Activation of p38 Mitogen-Activated Protein Kinase Drives Dendritic Cells to Become Tolerogenic in *Ret* Transgenic Mice Spontaneously Developing Melanoma

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Abstract

Purpose: The purpose of the study was to investigate signaling molecules involved in the acquisition of tolerogenic properties by dendritic cells (DC) in *ret* transgenic mice with spontaneous melanoma progression and to target these molecules to overcome the barrier for effective melanoma immunotherapy.

Experimental Design: DC functions and expression patterns of p38 mitogen-activated protein kinase (MAPK) in DCs were evaluated in a *ret* transgenic murine cutaneous melanoma model, which shows high similarity to human cutaneous melanoma with respect to clinical development. In contrast to transplantation melanoma models (like B16), this model allows the study of melanoma progression under conditions of natural interactions between tumor and host cells over time.

Results: We showed a strong tumor infiltration with immature DCs and a reduction in the number of mature DCs in lymphoid organs during melanoma progression. DCs from melanoma-bearing mice secreted significantly more interleukin 10 and less interleukin 12p70, and showed a decreased capacity to activate T cells compared with DCs from tumor-free animals. Observed DC dysfunction was linked to considerable activation of p38 MAPK. Inhibition of its activity in spleen DCs from tumor-bearing mice led to normalization of their cytokine secretion pattern and T-cell stimulation capacity.

Conclusions: Our data show a critical role of constitutively activated p38 MAPK in the acquirement of tolerogenic pattern by DCs during melanoma progression that contributes to the suppression of antitumor T-cell immune responses. We suggest that new strategies of melanoma immunotherapy can include inhibitors of p38 MAPK activity in DCs.

Dendritic cells (DC) are viewed as important regulators of effective adaptive immune responses against various tumors, including melanoma (1, 2). Considering high intrinsic melanoma immunogenicity (3, 4), DCs were loaded with defined melanoma antigens and applied for antitumor immunotherapy. Thus, such DCs were able to reject B16 melanoma and to stimulate antitumor T-cell reactions in mice (5, 6). Vaccination of melanoma patients could also result in the induction of cytotoxic tumor-specific CD8 T cells and in positive clinical effects in some patients (2, 7, 8). Despite the initial promising data, the overall results of clinical studies are not satisfactory (9). Insufficient antitumor reactivity could be due to the different mechanisms dealing with structural and functional changes both in tumor and stroma cells. Tumor-derived factors, like transforming growth factor (TGF)-β, interleukin (IL)-6, IL-10, and vascular endothelial growth factor (VEGF) have been described to induce tolerogenic DCs (10, 11), which stimulated the expansion of T_H2 cells or regulatory T cells. In particular, IL-10 accumulated in metastatic lymph nodes from melanoma patients blocked DC maturation, which resulted in the induction of anergic T cells and/or regulatory T cells (12). In addition, some human tumors stimulated the expression of inhibitory molecule B7-H4 on the DC surface, contributing thereby to the generation of an immunosuppressive tumor microenvironment (13). Furthermore, B16 mouse melanoma cells could recruit DCs to draining lymph nodes and stimulate them to produce IL-10 and TGF-β, which led to the regulatory–T-cell expansion (14).

There is thus an urgent need to develop innovative immunotherapeutic strategies that avoid tolerogenic effects mediated by DCs in the tumor-bearing host. In contrast to transplantation models (e.g., B16), in which tumor-host interactions are not comparable with the clinical situation, a

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Translational Relevance

Despite the well-known melanoma immunogenicity, the results of immunotherapy trials are not satisfactory. Insufficient antitumor reactivity could be due to tolerogenic dendritic cells (DC), which induce the anergy of tumor-specific T cells and expansion of immunosuppressive regulatory T cells. However, these data were obtained with the use of transplantation tumor models, in which the tumor development and tumor-host interactions are not comparable with the clinical situation. Moreover, the role of p38 mitogen-activated protein kinase (MAPK) was controversially discussed. In this study, we used *ret* transgenic mice spontaneously developing melanoma, which resembles human melanoma in histopathology and clinical development. Mechanistic investigations revealed that tumor-derived factors induced in tumor-bearing mice the p38 MAPK activation linked to DC dysfunctions. Inhibition of p38 MAPK activity in these DCs resulted in the normalization of their T-cell stimulation capacity. We suggest that novel melanoma immunotherapies can include p38 MAPK inhibitors to neutralize the immunosuppressive tumor microenvironment.

recently described MT/*ret* transgenic mouse model closely resembles human melanoma with respect to tumor genetics, histopathology, and clinical development (15, 16). Mice expressing the human *ret* transgene in melanocytes develop spontaneously malignant cutaneous melanoma metastasizing to the lymph nodes, lungs, brain, kidney, and spleen (16). This metastatic profile is similar to that observed in melanoma patients (17).

In the present study, we investigated alterations in DC functions during tumor progression with the use of a *ret* transgenic mouse model. We found that the DC population in tumor-bearing mice was characterized by decreased amounts of mature cells, higher IL-10 and lower IL-12 production, and by a diminished capacity to stimulate T cells compared with DCs from mice without macroscopic tumors or nontransgenic littermates. DCs from melanoma mice showed an increased expression of phosphorylated forms of p38 MAPK, signal transducers and activators of transcription 3 (STAT3), and small mothers against decapentaplegic homologue 3 (Smad3). Inhibition of p38 MAPK activity in DCs resulted in the reconstitution of their cytokine secretion profile and T-cell stimulation capacity. Therefore, constitutive activation of p38 MAPK by tumor-derived factors could induce a DC cytokine profile, which led to hampered antitumor T-cell responses and melanoma progression. Our study suggests that p38 MAPK inhibition in DCs emerges as a promising ingredient of new strategies for melanoma immunotherapy.

Materials and Methods

Mice. Animals (C57BL/6 background) expressing human *ret* proto-oncogene in melanocytes under the control of mouse metallothionein I promoter/enhancer (15) were kindly provided by Dr. Izuma Nakashima (Department of Biomedical Sciences, Chubu University, Aichi, Japan). OT-I mice, which express a transgenic T-cell receptor (Vα2/Vβ5) specific for the ovalbumin-derived peptide SIINFEKL, were kindly provided by Dr. Bernd Arnold (German Cancer Research Center, Heidelberg, Germany). All mice were crossed and kept under specific pathogen-free conditions in the animal facility of the German Cancer Research Center. Experiments were done in accordance with government and institutional guidelines and regulations. The survival and general performance of mice were monitored daily. Spontaneous tumor development was assessed macroscopically.

Antibodies and reagents. The media used were Ficoll (PAA) as well as RPMI 1640 (PAA) supplemented with 10% FCS (PAN Biotech) and 50 μmol/L β-mercaptoethanol (Sigma). The rat anti-mouse directly conjugated monoclonal antibodies (mAb; I-A^d/I-E^d-FITC, CD40-PE, CD80-PE, CD86-PE, CD11c-biotin, CD45.2-PerCP-Cy5.5, and isotype-matched control mAbs), streptavidin-allophycocyanin, and the mixture of mAbs against mouse Fc-receptors (Fc-block) used for the fluorescence-activated cell sorting staining were purchased from BD Biosciences. Purified rabbit antibodies against murine phosphorylated p38 MAPK, STAT3, Smad3, and extracellular signal-regulated kinase (Erk1/2) MAPK (Cell Signaling Technology), and Alexa Fluor 488 – conjugated secondary mAbs against rabbit IgG (Molecular Probes) were also used for flow cytometry. Neutralizing rat anti-mouse mAbs for IL-6 and TGF-β1, as well as purified goat antibodies against mouse VEGF were from R&D Systems. SB203580, a specific inhibitor of p38 MAPK, and lipopolysaccharide were purchased from Sigma. CpG oligodeoxynucleotide 1668 was purchased from MWG. The ovalbumin-derived peptide SIINFEKL was synthesized in the core facility of the German Cancer Research Center. Ovalbumin-specific $CD8^+$ T-cell line was established upon peptide immunization of C57BL/6 mice by SIINFEKL together with T-helper peptide (aa 128-140) derived from hepatitis B virus core antigen.

Preparation of single cell suspensions. Fresh bone marrow, spleen, lymph node, and tumor samples were immediately transferred into PBS, cut into small pieces, and filtered through a cell strainer. Bone marrow and spleen samples were depleted of erythrocytes by ammonium chloride lysis.

***DC isolation* ex vivo**. Single spleen cell suspension was digested in PBS supplemented with 2% FCS, 5 mg/mL DNase I (Sigma), and 10 mg/mL collagenase IV (Life Technologies) for 30 min at room temperature. Digested cells were then centrifuged at 400 g for 30 min in Ficoll followed by positive selection of DCs by CD11c MicroBeads isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. To test phosphorylation levels of transcription factors (p38 MAPK, STAT3, Smad3, and Erk1/2 MAPK) in DCs, *ex vivo* isolated splenocytes were directly fixed in 2% paraformaldehyde for 10 min at 37°C and permeabilized in 100% methanol for 30 min on ice followed by extensive washing with PBS. In some experiments, splenocytes were incubated for 15 min at 37°C in the RPMI 1640 medium supplemented with 10% FCS and 50 μmol/L β-mercaptoethanol, and containing supernatants from cultured Ret melanoma cells (Ret conditioned medium; 50%, volume for volume) followed by fixation and permeabilization as described above.

DC generation from the bone marrow. DCs were generated as previously described (18) with some modifications. Briefly, 2×10^6 bone marrow cells from *ret* transgenic mice or nontransgenic littermates were incubated for 8 d in RPMI 1640 medium supplemented with 50 μmol/L β-mercaptoethanol (Merck), 10 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (eBioscience), and 10 ng/mL recombinant mouse IL-4 (R&D Systems). In some experiments, Ret conditioned medium was added to the culture medium (50%, volume for volume) used for the generation of DCs from nontransgenic littermates. In addition, some culture samples were supplemented with neutralizing antibodies for IL-6, VEGF, or TGF-β1 (1.0, 0.4, and 1.0 μg/mL, respectively).

Flow cytometry. Single cell suspensions were treated with Fc-block and mAbs for 20 min at 4°C. To measure the expression of phosphorylated transcription factors, cells were incubated at room temperature with respective primary mAbs for 1 h and with secondary mAbs for 30 min. Acquisition was done by four-color flow cytometry

with the use of FACSCalibur with CellQuest software or FACSCanto II with FACSDiva 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 events. Data were expressed as dot plots or histograms.

Reverse transcription-PCR. Total RNA was extracted from primary tumors, Ret melanoma cells, and B16F10 melanoma cells with the use of a TRIzol reagent (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized with the use of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (Amersham Biosciences) for 2 h at 42°C. Reverse transcription products were amplified by PCR.

Detection of cytokines and growth factors. DCs generated from the bone marrow or *ex vivo* isolated DCs (5×10^5 cells/mL) were stimulated with 1 μg/mL lipopolysaccharide or 3 μg/mL CpG1668 for 24 h. Concentrations of IL-12p70 and IL-10 in supernatants were determined with the use of respective ELISA kits (both BD Biosciences) according to the manufacturer's protocols. In some experiments, *ex vivo* isolated spleen DCs were first incubated with the p38 MAPK inhibitor SB203580 (10 μmol/L) at 37°C for 1 h, and then stimulated with CpG1668 for 24 h followed by measurement of IL-12p70 and IL-10 via ELISA. In other experiments, isolated spleen DCs were incubated with or without SB203580 (10 μmol/L) during the loading with the ovalbumin-derived peptide SIINFEKL for 1 h. After extensive washing, DCs were cocultured with $CD8^+$ T cells isolated from OT-I mice for 3 d (T cell/DC ratio, 5:1), followed by measurement of IFN-γ levels in supernatants with the use of an ELISA kit (BD Biosciences). To measure tumor-derived factors, primary tumors and Ret tumor cells were treated by lysis solution (Bio-Rad) followed by centrifugation at 4,500 *g* for 6 min at 4°C. Protein concentration was determined through Bradford assay and adjusted to 500 μg/mL with the use of serum diluent (both Bio-Rad). Amounts of IL-6 and VEGF in tumor lysates and murine serum were measured with the use of multiplex technology (Bio-Rad), and levels of TGF-β1 in these samples were tested by a single plex kit (Millipore) according to the manufacturer's protocols.

IFN-γ enzyme-linked immunosorbent spot assay. The assay was done as previously described (19) with modifications. Briefly, *ex vivo* isolated spleen DCs were loaded with SIINFEKL (100 ng/mL) for 1 h at 37°C, washed, and cocultured with the ovalbumin-specific $CD8^+$ T-cell line for 40 h (T cell/DC ratio, 10:1). In another set of experiments, $CD8^+$ T cells were isolated from spleens of OT-I mice with the use of a $CD8^+$ T-cell isolation kit (Miltenyi Biotec) and coincubated with SIINFEKL-pulsed DCs for 72 h with the use of the same T cell/DC ratio. The number of IFN-γ–producing cells was detected with Bioreader 3000 (Biosys).

Data analysis. Statistical analyses were done with the use of parametric (Student's *t*test) and nonparametric (Mann-Whitney *U*test) tests. A value of $P < 0.05$ was considered statistically significant.

Results

Decrease in DC numbers in lymphoid organs of tumor-bearing mice and accumulation of immature DCs in advanced tumors. In this study, we used transgenic mice overexpressing the human oncogene *ret* (15) backcrossed at least six times with C57BL/6 wild-type mice. After a short latency (20-70 days of age), around 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 the lymph nodes, lungs, liver, and brain.

We investigated $CD11c^+MHCII^+$DCs in the spleen, bone marrow, and lymph nodes of *ret* transgenic mice with the use of flow cytometry (Fig. 1A to D). Compared with non-transgenic littermates and/or *ret* transgenic mice without visible tumors (control groups), transgenic animals with

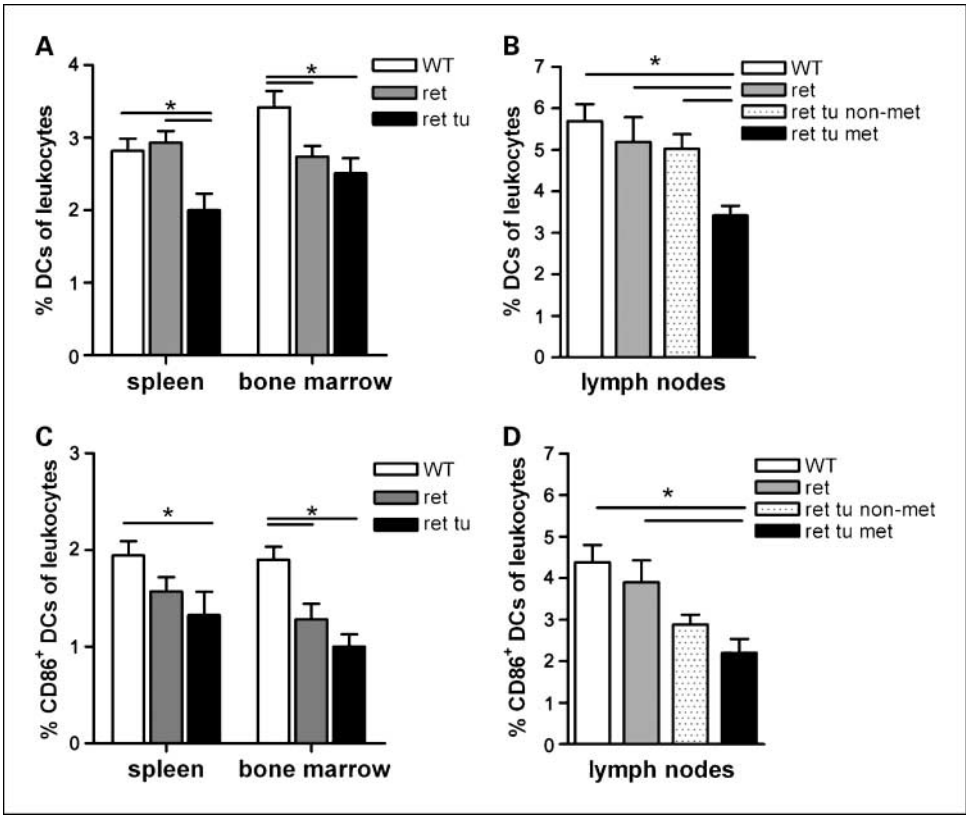

Fig. 1. Analysis of DCs in the spleen, bone marrow, and lymph nodes of *ret* transgenic mice. Cells from mice with macroscopic tumors (ret tu), without visible tumors (ret), and from nontransgenic littermates (wild type) were stained with mAbs for CD11c, MHC class II, and the leukocyte marker CD45.2 followed by flow cytometry. *A,* accumulative data for DCs in the spleen and bone marrow are expressed as the percentage within leukocytes. *B,* accumulative data for DCs in lymph nodes with macroscopic metastases (ret tu met) and without visible metastatic lesions (ret tu non-met) expressed as the percentage within leukocytes. *C,* amounts of mature $CD86^+$ DCs in the spleen and bone marrow expressed as the percentage among leukocytes. *D,* numbers of $CD86^+$ DCs in lymph nodes with macroscopic metastases and without metastases expressed as the percentage within leukocytes. Data, means ± SE from 5 to 20 mice. *, $P < 0.05$; lines, significant differences between groups.

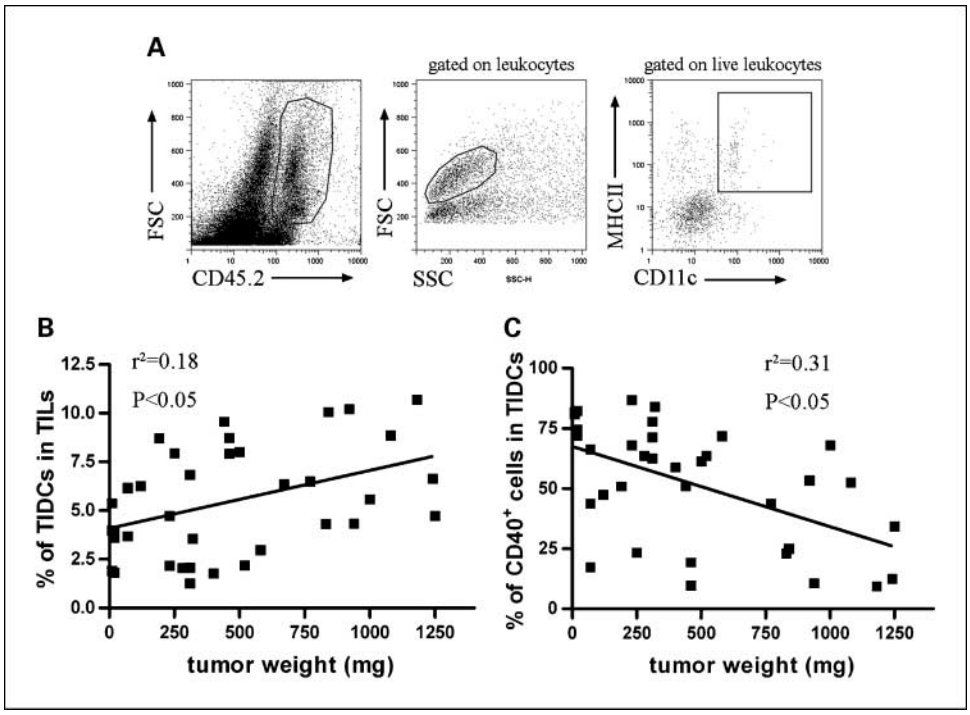

Fig. 2. Tumor infiltration with DCs is dependent on tumor progression. Single cell suspensions prepared from mouse tumors were stained with mAbs for CD11c, MHC class II, CD45.2, and CD40. *A,* representative dot plots are shown. *B* and *C,* the weight of each tumor sample ($n = 37$) was plotted against the percentage of TIDCs within CD45.2$^+$ TILs (*B*) or against the percentage of CD40$^+$ mature DCs within TIDCs (*C*). The correlation between the two variables was calculated through a linear regression analysis. TIDC, tumor-infiltrating DC; TIL, tumor-infiltrating leukocytes.

macroscopic tumors displayed in the spleen and bone marrow a significant decrease in total DC numbers within CD45.2$^+$ leukocytes ($P < 0.05$; Fig. 1A). Interestingly, in the bone marrow, this decrease was observed already in tumor-free transgenic mice compared with wild-type littermates. Moreover, DC amounts in metastatic lymph nodes were significantly lower than those in nonmetastatic lymph nodes from the same tumor-bearing mice or in lymph nodes from control groups ($P < 0.05$; Fig. 1B). We found also a profound diminution in numbers of CD86$^+$ DCs in all investigated lymphoid organs compared with control groups ($P < 0.05$; Fig. 1C and D). Furthermore, the level of CD86 expression on DCs was also reduced (data not shown). Similar changes were revealed in the distribution and expression of CD40 and CD80 costimulatory molecules on these DCs (data not shown). Thus, lymphoid organs from tumor-bearing mice show a systemic decrease in DC numbers and expression of costimulatory molecules. No statistical correlation between observed alterations and mouse age, tumor weight, or the dynamics of tumor growth was found (data not shown).

Next we studied DCs infiltrating primary skin tumors as a subset of tumor-infiltrating CD45.2$^+$ leukocytes (Fig. 2A to C). Increasing proportions of tumor-infiltrating DCs among tumor-infiltrating leukocytes were found to correlate with the increasing weight of primary melanomas ($r^2 = 0.18$; $P < 0.05$; Fig. 2B). Importantly, in larger tumors, significantly higher amounts of tumor-infiltrating DCs displayed immature phenotype according to the CD40 expression profile compared with DCs infiltrating smaller tumors ($r^2 = 0.31$; $P < 0.05$; Fig. 2C). These findings suggest that, in the process of tumor progression, the suppressive tumor microenvironment can block DC development at the immature stage.

Impaired DC functions in ret transgenic tumor-bearing mice. We investigated the secretion of IL-12p70 and IL-10 by *ex vivo* isolated spleen DCs after their stimulation with CpG 1668 with the use of an ELISA assay. DCs from *ret* transgenic mice with macroscopical tumors secreted significantly less IL-12p70 than DCs from mice of both control groups ($P < 0.05$; Fig. 3A). Moreover, the amount of IL-10 produced by tumor DCs was significantly increased compared with DCs from nontransgenic littermates ($P < 0.05$; Fig. 3B). This indicates an immunosuppressive profile of cytokines produced by DCs from tumor-bearing animals.

To address the question of whether the T-cell stimulating capacity of DCs from mice with macroscopic tumors was also impaired, *ex vivo* isolated spleen DCs were pulsed with the ovalbumin peptide SIINFEKL and cocultured with CD8$^+$ T lymphocytes isolated from OT-I mice followed by the detection of IFN-γ–producing cells via enzyme-linked immunosorbent spot assay. As shown in Fig. 3C, significantly reduced numbers of T cells were able to secrete IFN-γ after stimulation with DCs from mice with macroscopic tumors compared with DCs from nontransgenic littermates ($P < 0.05$). In another set of experiments, SIINFEKL-pulsed spleen DCs were coincubated with ovalbumin-specific CD8$^+$ T cells generated from ovalbumin-immunized mice. We detected substantially lower amounts of IFN-γ–producing T cells in the presence of DCs from mice with macroscopic tumors than in samples with DCs from control mice ($P < 0.05$; Fig. 3D). Taken together, the data on cytokine secretion and T-cell activation capacity suggest that DCs from transgenic tumor-bearing mice display a tolerogenic pattern.

Tumor-derived factors promote generation of DCs with a tolerogenic profile. To investigate which tumor-derived factors could be involved in the development of tolerogenic DCs during melanoma progression, we first examined the expression of some cytokines and growth factors, like IL-6, IL-10, VEGF, and TGF-β1, by reverse transcription-PCR at the

mRNA level in the cell line, which was established from primary skin melanomas isolated from *ret* transgenic mice (Ret melanoma cells). Whereas IL-6, VEGF, and TGF-β1 mRNAs were found in this cell line, the IL-10 mRNA expression was not detected (data not shown). Considerable amounts of VEGF and TGF-β1 proteins were shown in supernatants from cultured Ret melanoma cells by ELISA (data not shown). Next we analyzed the expression of all four above-mentioned factors in primary tumors removed from transgenic mice both at the mRNA and protein levels. Primary melanomas also expressed IL-6, VEGF, and TGF-β1 mRNAs, whereas IL-10 mRNA was not detectable (data not shown). At the protein level, we showed IL-6, VEGF, and TGF-β1 production in primary tumors with the use of bio-plex assay. Notably, the amount of VEGF displayed a significant positive correlation with the tumor weight (r^2 = 0.56; $P < 0.05$; Fig. 4A). Moreover, concentrations of IL-6 and VEGF were significantly elevated in the serum from transgenic tumor-bearing mice compared with wild-type littermates ($P < 0.05$; Fig. 4B).

To investigate a direct effect of these tumor-derived factors on DCs, supernatants from cultured Ret melanoma cell (Ret conditioned medium) were mixed with the culture medium (50%, volume for volume) used for the DC generation from normal bone marrow precursors. DCs generated under these conditions secreted significantly lower amounts of IL-12p70 upon lipopolysaccharide stimulation than DCs generated in the normal DC medium ($P < 0.05$; Fig. 4C). Furthermore, after adding neutralizing antibodies for IL-6, VEGF, or TGF-β1 to the DC medium supplemented with Ret conditioned medium, we found a significant increase in IL-12p70 production compared with DCs generated without these antibodies ($P < 0.05$; Fig. 4C).

p38 MAPK plays a key role in the development of tolerogenic DCs in tumor-bearing mice. Having shown a critical importance of IL-6, VEGF, and TGF-β1 for the acquirement of tolerogenic properties by DCs from tumor-bearing mice, we then addressed the question of which transcription factors regulating the functions of these substances are responsible for observed DC impairments. Phosphorylation levels of p38 MAPK, STAT3, Smad3, and Erk1/2 MAPK were examined in freshly isolated spleen DCs by flow cytometry (Fig. 5A). To prevent possible artificial effects induced by the DC isolation, spleen cells were immediately fixed in paraformaldehyde and permeabilized in ice-cold methanol followed by stainings with mAbs for CD11c and phosphorylated transcription factors. We found that DCs from transgenic mice with macroscopic tumors displayed a significant up-regulation in the expression of phosphorylated p38 MAPK, STAT3, and Smad3 compared with those in DCs from wild-type littermates ($P < 0.05$), whereas the level of phosphorylated Erk1/2 MAPK remained unchanged (Fig. 5A and B). To test if tumor-derived factors can directly modulate the p38 MAPK expression, normal spleen DCs were incubated in the RPMI 1640 medium supplemented with Ret conditioned medium (50%, volume for volume). The expression of p38 MAPK was significantly increased compared with DCs cultured in the normal RPMI 1640 medium ($P < 0.05$; Fig. 5C).

We next assessed whether the inhibition of p38 MAPK activity could reverse the altered pattern of cytokine production shown by DCs from transgenic tumor-bearing mice. Freshly isolated spleen DCs were incubated in the presence of SB203580, a p38 MAPK specific inhibitor, followed by the CpG stimulation. The suppression of p38 MAPK activity led to the profound decrease in IL-10 production compared with untreated cells ($P < 0.01$; Fig. 6A). To examine if SB203580 could restore the impaired capacity of DCs from transgenic melanoma-bearing mice to stimulate T cells, freshly isolated spleen DCs were loaded with SIINFEKL in the presence of SB203580. After washing out the inhibitor, tumor DCs were cocultured for 3 days with $CD8^+$ T cells isolated from spleens of OT-I mice followed by the measurement of IFN-γ in the supernatant via ELISA. DC pretreatment with SB203580 significantly stimulated IFN-γ production by T cells ($P < 0.05$; Fig. 6B). Thus, inhibition of the p38 MAPK activity in DCs from tumor-bearing mice led to the decrease in IL-10 production and to the restoration of the DC ability to stimulate $CD8^+$ T cells.

Taken together, our data suggest a crucial role of p38 MAPK activity in the generation of DCs with a tolerogenic pattern in *ret* transgenic tumor-bearing mice.

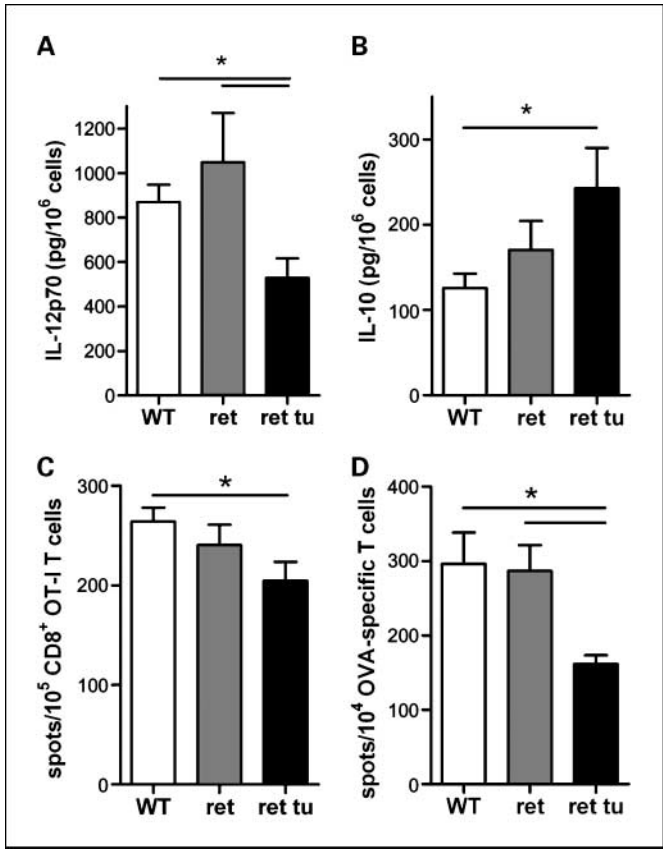

Fig. 3. Cytokine secretion and T-cell stimulation by DCs from tumor-bearing mice. *A* and *B*, spleen DCs simultaneously isolated *ex vivo* from tumor-bearing (ret tu), tumor-free (ret), and wild-type mice (WT) were stimulated with CpG1668 for 24 h, followed by detection of IL-12p70 (*A*) and IL-10 (*B*) in supernatants with the use of ELISA. Data, mean ± SE from 10 mice per experimental group. *, $P < 0.05$; differences between indicated groups. *C* and *D*, $CD11c^+$ DCs were isolated through positive selection, loaded with the ovalbumin peptide SIINFEKL at 37°C for 1 h, and cocultured for 3 d with $CD8^+$ T cells isolated from spleens of OT-I mice (*C*), or cocultured with an ovalbumin-specific T-cell line for 40 h (*D*). T-cell activation was evaluated by spot numbers in the IFN-γ enzyme-linked immunosorbent spot assay. Means ± SE from four animals per experimental group are shown. *, $P < 0.05$; differences between indicated groups.

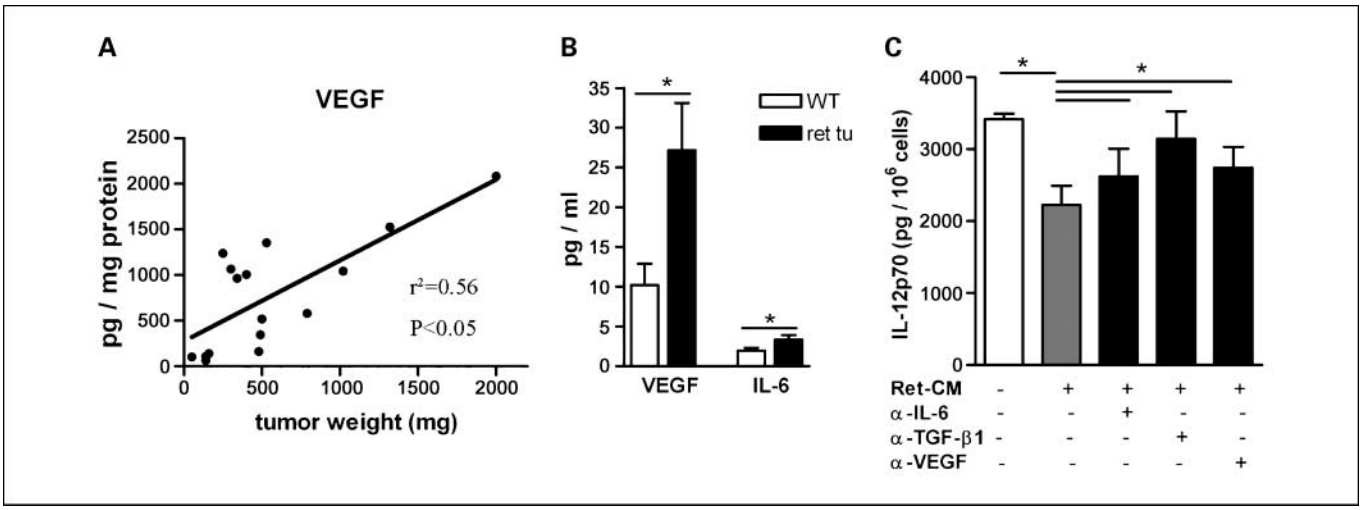

Fig. 4. Tumor-derived cytokines and growth factors induce down-regulation of IL-12 production by DCs. *A,* VEGF concentrations was measured in tumor lysates with the use of bio-plex technology, plotted against the weight of tumors (n = 15), and expressed as picograms per milligram protein. The correlation between the two variables was calculated through a linear regression analysis. *B,* amounts of VEGF and IL-6 were detected in serum of tumor-bearing and healthy mice. Data (means ± SE) from 6 to 12 mice per group are expressed as picograms per milliliter. *, $P < 0.05$; differences between indicated groups. *C,* DCs were generated from bone marrow precursors of nontransgenic mice in the presence of supernatants from cultured Ret melanoma cells (Ret conditioned medium; 50%, volume for volume) and/or neutralizing antibodies for IL-6 (α-IL-6), VEGF (α-VEGF), or TGF-β1 (α-TGF-β1) followed by lipopolysaccharide stimulation for 24 h. IL-12p70 levels were tested in supernatants via ELISA. Results (means ± SE) of four independent experiments are expressed as pg/10^6 cells. *, $P < 0.05$; differences between indicated groups.

Discussion

In the present study, we focused on the investigation of the molecular mechanisms of acquisition of tolerogenic properties by DCs in *ret* transgenic mice with spontaneous skin melanoma with high similarity to human melanoma (15, 16). In contrast to transplantation mouse melanoma models (e.g., B16), this transgenic model provides an opportunity to study mechanisms of melanoma-induced immunosuppression and therapeutic strategies for its neutralization under conditions relevant to the clinical situation.

First, we examined total MHCII$^+$CD11c$^+$ DC amounts in the spleen and bone marrow, and found a remarkable reduction of these cells in *ret* transgenic tumor-bearing mice. Numerous clinical studies reported a dramatic decrease in DC numbers in the peripheral blood of patients with squamous cell carcinoma of the head and neck, lung cancer, myeloma, invasive breast cancer, hepatocellular carcinoma, and leukemia (20–25). Moreover, the presence of metastases resulted in a more profound decrease in the numbers of circulating peripheral blood DCs in cancer patients (25, 26). In agreement with these reports, we showed a significant decrease in DC amounts in metastatic lymph nodes from tumor-bearing mice compared with those in nonmetastatic lymph nodes from the same mice or animals of both control groups.

An observed systemic reduction in DC numbers could be due to the induction of apoptosis in DCs and/or their precursors by tumor cells or soluble tumor-derived factors (27–29). However, we were not able to observe DC apoptosis upon generation from normal bone marrow precursors in the presence of melanoma-derived conditioned medium or after incubation of normal spleen DCs in this medium.[4] Another mechanism of the decreased DC frequency in tumor-bearing mice may be attributed to the inhibition of their maturation at a certain stage. It is known that mature DCs are characterized by high levels of costimulatory molecules, like CD80, CD86, and CD40. Investigating these markers on DCs from tumor-bearing transgenic mice, we found a significant decrease in the numbers of mature DCs expressing CD40, CD80, and CD86 in all studied lymphoid organs. Moreover, the growth of primary melanomas correlated with the accumulation of tumor-infiltrating DCs with the immature phenotype. These findings are in agreement with observations made in cancer patients (30–33). Blocking of normal DC differentiation and maturation can result also in the accumulation of cells with the properties of myeloid-derived suppressor cells (32). This heterogeneous population of myeloid cells has been recently found to induce a dramatic suppression of T-cell functions in mouse tumor models and in cancer patients (34, 35). We observed an accumulation of CD11b$^+$Gr-1$^+$ myeloid-derived suppressor cells in primary tumors and lymphoid organs from transgenic mice[4] that may be responsible for the loss of mature DCs.

Investigating the functional properties of DCs *ex vivo* isolated from spleens of transgenic tumor-bearing mice, we found less IL-12p70 and more IL-10 production upon appropriate stimulation than DCs from nontransgenic littermates. IL-12 has been described to be a critical cytokine for T-cell stimulation and for DC maturation and survival (36, 37). In contrast, IL-10 is known to stimulate regulatory–T-cell differentiation and induce T-cell anergy, and is characteristic for tolerogenic antigen-presenting cells (32, 38). Indeed, we showed here that coincubation of ovalbumin-specific CD8$^+$ T cells with SIINFEKL-loaded DCs isolated from *ret* transgenic mice with macroscopic tumors led to the drastic reduction in IFN-γ–producing cells in an enzyme-linked immunosorbent spot assay compared with T lymphocytes activated by normal DCs pulsed with the peptide.

[4] Unpublished data.

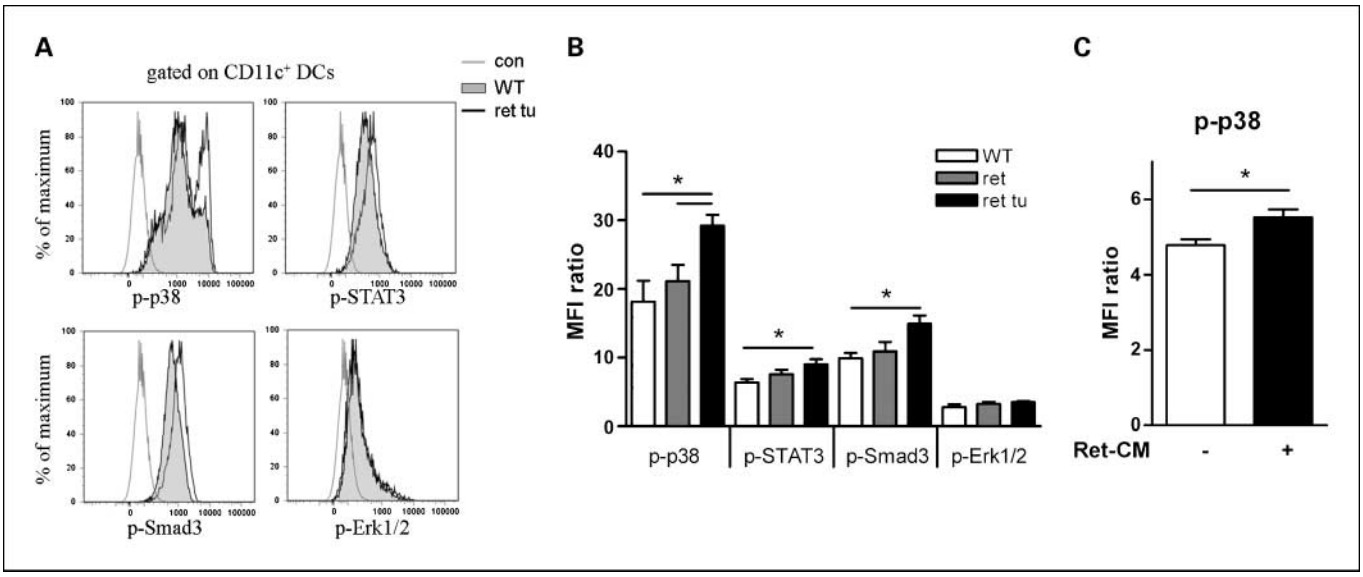

Fig. 5. Tumor-derived factors induce up-regulation of the expression of phosphorylated p38 MAPK in DCs. Spleen cells from tumor-bearing, tumor-free, or wild-type mice were fixed in paraformaldehyde and permeabilized in ice-cold methanol. *A,* expression of phosphorylated p38 MAPK (p-p38), STAT3 (p-STAT3), Smad3 (p-Smad3), and Erk1/2 MAPK (p-Erk1/2) was evaluated in $CD11c^+$ DCs after staining with respective primary and secondary mAbs with the use of flow cytometry. As a negative control (con), we used $CD11c^+$ DCs stained only with secondary mAbs. Results from one representative experiment of three are shown. *B,* accumulative data for transcription factors in spleen DCs (mean ± SE; 4-6 mice per group) are expressed as MFI ratio (MFI of experimental samples/MFI of respective negative controls). *, $P < 0.05$; differences between indicated groups. *C,* splenocytes from normal mice were first incubated for 15 min at 37°C in the medium supplemented with Ret conditioned medium (50%, volume for volume), then fixed and permeabilized as described above. Expression of p-p38 MAPK was evaluated in $CD11c^+$ DCs with the use of flow cytometry. Results (mean ± SE; 4-6 mice per group) are expressed as MFI ratio. *, $P < 0.05$; differences between indicated groups. MFI, mean fluorescence intensity.

Numerous growth factors and cytokines, like VEGF, TGF-β, IL-6, and IL-10, produced by human and mouse tumor cells have been reported to hamper DC maturation *in vitro* (11, 39–41). Tumor-derived VEGF can affect the early stage of DC maturation in the bone marrow and recruit immature DC from the bone marrow to the tumor microenvironment (32, 39, 41). Moreover, IL-6 knockout in *ret* transgenic mice resulted in the decrease of melanoma incidence and tumor size, indicating an importance of IL-6 for tumor progression in this melanoma model (42). In our studies, VEGF and IL-6 were found to be produced both in primary tumors and in Ret melanoma cells. We observed also significantly up-regulated serum levels of IL-6 and VEGF in transgenic tumor-bearing mice. Furthermore, DCs from these animals displayed an up-regulated surface expression of the IL-6 receptor α.[4] Finally, neutralizing antibodies for IL-6 or VEGF were shown to reduce the inhibitory effect of Ret conditioned medium on IL-12 production by stimulated DCs.

Although the list of tumor-derived factors involved in the impairment of DC functions is getting longer, they may utilize similar transcription factors and protein kinases, in particular STAT3 or p38 MAPK. Constitutive STAT3 activation in many human and mouse tumors was reported to inhibit DC maturation via STAT3 up-regulation that led to the impairment of antitumor T-cell responses (32, 43). In accordance with these data, we found a significant elevation of STAT3 expression in DCs isolated from *ret* transgenic tumor-bearing mice. In this melanoma model, we detected even a more considerable increase of the phosphorylated p38 MAPK expression. Furthermore, DC treatment with a specific p38 MAPK inhibitor SB203580 resulted in the decrease in IL-10 production and in the capability to stimulate ovalbumin-specific T cells. A role of p38 MAPK in DC tolerogenic functions during tumor progression has been controversially discussed in recent publications. Thus, Escors et al. (44) showed that constitutive activation of p38 MAPK in mouse DCs resulted in their maturation and stimulation of antitumor T-cell responses. On the other hand, in agreement with our findings reported here on the clinically relevant melanoma model, Wang et al. (45) found that the phenotype and T-cell stimulatory capacity of

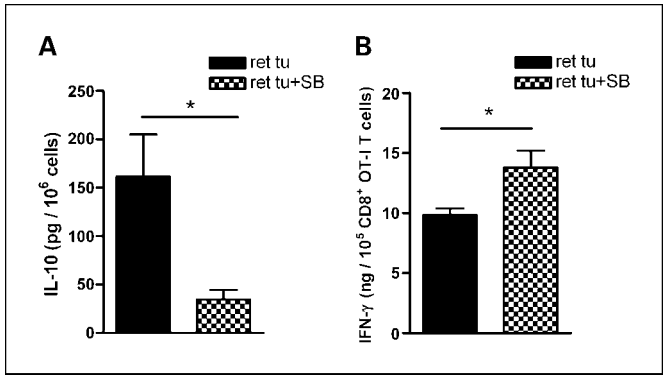

Fig. 6. Inhibition of p38 MAPK in DCs from tumor-bearing mice induces down-regulation of IL-10 production and increases T-cell stimulation. *A,* spleen $CD11c^+$ DCs were isolated from tumor-bearing mice with the use of MicroBeads isolation kit, incubated with or without p38 MAPK inhibitor SB203850 (10 μmol/L) for 1 h, and stimulated with CpG1668 for another 24 h. IL-10 production was measured in supernatants by ELISA. Data, mean ± SE from five independent experiments. *, $P < 0.05$; differences between indicated groups. *D,* freshly isolated spleen DCs were incubated with or without SB203580 during the loading with the ovalbumin peptide SIINFEKL for 1 h. After washing, DCs were cocultured with $CD8^+$ T cells from OT-I mice for 3 d, followed by measurement of IFN-γ in supernatants by ELISA. Means ± SE from three independent experiments are depicted. *, $P < 0.05$; differences between indicated groups.

monocyte-derived DCs in patients with multiple myeloma were considerably impaired and that they could be restored by inhibiting p38 MAPK activity in progenitor cells. In addition, suppression of p38 MAPK signaling in murine DCs was reported to enhance their ability for IL-12 production, to attenuate regulatory–T-cell induction, and to stimulate the antitumor therapeutic efficacy of DCs pulsed with tumor antigens (46). Application of p38 MAPK inhibitors for melanoma immunotherapy *in vivo* will require a thorough examination of their effects on host–T-cell antitumor reactions because the role of p38 MAPK in these cells is not completely clear. Whereas p38 activation was shown to be important for T-cell development and effector functions (47), other publications showed that p38 activity was a prerequisite for the regulatory–T-cell stimulation (48) or for apoptotic $CD8^+$ T-cell death (49). The antitumor effects of the p38 MAPK inhibitor in *ret* transgenic melanoma model *in vivo* are currently under investigation.

In conclusion, our findings provide evidence that constitutive activation of p38 MAPK is responsible for turning of DCs to display a tolerogenic profile during melanoma progression. We showed that suppression of p38 MAPK activity in DCs from *ret* tumor-bearing mice could reconstitute their impaired cytokine secretion and ability to stimulate T cells, suggesting thereby that such normalization of signaling pathways in DCs can represent an effective immunotherapeutic strategy in melanoma patients.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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