Skin Melanoma Development in ret Transgenic Mice Despite the Depletion of CD25+Foxp3+ Regulatory T Cells in Lymphoid Organs

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*J Immunol* published online 19 October 2009
http://www.jimmunol.org/content/early/2009/10/19/jimmunol.1.0900609
Skin Melanoma Development in ret Transgenic Mice Despite the Depletion of CD25\(^+\)Foxp3\(^+\) Regulatory T Cells in Lymphoid Organs

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CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells (Treg) known to mediate self-tolerance were also shown to contribute to tumor progression. In mouse melanoma transplantation models, Treg depletion resulted in the stimulation of antitumor immune responses and tumor eradication. To study Treg in conditions close to the clinical situation, we used a ret transgenic mouse spontaneous melanoma model, which, in contrast to transplantation models, resembles human melanoma regarding clinical development. Significantly higher numbers of Treg were found in skin tumors and metastatic lymph nodes at early stages of melanoma progression compared with more advanced stages accompanied by the elevated CCR4 expression on Treg and higher production of its ligand CCL2 in tumor lesions. Numbers of tumor infiltrating Treg inversely correlated with Treg amounts in the bone marrow, suggesting their possible recruitment to melanoma lesions from this organ. The immunosuppressive function of Treg from transgenic tumor-bearing mice was similar to that from transgenic tumor-free mice or nontransgenic littermates. Although anti-CD25 mAb injections resulted in the efficient Treg depletion from lymphoid organs of transgenic mice, melanoma development was not significantly delayed. Furthermore, the treatment of mice with macroscopical tumors also failed to inhibit tumor progression, which correlated with the inability to deplete intratumoral Treg. We suggest that in the autochthonous melanoma genesis, other immunosuppressive cells could play an important role and replace immunosuppressive, tumor-promoting functions of Treg. Therefore, effective melanoma immunotherapy should include the inhibition of Treg migration into the tumor combined with neutralization of other immunosuppressive cells and factors in the tumor microenvironment. The Journal of Immunology, 2009, 183: 0000–0000.
For example, IL-10 and/or TGF-β converted tumor-infiltrating effector CD4+ T cells into Tr1 or Th3 cells, respectively, which were considered as secondary Treg (17).

Elimination of CD25-expressing Treg using anti-CD25 mAbs has been reported to elicit potent immune responses to murine RL1 leukemia and B16 melanoma cells in vivo, leading to tumor eradication (18). Later, a similar antitumor effect of these mAbs was also demonstrated for other murine tumors (19–21). Importantly, all of these tumor models were based on the transplantation of tumor cells, in which the natural history of the disease and tumor-host interactions are not comparable with the clinical situation. In contrast to transplantation models, a recently described ret transgenic mouse model closely resembles human melanoma regarding tumor genetics, histopathology, and clinical development (22, 23). Mice expressing the human ret transgene in melanocytes controlled by the mouse metallothionein-I promoter-enhancer develop spontaneously malignant cutaneous melanoma lesions metastasizing to lymph nodes (LN), lungs, brain, kidney and spleen (23). This metastatic profile is similar to that observed in melanoma patients (24).

In the present study, we investigated the role of Treg in ret transgenic mice that spontaneously develop skin melanoma. We showed that Treg preferentially infiltrate primary tumors and metastatic LN at the early stage of tumor progression that correlated with an increased CCR4 expression on Treg and CCL2 production as well as with decreased Treg numbers in the bone marrow (BM), suggesting a Treg capacity to migrate between the BM and melanoma lesions. Furthermore, a similar suppressive effect on the proliferation of conventional (helper) CD4+ T cells (Tcon) was demonstrated for Treg isolated from tumor-bearing mice and from nontransgenic littersmates or transgenic mice without macroscopical tumors. Additionally, Treg depletion in ret transgenic mice by anti-CD25 mAbs did not significantly modulate melanoma progression and mouse survival. We suggest that in the autochthonous melanoma genesis, other immunosuppressive cells could play an important role and replace immunosuppressive, tumor-promoting functions of Treg.

Materials and Methods

**Mice**

Mice (C57BL/6 background), which express human ret transgene in melanocytes under the control of mouse metallothionein-I promoter-enhancer (22) were provided by Dr. I. Nakashima (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 performed in accordance with government and institutional guidelines and regulations. The survival and general performance of mice was monitored daily. Spontaneous tumor development was assessed macroscopically.

**Abs and reagents**

The following directly conjugated rat anti-mouse mAbs were used for the FACS staining: CD3-PerCP-Cy5.5, CD4-FITC, CD25-allophycocyanin, CD45.2-PerCP-Cy5.5, IFN-γ-PE, and isotype-matched control mAbs (all from BD Biosciences), as well as Foxp3-PE and IL-17-FITC (both from eBiosciences). Additionally, directly conjugated hamster anti-mouse CCR4 (CD194)-allophycocyanin and CXCXR3 (CD183)-PE (both from BioLegend) as well as mouse anti-human Ki67-FITC (cross-reacting with mouse) mAbs (BD Biosciences) were used. For immunohistochemical staining, purified rat anti-mouse CCR4 (BD Biosciences) and biotinylated rat anti-mouse Foxp3 (eBioscience) Abs were utilized. Biotinylated rabbit anti-rat IgG (Dako) served as a secondary Ab for the CD4 staining. Both CD4 and Foxp3 were visualized using either streptavidin-conjugated HRP or avidin-conjugated alkaline phosphatase and corresponding substrates (all from Vector Laboratories). Tcon were stimulated with rat anti-mouse CD3 and CD28 mAbs (BD Biosciences). CD25-blocking mAbs (clone PC61) were purified from the culture supernatant of hybridoma (provided by Dr. E. Suri-Payer, German Cancer Research Center). The medium used was RPMI 1640 supplemented with 2 mM l-glutamine (PAA Laboratories), 10% FCS (PAN Biotech), and 50 μM 2-ME.

**Preparation of single-cell suspensions**

Fresh BM, spleen, LN, and tumor samples were immediately transferred into PBS 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 and LN were dissociated and filtered. BM and spleen samples were depleted of erythrocytes by ammonium chloride lysis and washed twice.

**Flow cytometry**

Single-cell suspensions were treated with Fc-block and stained with mAbs for 20 min at 4°C. Acquisition was performed by four- or five-color flow cytometry using a FACS-Calibur with CellQuest software or a FACS-Canto II with FACS-Diva software (both from 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 events. Data were expressed as dot plots.

**In vitro proliferation assay**

Splenocytes from ret transgenic mice with or without macroscopical skin tumors and from nontransgenic littersmates were sorted for CD4+ CD25+ Treg and CD4+ CD25− Tcon using the MACS Treg isolation kit (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of both sorted CD4+ T cell subsets checked by flow cytometry was ~80%. Additionally, isolated CD4+ CD25− T cells expressed Foxp3. CD4+ CD25− Tcon (103 cells/well) were plated in 96-well plates (Greiner Bio-One) and stimulated with soluble anti-CD3 and anti-CD28 mAbs (0.5 μg/ml each) followed by the coculture with CD4+ CD25+ Treg in triplicates at the Treg-to-Tcon ratios of 1:1, 1:2, 1:4, and 1:10. As a control, CD4+ CD25+ Tcon were incubated alone with or without stimulating mAbs. After 48 h, 1 μCi of [3H]thymidine (PerkinElmer) was added to each well and cells were cultured for an additional 16 h. Then, cells were harvested and thymidine uptake in CD4+ CD25+ T cells was counted with a beta counter (TopCount NXT; PerkinElmer). In some experiments, triplicates were incubated in parallel without [3H]thymidine for 96 h. These culture supernatants were collected and stored at −20°C for the measurement of IFN-γ by ELISA.

**IFN-γ ELISA**

The concentration of IFN-γ in culture supernatants was determined using an IFN-γ ELISA kit (BD Biosciences) according to the manufacturer’s instructions.

**BioPlex array assay**

Snap-frozen primary tumor samples were mechanically disrupted and treated by lysis solution (Bio-Rad). After sonication, samples were centrifuged at 4500 × g for 6 min at 4°C. Protein concentration in the supernatant was determined using Bradford assay and adjusted to 500 μg/ml using serum diluent (both from Bio-Rad). Amounts of chemokines CCL2/MCP-1 and CXCL9/MIG in tumor lysates were measured using multiplex technology (Bio-Rad) according to the manufacturer’s protocols.

**In vivo depletion of CD25+ T cells**

Ret transgenic mice with macroscopic melanomas or without visible tumors were injected i.p. twice (at day 0 and day 21) with 400 μg of anti-CD25 mAbs in 200 μl of PBS and monitored for tumor progression. The depletion efficacy was checked by testing Treg amounts in the peripheral blood, secondary lymphoid organs, or primary tumors using flow cytometry.

**Immunohistochemistry**

Tumor samples from ret transgenic mice were fixed in zinc fixative solution (BD Biosciences) overnight at room temperature followed by paraffin embedding. Consecutive cryostat sections 5 μm in thickness were air dried, incubated overnight at 60°C for Ag recovery, and blocked with PBS containing 10% goat serum, 2% BSA, and 0.05% Tween 20 for 30 min at 37°C. Sections were stained with rat anti-mouse CD4 mAbs followed by secondary biotinylated rabbit anti-rat Abs. After staining with streptavidin-conjugated HRP complex (Vectastain ABC kit; Vector Laboratories) and with peroxidase substrate 3,3′-diaminobenzidine (Dako), the sections were washed, incubated with biotinylated rat anti-mouse Foxp3 mAbs, and treated with Vectastain ABC-AP and Vector red alkaline phosphatase substrate kits (both from Vector Laboratories) according to the manufacturer’s protocol and counterstained with hemaluna (Roth).
FIGURE 1. Treg infiltrate primary skin tumors of ret transgenic mice. A, Consecutive paraffin sections of tumors were stained with anti-mouse CD4 (brown) and Foxp3 (black) mAbs and counterstained with hemalaun. Sections stained only with second Abs are presented on the left panel. Arrow indicates CD4+/Foxp3+ Treg. Original magnifications, ×200 (upper panel) and ×400 (lower panel). B, Single-cell suspensions prepared from tumors of ret transgenic mice were stained with mAbs for CD4, CD25, Foxp3, and the leukocyte marker CD45.2 followed by flow cytometry. Representative dot plots are shown. C, The weight of each tumor sample was plotted against the percentage of tumor-infiltrating CD4+CD25+Foxp3+ Treg within CD45.2+ cells (n = 20). The correlation between the two variables was calculated using a linear regression analysis (r² = 0.258, p = 0.011). D, Tumor samples shown in C were separated according to the tumor weight (ret tu I: <400 mg, n = 14; ret tu II: >400 mg, n = 6). Data are shown as means ± SEM. *, p = 0.001, significant differences between groups indicated with the lines.

Real-time PCR

Total RNA was extracted from mouse primary tumor and adjacent skin tissue with the use of a TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Amplification of the gene coding CCL22 and CCL17 was done with primer sets (Applied Biosystems) according to the manufacturer’s protocol. Amplification of the gene coding CCL22 and CCL17 was done with primer sets (Applied Biosystems) according to the manufacturer’s protocol.

Statistical analysis

All data are shown as means ± SEM for the indicated number of independent experiments. Results were assessed with a Student’s t test and Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism software. All statistical tests were two-sided. A value of p < 0.05 was considered statistically significant.

Results

Treg infiltrate primary skin tumors in ret transgenic mice

In this study, we used transgenic mice overexpressing the oncogene ret (22) back-crossed at least six times with C57BL/6 wild-type mice. After a short latency (20–70 days of age), ~25% of all transgenic mice develop skin tumors on the face (nose, ears, eyes, and neck), back, or on the tail. Tumor-bearing mice developed metastases in LN and some distant organs such as lungs and liver.

Paraffin sections of primary skin tumors from ret transgenic mice were double stained with Abs against mouse CD4 and Foxp3. Immunohistochemical analysis of these sections revealed the presence of CD4+/Foxp3+ Treg located mainly in the tumor stroma (Fig. 1A). To quantify tumor-infiltrating Treg, single-cell suspensions prepared from tumors at different stages of melanoma progression were tested for CD4+CD25+Foxp3+ Treg as a tumor-infiltrating lymphocyte subset by flow cytometry (Fig. 1B). Treg numbers varied between 0.2 and 12.0% within the CD45.2+ T cells. Decreasing proportions of Treg were found to correlate with the increasing weight of primary melanomas (r² = 0.258, p = 0.011; Fig. 1C). Tumors at earlier stages of progression (<400 mg of weight; ret tu I) were infiltrated with significantly higher amounts of Treg as compared with larger tumors (6.2 ± 2.5% and 3.2 ± 2.6%, respectively, p = 0.001; Fig. 1D).

Distribution of Treg in lymphoid organs of ret transgenic mice

It has been shown that the human BM can serve as a reservoir for CD4+CD25+Foxp3+ Treg (25). To address the question whether Treg can migrate from the BM to the tumor site at early stages of melanoma progression, we investigated CD4+CD25+Foxp3+ Treg in the BM isolated from ret transgenic mice bearing tumors of different weight by flow cytometry (Fig. 2A). For comparison, BM samples were taken also from ret transgenic mice without macroscopical tumors and from nontransgenic littermates (wild-type controls). Treg numbers in ret transgenic tumor-bearing mice ranged from 6.5 to 39.4% of total CD3+CD4+ T cells. Importantly, the BM from mice with smaller tumors (<400 mg of weight; ret tu I) contained significantly less Treg amounts (p < 0.05) than did that from animals with larger tumors (>400 mg of weight, ret tu II; Fig. 2B). Interestingly, Treg numbers in the BM from the ret tu I group were at the level detected in the BM from ret transgenic
mice without macroscopical tumors or in wild-type animals (Fig. 2B). The observed inverse correlation between Treg amounts infiltrating primary tumors and their numbers in the BM during melanoma progression suggest that Treg may be recruited from the BM of these transgenic mice into the tumor at early stages of the disease.

Next we studied Treg accumulation in other lymphoid organs of ret transgenic mice using flow cytometry (Fig. 3, A and C). We observed an increase in Treg numbers in spleens of ret transgenic mice with macroscopical tumors as compared with transgenic mice without visible melanomas ($p < 0.05$; Fig. 3B). Interestingly, no significant differences in Treg amounts were detected in mice bearing tumors at different progression stages (ret tu I and ret tu II; Fig. 3B). Similar to spleens, we found an increase in Treg amounts in metastatic LN (macroscopically determined by their dark color typical for melanoma cells) from tumor-bearing mice compared with nonmetastatic LN from animals without visible tumors ($p < 0.05$; Fig. 3D). Data are means ± SEM from 5–10 mice per experimental group. * $p < 0.05$, differences between indicated groups.

Chemokine production in primary tumors and metastatic LN of transgenic mice

Treg are known to be recruited into the tumor site through certain chemokines released by tumor and stroma cells (16). Therefore, we investigated the production of chemokines CCL2/MCP-1 and CXCL9/MIG in lysates prepared from primary tumor and adjacent normal skin tissues as well as from metastatic and normal LN using a BioPlex array assay. As seen in Fig. 4A, CCL2/MCP-1 concentration was found to be significantly higher in metastatic than in normal LN ($p < 0.05$). Moreover, we observed decreased chemokine levels in primary tumor samples from mice with more advanced melanomas (ret tu II) compared with corresponding normal skin tissues (Fig. 4A; $p < 0.05$). Interestingly, CXCL9/MIG was also accumulated in skin samples from the same melanoma mice (ret tu II) compared with respective primary tumors (Fig. 4B; $p < 0.05$). Additionally, amounts of this chemokine were demonstrated to be larger than those of CCL2/MCP-1 in the lysates studied.

Next we investigated other chemokines such as CCL22 and CCL17 that are critical for Treg migration. Since commercially available BioPlex kits for these mouse cytokines are absent, we...
Results (means ± SEM) show thymidine incorporation in proliferated cells expressed as the percentage of suppression of proliferative activity. Data (means ± SEM; three to six mice per group) are expressed as cpm × 1000. One representative experiment of three is presented. B, Inhibition of Tcon proliferation by Treg from mice with melanoma at earlier and later progression stages (ret tu I and ret II, respectively). Data (means ± SEM) show thymidine incorporation in proliferated cells and are expressed as cpm x 1000. One representative experiment of three is presented. B, Inhibition of Tcon proliferation by Treg from mice with melanoma at earlier and later progression stages (ret tu I and ret II, respectively). Data (means ± SEM; three to six mice per group) are expressed as the percentage of suppression of proliferative activity.

measured their expression in primary tumors, metastatic LN, and corresponding normal tissues by real-time PCR technique. Both chemokines showed extremely low expression in all samples tested (data not shown).

By analyzing the expression of receptors for the above-mentioned chemokines on Treg, we revealed a significant increase in the amount of CXCR4/CX194-positive cells in primary tumors and metastatic LN from mice with melanomas at earlier progression stages as compared with animals with more advanced tumors (ret tu I and ret tu II, respectively; p < 0.05; Fig. 4C). Additionally, in metastatic LN from ret tu I mice, we also detected significantly more (p < 0.05) Ki67+ proliferating Treg (data not shown). This correlates with the described Treg accumulation in less advanced melanoma lesions. In contrast, numbers of Treg bearing another chemokine receptor CXCR3/CX183 did not differ in tumors or metastatic LN from mice at different stages of melanoma progression (data not shown).

Treg from tumor-bearing and tumor-free mice display a similar immunosuppressive activity

To investigate an immunosuppressive function of CD4+CD25+ Treg isolated from spleens of transgenic mice with macroscopical tumors, we coincubated them in vitro with splenic CD4+CD25+ Tcon from wild-type mice in the presence of stimulating anti-CD3 and anti-CD28 mAbs. Tcon proliferation and its inhibition by Treg were evaluated by [3H]thymidine incorporation assay. As controls, we utilized splenic CD4+CD25+ Treg from transgenic mice without macroscopical tumors or from nontransgenic littermates. The capacity of Treg from melanoma-bearing mice to inhibit Tcon proliferation did not significantly differ (at all Tcon-to-Treg ratios used) from that of Treg from control groups (p > 0.05; Fig. 5A). Furthermore, we observed no differences between splenic Treg immunosuppressive activities in mice with melanoma at earlier and later progression stages (ret tu I and ret tu II, respectively; p > 0.05; Fig. 5B). Since IFN-γ production is known to be an indicator of T cell activation, we also measured its concentration in coculture supernatants using ELISA. Spleen Treg isolated from ret transgenic mice were demonstrated to suppress IFN-γ production by Tcon to a similar extent as Treg derived from wild-type mice (data not shown). These data corroborate the above-mentioned results obtained with the [3H]thymidine incorporation assay and show that Treg from ret transgenic tumor-bearing and tumor-free mice display a comparable immunosuppressive activity.

Effects of CD25+ Treg depletion in ret transgenic mice on melanoma progression

To test the efficacy CD25+ Treg depletion in vivo, ret transgenic mice animals were injected i.p. once with 400 μg of anti-CD25 mAbs (clone PC61; volume, 200 μl). We observed a drastic decrease or even complete disappearance of this T cell subset from the peripheral blood and lymphoid organs (spleen, BM, and LN) at day 5 after the treatment (data not shown). Then, ret transgenic mice with macroscopical skin tumors were inoculated i.p. with above-mentioned amounts of anti-CD25 mAbs. As controls, age-matched ret transgenic mice bearing skin tumors of similar size were injected i.p. with PBS. Five days after the treatment, no CD4+CD25+Foxp3+ Treg could be detected in the mouse peripheral blood by FACS analysis (Fig. 6A). We checked the presence of Treg in the peripheral blood further and found a slow recovery of these cells. At day 21 after the initial treatment (when the CD25+ cell compartment almost reached its initial level), mice were injected again with 400 μg of anti-CD25 mAbs, which resulted in a complete Treg depletion in the peripheral blood. Analysis of the therapeutic efficiency of this treatment revealed no statistically significant differences in the tumor progression and survival of treated and nontreated melanoma-bearing mice (Fig. 6B). Similar effects were obtained if 400 μg of anti-CD25 mAbs was administered six times weekly (data not shown).

Next, we investigated whether the treatment with anti-CD25 mAbs in the prophylactic setting could modulate a spontaneous melanoma development in transgenic mice. Animals without macroscopical skin tumors were inoculated i.p. with 400 μg of mAbs. The depletion of CD4+CD25+Foxp3+ Treg in the peripheral blood was regularly monitored by flow cytometry and appeared to be successful (data not shown). Similar to the above-described experiments, a second injection of anti-CD25 mAbs was performed at day 21 after the first inoculation. We observed a tendency for the delay of tumor development and the survival prolongation in the treated transgenic mice as compared with the nontreated group; however, this tendency was not statistically significant (Fig. 6, C and D).

Since the main immunosuppressive effect of Treg is expected to be present at the tumor site, we studied whether anti-CD25 mAbs were able to deplete tumor-infiltrating Treg. To address this question, we tested CD25+Foxp3+ Treg frequencies within CD3+CD4+ T cells in the peripheral blood, BM, metastatic LN, spleens, and skin tumors of the same transgenic mice at day 5 after the i.p. mAb administration. An almost complete Treg depletion was demonstrated in all lymphoid organs, whereas the frequency of this cell subset among tumor-infiltrating CD3+CD4+ T lymphocytes did not significantly differ from that in nontreated animals (Fig. 6E).

Comparing the phenotype of CD3+CD4+ Tcon in lymphoid organs and primary tumors of treated and nontreated animals with the use of intracellular stainings for IFN-γ and IL-17, we observed no changes in amounts of Th1 or Th17 cells (data not shown).
These findings could partly explain the observed lack of antitumor effects after the treatment of ret transgenic mice with macroscopic melanomas.

**Discussion**

It has been clearly shown during the last decade that Treg play critical roles in the maintenance of peripheral tolerance by suppressing immune responses against “self” such as autoantigens or “non-self Ags” such as tumor and pathogenic Ags (6–11). Recent studies have demonstrated the accumulation of CD4+CD25+Foxp3+ Treg at tumor sites, implying that these cells induce Ag-specific and local immune tolerance. In a number of mouse models, Treg inhibition or depletion before tumor challenge has been reported to induce efficient immune responses against transplantation tumors (18–21, 26). Moreover, Treg removal could stimulate strong antitumor immunity also in the case of established murine tumors (27, 28). Additionally, adoptive transfer of CD4+CD25+ Treg cells, but not CD4+CD25– Tcon cells, effectively prevented CD8+ T cell-mediated destruction of transplanted B16 melanoma (29). Notably, most of these studies were performed using conventional mouse B16 melanoma model, which is based on the tumor cell transplantation and is not comparable with the clinical situation. In contrast, ret transgenic mice used in this study serve as an excellent model for spontaneous skin melanoma, closely resembling human melanoma with respect to clinical development (22, 23). Additionally, this model permits investigations under conditions of natural tumor-host interactions.

Studying the distribution of CD4+CD25+Foxp3+ Treg in lymphoid organs and primary tumors from ret transgenic mice, we detected a remarkable elevation of Treg frequencies within CD4+ T cells at the earlier stage of tumor growth followed by their significant decrease during the later phase of melanoma progression. Similar changes in Treg frequencies were observed also in metastatic LN from respective mouse groups. Tumor infiltration with Treg has been also reported for patients with melanoma, non-small cell lung, ovarian, pancreatic, and breast cancers (30–33). However, we demonstrated for the first time an inverse correlation between the Treg numbers infiltrating tumors and those located in the BM of the same mice. The human BM has been previously described as a significant reservoir for Treg that can migrate to the periphery through chemokine signals (25). It has been recently reported that the BM in mice with renal cell carcinoma tumors contained a lower proportion of Treg than in normal animals (34). We did not observe significant changes in Treg frequencies in the BM from mice with tumors at the earlier progression stage as compared with nontransgenic littermates. This discrepancy could be due to the differences between the transplantable renal cell carcinoma tumor model and the autochthonous melanoma model used in our study.

Human Treg are known to express chemokine receptors CCR4 and CCR8, which bind chemokines produced by monocytes/macrophages such as CCL22, CCL17, and CCL2/MCP-1 (35). Moreover, CCL2/MCP-1 was shown to be produced by human melanoma cells and to be implicated in the tumor development (36).
Furthermore, this chemokine has been shown to induce migration of IL-10-producing CD4\(^+\) regulatory T lymphocytes (37). We therefore suggested that in ret transgenic mice, Treg may also migrate from the BM to the tumor at the beginning of melanoma progression, being attracted by tumor-derived CCL2/MCP-1. To address this question, we measured concentrations of CCL2/MCP-1 and CXCL9/MIG in primary skin melanomas and metastatic LN at different stages of progression. Metastatic LN showed higher CCL2 concentrations than did normal LN. More advanced melanomas (>400 mg of weight) were found to contain fewer amounts of both chemokines than was adjacent normal skin. Moreover, significantly lower numbers of Treg that infiltrated these tumors expressed CCR4 than did those in less advanced tumors (<400 mg of weight). These data may explain the observed accumulation of Treg in primary tumors and metastatic LN at earlier stages of melanoma development. Similar observations on the pivotal role of CCL2 in Treg migration via CCR4 receptors have been recently demonstrated in glioma patients (38). Additionally, Treg enrichment in metastatic LN from transgenic mice could be partly due to the increased proliferation of these cells detected by the Ki67 staining. It has been reported that another chemokine, MCP-1 and CXCL9, also contributes to reduced survival (32). However, the expression of this chemokine at the mRNA level tested by real-time PCR was hardly detectable both in primary tumors and metastatic LN, suggesting thereby a major importance of CCL2 in Treg recruitment in the ret transgenic melanoma model.

In contrast to the BM, we found a significant accumulation of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Treg in the spleen of transgenic mice bearing tumors at both early and late progression stages. These data are in agreement with clinical studies reporting a dramatic increase in Treg numbers in the peripheral blood of patients with melanoma and other tumor types (33, 39, 40). Studying the capacity Treg from tumor-bearing mice to inhibit the proliferation of conventional CD4\(^+\)CD25\(^-\) T cells, we used spleens as a source of Treg due to insufficient cell amounts, which could be isolated from primary skin tumors. Surprisingly, the immunosuppressive activity of Treg in transgenic mice with macroscopical tumors did not significantly differ from that in transgenic mice without macroscopic tumors or nontransgenic littermates. Additionally, we found no correlation between Treg immunosuppressive activities in spleens from melanoma-bearing mice and tumor progression stages.

To address the question of the Treg importance for tumor initiation and progression in ret transgenic melanoma model, we injected anti-CD25 mAbs not only into animals with visible tumors (therapeutic approach) but also at the preclinical phase of spontaneous melanoma growth (in the absence of macroscopical skin tumor lesions), which is not possible in the human melanoma situation. When testing CD25\(^+\)Foxp3\(^+\) cells within CD3\(^+\)CD4\(^+\) T cells in the peripheral blood and lymphoid organs, we found that Treg depletion was successful. However, in the therapeutic approach, we were not able to demonstrate statistically significant delay of tumor progression and increased survival of treated melanoma-bearing mice as compared with nontreated animals. These results might be partly due to the observed insufficient Treg depletion in mouse tumors, where Treg can directly inhibit tumor-specific T cells (41). Our data are in agreement with recently published data from clinical trials showing that the treatment of melanoma patients with the fusion protein of IL-2 and diphtheria toxin (Ontak) did not result in the decrease of Treg numbers and function as well as in objective clinical responses (42, 43). After the Treg depletion in ret transgenic mice without macroscopic skin tumors, we found only a little, statistically nonsignificant antitumor effect. These data suggest that other immunosuppressive cells and tumor- or stroma-derived factors could replace Treg-mediated inhibition of tumor specific T cells, at least in our autochthonous melanoma model. In particular, recently described myeloid-derived suppressor cells and tolerogenic dendritic cells were found to induce a dramatic suppression of T cell-mediated antitumor immune responses in mouse tumor models and in cancer patients (44, 45). An increased number and function of these cells was observed also in primary tumors and lymphoid organs from ret transgenic mice (our unpublished observations).

Taken together, using a transgenic mouse skin melanoma model with high similarity to human cutaneous melanoma, we demonstrated a Treg accumulation in primary tumors and metastatic LN at the early stage of melanoma development associated with the increased CCR4 expression on Treg and higher CCL2 production in tumor lesions. Although the administration of anti-CD25 mAbs led to the efficient depletion of CD25\(^+\)Foxp3\(^+\) Treg in lymphoid organs, melanoma development was not delayed, indicating that Treg functions might be replaced by other immunosuppressive cells and factors during melanoma progression. Inability of anti-CD25 mAbs to deplete Treg from macroscopic skin melanoma lesions requires the development of therapeutic strategies based on the blockage of their migration into the primary tumor and/or on the modulation metabolic pathways involved in their inhibitory functions. We suggest that for effective melanoma immunotherapy, these Treg-suppressing strategies should be combined with neutralization of other immunosuppressive cells (such as myeloid-derived suppressor cells or tolerogenic dendritic cells).

**Acknowledgments**

We thank Dr. Izumi Nakashima for initial providing ret transgenic mice, Dr. Elisabeth Suri-Payer for providing the hybridoma-producing mAbs for CD25, Dr. Axel Benner for help with the statistical analysis, and Ludmila Umansky for excellent technical assistance.

**Disclosures**

The authors have no financial conflicts of interest.

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