Tyr369 and TRP2180 were detected in all mice. But some epitopes (e.g., Mart127, and gp100154, gp100476) were only recognized by a limited number of mice. Moreover, we cannot exclude that mice might also react to other MDA- or cancer/testis-derived peptides. All together, whereas all tumor-bearing mice responded to the melanoma cell line B16AAD, our data reveal important differences in the specificity and breadth of the response against melanoma Ags.

Each tumor displays a unique HLA or MDA expression profile

Tumor growth in the presence of a vigorous melanoma-specific immune response may seem paradoxical, but is indeed frequently observed in melanoma patients. Loss of MHC class I molecules...
has been one of the most frequent escape mechanisms reported in these patients. We therefore looked for such an event in cutaneous tumors (facial or back nodules) and in visceral metastases. Because MHC down-modulation often occurs at a post-transcriptional step, we analyzed MHC expression at the protein level. To obtain a sufficient number of tumor cells, we derived short-term cultures that we expanded for no more than 1 or 2 wk. Using the BB7.2 mAb we stained 18 of these Melan-ret/AAD tumor cell lines (eight from cutaneous tumors of the face, four from cutaneous tumors of the back, and six from visceral metastases) isolated from 10 mice. AAD expression was quite heterogeneous, with fluorescence index (FI) ranging from 1 (no expression) to 10. There was no significant difference in AAD expression between cutaneous tumors derived from cutaneous tumors of the face, four from cutaneous tumors of the back, and six from visceral metastases) isolated from 10 mice. AAD expression was quite heterogeneous, with fluorescence index (FI) ranging from 1 (no expression) to 10. There was no significant difference in AAD expression between cutaneous tumors derived from the same mouse (mean FI 1.65 ± 0.8, not shown). However, visceral nodules expressed significantly less AAD than cutaneous tumors (mean FI 1.65 vs 3.75, respectively; p < 0.01). Moreover, as shown in Fig. 4A, AAD expression was also significantly lower in visceral metastases than in cutaneous tumors derived from the same mouse (mean FI 1.65 vs 2.65, respectively; p < 0.01). These results indicate that tumor cell lines derived from the same mouse but from different nodules could express different levels of AAD and that AAD expression is preferentially down-modulated on visceral metastases.

Another escape mechanism by which human tumors escape the immune system is through down-modulation of their tumor Ags. Such event is more frequently observed for aggressive tumors such as distant metastases. We therefore compared the expression of five tumor Ags in the different nodules isolated from individual mice using real-time quantitative RT-PCR and normalized with regard to the ocular tumor, which is the first site of tumor occurrence. Because of the strong homology between murine and human MDA, we focused on these Ags. To avoid possible biases due to in vitro culture, this analysis was performed ex vivo, on fresh tumors resected from two MT-ret/AAD mice (referred to as K138 and K171). Twenty tumors were analyzed for Melan-A/Mart1, gp100, tyrosinase, TRP1, and TRP2. Tumors included superficial cutaneous nodules (eye, nose, neck, cheek, ear, legs, sacrum, and tail) as well as visceral tumors (mediastinal lymph nodes, liver, and lung). Each tumor displayed a unique MDA expression profile. For most cutaneous tumor samples, alterations in MDA expression were rather modest, and both down-modulation and overexpression were observed as illustrated for tyrosinase and TRP2 in eight representative tumors (Fig. 4B). Interestingly, much stronger variations were observed for visceral metastases: in mouse K171, the relative abundance of tyrosinase transcripts was reduced 90- and

![FIGURE 3](image-url) Specificity of the antitumor T cell response in MT-ret/AAD mice. A. Splenocytes from MT-ret or MT-ret/AAD mice were restimulated with either Dβ- and Kβ-restricted (gp10025 and TRP180) or A2-restricted peptides (Mart127, gp100154, gp100476, Tyr369) for 5 days, before being assessed for IFN-γ secretion using the same peptide pools. B. Splenocytes from 8-mo-old MT-ret/AAD mice bearing cutaneous tumors (K246, K250, and K251) were restimulated for 5 days in vitro and then tested by IFN-γ ELISPOT assay using individual A2-restricted melanoma peptides (Tyr369, Mart127, Mart132, Mart156, gp100154, gp100476, TRP2180). These results are representative of 10 mice analyzed.

![FIGURE 4](image-url) AAD and tumor Ag expression. A, HLA-A2 modulation in Melan-ret/AAD tumor cell lines. Melan-ret/AAD cell lines derived from cutaneous (n = 5) or visceral (n = 6) nodules derived from five tumor-bearing mice were stained with BB7.2 and analyzed by flow cytometry. Fluorescence index corresponds to the ratio between the mean fluorescence intensity after staining with the BB7.2 Ab and the isotype control. Significant reduction of AAD expression was observed in visceral nodules (p < 0.01; paired t test). B, Tumor Ag down-modulation in visceral tumors. The expression of tyrosinase and TRP2 in 12 representative freshly resected tumor nodules was measured by real-time quantitative RT-PCR. Shown are eight cutaneous tumors (1: eyelid; 2, 3, and 4: cheek; 5: ear; 6: leg; 7: sacrum and 8: back) from K171 and four distant metastases (9: liver from K171; 10: lung from K171; 11: lung from K138 and 12: mediastinum from K100). Values are expressed as fold increase over the primary ocular tumor. Significant reduction of tyrosinase and TRP2 expression was observed in visceral nodules (p = 0.002 for both Ag, by Mann-Whitney U test). C, Tumor Ag down-modulation in visceral tumors. Tyrosinase and TRP2 were measured at the protein level by Western blotting.
630-fold in lung and liver, respectively, whereas TRP2 transcripts were reduced 850- and 600-fold, respectively. Similarly, in mouse K138, a lung metastasis displayed 14- and 7-fold reduction in tyrosinase and TRP2 expression, respectively. Strong down-modulation of tyrosinase and TRP2 transcripts (550- and 900-fold, respectively) was also observed in a mediastinal tumor isolated from a third mouse (mouse K100). Down-modulation of tyrosinase and TRP2 expression was further confirmed by Western blot analysis (Fig. 4C). All together, our data show that the profiles of MHC and MDA are unique to each tumor and that the most significant down-modulations are in visceral lesions and concern tyrosinase and TRP2 Ags.

Most cutaneous tumor cell lines derived from MT-ret/AAD mice are recognized by tumor-specific T cells

We next assess whether these modulations of MHC and MDA could explain the tumor escape from the T cell response. We first isolated splenocytes from tumor-bearing mice and showed that they efficiently recognize B16AAD (Fig. 2 and data not shown). We next tested these splenocytes against 24 short-term cultures derived from independent nodules of 12 different mice in an IFN-γ ELISPOT assay. Twelve Melan-ret/AAD cutaneous tumor cell lines (of 14) were efficiently recognized (Fig. 5). One cutaneous line (K4-back) failed to be recognized, although another line derived from a nodal nodule on the same mouse (K4-face) efficiently induced IFN-γ secretion. Interestingly, cell lines derived from visceral metastases were among the less immunogenic and were significantly less recognized than cutaneous tumors derived from the same mice (p < 0.001). Therefore, these data show that although some cutaneous tumors modulate expression of their MHC and MDA, this modulation does not prevent T cell recognition and MHC or MDA modulation is unlikely to explain cutaneous tumor escape from T cells. In contrast, the reduced expression of AAD or MDA on visceral nodules (Fig. 4) may contribute to their poor immunogenicity (Fig. 5).

CD8 T cells do not prevent melanoma outgrowth, but delay disease progression

Our previous data showed that naturally occurring CD8+ T cells protected MT-ret mice against a challenge with syngeneic melanoma cells (28). To determine whether these cells also prevent the onset of spontaneous melanoma, we compared tumor outgrowth in MT-ret/AAD animals treated with anti-CD8 mAb (Ab 2.43) and in control mice (either untreated or treated with the purified rat IgG control Ab). Depletion experiments were conducted starting at 8–11 days of age. The median age at which tumors developed was similar in both groups (p = 0.54, log-rank test) (Fig. 6A). No significant difference was observed in the incidence of vitiligo. By contrast, CD8 T cell-depleted animals had significantly shorter survival than the control mice (p < 0.0001, log-rank test; median survival 160 days) (Fig. 6B). As early as 3 mo of age, depleted mice began to die, and by 6 mo >70% of them had died, whereas >90% of the control mice were still alive. No significant increase in the size or number of the cutaneous nodules was observed in the depleted mice. When feasible, dead mice were examined postmortem. All analyzed mice displayed lung or other visceral metastases, suggesting that visceral but not cutaneous tumors were the cause of the death.

CD8 T cell delay the spread of visceral metastases

The systematic presence of visceral metastases associated with premature death in CD8-depleted mice suggested that CD8+ T cells prevent the spread of metastases in control animals, for example by eliminating circulating cancer cells. Alternatively, CD8+ T cells could prolong mouse survival without affecting the kinetics of development of visceral metastases. To distinguish between these two possibilities, 96 tumor-bearing mice between 162 and 367 days of age were culled and examined for the presence of visceral metastases postmortem. The vast majority of these mice (87 of 96) were found free of visceral metastases. Therefore, we estimated that by the age of 162 days, only 15% of the CD8-sufficient mice had died (six mice) or had visceral metastases (nine mice), whereas 93% of the CD8-depleted mice has succumbed to metastatic disease (p < 0.0001, Fisher’s exact test) (Fig. 7). Moreover the kinetics of appearance of visceral metastases in CD8-sufficient mice was not significantly different from that of survival (data not shown), reinforcing the interpretation that visceral metastases were the proximal cause of death in MT-ret/AAD mice. Because these CD8-sufficient mice were analyzed at a time were most CD8-depleted mice had succumbed to metastatic disease, these data demonstrate that CD8 T cells delay the spread of visceral metastases.
Discussion

One of the major paradoxes in tumor immunology is the limited effect of functional immune responses in cancer patients. This applies for both humoral and cellular immunity. In the present study, we questioned the role of CD8\(^+\) T cells in a new model of spontaneous melanoma. It is usually proposed that such tumor-specific T cells may be ineffective in rejecting tumors because 1) their frequency is too low (34); 2) they are functionally deficient or suppressed by regulatory mechanisms (35–37); 3) they are unable to access the tumor site or lose their functionality locally (18); or 4) tumor cells are selected to escape T cell recognition (38–40). Recently Willimsky and Blankenstein (41) reported a model in which immunogenic tumors escape the immune response without losing immunogenicity, but instead by inducing systemic T cell tolerance. To further address the relative contribution of the various escape mechanisms, we developed a novel double-transgenic model, the MT-ret/AAD mouse, which recapitulates the natural history of human melanoma and is particularly suitable for CD8\(^+\) T cell analysis. In this model, we found that most mice develop a strong tumor-specific CD8\(^+\) T cell response and that these T cells efficiently recognize tumor cell lines derived from cutaneous nodules. Therefore systemic ignorance (11), anergy (35), or immunoediting (38) alone cannot explain the growth of cutaneous tumors. The escape of cutaneous tumor from the immune system involves some local suppression that deserves further exploration.

Systemic ignorance and anergy were excluded because most, if not all, tumor-bearing mice developed systemic melanoma-specific T cell responses spontaneously. Although these responses were detected ex vivo (i.e., before any restimulation), one could argue that they would not be functional in vivo. We do not favor this explanation because, as shown in the present study, CD8\(^+\) T cell depletion resulted in accelerated disease progression and death. Moreover, as shown previously in the single-transgenic MT-ret model, a large proportion of mice was able to reject and control transplanting syngeneic tumor cells, whereas this protection was lost upon CD8\(^+\) T cell depletion (28). We therefore are confident that tumor-specific CD8\(^+\) T cells that we detect ex vivo are indeed functional in vivo.

One of the striking observations made in the present study is the lack of effect of CD8\(^+\) T cell depletion on tumor appearance. The most likely explanation for this finding is the absence of melanoma-specific CD8\(^+\) T cells at the time when the depletion is initiated. Indeed, we did not detect tumor-specific T cells before 6 wk of age. It is important to note that the genetic defect in the MT-ret/AAD model is congenital and that the melanoma develops early in life. Tumors probably need to reach a minimal size and the immune system needs to mature before a significant immune response can be induced. Therefore, at early stages, tumors may be ignored by the immune system or the immune response may be too weak. Additionally, CD8\(^+\) T cells are most likely inactive against established cutaneous tumors as already observed in the single-transgenic MT-ret mice in which autochthonous tumors continue to grow, whereas transplanted tumors are rejected (28).

Vitiligo was detected in 40% of the MT-ret/AAD mice and was associated with a significant delay in melanoma onset. In response to increased melanogenesis induced by the RET oncogene (42), some mice may develop a melanocyte-specific immune response even before tumors develop. Whether this response is mediated by T cells, NK cells, or even Abs (possibly of maternal origin) remains to be determined. The fact that we did not detect melanoma-specific splenocytes before tumor development would suggest that CD8\(^+\) T cells are not involved in the induction of vitiligo, at least initially. The lack of effect of CD8\(^+\) T cell depletion on vitiligo onset would fit this interpretation. In humans, both humoral and cellular immunity have been associated with vitiligo (43, 44). In any case, this early melanocyte-specific immune response results in vitiligo and somehow delays the onset of the melanoma. But, as shown in Fig. 1C, this response is too weak to fully prevent the development of the disease and, eventually all animals develop tumors.

We focused our analysis of T cell specificity on four MDA: tyrosinase, MelanA/MART-1, gp100, and TRP2. Comparison of B16AAD recognition with that of seven peptides derived from these MDA suggests that MDA are not the only melanoma Ags recognized. In fact, the spontaneous melanoma-specific immune response that we observed was remarkably broad in most mice. When we compared various tumor nodules for the expression of MDA and AAD molecules, some variations were observed. AAD expression was measured on short-term Melan-ret/AAD cell lines and we cannot fully exclude that these differences appeared during the short culture in vitro. In any case, the differences observed in cutaneous nodules could not account for immune escape because most of these cultures efficiently stimulated melanoma-specific T cells. Therefore, immunoediting can be excluded as explanation for the growth of cutaneous tumors in the presence of a functional immune response.

The situation is different for visceral metastases in which modulations of MHC class I AAD expression or of MDA gene expression were observed. In particular, in mouse K171, TRP2 and tyrosinase expression was strongly reduced in both liver and lung tumors. The most likely explanation for this observation is that visceral metastases, unlike earlier cutaneous tumors, are subjected to immunoediting through the selective pressure exerted by T cells. During disease spreading, cancer cells migrate from the primary tumor site to the various visceral locations, which in our model is mostly the lungs and the liver. Such migrating cells are exposed to systemic T cells, a situation similar to the transplanted tumor cell suspensions that are sensitive to CD8\(^+\) T cells (28). This explanation would also account for the accelerated disease progression upon CD8 depletion. Indeed, the vast majority of CD8-depleted mice have succumbed to metastatic disease by the age of 160 days, whereas at this age, most CD8-sufficient mice are still alive and free of visceral metastases. One would predict that tumor cells that escape T cell selection would be more resistant to immunotherapy. Indeed, human patients with visceral metastases (M1b or M1c) have the worst prognosis and respond very infrequently to immunotherapy, whereas patients with in-transit or nonvisceral (stage III
and M1a) metastases are more likely to benefit from such treatments (45, 46). Interestingly, the strongest down-modulations in our model concern the tyrosinase and TRP2 Ags. This observation is reminiscent of that made by Sanchez-Perez et al. (47) who observed preferential loss of TRP2 and tyrosinase in B16 melanoma variants selected by immunoeediting in vivo. Alternatively primary tumors that have down-regulated the expression of their MDA and MHC may have a higher propensity to metastasize.

Finally, we propose a scheme in which the immaturity of the immune system facilitates the initial development of tumors. As mice age and their immune system matures, growing tumors induce a melanoma-specific CD8+ T cell response. But some local suppression mechanism precludes these CD8+ T cells from efficiently preventing tumor growth. Nevertheless, CD8+ T cells control tumor cells that migrate to visceral sites and delay the development of metastases until antigenic variants are eventually selected. In conclusion, in this model, ignorance, local suppression and tumor immunoeediting are involved sequentially during melanoma development.

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Disclosures

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