Melanoma-Specific Memory T Cells Are Functionally Active in Ret Transgenic Mice without Macroscopic Tumors

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Abstract
We previously reported that bone marrows of breast cancer patients contained tumor antigen–specific CD8+ T cells with central or effector memory phenotype. Using a recently developed ret transgenic mouse melanoma model, we now show that bone marrows and tumors of transgenic mice contain high frequencies of CD8+ T cells specific for the melanoma antigen tyrosinase-related protein 2 and showing mostly effector memory phenotype. Moreover, increased numbers of bone marrow tyrosinase-related protein-2–specific effector memory CD8+ T cells are also detected in transgenic animals older than 20 weeks with disseminated melanoma cells in the bone marrow and lymph nodes but showing no visible skin tumors and no further melanoma progression. After a short-term coinoculation with dendritic cells generated from the bone marrow and pulsed with melanoma lysates, bone marrow memory T cells from mice without macroscopic melanomas produced IFN-γ in vitro and exerted antitumor activity in vivo after adoptive transfer into melanoma-bearing mice. Our data indicate that functionally active bone marrow–derived melanoma-specific memory T cells are detectable at the phase of microscopic tumor load, suggesting that thereby they could control disseminated melanoma cells. [Cancer Res 2008;68(22):9451–8]

Introduction
Immunogenicity of human malignant melanoma is well documented, including an identification of large numbers of melanoma antigens and development of spontaneous tumor regressions in some patients thereby providing direct evidence for the induction of antitumor immunity, in which T cells play a key role (1–3). In this respect, melanoma antigen–specific memory T cells are of special interest because they are known to react to tumor antigens indifferents forms (tumor cell lysate, tumor specific proteins, or peptides) were shown to produce IFN-γ (8, 9, 14–16) and to acquire antitumor cytotoxicity in vitro and in vivo (8, 9, 14, 15). Naive BM T cells were shown to be primed in conditions of altered lymphocyte trafficking in splenectomized mice (17) or in animals with normal lymphoid organs resulting in generation of cytotoxic T cells, protective antitumor immunity, and immunologic memory (18). This priming is believed to be induced by resident dendritic cells, which form clusters with T cells in mouse BM (18). Thus, it was suggested that in vitro reactivated and expanded BM-derived autologous tumor antigen–specific memory T cells can be applied for effective adoptive immunotherapy of cancer patients (8, 9). However, the question at which stage of tumor progression (before or after development of macroscopic lesions) tumor antigen–specific memory T cells appear has not been systematically investigated.

To address this question, it is essential to establish reliable animal tumor (in particular, melanoma) models. Conventional mouse melanoma models (e.g., B16) are based on the transplantation of tumor cells, in which the natural history of the disease is not comparable with the clinical situation. In contrast to transplantation models, recently described MTX/ret transgenic mouse model showed similarity to human melanoma with respect to histopathology and clinical development (19, 20). Mice expressing the human ret transgene in melanocytes controlled by the mouse metallothionein I promoter-enhancer develop spontaneously malignant cutaneous melanoma metastasizing to lymph nodes, lungs, brain, kidney, and spleen (19). This metastatic profile resembled that of human malignant melanoma (21). Ret kinase belongs to the family of receptor tyrosine kinases (22) and is activated in melanoma developing in vivo by dendritic cells loaded with melanoma restimulated in vitro by dendritic cells loaded with tumor antigens in different forms (tumor cell lysate, tumor specific antigens (including tumor antigens) faster and stronger than naive T cells by increased proliferation; they release a broader spectrum of cytokines and need less costimulation by professional antigen–specific memory T cells appear has not been systematically investigated.

Applying this ret transgenic mouse melanoma model, we performed a detailed characterization of melanoma antigen–specific memory T cells in primary tumors as well as in BM, spleens, and lymph nodes obtained from mice with and without macroscopic melanoma burden. Using tyrosinase-related protein 2 (TRP-2) as a model melanoma antigen, increased frequencies of TRP-2–specific memory CD8+ T cells (with mostly EM phenotype) were detected in BM and tumors of transgenic mice. TRP-2–specific memory CD8+ T cells were also found in the BM of ret transgenic mice without macroscopic tumors but showing microscopic melanoma lesions in lymph nodes. After restimulation with melanoma antigen–loaded dendritic cells, BM-derived memory T cells from macroscopically tumor-free animals could produce IFN-γ in vitro and exert antimelanoma effects in vivo. Our results

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show that functionally active melanoma antigen–specific memory T lymphocytes develop already at the early stage of melanoma progression in the absence of clinically visible cutaneous tumors.

Materials and Methods

Mice. Mice (C57BL/6 background), which express human ret transgene in melanocytes under the control of mouse metallothionein I promoter-enhancer (19), were kindly provided by Dr. I. Nakashima (Department of Biomedical Sciences, Chubu University, Aichi, Japan). All mice were crossed and kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg, Germany). Experiments were done in accordance with government and institute guidelines and regulations. The survival and general performance of mice were monitored daily. Spontaneous tumor development was assessed macroscopically.

Antibodies and reagents. The medium used was RPMI 1640 supplemented with 2 mmol/L L-glutamine (PAA) and 10% FCS (PAN Biotech). The following rat anti-mouse directly conjugated monoclonal antibodies (mAb) were used for the fluorescence-activated cell sorting (FACS) staining: CD3-PerCP-Cy5.5, CD4-PE, CD8-FITC, CD44-PE, CD45RB-FITC, CDE2L-APC, and isotype-matched control mAbs (all from BD Biosciences). Rabbit anti-mouse polyclonal antibodies against TRP-2 were kindly given by Dr. V. Hearing (Laboratory of Cell Biology, National Cancer Institute, Bethesda, MD). Mouse APC-conjugated tetrameric complexes containing K\(^{\alpha}\) and peptide SVYDFFVWL derived from model MA TRP-2 were kindly provided by Dr. T. Schumacher (Division of Immunology, The Netherlands Cancer Institute, Amsterdam, the Netherlands). Mixture of mAbs against mouse Fc-receptors (Fc-block) was purchased from BD Biosciences.

Single-cell suspension preparation. Fresh tumor, spleen, and BM samples were immediately transferred into serum-free RPMI medium and stored on ice. After removal of necrotic tissue and fat, tumor biopsies were cut into small pieces and filtered through the cell strainer. Whole spleens were dissociated and also filtered. Samples from these three tissues were depleted of RBC by ammonium chloride lysis, washed twice, and used for FACS staining.

Flow cytometry. Single cell suspensions were treated with Fc-block and stained with K\(^{\alpha}\)/TRP-2 tetramers and/or mAbs for 40 min at 4°C. Acquisition was done by four- or five-color flow cytometry using a FACS-Calibur with CellQuest software or FACS LSRII with FACS Diva Software (both BD Biosciences) with dead cell exclusion based on scatter profile or propidium iodide inclusion. FlowJo software (Tree Star) was used to analyze at least 100,000 or 300,000 (if tetramer staining was included) events. Data were expressed as dot plots.

Isolation of BM-derived T cells and dendritic cells. To purify T cells, BM mononuclear cells from transgenic tumor-bearing or macroscopically tumor-free mice (pooled for each functional assay from at least five animals) were treated with Mouse T-Cell Negative Isolation Kit (Dynal) according to the manufacturer’s protocol. After negative selection, the suspension contained ~70% CD3+ T cells. For the generation of dendritic cells, BM mononuclear cells from nontransgenic littermates were cultured for 8 d in RPMI 1640 with 10% FCS and 200 units/mL mouse granulocyte macrophage colony-stimulating factor (eBioscience) as described (23). Then dendritic cells were stimulated with 3 μg/mL CpG oligodeoxynucleotide 1668 (MWG) for 6 h, followed by pulsing for 20 h with lysates of melanomas from ret transgenic mice (500 μg protein/10\(^6\) cells/mL). Tumor lysis was done by five freeze-thaw cycles as described (8).

IFN-γ ELISPOT assay. The assay was done as previously described (8). Briefly, dendritic cells loaded with melanoma cell lysates or lysate unloaded were coincubated with BM T cells (T cell/dendritic cell ratio, 5:1) for 40 h. The number of IFN-γ–producing cells was detected using a Bioreader 3000 (Biosys). Spots measured in the presence of unpulsed dendritic cells were considered as nonspecific background (negative control). Samples were considered to contain melanoma antigen–reactive T cells producing IFN-γ when spot numbers in experimental triplicates were significantly higher than the numbers in negative control triplicates (P < 0.05). The quantity of melanoma antigen–reactive BM T cells was calculated as follows: spot numbers in wells with lysate-loaded dendritic cells minus spot numbers in negative control wells.

Adoptive immunotherapy. Adoptive transfer of T cells restimulated with dendritic cells was done as described with some modifications (8). Briefly, BM T cells were isolated by negative selection from BM mononuclear cells pooled from 20 transgenic mice without macroscopic tumors and older than 20 wk. T cells were then cocultured with tumor-pulsed or unpulsed dendritic cells for 20 h (T cell/dendritic cell ratio, 10:1) followed by i.p. injections of 4.5 × 10\(^5\) T cells and 0.45 × 10\(^5\) dendritic cells into transgenic mice with established, growing cutaneous melanoma lesions.

Immunohistochemistry. Lymph nodes from ret transgenic mice without visible tumors at the age of 20 wk and older were fixed in 4% formalin solution for 24 h at 4°C followed by paraffin embedding. Consecutive cryostat sections 5 μm in thickness were air-dried, treated with xylol (J.T. Baker) and ethanol, and stained with polyclonal antibodies against TRP-2 as previously described with some modifications (24). After staining with secondary biotinylated goat anti-rabbit antibody (Vector Laboratories), the sections were treated with Vectastain ABC-AP and Red Alkaline Phosphatase Substrate kits (both from Vector Laboratories) according to the manufacturer’s protocol and counterstained with hemalun (Roth).

BM cells from transgenic mice without visible tumors at the age of 20 wk and older were spun at 450 rpm. Cytospin slides were stained to detect TRP-2–positive cells as described above and screened by microscopy.

Real-time PCR. Total RNA was extracted from mouse BM and lymph node cells by using the Qiagen BNeasy mini kit (Qiagen) according to the manufacturer’s protocol. Amplification of the gene coding a model melanoma antigen tyrosinase was done with primer sets (Applied Biosystems) according to the manufacturer’s protocol.

Data analysis. Student’s t test was used to determine statistical significance between control and test groups.

Results

Phenotype analysis of memory T cells in lymphatic organs and tumors of ret transgenic mice. In this study, we used transgenic mice overexpressing the human oncogene ret (19) back-crossed at least six times with C57BL/6 wild-type mice. After a short latency (20–70 days; mean, 40 days), ~20% of all transgenic mice developed skin tumors on the face, nose, eyes, and neck, back, or tail. No new tumor development was ever observed in animals older than 20 weeks. Tumor-bearing mice developed metastases in lymph nodes and some distant organs like lungs, liver, and brain. Histologic analysis of primary tumors and metastases revealed the morphology of malignant melanoma (data not shown).

Memory CD4+ and CD8+ T-cell subsets (CM and EM) were evaluated in ret transgenic tumor-bearing mice in comparison with age-matched transgenic macroscopically tumor-free mice and nontransgenic littermates (control groups) by flow cytometry using respective markers (Fig. 1A–D). All mice were younger than 20 weeks. BM memory CD4+CD3+ T cells from all three groups contained significantly higher proportion of EM (CD45RB CD62L+) than CM (CD45RB CD62L+) cells (P < 0.001; Fig. 1B). The latter subset was equally presented in all studied groups, whereas the fraction of EM CD4+ T cells was considerably lower in tumor-bearing mice as compared with macroscopically tumor-free and nontransgenic animals (P < 0.0001). A similar pattern was detected in memory CD8+CD3+ T-cell population with a significant decrease of EM cells (CD44\(^{high}\)CD62L\(^{low}\)) in the BM of tumor-bearing mice as compared with both control groups (P < 0.0001) and with a similar distribution of CM T cells (CD44\(^{low}\)CD62L\(^{low}\)) among studied groups (Fig. 1C).
Figure 2 illustrates the distribution of memory T cells among tumor-infiltrating lymphocytes (TIL) from transgenic mice in comparison with the pattern observed in lymphatic organs of the same animals. Of all CD4+ T lymphocytes, the number of EM cells was markedly higher than that of CM cells (P < 0.02) that corresponded to the pattern of memory T cells in the BM of these mice (Fig. 2A). In contrast, both subsets of spleen memory CD4+ T cells were presented almost at the same level. Analysis of CD8+ TILs also revealed an increase of EM as compared with CM cells, although it was not statistically significant (Fig. 2B). In both lymphatic organs studied, we detected a higher proportion of CM than EM CD8+ T cells (Fig. 2B).

Analysis of BM, spleen, and tumor samples from tumor-bearing transgenic mice showed no statistical correlation between the observed memory T-cell phenotype in these tissues and mouse age, tumor weight, or the dynamics of tumor growth (data not shown).

TRP-2–specific memory CD8+ T-cell subsets from lymphatic organs and tumors of ret transgenic tumor-bearing mice. First we determined the frequencies of BM CD8+ T lymphocytes from tumor-bearing mice with specificity for TRP-2 as a model melanoma antigen using tetrameric complexes containing Kb and TRP-2–derived peptide SYVYDFVWL (Fig. 3A–C). As a negative control, we used the staining of BM samples from nontransgenic littermates (n = 20) with the same tetramers. The background level was always <0.2% of total CD8+ T cells (Fig. 3B). Of tumor-bearing mice, 80% contained TRP-2–specific BM lymphocytes with frequencies between 0.21% and 13.9% of total CD8+ T cells (Fig. 3B). To define CD8+ T-cell subsets with TRP-2 specificity, we also stained some of these samples with mAbs against CD44 and CD62L. There were significantly more tetramer-positive CD8+ T cells with CD44high memory than CD44low naive phenotype (P < 0.0005; Fig. 3C). In contrast to the memory pattern of the total CD8+ T-cell population, we detected within TRP-2–specific CD8+ T lymphocytes significantly more EM than CM cells (P < 0.0001; Fig. 3C). In addition, these melanoma antigen–specific lymphocytes contained much lower fraction of naive cells compared with total CD8+ T cells (P < 0.0001).

TRP-2–specific CD8+ T lymphocytes were also detected in 69% of tumor samples isolated from transgenic mice. The frequencies of these cells varied between 0.26% and 12.5% of total CD8+ TILs (Fig. 4A). Similar to the BM, a majority of TRP-2–specific CD8+ TILs showed memory phenotype (P < 0.0001; Fig. 4B). Analysis of memory cells in tumor samples revealed an accumulation of EM cells within TRP-2–specific CD8+ TILs as compared with total CD8+ TILs (P < 0.05), whereas the proportion of CM cells within melanoma antigen–specific CD8+ TILs was very low (Fig. 4B). TRP-2–specific CD8+ T lymphocytes were found also in spleens in 58% of tumor-bearing mice (Fig. 4A). However, in striking contrast to BM or tumor samples, spleens contained only between 0.22% and 0.77% melanoma antigen–specific T cells of total CD8+ T cells. Furthermore, mostly naive cell markers were expressed, and CM T cells dominated within the memory subset (P < 0.05; Fig. 4C).

Investigating lymph nodes, we detected in five of eight mice 1.6% to 3.2% CD8+ T lymphocytes specific for TRP-2 (data not shown).

Thus, EM cells were significantly enriched within TRP-2–specific CD8+ T cells (in contrast to total CD8+ T lymphocytes) in BM and tumors from melanoma-bearing transgenic mice. No correlation of TRP-2–specific CD8+ T-cell numbers or their memory pattern in lymphoid organs and tumors was found in relation to mouse age, tumor weight, or the speed of tumor development.
TRP-2–specific memory CD8+ T cells from lymphatic organs of transgenic tumor-free mice. To address the question at which stage of melanoma progression in transgenic mice (before or after formation of visible tumors) could melanoma antigen–specific memory T cells be detected, we studied BM samples from animals of different ages without macroscopic tumors. We found no TRP-2–specific CD8+ T cells in mice at the age of 5 to 19 weeks (data not shown). Only in mice at the age of 20 weeks and older was it possible to detect these melanoma antigen–specific T cells in 54% of tested mice (Fig. 5A). The percentage of TRP-2–specific cells within the total BM CD8+ T cells varied from 0.7% to 6.9% and did not correlate with the mouse age. Similar to findings in tumor-bearing mice, there was a significantly larger proportion of memory TRP-2–specific CD8+ T cells than naïve cells in tumor-free animals (P < 0.0001; Fig. 5B). More detailed analysis revealed that this memory subset was characterized by mostly EM phenotype (32 ± 3% and 38 ± 2% within total memory CD8+ T cells; P < 0.05; Fig. 5B). The amount of CM cells among the TRP-2–specific subset was also higher than within total memory CD8+ T cells (P < 0.0005). Therefore, TRP-2–specific CD8+ T cells with largely EM phenotype can be detected also in the BM of transgenic animals, which showed no macroscopically visible melanomas and which were at the age of 20 weeks or more. CD8+ T cells specific for TRP-2 were also tested in spleens and lymph nodes from some of these mice. Spleens from 5 of 8 animals (63%) and lymph nodes from 6 of 8 animals (75%) contained TRP-2–specific T cells, the frequency of which varied from 0.28% to 0.74% and 1.9% to 6.2%, respectively (data not shown).

Next we asked whether transgenic mice of 20 weeks and older, in which TRP-2–specific CD8+ T cells could be shown in the BM and lymph nodes by tetramer staining, might contain microscopic tumor lesions in these organs. To answer this question, we studied TRP-2 expression by immunohistochemical staining of BM cytosin slides or consecutive sections of whole lymph nodes with respective polyclonal antibodies. Screening of cytosin slides revealed the presence of disseminated TRP-2–positive melanoma cells in the BM from 6 of 10 animals tested (average, 6 tumor cells among 10^6 BM cells; range, 1–14 cells; Fig. 6A). Melanoma cells were also detected in the BM from these mice by real-time PCR technique (data not shown). Analyzing consecutive sections of whole lymph nodes from animals without macroscopic tumors, we found that in 8 of 10 mice studied, lymph nodes contained clusters of TRP-2–positive melanoma cells (Fig. 6A). The numbers of clusters varied from 1 to 13 per mouse (mean, 5) and the area of melanoma metastases ranged from 332 to 14,625 μm^2 per mouse (average, 4,560 μm^2). Metastatic melanoma lesions were also found in lymph nodes of these mice using real-time PCR (data not shown).

Thus, the presence of TRP-2–specific T cells in macroscopically tumor-free animals at the age of 20 weeks and more argues that melanoma development can be efficiently controlled in these mice for the rest of the life.

Melanoma-specific reactivity of reactivated BM memory T cells in vitro and in vivo. Having shown the presence of detectable numbers of BM melanoma antigen–specific T cells both in tumor-bearing and macroscopically tumor-free (older than 20 weeks) ret transgenic mice, we then tested whether these cells produced IFN-γ after a short-term in vitro stimulation with dendritic cells pulsed with lysates from mouse melanomas to elicit secondary, but not primary, immune responses. Dendritic cells were generated from the BM of nontransgenic littermates. Because mouse BM contains only 6% to 7% CD3+ T lymphocytes, we pooled for each experiment BM mononuclear cells from at least five animals and enriched T cells by negative selection. In three of six independent experiments (with five mice in each), BM memory T cells could produce IFN-γ as detected by ELISPOT assay (Fig. 6B and C). The frequency of memory T cells among total T lymphocytes from the BM reacting to melanoma antigen varied from 66 to 404 per 10^6 cells (Fig. 6C).

We next studied antitumor reactivity of BM memory T cells from transgenic mice older than 20 weeks without visible tumor. In two of six independent experiments (with 30 mice in total), we detected specific IFN-γ responses after a short-term incubation with dendritic cells generated from nontransgenic littermates and loaded with melanoma lysates. In these two experiments, the frequencies of IFN-γ–producing memory T cells within total BM T lymphocytes were 132 and 136 per 10^6 cells (Fig. 6C). To study the therapeutic potential of these memory T cells in vivo, we pooled BM mononuclear cells from 20 macroscopically tumor-free transgenic mice older than 20 weeks, purified T cells using negative selection, cocultured them with dendritic cells that were loaded with melanoma lysates (therapy group) or remained unloaded (control group), followed by i.p. injection into transgenic mice with established growing tumors. All mice of control groups showed progressive tumor growth resulting in the fast death (75% of animals died until day 22 after the cell transfer), whereas we observed a stabilization of tumor development in mice injected with melanoma reactive effector T cells (Fig. 6D). At day 39 after adoptive cell transfer, the survival of mice in the therapy group was significantly better than in the control group (P < 0.05; Fig. 6D).
addition, we observed a tendency for the elevation of numbers of tumor-infiltrating TRP-2–specific CD8+ T cells in the therapy group as compared with the control group (data not shown).

Therefore, ret transgenic mice without macroscopic primary melanomas contained in their BM functional melanoma-specific memory T cells that could be restimulated with melanoma antigen–loaded dendritic cells to produce IFN-γ in vitro and to exert antitumor effects in vivo.

Discussion

This study addresses an important issue of the development of tumor-specific memory T cells in the process of melanoma progression from the preclinical stage to visible tumor lesions. To study these cells in conditions maximally close to the clinical situation, we used ret transgenic mice, which spontaneously develop cutaneous melanomas (19, 20) with high similarity to human cutaneous melanoma, including the pattern of metastasis to lymph nodes and distant organs (21). In contrast to classic transplantation melanoma models (e.g., B16), different stages of spontaneously developing melanoma in ret transgenic mice can be investigated under conditions of natural interactions between tumor and host cells over time.

First we studied the distribution of total memory T cells and found a remarkable enrichment of these cells in mouse BM. These findings are in agreement with previous publications (7–12) and might be explained by the presence of cytokines such as interleukin (IL)-7 and IL-15 that are critical for memory T-cell survival and are produced by BM stroma cells (25, 26). Moreover, observed memory T lymphocyte accumulation could be mediated by interactions between very late activation antigen-4 and vascular cell adhesion molecule 1 or between α4β7 integrin and mucosal addressin cell adhesion molecule 1 or between α4β7 integrin and mucosal addressin cell adhesion molecule 1 expressed on memory T cells and stromal BM cells, respectively, as previously described (8, 27, 28). In addition, an important role of the chemokine CXCL12 expressed by BM sinusoidal endothelium in CM T-cell attraction has recently been reported (29).

Investigating BM T cells from tumor-bearing transgenic mice, we found that 80% of animals contained TRP-2–specific CD8+ T lymphocytes with mostly memory phenotype thereby confirming clinical and experimental data published by us and others (8, 9, 11, 14, 15, 30). In contrast to memory cells within the total CD8+ subset, majority of melanoma antigen–specific memory CD8+ T cells showed an EM phenotype, which was also previously described in breast cancer and melanoma patients (9, 11). The observed phenomenon might be attributed to proliferative and survival signals provided both by IL-7 and IL-15 cytokines (31, 32) as well as to the preferential expression of chemokine receptors CXCR4 and CCR5 on antigen-specific EM CD8+ T lymphocytes interacting with their respective ligands, chemokines CCL3 and CCL5 in the BM stroma (11, 12). Interestingly, although 58% of tested tumor-bearing mice showed TRP-2–specific CD8+ T lymphocytes also in spleens, only a minority of these cells displayed memory markers, and their proportion within total CD8+ T cells was much lower than that in BM. These data corroborate previous reports on the higher frequency of tumor antigen–specific memory T cells in the BM than in the peripheral blood of patients with breast cancer and melanoma (8, 11). It has been recently reported that ret transgenic melanoma mice also contained melanoma antigen–specific CD8+ T cells (without studying their phenotype) in the peripheral blood and spleen,

![Figure 3.](image-url)
which correlated with spontaneous vitiligo (33, 34). However, we were not able to observe the development of melanoma-associated vitiligo in our transgenic animals.

We showed that TILs from 68% of studied melanoma mice also contained TRP-2–specific memory CD8+ T cells predominantly of EM phenotype. Similar to the BM, the number of EM cells in TRP-2–specific CD8+ TILs was significantly increased as compared with numbers within total CD8+ TILs. These data are in agreement with the reported accumulation of tumor antigen–specific EM CD8+ T cells in different types of human tumors (9, 35, 36). It is worthwhile to note that in patients with colorectal cancer, significant tumor infiltration with EM CD8+ T lymphocytes has been reported to correlate with an absence of early metastatic invasion, a less advanced pathologic stage, and increased survival (35). Using NOD/SCID mice, we have recently shown that the homing of EM T cells to breast cancer transplant could be particularly mediated via interactions between P-selectin and P-selectin glycoprotein ligand 1 expressed in tumor tissue and T lymphocytes, respectively (9). In most cases, however, memory CD8+ T cells infiltrating tumors (like melanoma or colon cancer) exhibit decreased antitumor cytotoxic activity (37, 38). This inhibition is reported to be mediated by immunosuppressive tumor microenvironment represented, in particular, by soluble factors (e.g., transforming growth factor-β, IL-10, or nitric oxide) and host cells like regulatory T cells or myeloid-derived suppressor cells (39–42). Only if memory T cells were removed from the suppressive environment could their anergic phenotype be reversed after appropriate reactivation (8, 9).

A key aspect of our investigation was the development of melanoma antigen–specific memory T cells during melanoma progression. This ret transgenic mouse model allowed us to address this question also at the preclinical phase of spontaneous melanoma growth (in the absence of visible skin tumor lesions), which is not possible in human melanoma situation. We could not find TRP-2–specific CD8+ T cells in BM from macroscopically tumor-free transgenic mice at the age of 5 to 19 weeks by the staining with Kb/peptide tetramers. However, in 54% of mice at the

Figure 4. Detection of TRP-2–specific memory T cells in tumors and spleens of ret transgenic tumor-bearing mice. Single tumor cell suspensions and splenocytes were stained with tetramers and mAbs for CD3, CD8, CD44, and CD62L followed by FACS analysis. A, frequencies of TRP-2–specific CD8+ T cells from mouse tumors (n = 26) and spleens (n = 19) as compared with BM samples. Solid line, maximal level of nonspecific bindings in BM or spleens of nontransgenic mice. B, accumulative data for tetramer-positive CD8+ TILs and total CD8+ TILs expressing naive and memory markers. Columns, mean of nine mice; bars, SE. *, P < 0.05, TRP-2–specific EM CD8+ T cells versus total EM CD8+ T cells. **, P < 0.05, total CM CD8+ versus TRP-2–specific CD8+ T cells. C, accumulative data for spleen tetramer-positive CD8+ T cells and total CD8+ T lymphocytes expressing naive and memory markers. Columns, mean of 10 mice; bars, SE. *, P < 0.05, CM versus EM TRP-2–specific CD8+ T cells.

Figure 5. TRP-2 specificity of BM memory T cells from macroscopically tumor-free ret transgenic mice. Cells were stained with tetramers and mAbs for CD3, CD8, CD44, and CD62L and analyzed by flow cytometry. A, frequencies of TRP-2–specific CD8+ T cells from BM of transgenic tumor-free (ret; n = 26) and wild-type (n = 20) mice. Solid line, maximal level of nonspecific bindings. Columns, mean of five mice; bars, SE. *, P < 0.05, TRP-2–specific EM versus total EM CD8+ T cells. **, P < 0.0005, total CM versus TRP-2–specific CM CD8+ T cells.
age of 20 weeks and older, these cells were enriched to the levels, which became detectable by tetramer stainings, and showed predominantly EM phenotype. Interestingly, a similar frequency of T cells specific for TRP-2 was found also in lymph nodes of these mice. After appropriate restimulation, BM melanoma antigen–specific memory T cells could produce in vitro IFN- \( \gamma \) (in 33% of cases) similar to corresponding T cells from transgenic mice with visible tumors. In addition, these reactivated cells were found to exert an antitumor effect in vivo, being adoptively transferred into transgenic mice with visible, aggressively growing melanoma. Importantly, a majority of macroscopically tumor-free transgenic mice older than 20 weeks contained TRP-2–positive disseminated melanoma cells both in the BM and lymph nodes and showed no visible tumors and no further melanoma progression. The presence of dormant tumor cells being under the control of host immune system has been previously documented in experimental and clinical studies (10, 43). These reports and our data argue for the potential role of dormant tumor cells in the maintenance of a long-term persistence of tumor antigen–specific memory T cells, which could restrict tumor growth in vivo.
Findings in transgenic mice without visible tumor lesions presented here are in line with the recently developed concept of cancer immunoediting (including elimination, equilibrium, and escape processes; refs. 44, 45). Melanoma progression in these mice corresponds most probably to the described equilibrium process because we detected melanoma antigen–specific CD8+ T lymphocytes and disseminated melanoma cells both in the BM and lymph nodes. Some transgenic mice develop both palpable skin primary tumors and macroscopic lymph node metastases, indicating thereby that these mice may be at the escape phase of cancer immunoediting. Molecular tumor- and host-mediated mechanisms of the development of this phase in our melanoma model are currently under investigation.

In conclusion, we have shown that not only ret transgenic mice with macroscopic primary tumors but also ret transgenic mice without them contained BM melanoma antigen–specific CD8+ T cells mostly of EM phenotype. On appropriate activation with melanoma antigen–pulsed dendritic cells, these cells from tumor-free were shown to produce IFN-γ in vitro and to exert antitumor effects in vivo, implying thereby the development and maintenance of functionally active melanoma antigen–reactive memory T cells at the preclinical stage of melanoma progression. We suggest that these cells could be used for adoptive melanoma immunotherapy in vivo, which should be combined with neutralization of immunosuppressive tumor microenvironment to prevent the development of anergy in transferred activated T cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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