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**Tolerogenic dendritic cells**  
**in *ret* transgenic mouse model of spontaneous melanoma**

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## Summary

To investigate mechanisms of the dendritic cell (DC) dysfunction during tumor progression, a transgenic murine model of spontaneous melanoma was used. *Ret* transgenic mice overexpress the human proto-oncogene *ret* in melanin containing cells and develop skin malignant melanoma which closely resembles human melanoma with respect to tumor genetics, histopathology and clinical development.

Numbers of total DCs (MHCII<sup>+</sup> CD11c<sup>+</sup> cells) and mature DCs (DCs expressing CD40, CD80, or CD86) were found to be significantly decreased in the spleen, lymph nodes and bone marrow of tumor bearing mice as compared to *ret* transgenic tumor free or wild type mice (control groups). Moreover, tumors recruited more DCs during progression but the tumor infiltrating DCs were blocked at the immature stage.

After stimulation *in vitro*, *ex vivo* isolated DCs or those generated from bone marrow precursors from tumor bearing mice produced significantly more IL-10 and less IL-12 than DCs from control mice, showing a tolerogenic cytokine pattern. DCs from tumor bearing mice displayed also significantly less capacity to stimulate CD8<sup>+</sup> T cells to produce IFN- $\gamma$ . Therefore, the phenotype and function of DCs in *ret* transgenic mice showed the characteristics of tolerogenic DCs.

Melanoma-derived factors in *ret* transgenic mice were demonstrated to be involved in the acquisition of tolerogenic properties, since DCs generated from bone marrow precursors in medium supplemented with mouse melanoma cell conditioned medium produced significantly less IL-12. Moreover, when activity of VEGF, IL-6, or TGF- $\beta$  was blocked with the respective neutralizing antibodies, IL-12 production by DCs was significantly upregulated.

The p38 mitogen-activated protein kinase (MAPK) can be activated by different tumor-derived factors. A considerably elevated expression of the phosphorylated form of p38 MAPK was detected in DCs from tumor bearing mice. Suppression of p38 MAPK activity in DCs from tumor bearing mice *in vitro* was found to lead to normalization of their cytokine expression pattern and T-cell stimulation capacity.

Taken together, constitutive activation of p38 MAPK is responsible for turning of DCs

to display a tolerogenic profile in the process of melanoma development. We have demonstrated that suppression of the p38 MAPK activity in DCs from *ret* tumor bearing mice can reconstitute their impaired cytokine secretion pattern and ability to stimulate T cells suggesting thereby that such normalization of signaling pathways in DCs could represent an effective immunotherapeutic strategy in melanoma patients.

# **I. Introduction**

## **1. Immune system and antitumor immune response**

The immune system defends host against infection. Serving as the first line of defense, innate immunity is essential for the control of common bacterial infections. However, it lacks the ability to recognize certain pathogens and to provide the specific protective immunity that prevents reinfection.

In the adaptive immune response, lymphocytes, which express diverse antigen-specific receptors, enable the immune system to recognize any foreign antigen. Besides eliminating pathogens, the adaptive immune response can generate increased numbers of memory lymphocytes, which allow a more rapid and effective reaction upon reinfection.

### **1.1. Innate immunity**

#### ***Phagocytes***

The phagocytic cells of the immune system include macrophages, neutrophils, and dendritic cells (DCs). Macrophages are large phagocytic leukocytes, which are able to move outside of the vascular system by moving across the cell membrane of capillary vessels and entering the areas between cells in pursuit of invading pathogens. In tissues, organ-specific macrophages are differentiated from phagocytic cells present in the peripheral blood called monocytes. Macrophages are the most efficient phagocytes, and can phagocytose substantial numbers of bacteria or other cells or microbes. The binding of bacterial molecules to receptors on the surface of a macrophage triggers it to engulf and destroy the bacteria through the generation of a “respiratory burst”, causing the release of reactive oxygen species. Pathogens also stimulate macrophages to produce chemokines, which summon other cells to the site of infection.

Neutrophils eosinophils and basophils are known as granulocytes due to the

presence of granules in their cytoplasm, or as polymorphonuclear cells due to their distinctive lobed nuclei. Neutrophil granules contain a variety of toxic substances that kill or inhibit growth of bacteria and fungi. Similar to macrophages, neutrophils attack pathogens by activating a respiratory burst.

DCs are phagocytic cells located in tissues that are in contact with the external environment, mainly in the skin (where they are often called Langerhans cells), and in the inner mucosal lining of the nose, lungs, stomach and intestines. They are named for their resemblance to neuronal dendrites, but DCs are not connected to the nervous system. DCs are very important in the process of antigen presentation, and serve as a link between the innate and adaptive immune systems.

### ***Natural killer (NK) cells***

As a component of the innate immune system, NK cells attack host cells that have been infected by microbes, but do not directly attack invading microbes. For example, NK cells attack and destroy tumor and virus-infected cells through a process known as "missing-self" (1). This term describes cells with low level expression of major histocompatibility complex (MHC) class I, a situation that can arise in viral infections of host cells. They were named "natural killer" because of the initial notion that they do not require activation in order to kill cells that are "missing self."

### ***Mast cells***

Mast cells reside in the connective tissue and mucous membranes, and are intimately associated with defense against pathogens, wound healing, but are also often associated with allergy and anaphylaxis (2). When activated, mast cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators, and chemokines, or chemotactic cytokines into the environment.

### ***Basophils and eosinophils***

Basophils and eosinophils are cells related to the neutrophils. When activated by a pathogen encounter, basophils releasing histamine is important in defense against parasites, and plays a role in allergic reactions such as asthma. Upon activation, eosinophils secrete a range of highly toxic proteins and free radicals that are highly effective in killing bacteria and parasites, but are also responsible for tissue damage occurring during allergic reactions.

### ***$\gamma\delta$ T-cells***

Like other 'nonconventional' T-cell subsets bearing invariant T-cell receptors (TCRs) such as CD1d-restricted NK T cells,  $\gamma\delta$  T cells exhibit characteristics that place them at the border between innate and adaptive immunity. On one hand,  $\gamma\delta$  T cells may be considered as a component of adaptive immunity since they rearrange TCR genes to produce junctional diversity and develop a memory phenotype. On the other hand, they may function as a part of the innate immune system where a restricted T-cell or NK receptors may be used as a pattern recognition receptor.

### ***Complement***

The complement system is a biochemical cascade of the immune system that helps or “complements” the ability of antibodies to clear pathogens or mark them for destruction by other cells. The cascade is composed of many plasma proteins, synthesized in the liver, primarily by hepatocytes. The proteins work together to 1) trigger the recruitment of inflammatory cells; 2) "tag" pathogens for destruction by other cells via opsonizing the surface of the pathogen; 3) disrupt the plasma membrane of an infected cell, resulting in cytolysis of the infected cell, causing the death of the pathogen; 4) rid the body of neutralized antigen-antibody complexes.

## **1.2. Adaptive immunity**

### ***CD8<sup>+</sup> T lymphocytes***

Cytotoxic T cells (CTL, CD8<sup>+</sup> T cells) are a subgroup of T cells, which induce the death of cells that are infected with viruses (and other pathogens), or are otherwise damaged or dysfunctional. Naive CD8<sup>+</sup> T cells are activated when their TCR strongly interacts with a peptide-bound MHC class I molecule. This affinity depends on the type and orientation of the antigen/MHC complex and keeps the CTL and infected cell bound together. Once activated, the CD8<sup>+</sup> T cell undergoes a process called clonal expansion, in which it gains functionality and divides rapidly into effector cells. Activated CD8<sup>+</sup> T cells will then travel throughout the body in search of cells bearing that unique MHC class I peptide.

When exposed to these infected or dysfunctional somatic cells, effector CD8<sup>+</sup> T cells release perforin and granulysin, which form pores in the target cell plasma membrane allowing ions and water to flow into the infected cell and causing it to burst or lyse. CD8<sup>+</sup> T cells release granzyme, a serine protease that enters cells via pores to induce apoptosis. To limit extensive tissue damage during an infection, CD8<sup>+</sup> T cells activation is tightly controlled.

Upon resolution of the infection, most of the effector CD8<sup>+</sup> T cells die and are removed away by phagocytes, but a few of these cells are retained as memory cells. Upon a later encounter with the same antigen, these memory cells quickly differentiate into effector cells, dramatically shortening the time required to mount an effective response.

### ***T helper (T<sub>H</sub>, CD4<sup>+</sup> T) cells***

CD4<sup>+</sup> T cells are immune response mediators and play an important role in establishing and maximizing the capabilities of the adaptive immune response. These cells have no cytotoxic or phagocytic activity and can not kill infected cells or clear pathogens as CD8<sup>+</sup> T cells, but they can direct other cells to perform these tasks.

CD4<sup>+</sup> T cells express TCRs that recognize antigen bound to major

histocompatibility complex (MHC) class II molecules. The activation of a naive  $CD4^+$  T cell causes it to release cytokines, which influences the activity of many cell types.  $CD4^+$  T cells require a much milder activation stimulus than CTLs and can provide extra signals that "help" to activate cytotoxic cells.

Two types of effector  $CD4^+$  T cell responses can be induced by a professional antigen-presenting cell (APC) designated as  $T_H1$  and  $T_H2$  each designed to eliminate different types of pathogens. The  $T_H1$  response is characterized by the production of interferon (IFN)- $\gamma$ , which activates the bactericidal activities of macrophages, induces B cells to make opsonizing antibodies and leads to cell-mediated immunity. The  $T_H2$  response is characterized by the release of interleukin (IL)-4, which results in the activation of B cells to make neutralizing antibodies, leading to humoral immunity. Generally,  $T_H1$  responses are more effective against intracellular pathogens (viruses and bacteria that are inside host cells), while  $T_H2$  responses are more effective against extracellular bacteria, parasites and toxins. Like CTLs, most of the  $CD4^+$  T cells die upon resolution of infection with a few remaining as  $CD4^+$  memory T cells.

### ***Regulatory T cells (Tregs)***

Tregs may control aberrant immune responses to self-antigens, in particular the development of autoimmune diseases, such as type 1 diabetes, and some chronic inflammatory diseases like asthma. However, Tregs also block beneficial responses by preventing sterilizing immunity to certain pathogens and limiting antitumor immunity (3). Accumulation and activation of Tregs was reported in both tumor patients and tumor animal models (4-8). From the functional perspective, the various potential suppression mechanisms used by Tregs can be grouped into four basic 'modes of action': 1) suppression by inhibitory cytokines like IL-10, IL-35 and TGF- $\beta$ ; 2) suppression by cytotoxicity via granzymes and perforin; 3) suppression by 'metabolic disruption' of effector T cells through adenosine or cyclic AMP; 4) suppression by modulation of DC maturation and function (9).

## ***APCs***

Host's cells express "self" antigens. These antigens are different from those on the surface of bacteria ("non-self" antigens) or on the surface of virally infected host cells ("missing-self"). The adaptive response is triggered by recognizing non-self and missing-self antigens.

With the exception of non-nucleated cells (including erythrocytes), all cells are capable of presenting antigen and of activating the adaptive immune response. Some cells are specially equipped to present antigen, and to prime naive T cells. DCs, macrophages and B cells are equipped with special immunostimulatory receptors for activation of T cells and are termed professional APCs.

## ***B cells***

B cells are major cells involved in the creation of antibodies that circulate in blood plasma and lymph known as humoral immunity. Antibodies (or immunoglobulins, Ig), are large proteins used by the immune system to identify and neutralize foreign objects. In mammals, there are five types of antibody, namely IgA, IgD, IgE, IgG, and IgM, which differ in biological properties. Upon activation, B cells produce antibodies, each of which recognizes a unique antigen and neutralizes specific pathogens.

Like the T cells, B cells express a unique B cell receptor (BCR), which recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell "sees" an antigen. T cells recognize their cognate antigen as a peptide in the context of an MHC molecule, while B cells recognize antigens in their native form. Once a B cell encounters its specific antigen and receives additional signals from T<sub>H</sub>2 cells, it further differentiates into an effector cell, a plasma cell.

Plasma cells are short lived cells (2-3 days), which secrete antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells will survive to become long-lived antigen specific memory B cells. Already primed to produce specific antibodies, these

cells can be called upon to respond quickly if the same pathogen re-infects the host.

### **1.3. Evidences of antitumor immunity in animal models and cancer patients**

The idea that the immune system may have a protective role during tumor growth was embodied in the cancer immunosurveillance hypothesis of Burnet and Thomas (10, 11). Large amount of data obtained from animal experiments demonstrated that the immune system was capable of recognizing and eliminating primary tumors and that lymphocytes and cytokines produced by these cells were important in the process. In humans, early follow-up studies of transplant patients who were immunosuppressed (12) and individuals with primary immunodeficiencies (13) showed that they had a significantly higher risk of cancer development. A review of data accumulated by the Cincinnati Transplant Tumor Registry from 1968 to 1995 found a two fold greater risk in transplant patients for developing melanoma than that of the general population (14). In Australia and New Zealand, the tracking of 925 patients who received renal transplants from 1965 to 1998 showed an increased risk of the development of colon, pancreatic, lung and endocrine tumors as well as malignant melanomas (15). In addition, assessment of 5,692 renal transplant patients from 1964 to 1982 in Finland, Denmark, Norway and Sweden showed higher standardized cancer incidence ratios for colon, lung, bladder, kidney, ureter and endocrine tumors as well as malignant melanomas as compared with the general population (16).

A positive correlation between the presence of lymphocytes in the tumor and increased patient survival has also been shown by numerous studies (17-20). One of the most convincing evidence came from the study of cutaneous melanomas. Sorting more than 500 patients with primary melanoma who had 5-, 8- or 10-year follow-ups and comparing their survival statistics, it was shown that patients with tumor infiltrating lymphocytes survived 1.5-3 times longer than patients without tumor infiltrating lymphocytes (21). Similar correlations between the presence of tumor infiltrating lymphocytes and patient survival have been made that involved more than 3,400 patients with cancer of the breast, bladder, colon, prostate, ovary or rectum cancer and for neuroblastoma (17-25). In some cases, the correlation has been refined

to show that CD8<sup>+</sup> T cells are the relevant lymphocyte population that affects survival (20).

However, the immune surveillance in tumor patients is mostly insufficient to control tumor growth, which is attributed to tolerance induced by many cells and soluble factors, such as regulatory T cells, myeloid derived suppressor cells (MDSCs), immunosuppressive cytokines and growth factors derived from tumor and host cells, and dysfunctional NK cells and DCs.

## **2. Dendritic cells**

DCs engulf exogenous pathogens, such as bacteria, parasites or toxins in the tissues and then migrate, via chemotactic signals, to the T-cell enriched lymph nodes. During migration, DCs undergo a process of maturation in which they lose most of their ability to engulf other pathogens and develop an ability to communicate with T cells. DCs use enzymes to chop the pathogen into antigens. In the lymph node, DC will display these "non-self" antigens on their surfaces by coupling them to a "self"-receptor called the MHC (also known in humans as human leukocyte antigen (HLA)). This MHC:antigen complex is recognized by T cells passing through the lymph node. Exogenous antigens are usually displayed on MHC class II molecules, which activate CD4<sup>+</sup> T<sub>H</sub> cells. Endogenous antigens are produced by viruses replicating within a host cell. The host cells use enzymes to digest virally associated proteins, and display these pieces on their surfaces to T cells by coupling them to MHC. Endogenous antigens are typically displayed on MHC class I molecules, and activate CD8<sup>+</sup> cytotoxic T cells.

Binding of MHC:antigen complex by TCR does not stimulate on its own the proliferation and differentiation of naive T cells into armed effector T cells. The antigen-specific clonal expansion of naive T cells requires a second or costimulatory signal, which is delivered by binding of CD80/CD86 on DCs to CD28 on T cells. CD40 is also a costimulatory molecule which binds to CD40 ligand (CD40L) on T cells, transmits signals to the T cell, activates DCs to express CD80 and CD86, and thus stimulates further T-cell proliferation. Costimulatory signals promote not only

transcription of IL-2 but also stabilize IL-2 mRNA. An autocrine mechanism is used by IL-2 to stimulate T-cell proliferation. In contrast, antigen recognition in the absence of costimulation leads to T-cell anergy, a state in which T cells are unable to produce IL-2 (26). In recent year, a cytokine profile secreted by DCs has been described as a “third signal” (27). For example, naive CD4<sup>+</sup> T cells stimulated in the presence of IL-12 tend to develop into T<sub>H</sub>1 cells, whereas environmental IL-10 drives the generation of Tregs (28). It means that these three DC-derived signals together determine the stimulation or anergy of naive CD8<sup>+</sup> T cells and the development of T<sub>H</sub>1, T<sub>H</sub>2 cells or Tregs.

Different DC subsets are generated from different precursor cells. For example, myeloid DCs (MDCs) are differentiated from common myeloid precursors (CMPs), whereas plasmacytoid DCs (PDCs) are from common lymphoid precursors (CLPs). MDCs are the main IL-12 producer (26) and the most efficient APCs able to prime directly naïve T cells and can be immunogenic or tolerogenic under different conditions (29). It is assumed that MDCs are important for the induction of the specific cytotoxic T-cell responses as well as T<sub>H</sub>1 polarized responses which are more efficient in anti-tumor immune response (30). De Vries et al. demonstrated that for the generation of an effective antimelanoma T-cell response, high numbers of sufficient activated MDCs are essential (31). Moreover, local priming of melanoma-specific CD8<sup>+</sup> T cells, in stage I melanoma patients is associated with a high MDC content in the sentinel lymph node (32).

PDCs can produce large amounts of type I IFNs in response to viruses and other pathogens. Under steady-state conditions, PDCs appear to play a key role in maintaining peripheral immune tolerance, and may be considered as naturally occurring tolerogenic DCs (26). For example, activating-PDC-TLR ligands upregulated the expression of inducible costimulator ligand (ICOS-L), which led to the generation of IL-10 producing T regulatory cells (33). However, if stimulated with microbial pathogens, PDCs differentiate into potent APCs with type I IFN production capacity (34, 35). After viral stimulation, murine PDCs take part in both innate and adaptive immune responses by directly priming naive CD8<sup>+</sup> T cells *in vivo* (36). Zuniga et al. reported that bone marrow PDCs can differentiate into MDCs upon virus infection (37). Moreover, PDCs help other DC subsets to induce T-cell responses. It

has been demonstrated that PDCs synergize with MDCs in the induction of antigen-specific antitumor immune responses *in vivo* (38). PDCs can also improve the activity of lymph node DCs to stimulate CTLs (39).

### **3. Tolerogenic dendritic cells and tumor escape**

Numerous clinical studies reported a dramatic decrease in DC numbers in the peripheral blood of patients with different types of tumors such as squamous cell carcinoma of the head and neck, lung cancer, myeloma, invasive breast cancer, hepatocellular carcinoma and leukemia (40-46). Moreover, the presence of metastases resulted in a more profound decrease in numbers of circulating peripheral blood DCs in cancer patients (46, 47).

Furthermore, impaired capacity of DCs to prime effective antitumor responses has also been observed, which is due to the lack of an efficient immune synapse between DCs and T cells (48).

#### **3.1 Generation of tolerogenic dendritic cell**

DC functions in tumor patients are quite often found to be abnormal and such DCs induce normally immune tolerance to tumor cells. Therefore, understanding the mechanism of the generation of tolerogenic DCs in tumor progression will benefit the current immunotherapies in particular DC vaccination in tumor patients.

##### **3.1.1. Alteration of DC functions in cancer**

Tumor cells can directly modulate DC function through different mechanisms, among which an induction of DC apoptosis and a blockade of DC differentiation are mostly investigated.

Tumor induced DC apoptosis has been reported to be mediated by cytochrome c release, which further leads to cytoplasm shrinking, caspase-3 activation, upregulation

of pro-apoptotic protein Bax and down-regulation of anti-apoptotic proteins Bcl-xL and Bcl-2 (49-51). Furthermore, growth of RM-1 prostate cancer cells could be significantly inhibited in mice treated intratumorally with DCs which were transduced with murine Bcl-xL gene (50). Other anti-apoptotic molecules are also shown to be able to inhibit tumor-mediated DC apoptosis, such as Fas associated death domain (FADD)-like ICE inhibitory proteins (FLIP) which blocks binding of procaspase-8 to FADD (52).

Numerous studies have shown that DCs in tumor patients or tumor animal models are phenotypically or functionally immature. CD83<sup>+</sup> DCs obtained from progressing, chemotherapy-resistant melanomas revealed a marked downregulation of CD86 expression and induced syngeneic CD4<sup>+</sup> T-cell anergy (53). In breast cancer, DCs failed to stimulate proliferation of allogeneic T cells (54). Human basal cell carcinoma associated DCs were deficient in CD80 and CD86 expressions as well as in their ability to stimulate T-cell proliferation (55). Moreover, DCs isolated from human non-small cell lung cancer were blocked at immature stage (56). Similar data have also been shown in patients, such as multiple myeloma, colon carcinoma, head and neck cancer, and lung cancer (57-59).

Tumors can also affect the process of DC differentiation from their precursor cells. DCs derived from monocytes of myeloma patients expressed significantly lower levels of CD40, CD80, and HLA-DR and were not able to activate alloreactive and autologous antigen-specific T cells (60). *In vitro* studies showed that myeloma cells or myeloma cell conditioned medium could inhibit differentiation and function of murine bone-marrow derived DCs (61). The blockade of DC differentiation at the level of immature myeloid cells, an immune suppressive population, which comprises of immature macrophages, granulocytes, DCs and myeloid cells at early stages of differentiation has been found in a large number of different tumors including breast, lung, and head and neck cancer (40).

### **3.1.1. Tumor-derived factors and impairment of DC functions in cancer**

In addition to the modulation of DC functions induced by tumors through cell-cell contact, tumors produce several immunosuppressive factors, which can also affect DC functions.

#### ***TGF- $\beta$***

TGF- $\beta$  is involved in the regulation of numerous processes including hematopoiesis, cell proliferation, differentiation and activation (62). It is also known as the most potent immunosuppressive cytokine described to date. TGF- $\beta$  can be secreted by many types of tumor cells. It is associated consistently with an aggressive tumor phenotype. For example, transfection of a regressor skin tumor cell with the gene for TGF- $\beta$  enabled evasion of immunological destruction resulting in tumor progression *in vivo* (63). By modulating activity of NK cells, T cells, macrophages, and DCs, TGF- $\beta$  affects initiation and effector phases of both primary and secondary immune responses (64).

Mechanisms of TGF- $\beta$  in regulating DC functions have been described in several studies. Although it has been shown that TGF- $\beta$  promoted the generation of Langerhans cells (65), data from most studies demonstrated TGF- $\beta$  as a negative regulator for the immunogenic function of DCs. TGF- $\beta$  induced the generation of DCs with an immature phenotype both in human and murine studies (66, 67). When added to LPS-stimulated DC culture, TGF- $\beta$  inhibited the expression of MHC class II and costimulatory molecules on DCs (69). In addition, TGF- $\beta$  could also suppress DC maturation and IL-12 production induced by IL-1 and TNF- $\alpha$  but not by CD40 ligand or anti-CD40 antibody (69, 70). Moreover, Byrne et al. showed that TGF- $\beta$  producing progressive tumor recruited tumor infiltration of macrophages while the number of tumor-infiltrating DCs decreased, altering thereby proportion of APCs in favor of macrophages that enables tumor evasion of the host immune system (68).

***IL-10***

Various tumor cells express and release IL-10. This cytokine is also produced by tumor-infiltrating lymphocytes and macrophages (71). DC dysfunction in tumor bearing mice was shown to be induced by tumor-derived IL-10, whereas improvement of DC function was observed in tumor-bearing IL-10-deficient mice (72). IL-10 can convert immature DC into tolerogenic DC through decreased expression of costimulatory molecules (73). Moreover, DCs derived from transgenic mice which overexpress IL-10 markedly suppressed allogeneic T-cell responses and IL-12 production (74). Human DCs generated from CD34<sup>+</sup> hematopoietic progenitors were sensitive to IL-10 treatment at all stages of differentiation and IL-10 inhibited the primary and secondary proliferative responses of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells induced by allogeneic CD1a<sup>+</sup> DCs (75). Steinbrink et al. reported that IL-10-treated human DCs induced CD4<sup>+</sup> and CD8<sup>+</sup> T cells that suppressed antigen-specific proliferation of other T cells (76). Notably, IL-10 worked also as a mediator of prostaglandin E2 (PGE2), which could suppress IL-12 production by DCs (77). Finally, IL-10 was reported to skew the differentiation of monocytes into macrophages instead of DCs (78).

***Vascular endothelial growth factor (VEGF)***

VEGF is produced by most tumors and promotes tumor development, in particular tumor angiogenesis (79). A correlation between VEGF expression and microvessel density could be observed in many malignancies (79). Increased plasma levels of VEGF were reported in tumor patients compared with healthy donors; high VEGF levels were associated with a poor outcome in small cell lung carcinoma and breast cancer patients (80-82). Production of VEGF by human tumors inhibits the functional maturation of DCs from precursors and affects the function of relatively mature DCs (83). Saito et al. reported an inverse correlation between the DC density and the VEGF expression within tumor tissue and peripheral blood of cancer patients (84).

The role of tumor-derived VEGF in DC differentiation have been demonstrated by *in vitro* experiments, in which neutralizing antibody against VEGF abrogated the negative effect of tumor cell conditioned medium on DC differentiation from their

precursors (83), and the *in vivo* data further confirmed this conclusion (85). Moreover, continuous infusion of VEGF into naïve animals resulted in a dramatic inhibition of DC development and an increase in numbers of B cells and immature myeloid cells (85). Consistent with these observations, administration of neutralizing VEGF-specific antibody to tumor-bearing mice improved DC differentiation and increased the number of mature DCs (86, 87). Furthermore, an inhibitory effect of VEGF on DC differentiation has been shown in patients with gastric cancer or non-small-cell lung cancer (88).

Mechanism of VEGF-induced DC dysfunction remains unclear. More recent studies indicated that VEGF receptor 1 is the primary mediator of the VEGF inhibition of DC maturation (89), whereas VEGF receptor 2 is crucial for early hematopoietic differentiation, and only marginally affects final DC maturation. Although several inhibitors of VEGF receptors are undergoing clinical trials (90), a recent study showed that the defective differentiation of DCs in advanced cancer patients could not be normalized by blocking VEGF signaling through VEGF receptor inhibitors (88).

## ***IL-6***

IL-6 is a pleotropic cytokine that can be produced by several malignant tumors (91). In plasmacytoma and chronic lymphatic leukemia patients who showed overproduction of IL-6, the development and functional maturation of DCs was impaired (92). In the recent study, IL-6 was found to suppress DC maturation *in vivo* (93).

IL-6 skewed *in vitro* differentiation of monocytes into phenotypically mature but functionally impaired DCs and inhibited the chemotactic response of DCs through blocking the upregulation of CCR7 expression on their surfaces (94). IL-6 secreted by stroma cells upregulated the expression of macrophage-colony stimulating factor (M-CSF) receptors on monocytes, and together with M-CSF, it could directed monocyte differentiation rather to macrophages than to DCs (95). Involvement of IL-6 in the inhibition of DC differentiation has also been shown in multiple myeloma patients (53). Human renal cell carcinoma cells released large amounts of M-CSF and

IL-6, which inhibited myeloid progenitor cell to differentiate into DCs (96). Bharadwaj et al. reported that IL-6 and G-CSF in human pancreatic cancer cell conditioned medium are responsible for the suppression of DC differentiation, maturation, and antigen presentation due to the aberrant activation of STAT3 (97).

### ***M-CSF***

The inhibition of DC differentiation by renal-cell carcinoma cells conditioned medium was preceded by inducing M-CSF receptor and losing granulocyte-macrophage colony stimulating factor (GM-CSF) receptor  $\alpha$  expression at the surface of CD34<sup>+</sup> cells (96). Interestingly, IL-4 and IL-13 could reverse such inhibitory effect of IL-6 and M-CSF on the phenotypic and functional differentiation of CD34<sup>+</sup> cells into DCs (98). The protecting effect of IL-4 on DC differentiation was found to be due to the down-regulation of M-CSF receptor on surface of DC progenitors, the decrease in M-CSF production, and the maintenance of GM-CSF receptor  $\alpha$  expression.

### ***GM-CSF***

In the physiologic situation, GM-CSF is required for normal myelopoiesis and DC differentiation. However, excessive amounts of GM-CSF can mediate immunosuppression via induction of tolerogenic DCs and immature myeloid cells (99, 100, 104). Gaudreau et al. reported that GM-CSF could prevent diabetes development in NOD mice by inducing tolerogenic DCs, which sustain the suppressive function of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (99). Chronic administration of GM-CSF to mice resulted in the generation of immunosuppressive Gr-1<sup>+</sup> CD11b<sup>+</sup> cell population which resembled morphology of granulocyte-monocyte progenitors (100). These cells could further differentiate to fully mature APCs in the presence of IL-4 and GM-CSF.

In mice bearing Lewis lung carcinoma, the administration of anti-GM-CSF and anti-IL-3 antibodies abrogated the accumulation of tumor-induced immune suppressive granulocyte/macrophage progenitor cells (101). Interestingly, GM-CSF

has shown its therapeutic potential as a component of cancer vaccines (102). In a randomized trial in patients with high-risk melanoma, GM-CSF treatment was associated with a transient increase in mature DCs (103). However, it seems that production of large amounts of GM-CSF by some tumors is detrimental to the host immune system. Serafini et al. (104) showed that vaccination of mice with tumor cells producing large amounts of GM-CSF generated a large number of Gr-1<sup>+</sup> immunosuppressive cells which consequently have a negative impact on vaccination.

### **3.1.3. Role of regulatory T cells in the impairment of DC functions in cancer**

It has been reported that the co-culture of mouse CD4<sup>+</sup> CD25<sup>+</sup> Tregs with mouse bone marrow-derived DCs prevented upregulation of costimulatory molecules and resulted in downregulation of DC-mediated T-cell function (105). *In vivo*, the function of mature DCs is also under the control of naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> Tregs. Thus, depletion of CD4<sup>+</sup> CD25<sup>+</sup> Tregs enhanced the development of MHC class I and II restricted IFN- $\gamma$  producing cells and induced higher cytotoxic activity of CD8<sup>+</sup> T cells (106). In a murine melanoma model, depletion of CD4<sup>+</sup> CD25<sup>+</sup> Tregs elicited long-lasting protective tumor immunity induced by DCs loaded with stressed tumor cells (107). In humans, the coculture of CD4<sup>+</sup> CD25<sup>+</sup> Tregs with monocyte-derived DCs rendered DC inefficient as APCs despite of their prestimulation with CD40 ligand. Moreover, DCs cultured with Tregs were prevented from maturation (108) and produced more IL-10 (109). Accumulation of immunosuppressive DCs and regulatory T cells has been found in melanoma and other tumor patients (110). In the peripheral blood of patients with squamous cell carcinoma of the head and neck, expression levels of HLA-DR on myeloid and total DCs positively correlated with the ratios of T<sub>H</sub>1 and T<sub>H</sub>2, and the percentage of total circulating DCs inversely correlated with that of CD4<sup>+</sup> CD25<sup>+</sup> Tregs (111).

Although the cellular and molecular events that play a role in the DC regulation by CD4<sup>+</sup> CD25<sup>+</sup> Tregs are still not completely clear, it seems that cell-cell contacts and immunoregulatory cytokines are involved. For example, CTLA-4 on CD4<sup>+</sup> CD25<sup>+</sup> Tregs could upregulate indoleamine 2,3-dioxygenase (IDO) expression in DCs (112). Larmonier et al. (113) demonstrated that CD4<sup>+</sup> CD25<sup>+</sup> Tregs derived from a leukemia

mouse model suppressed DC functions, which required TGF- $\beta$  and IL-10 and was associated with induction of Smad signaling pathway and activation of STAT3.

### **3.1.4. Transcription factors in tolerogenic DCs**

#### ***STAT3***

STAT3 was found to be activated in diverse cancers (114). Constitutively activated STAT3 enhanced tumor cell proliferation and prevented apoptosis (115). Inactivation of STAT3 by certain platinum compounds inhibited tumor cell growth and induced apoptosis (115). It is important to note that constitutive STAT3 activity in tumors induced the release of factors such as VEGF and IL-10 that inhibited DC maturation through activation of STAT3 in DCs (116). Inhibiting STAT3 signaling in DC progenitors has been shown to reduce accumulation of immature DCs by tumor-derived factors *in vitro* (117). Ablation of STAT3 in hematopoietic cells enhanced DC maturation in tumor-bearing mice and resulted in a multicomponent antitumor immunity (118).

#### ***Extracellular signal regulated kinase-1/2 mitogen-activated protein kinase (Erk 1/2 MAPK)***

Erk, a member of MAPK family, regulates essential cellular functions like proliferation, differentiation, cell survival, and cell death (119, 120). Constitutive activation of Erk pathway has been described in cell lines derived from pancreas, colon, lung, ovary, and kidney cancers (120). In melanoma, Erk1/2 activation was observed in 54% of primary and 33% of metastatic melanomas (121). Furthermore, Erk protected melanoma cells from cisplatin-mediated apoptosis and was shown to be essential for melanoma-immune evasion (122,123).

Activation of Erk in DCs has been demonstrated to promote IL-10 production and mediated negative feedback regulation of IL-12 production (124). Moreover, Jackson et al. (125) showed that melanoma lysate could suppress IL-12 production by DCs through activating Erk1/2 MAPK in DCs and blockade of Erk1/2 activation restored

IL-12 production and generation of T<sub>H</sub>1 cells by DCs. Inhibition of Erk function could also promote the phenotypic and functional maturation of murine resident Langerhans cells (126).

### ***p38 MAPK***

A significant role of p38 MAPK has been reported in several types of tumors, such as follicular lymphoma, chronic B cell lymphocytic leukemia, and melanoma (127-129). Activation of p38 in malignant B lymphoma cells upregulated IL-10 gene expression and promoted lymphomagenesis (130). In addition, human malignant glioma cells secrete high level of VEGF through constitutive activation of p38 MAPK (131).

It was reported that blocking p38 MAPK pathway could significantly up-regulate IL-12 production in mature DCs (132, 133). Wang et al. (60) reported that the impaired function of monocyte-derived DC from multiple myeloma patients could be restored by inhibiting p38 MAPK in progenitor cells. In addition, inhibition p38 MAPK activity in the bone marrow (BM) cells cultured in the presence of tumor culture conditioned medium restored the generation of functional BMDCs (61). Importantly, regulatory T-cell induction could be attenuated by inhibiting p38 MAPK signaling in DCs (134).

## **3.2. Tolerogenic DCs induce T-cell tolerance**

DC maturation in tumor patients is often blocked at the immature stage which may tolerize peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells by inducing deletion, anergy or regulation (135-138). This was attributed to the lack of costimulatory molecules on DC surface.

However, the CD80 and CD86 molecules could also limit the activation of T cells by binding with CTLA4 expressed on T cells. In these DCs, activity of indoleamine 2,3-dioxygenase (IDO) was upregulated by the autocrine secretion of IFN- $\gamma$  following the engagement of CD80/CD86 by CTLA4 (139). IDO is an enzyme that exists in DCs within mouse and human lymphoid tissues and catalyzes the depletion of the

essential amino acid tryptophan, resulting in the inhibition of T-cell proliferation and produces tryptophan-derived metabolites that promote T-cell apoptosis (140). In mice, PDCs could downregulate T-cell responses through the upregulation of IDO (141). Moreover, Baban et al. have characterized a small subpopulation of DCs in mouse spleen that synthesized large amounts of IDO and downregulate T-cell function (142). Such IDO<sup>+</sup> CD19<sup>+</sup> DCs increased IDO production following CD80/CD86 ligation by CTLA4 or TLR9 ligation.

In addition, a role of B7-H1 and B7-H4 molecules (inhibitory members of B7 family) on DCs has been recently studied. Investigations in patients with breast cancer, colon cancer, melanoma and multiple myeloma showed that the overexpression of B7-H1 and B7-H4 led to the inhibition of T-cell function and was associated with poor prognosis (143). Furthermore, MDCs in human tumors and tumor-draining lymph nodes expressed a high level of B7-H1 (144, 56). The interaction between B7-H1<sup>+</sup> MDCs and tumor-associated T cells led to the downregulation of IL-12 and upregulation of IL-10 production by MDCs in a B7-H1-dependent manner. Blockade of B7-H1 on tumor-infiltrating MDCs resulted in the increase of IFN- $\gamma$  production by T cells (144). Adoptive transfer of such T cells improved the clearance of human tumors in xenotransplanted mice, supporting the idea that B7-H1 plays a role in the downregulation of DC-mediated tumor immunity (144).

### **3.3. Tolerogenic DCs induce the differentiation of T<sub>H</sub>2 cells or Tregs**

Steinman et al. reported that immature DCs may maintain the immune tolerance by inducing T<sub>H</sub>2 response (145). In cancer patients, the T<sub>H</sub>1 skewing has been correlated with improved clinical outcomes (146-148). Investigations on tumor-specific T-cell immunity in renal cell carcinoma (RCC) and melanoma patients have demonstrated that patients with active melanoma or RCC displayed strongly polarized T<sub>H</sub>2-type reactivity, whereas healthy donors and patients that were disease-free following therapeutic intervention exhibited either weak mixed T<sub>H</sub>1/T<sub>H</sub>2 or strongly-polarized T<sub>H</sub>1 (146).

Immature DCs may also induce expansion of IL-10 producing Treg (Tr1) and CD4<sup>+</sup> CD25<sup>+</sup> Tregs (149-151). Furthermore, tumor-associated immature DCs induced CD4<sup>+</sup>

CD25<sup>+</sup> Tregs from naïve T cells, and suppress T-cell proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (152). It has been shown that DCs induced Tr1 through IL-10 (149, 153, 154). Finally, TGF- $\beta$  secreted by a DC subpopulation has also been implicated in the generation of CD4<sup>+</sup> regulatory T cells (155).

## **4. Malignant melanoma**

Melanoma is a malignant tumor due to the uncontrolled growth of transformed melanocytes, which are found predominantly in skin but also in the bowel and the eye.

### **4.1. Epidemiology and diagnosis**

#### **4.1.1. Risk factors**

Major risk factors for melanoma development include excessive sun exposure, number of melanocytic nevi, cutaneous phenotype, and family history of melanoma. Unlike the more common skin cancers that are associated with high cumulative doses of ultraviolet light, most melanomas appear to be associated with intense, intermittent exposure, particularly during childhood and adolescence.

Epidemiologic studies have repeatedly shown an increased risk of melanoma in persons with large numbers of melanocytic nevi (156). Individuals with clinically atypical or "dysplastic" nevi have a higher risk, particularly in the setting of a positive family history of melanoma. In one study, the risk of melanoma was double in individuals with 50 to 99 small nevi compared to those with less nevi's numbers (157). About 10% of all melanoma patients have a positive family history. Genetic linkage studies have identified a gene CDKN2 on chromosome 9p21, which encodes the tumor suppressor protein p16 and is involved in melanoma development. Mutations in this gene have been documented in 50% of familial melanoma patients (158).

### **4.1.3. Clinical presentation, diagnosis, and prognosis**

Melanoma generally arise from preexisting nevi and demonstrate asymmetry, pigment variegation, and irregular borders. Nodular melanomas are the second most common form of melanoma. They may be evenly pigmented and have regular borders and be easily mistaken for a basal cell carcinoma, seborrheic keratosis, or a benign nevus. Lentigo maligna melanoma occurs on sun-exposed skin, especially the face of elderly patients. In contrast to superficial spreading and nodular melanomas, this subtype is associated with high cumulative doses of the ultraviolet light. They tend to grow slowly and often are confined to the epidermis (lentigo maligna) for years before dermal invasion occurs (lentigo maligna melanoma).

The prognosis of melanoma is related to tumor thickness. Melanoma that is confined to the epidermis (in-situ) is greater than 99% curable, and patients with thin lesions ( $< 0.75$  mm) have a 5-year survival rate of greater than 98%. This is in contrast to patients with thicker lesions ( $> 4$  mm) who have 5-year survival rate of less than 50%.

## **4.2. Melanoma therapies**

### **4.2.1 Surgery**

Complete surgical excision with adequate margins and assessment for the presence of detectable metastatic disease along with short- and long-term followup is a standard procedure.

### **4.2.2. Chemotherapy**

Various chemotherapeutic regimens have been tried without significantly increasing overall survival rates of metastatic melanoma patients (159). An increasing number of small molecules have been developed in the last years, which may be promising for melanoma therapy (160-162). They aimed to specifically target different specific features of melanoma cells like proliferation, metastasis,

angiogenesis and in particular apoptosis deficiency. MAPK and PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase B) signalling pathways appear to be especially promising targets for the treatment of advanced melanoma (163).

#### **4.2.3. Radiotherapy**

Melanoma has long been considered to be relatively radio-resistant and therefore the mainstay of treatment has been surgery. Although radiotherapy has a relatively low probability of achieving long-term control in patients with incompletely resectable locoregional disease, it has been increasingly employed postoperatively to improve locoregional control in patients at a high risk for residual subclinical disease after surgery (164).

#### **4.2.4. Immunotherapy**

Immunotherapy has a long history in patients with melanoma, including a number of different strategies of vaccines utilizing whole tumor cells, peptides, DCs, DNA and RNA, and antibodies. To date, IL-2 and IFN- $\alpha$ 2b are the only approved immunotherapeutic agents for melanoma. Studies have demonstrated that IL-2 allowed a complete and long-lasting remission in this disease, although only in a small percentage of patients (165). For patients who are at a high risk of developing recurrent disease, the treatment with IFN- $\alpha$ 2b in the adjuvant setting has been shown to improve the overall survival from 2.8 to 3.8 years (166).

#### **4.2.5. DC vaccination**

Earlier small clinical trials using DCs showed promising results, with frequent induction of anti-cancer immune reactivity and clinical responses (167). In recent years, additional trials have been carried out in melanoma patients.

***DC sources***

The addition of DCs to the adjuvants for cancer immunotherapy has been possible due to the introduction of new methods of DC isolation and generation. A breakthrough was achieved after the discovery that DCs might be generated from peripheral monocytes or CD34<sup>+</sup> bone marrow precursors in the presence of GM-CSF and IL-4 (168). The most commonly used factors for DC maturation are cytokine cocktails, including PGE2, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (169). PGE2 is believed to enhance the migratory/homing capacity of the DC (170) by upregulating the expression of chemokine receptor CCR7. However, PGE2 possibly also mediated T<sub>H</sub>2 polarisation and secretion of the immunosuppressive cytokine IL-10 (171). Furthermore, Banerjee et al. (172) showed that cytokine production of DCs matured by these cytokine cocktail were more prone to expand immunosuppressive T regulatory cells than immature DCs.

An improved protocols for maturation of DC inducing of TNF, IL-1 $\beta$ , PolyI:C, IFN- $\alpha$  and IFN- $\gamma$  ( $\alpha$ DC) has been proposed (173).  $\alpha$ DC cocktail-matured DC displayed superior immunogenic abilities compared to standard cocktail-matured DC and have retained lymph node migratory capacities *in vitro* even though PGE2 was not added. Nevertheless, the published advantages of  $\alpha$ DC1 maturation were not reproducible when DCs were prepared in plasma containing medium (174).

As an alternative to the well-defined but expensive cytokine cocktails monocyte-derived conditioned medium has been used for DC maturation (175). Presumably, this supernatant from activated monocyte cultures contains critical maturation factors (176).

***Critical issues for optimal DC vaccination***

Apart from choosing the right source of DCs and the right method of DC maturation, critical issues for successful vaccination involved the choice of antigen, the antigen loading, and the route of administration.

A wide range of antigenic preparations are available for loading of DC, including

including peptides, whole proteins, tumor lysate, or apoptotic tumor cells. DCs could be transfected with mRNA or cDNA encoding tumor antigens or fused with whole tumor cells (177).

After pulsing with the tumor antigen, DCs need to be administered to the cancer patients. Several possible injection modes were used, such as intravenous (i.v.), intradermal (i.d.), subcutaneous (s.c.), intralymphatic (i.l.) or intranodal (i.n.) injections. Morse et al. (178) showed that DC injected i.v. primarily accumulated in the lungs and subsequently redistributed to the liver, spleen and bone marrow, while DC migrated to the regional lymph nodes after i.d. or s.c. injection. In another study, it was demonstrated that i.d. and i.l. administration induced a T-cell IFN- $\gamma$  response, whereas i.v. injection resulted in a humoral response (179), suggesting that the nature of the immune response varied with the route of injection. Intranodal injections have been previously claimed to be superior to i.v. or i.d. injection (180). However, Kyte et al. (181) found i.d. injection to be significantly better in the inducing immune responses compared to i.n. inoculation. Thus, the most favourable combination of DC maturation status and routes of injection still need to be clarified.

### ***IL-2 and helper antigen***

IL-2 was frequently used as the DC vaccination adjuvant. It can potently stimulate T-cell growth and was administered in combination with DCs for enhancement of T-cell proliferation and differentiation into effector cells to improve vaccine efficacy (182). On the other hand, high dose IL-2 therapy has been found to promote expansion of regulatory T-cells and could thereby potentially limit antigen specific immune responses (183). Even low doses of IL-2 for a period of two weeks were able to expand CD4<sup>+</sup>CD25<sup>+</sup> Tregs in cancer patients (184).

Helper antigens in the form of microbial components such as keyhole limpet haemocyanin (KLH), tetanus toxoid (TT) and hepatitis B virus antigen (HBsAg) were also added to the vaccine. These compounds interact with the Toll like receptors and promote cytokine secretion and inflammation. In addition, KLH amplified a T<sub>H</sub>1-type cellular tumour-specific response when added to the lysate, suggesting that helper T-cell epitopes contained within KLH may enhance the ability of DCs to induce CTL

responses (185).

### ***DC clinical trials***

During the past decade a large number of DC vaccination studies have been performed (186). Although no significant correlation with objective response was found, clinical responses (complete response, CR; partial response, PR; stable disease, SD) significantly correlated with an induction of vaccine specific T cells as measured by ELISPOT (187). These findings are very encouraging for the ongoing work to optimize immune monitoring in clinical vaccination trials including defined standards for monitoring CD8<sup>+</sup> T-cell response against the used tumor antigen during vaccination (188, 189). However, it is still questionable if these T cells are really able to kill their tumor target *in vivo*, since it was demonstrated that vaccine specific memory CD8<sup>+</sup> T cells could secrete IFN- $\gamma$  and proliferated also in patients without objective clinical responses (190)

A few trials showed correlation between survival and immunologic response. For example, Kyte et al. (181) showed that DC vaccinated patients survived longer than non-vaccinated when retrospectively compared to a control group of patients receiving standard treatment. In addition, subgroup analyses indicated that vaccination treated HLA-A2+/HLA-B44- patients survived longer than dacarbazine treated patients (191). Even though the data lack sufficient statistical strength, they emphasize the importance of careful patient selection for these kinds of trials.

Taken together, there is no doubt that DC vaccines can prime and boost antigen-specific T-cell responses in patients. Although definite advantages in using DC vaccines still could not be observed, it is believed that the full potential of these potent immunostimulatory cells has not yet been entirely exploited. Larger clinical trials comparing DCs with other vaccination strategies in patients with similar stages of disease and using standardized immunomonitoring protocols would be beneficial for tumor immunotherapy.

## 5. *Ret* transgenic spontaneous melanoma mouse model

In contrast to human situation with a strong association between UV exposure and melanoma development (192), normal adult mice do not develop melanomas, even after chronic exposure to UV radiation. Shaved or hairless mice have been exposed to various acute (intense, short-term) and chronic (low level, long-term) UV treatments to simulate human sun exposure. These treatments promote various types of skin cancers including squamous cell carcinoma, papilloma, and fibrosarcoma, but not melanoma (193). Even a combined treatment with carcinogens like 7,12-dimethylbenz[a]anthracene (DMBA) promoted little or even no melanoma development, although there was a significant increase in the frequency of other skin cancers (194).

The conventional animal melanoma models are based on the transplantation of tumor cells (e.g., B16), 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 with respect to tumor genetics, histopathology and clinical development (195).

*Ret* transgenic mouse model (C57BL/6 background) was described by Kato et al. in 1998 (195). By introducing the recombinant human proto-oncogene *ret* fused to mouse metallothionein-I (MT) promoter-enhancer, primary melanoma in *ret* transgenic mice develops subcutaneously. Tumors at the late stage of development metastasize to the lymph nodes, lung, brain, kidney, liver and spleen (195), which corresponds well to that in human skin malignant melanomas, in which the lymph nodes and lung are the most common sites of distant metastasis (196)

The mechanism of melanoma development in *ret* transgenic mice is still under investigation. Kato et al. (195) reported that the level of Ret protein expression and activity at the benign stage was much higher than those in the skin with melanosis at the tumor-free stage. And the expression level and kinase activity of Ret in tumors at the malignant stage were further elevated, which lead to the activation of its downstream signaling molecules, such as Erk2 and c-Jun. Tumors at the malignant stage showed also a high activity of matrix metalloproteinase-9 (MMP-9) and MMP-2,

which are indicators of the potential capability for tumor invasion and metastasis (195).

Finally, Felbert et al. (197) showed that IL-6 gene ablation in *ret* transgenic mice could lead to a decrease of both melanoma incidence and tumor size. The mechanism of IL-6-induced tumor progression is probably due to constitutive activation of PI3K in tumors.

## **6. Aims of the study**

DC dysfunctions have been reported in many types of human tumors and tumor animal models. However, the mechanisms of the DC impairment during tumor progression are still not completely clear. The objective of this work was to investigate the mechanisms of the acquirement of tolerogenic properties by DCs in the process of melanoma development. Using *ret* transgenic mouse model of spontaneous melanoma, which closely resembles human melanoma as regard to tumor genetics, histopathology and clinical development, the following questions were addressed:

- 1) whether DCs from *ret* transgenic tumor bearing mice display a tolerogenic pattern of phenotype and function during tumor progression;
- 2) which tumor-derived cytokines and growth factors as well as transcription factors from their signaling pathways are involved in the driving of DCs to become tolerogenic;
- 3) how to reconstitute the impaired function of DCs and to improve thereby anti-tumor immune responses in tumor bearing mice.

By addressing these questions, we hope to find a strategy which may benefit immunotherapy in melanoma patients.

## **II. Materials and methods**

### **7. Mice and cell lines**

All mice were crossed and kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg). Experiments were performed in accordance with government and institute guidelines and regulations.

#### **7.1. *Ret* transgenic spontaneous melanoma mouse model**

*Ret* transgenic mice (C57BL/6 background), which express human *Ret* proto-oncogene in melanocytes under the control of mouse metallothionein-I promoter-enhancer were kindly provided by Dr. I. Nakashima (Japan) (185). The survival and general performance of mice was monitored at least twice a week. Spontaneous tumor development was assessed macroscopically.

#### **7.2. OT-I transgenic mice**

Homozygous OT-I mice, which express transgenic TCR (V $\alpha$ 2/V $\beta$ 5) specific for ovalbumin (OVA)-derived peptide SIINFEKL, were kindly provided by Dr. B. Arnold (Germany).

#### **7.3. Cell lines**

Cells were all cultured in 5% CO<sub>2</sub> at 37 °C.

##### ***Ret* melanoma cell line**

*Ret* melanoma cell line was established from the primary skin tumor of *Ret* transgenic mouse. Cells were cultured in RPMI-1640 medium supplemented with

10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, L-glutamin and  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol (2-ME).

Ret melanoma cells ( $2 \times 10^5$  cells/ml) were cultured in complete medium for 48h. Supernatants were then collected and stored at -20 °C.

### ***B16F10 cell line***

B16F10 melanoma cell line was cultured in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and L-glutamin.

## **8. Chemicals, buffers/medium, antibodies/cytokines**

### **8.1. Chemicals**

|  |  |
|--|--|
| 3-Amino-9-ethyl-carbazol (AEC) tablets                 | Sigma, Cat.#A6926                      |
| Agarose  | Roth, Cat.#2267.2                      |
| Ammonium chloride (NH <sub>4</sub> Cl)                 | Merck, Cat.#101141                     |
| Bovine serum albumin (BSA)                             | Sigma, Cat.#7030-50G                   |
| Dimethylsulfoxid (DMSO)                                | Merck, Cat.#109678                     |
| GeneRuler™ 100bp DNA Ladder Plus                       | Fermentas, Cat.#SM0321                 |
| EDTA disodium  | GERBU, Cat.#1034                       |
| 0,5M EDTA (pH 8.0)                                     | GIBCO, Cat. #15575-098                 |
| 100% Acetic acid (CH <sub>3</sub> COOH)                | Merck, Cat.#100063                     |
| 1% Ethidium bromide                                    | Merck, Cat.#111608                     |
| Fatal bovine serum (FBS)                               | PAN Biotech GmbH,<br>Cat.#3702-P260718 |
| 30% Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ) | Sigma, Cat.#H1009                      |

|  |                            |
|--|----------------------------|
| Isofluran  | DeltaSelect                |
| 2-mercaptoethanol (2-ME)                         | Merck, Cat.#444203         |
| Potassium hydrogen carbonate ( $\text{KHCO}_3$ ) | Roth, Cat. #P748           |
| Sodium acetate ( $\text{CH}_3\text{COONa}$ )     | neoLab, Cat.#4720          |
| Sodium azide ( $\text{NaN}_3$ )                  | Roth, Cat.#K305            |
| Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ),   | AppliChem, Cat.#A3900      |
| Sodium chloride ( $\text{NaCl}$ )                | Fluka, Cat.#71379          |
| Sodium hydrogen carbonate ( $\text{NaHCO}_3$ )   | AppliChem, Cat.#A3084      |
| N,N – Dimethylformamid(DMF)                      | Sigma, Cat.#D-4551         |
| 1x PBS   | PAA, Cat.#H15-002          |
| 100x Penicillin / Streptomycin                   | PAA, Cat.#P11-010          |
| Phenol / Chloroform                              | Roth, Cat.#A156-1          |
| Potassium bicarbonate ( $\text{KHCO}_3$ )        | Sigma, Cat.#P9144          |
| Sodium Dodecyl sulfate (SDS)                     | Gerbü, Cat.#1212           |
| Proteinase K                                     | Sigma, Cat.#P6556          |
| Tris   | Roth, Cat.#4855.1          |
| Trizol   | Invitrogen, Cat.#15596-026 |
| 0.4% Trypan blue solution                        | Sigma, Cat.#T8154          |
| Tween20  | Sigma, Cat.#P-2287         |
| Xylene   | J.T. Baker, Cat.#3410      |
| Goat serum                                       | GIBCO, Cat.#PCN5000        |

## **8.2. Buffers / medium**

### ***Enzyme-linked immunosorbent assay (ELISA)***

#### *Assay Diluent*

1x PBS containing 10% FBS (v/v)

#### *Wash buffer*

1x PBS containing 0.05% Tween20 (v/v)

### ***Enzyme-linked immunosorbent spot (ELISPOT)***

#### *AEC buffer*

A tablet of AEC (20 mg) was dissolved into 2.5 ml dimethylformamide in a 50 ml Falcon tube. 8.4 ml 0.2 M sodium acetate, 3.5 ml 0.2 M acetate acid and 35.6 ml H<sub>2</sub>O were then added. After mixed well, solution was filtered with 0.45 µm filter and 25 µl H<sub>2</sub>O<sub>2</sub> was added. The prepared AEC buffer should be kept in dark and used within one month after preparation.

#### *Coating buffer*

Buffer A: 1,59 g Na<sub>2</sub>CO<sub>3</sub> dissolved in 100 ml H<sub>2</sub>O

Buffer B: 2,93 g NaH<sub>2</sub>CO<sub>3</sub> dissolved in 100 ml H<sub>2</sub>O

Working coating buffer (pH 9.6) was prepared by adding 1 ml A, 1 ml B and 8 ml H<sub>2</sub>O into a 15 ml Falcon tube.

#### *Wash buffer*

1x PBS containing 0.05% Tween20 (v/v)

### ***Flow cytometry (FACS)***

#### **FACS**

1 x PBS containing 2% FBS and 0.2% NaN<sub>3</sub>

#### **FACS blood buffer**

1x PBS containing 2% FBS, 0.2% NaN<sub>3</sub>, and 0.02% Liquemin N

#### **Red blood cell lysis buffer**

8,29 g NH<sub>4</sub>Cl, 1 g KHCO<sub>3</sub>, and 37,2 mg EDTA were dissolved in 1 L H<sub>2</sub>O pH (7.2-7.4) as a 10-time stock solution. 1-time working buffer was prepared by diluting stock solution with H<sub>2</sub>O.

### ***Agarose gel electrophoresis***

#### **DNA loading buffer**

6 x loading buffer (0.25% bromphenol blue and 30% glycerol) was diluted with H<sub>2</sub>O to make 1x loading buffer

#### **TAE-Puffer**

242 g Tris, 100 ml 0.5 M EDTA, and 57.1 ml pure acetic acid were mixed with H<sub>2</sub>O to reach a final volume of 1 liter.

### ***Immunohistochemistry***

#### **Blocking buffer**

1x PBS containing 10% goat serum, 2% BSA and 0.05% Tween-20

#### **Antibody diluent**

1x PBS containing 2% goat serum, 2% BSA and 0.05% Tween-20

Wash buffer

1x PBS containing 0.05% Tween-20

***Polymerase chain reaction (PCR)***

PCR Mix

To prepare 50 µl PCR mix, 5 µl 10x Reaction Buffer, 1 µl 10 mM dNTPs, 2 µl of each primer stock, 0.5 µl Taq Polymerase, 3 µl templates and 37 µl ddH<sub>2</sub>O were mixed

Digesting buffer

50 mM Tris, 20 mM NaCl, 1 mM EDTA and 1% SDS in H<sub>2</sub>O

***Magnetic activated cell sorting (MACS) buffer***

PBS with 0.5% BSA and 2 mM EDTA

**8.3. Medium**

Complete culture medium

RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, L-glutamin and  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol (2-ME) was used for cell culture unless especially mentioned.

DC medium

RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, L-glutamin,  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol (2-ME), 10 ng/ml recombinant mouse GM-CSF, and 10 ng/ml recombinant mouse IL-4.

10x Trypsin / EDTA

10x Trypsin / EDTA was purchased from PAA (Cat.#L11-003)

## **2.4. Antibodies**

All antibodies for flow cytometry were purchased from BD Biosciences: biotin-conjugated hamster anti-mouse CD11c (clone HL3); fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse I-A/I-E (MHC class II, clone 2G9); R-phycoerythrin conjugated rat anti-mouse CD40 (clone 3/23), hamster anti-mouse CD80 (clone 16-10A1), rat anti-mouse CD86 (clone GL1); PerCP-Cy5.5 mouse anti-mouse CD45.2 (clone 104).

Rat monoclonal antibody against mouse interferon gamma (IFN- $\gamma$ ) (clone RMMG-1) was bought from Biosource. Stock concentration was 2.5 mg/ml. Aliquots were stored at -20 °C.

Rat anti-mouse IFN- $\gamma$  (clone XMG1.2, BD Biosciences, Cat. #554410) was used as a detection antibody for ELISPOT.

Monoclonal antibody against IL-6 (Cat. #MAB406), TGF- $\beta$ 1 (Cat. #MAB240), and VEGF (Cat. #AF-493-NA) were purchased from R&D Systems.

Antibodies against phosphorylated p38 MAPK (Thr180/Tyr182) (Cat. #9215), phosphorylated STAT3 (Tyr705) (Cat. #9131), phosphorylated Smad3 (Ser423/425) (Cat. #9520), and phosphorylated Erk1/2 MAPK (Thr202/Tyr204) (Cat. #4377) were purchased from Cell Signaling Technology.

## **2.5. Cytokines**

Recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF, eBioscience) was shipped as 100  $\mu$ g/ml stock and stored at -20 °C in 10  $\mu$ l aliquots.

Recombinant mouse IL-4 (R&D systems) was reconstituted in sterile PBS containing 0.1% bovine serum albumin to reach a stock concentration of 100  $\mu$ g/ml, and stored at -20 °C in 10  $\mu$ l aliquots.

## **8.6. Other reagents**

SIINFEKL peptide was synthesized in the core facility of German Cancer Research Center (Heidelberg).

Phosphothioate (PTO)-modified CpG ODN 1668 (5'-TCC ATG TTC CTG ATG CT-3') and primer pairs for RT-PCR were obtained from MWG.

Lipopolysaccharide (LPS, Sigma-Aldrich, Cat. #L2654) was dissolved in PBS at a 1mg/ml concentration and aliquots were stored at -20°C.

SB203580 (a specific p38 MAPK inhibitor, Sigma-Aldrich, Cat. #S8307) was dissolved in DMSO to make a 10 mM stock concentration and aliquots were stored at -20 °C.

## **9. Reagent kits**

### **9.1 MACS kit**

CD11c (N418) Microbeads (Cat. #130-052-001) and CD8a<sup>+</sup> T-cell isolation kit (Cat. #130-090-859) were purchased from Miltenyi Biotec.

### **9.2. ELISA kit**

ELISA kits for mouse IL-12p70 (Cat. #555256), IL-10 (Cat. #555252), and IFN-γ (Cat. #555138) were BD OptEIA™ Sets purchased from BD Biosciences.

### **9.3. 3,3',5,5' tetramethylbenzidine (TMB) substrate reagent kit**

TMB substrate reagent kit is used together with BD OptEIA™ ELISA sets. Substrate Reagent A in the kit contains hydrogen peroxide, and Substrate Reagent B contains TMB.

## **9.4. Luminex cytokine assay**

Kits for luminex cytokine assay include 23-plex Bio-Plex mouse cytokine (Bio-Rad, Cat. #171-F11241), single plex mouse cytokine for VEGF (Bio-Rad, Cat. #XD0000007B), M-CSF (Bio-Rad, Cat. #XD0000000G), and TGF- $\beta$ 1 (Millipore, Cat. #TGFB-64K-01).

## **9.5. Immunohistochemistry**

|                                    |                                   |
|------------------------------------|-----------------------------------|
| Vectastain ABC-AP Kit              | Vector Laboratories, Cat.#AK-5000 |
| Alkaline Phosphatase Substrate Kit | Vector Laboratories, Cat.#SK-5100 |

## **10. Routine laboratory materials**

### **10.1. Devices**

|                               |                                   |
|-------------------------------|-----------------------------------|
| ELISA Reader                  | Rainbow Thermo (SLT)              |
| ELISPOT Reader                | Bioreader 3000 (Biosys)           |
| FACS machine                  | FACSCalibur 4-Farben (BD)         |
|                               | FACSLSR II 6-Farben (BD)          |
| Fume hood                     | STA 120 1297 (Prutcher)           |
| Refrigerator (-80 °C)         | HeraFreeze (Heraeus)              |
| Gel chamber                   | 40-1214 (PeqLab)                  |
| Gel chamber power supply unit | EC105 (Apparatus Corporation)     |
| Gel photo chamber             | Gel Jet Imager INTAS (UV Systems) |
| Warming block                 | Thermomixer compact (Eppendorf)   |

|                               |   |
|-------------------------------|---|
| Incubator                     | HeraCell (Heraeus)                      |
| Refrigerator (-20 °C)         | Premium (LiebHerr)                      |
| Microscopes                   | DMIL (Leica)                            |
|                               | DMRE (Leica)                            |
| Microscope camera             | DC500 (Leica)                           |
| Microtome                     | RM 2125 RT (Leica)                      |
| Microtome water bath          | HI 1210 (Leica)                         |
| Microwave oven                | R-352 (Sharp)                           |
| PCR-System                    | DNA-Engine DYAD (MJ Research)           |
| pH meter                      | 766 (Calimatic)                         |
| Photometer                    | UltroSpec 3100pro (Amersham)            |
| Laminar flow                  | Hera Safe (Thermo Electron Cooperation) |
| Container for liquid nitrogen | Isotherm (KGW)                          |
| Liquid nitrogen tank          | Biosafe MD (Messer)                     |
| Timer                         | Oregon Scientific                       |
| Vortex                        | REAX top (Heidolph)                     |
|                               | Vortex Genie 2 (Scientific Industries)  |
| Weighing machine              | BP 3100P (Sartorius)                    |
| Water bath                    | DC3 (HAAKE / GFL)                       |
| Centrifugers                  | Labofuge 400R (Heraeus)                 |
|                               | Biofuge pico (Heraeus)                  |

Biofuge primo R (Heraeus)

Varifuge K (Heraeus)

RT 7 Plus (Thermo Electron Cooperation)

## **10.2. Routine laboratory materials**

|                                |   |
|--------------------------------|---|
| ELISA Plates                   | MaxiSorp 96-well, Nunc, Cat.#442404                           |
| ELISPOT Plates                 | Silent Screen Plate 96-well clear w/o Lid., Nunc, Cat.#256154 |
| Needles                        | 0,4x19 mm Mikrolance (BD)<br>0,3x13 mm Mikrolance (BD)        |
| Cryo tubes                     | Cryo.s (Greiner)  |
| Pipets with adjustable volumes | 2-20 µl, 20-200 µl, 200-1000 µl; Rainin                       |
| Objekt carrier                 | 76x26 mm SuperFrostPlus<br>(Menzel-Gläser) Cat. J1800AMNZ     |
| Coverglass                     | 24 x 24 mm (Roth)   |
| Tubes 15 ml / 50 ml            | Polypropylen (Falcon)   |
| 0.5 ml Eppendorf tubes         |   |
| 1.5 ml Eppendorf tubes         |   |
| 2 ml Eppendorf tubes           |   |
| Syringes                       | 1 ml Plastipak (BD)<br>5 ml (Terumo)                          |

|                     |                          |
|---------------------|--------------------------|
|                     | 50 ml (Terumo)           |
| Cell culture plates | 96-well-Platte (Greiner) |
|                     | 24-well-Platte (TPP)     |
|                     | 6-well-Platte(Greiner)   |
| Cell strainer       | 100µm; Nylon (BD Falcon) |

## 11. Softwares for data analysis

Flowjo (Version 7.2.4), Tree Star, Inc., Ashland, USA

GraphPad PRISM (Version 4), GraphPad Software, Inc., San Diego, USA

## 12. Methods

### 12.1. Genotypization of *ret* transgenic mice

Each tail biopsy of *ret* transgenic mouse was digested in 20 µl Proteinase K digestion buffer at 56 °C for 3 h. Afterwards 300 µl ddH<sub>2</sub>O was added and tubes containing digested biopsies were put at 99 °C for 3 min to inactivate the enzyme activity. 3 µl freshly isolated DNA was added into 47 µl PCR Mix buffer and then PCR was performed. The PCR program was set as following:

- Step 1: 95 °C 5 min
- Step 2: 94 °C 1 min
- Step 3: 58 °C 1 min
- Step 4: 72 °C 1.5 min
- Step 5: back to Step 1 for another 34 cycles
- Step 6: 72 °C 10 min

- Step 7: 4 °C for ever

The primer sequences used for detecting *ret* gene expression in PCR mix were 5'-AAA ATG CAG TCA GAT ATG GA-3', 5'-ACT CGG GGA GGG GTT C-3'.  $\beta$ -actin is used as house keeping gene to qualify PCR process. The sequences for  $\beta$ -actin primers are 5'-CAC CGG AGA ATG GGA AGC CGA A-3', 5'-TCC ACA CAG ATG GAG CGT CCA G-3'.

Results for PCR were tested by 1.5% agarose gel electrophoresis. The gel was prepared by dissolving 1.5% agarose in 100 ml TAE buffer in microwave oven and 4  $\mu$ l ethidiumbromid was added to the heated agarose containing TAE buffer before watering the buffer to gel chamber.

10  $\mu$ l PCR product from each sample was loaded to prepared gel after mixed with 3  $\mu$ l 6x loading buffer and DNA marker was used to locate *ret* and  $\beta$ -actin genes. Potential of power adaptor is set at 100 V. PCR results were then photographed by gel photo chamber.

## **12.2. Preparation of single cell suspension from mouse organs**

Mice were killed by cervical dislocation after anastesy with isofluran.

### **12.2.1 Spleen**

Single cell suspension from mouse spleen was prepared using 9 cm petri dishes with 8-10 ml cold PBS. Spleens were damaged by a 5 ml syringe plunger and filtered through cell strainer (100  $\mu$ m). Cells were washed once with cold PBS at 400 g for 5 min. Red blood cells were lyzed with 1ml lysis buffer at room temperature for 3 min followed by adding 9 ml PBS to stop the reaction. After centrifugation (400 g, 5 min), cell pellets were resuspended in appropriate buffers to perform different assays.

### **12.2.2 Lymph nodes**

Freshly isolated mouse lymph nodes were smashed between two object carriers. The object carriers were then washed with cold PBS to completely remove the remaining cells. Cells were then washed once with cold PBS at 400 g for 5 min and resuspended in FACS buffer for the following flow cytometry.

### **12.2.3 Bone marrow**

Bone marrow cells were obtained from femurs and tibiae and lysed with 1 ml lysis buffer after centrifugation. Then 9 ml PBS was added to stop the reaction. After centrifugation (400 g, 5 min), cell pellet was resuspended in appropriate buffers to perform different assays.

### **12.2.4 Tumor**

Freshly removed tumor mass was pressed by a 5 ml syringe plunger through a cell strainer into a 50 ml Falcon tube. Cells were washed with cold PBS at 400 g for 5 min. Cell pellet was resuspended in appropriate buffers to perform different assays.

## **12.3. Flow cytometry**

For phenotype analysis of DCs in lymphoid organs and primary tumors, single cell suspensions were treated with Fc-block solution followed by incubation with mAbs against MHC classII-FITC, biotinylated CD11c, CD40-PE, CD80-PE, and CD86-PE for 20 min at 4 °C. After washing with FACS buffer, cells were treated with streptavidin-APC for 10 min at 4 °C. If measurements need to be performed on other day, cells were fixed in 2% paraformaldehyde (PFA) until measurement. Before measured in flow cytometer, cells were washed and resuspended in PBS.

To test phosphorylation levels of p38, Erk1/2, STAT3, and Smad3 *ex vivo*, freshly isolated splenocytes were fixed in 2% PFA for 10 min at 37 °C and permeabilized in

100% methanol for 30 min on ice followed by extensive washing with PBS. Purified rabbit anti-mouse antibody against p38, Erk1/2, Stat3, or Smad3 were added to the cell suspension together with biotinylated CD11c mAbs for 1 h at room temperature. After being washed, cells were incubated with goat anti-rabbit antibodies labeled with Alexa Fluor 488 and streptavidin-APC antibodies for 30 min at room temperature. Afterwards, cells were washed and measured in flow cytometer.

Acquisition was performed by four-color flow cytometry using a FACSCalibur with CELL-Quest software (BD Biosciences) or FACSCantoII with Diva software. Dead cells were excluded 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.

#### **12.4. Bone marrow-derived DC culture**

Bone marrow cells were cultured in complete culture medium supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 in 9 cm petri dishes ( $2 \times 10^6$  cells in 10 ml medium). On day 3, another 10 ml fresh medium was added. On day 6 and 8, 10 ml of supernatant from each plate was collected and centrifuged followed by resuspending cell pellet with 10 ml fresh medium. Cells were ready to be used as immature DCs at day 8-10.

#### **12.5. Isolation of DCs from spleen**

DCs were isolated from spleens using CD11c magnetic beads according to manufacturer's instruction. Cells were centrifuged at 400 g for 5min, resuspended in 1 ml ice cold sterile PBS containing 2% FCS, 25  $\mu$ l 40 mg/ml collagenase IV, 250  $\mu$ l 20 mg/ml DNase and incubated for 25 min at room temperature. Then, 5 ml PBS containing 2% FCS was added to stop the reaction. Cells were then passed through cell strainer and carefully overlayed on 3 ml Lymphoprep solution in a 15 ml tube. After 30 min centrifugation (400 g, no shake), supernatant from upper phase and interphase was transferred into a 15 ml tube and centrifuged at 300 g for 8 min at 4°C.

Cell pellets were resuspended in 400 µl MACS buffer and 100 µl CD11c Microbeads and incubated for 15 min at 4°C. After washing with 2 ml MACS buffer (400 g, 8 min, 4°C), cells were resuspended in 500 µl MACS buffer and put onto pre-washed mini-columns. After the unlabeled cells flew through, columns were washed three times to completely remove unbounded cells. The magnetically labeled cells were flushed out by firmly applying the plunger supplied with the column. After centrifugation (400 g, 8 min, 4°C), cells were resuspended in the complete culture medium.

## **12.6. DC cytokine secretion**

For investigating cytokine secretion by bone marrow derived DCs, cells ( $5 \times 10^5$  cells/ml) were stimulated with 1 µg/ml LPS for 24 h at 37 °C.

To study cytokine secretion by freshly isolated spleen DCs, cells ( $5 \times 10^5$  cells/ml) were stimulated with 3 µg/ml CpG 1668 for 24 h at 37 °C.

Supernatants were collected and stored at -20 °C in aliquots. IL-12p70 and IL-10 levels in supernatants were tested by ELISA kit.

## **12.7. T-cell priming and restimulation**

*Ex vivo* isolated spleen DCs were loaded with SIINFEKL (100 ng/ml) for 1h at 37°C, then washed and co-cultured with T cells (T cell/DC ratio = 10:1). In some experiments, DCs were co-incubated with naive CD8<sup>+</sup> T cells isolated from spleens of OT-1 mice ( $1 \times 10^5$  T cells/well) for 3-4 days. In another set of experiments, DCs were co-cultured with OVA-specific CD8<sup>+</sup> T cells for 40 h.

To test effect of SB203580, a p38 MAPK inhibitor, on the DC capacity to activate T cells, spleen DCs isolated using CD11c<sup>+</sup> magnetic beads were loaded with SIINFEKL (100 ng/ml) with or without 10 µM SB203580 for 1 h at 37 °C. Then cells were washed for 3 times with complete culture medium to remove extra peptide.

Isolated CD8<sup>+</sup> T cells from spleen of OT-I mouse ( $1 \times 10^5$  cells in 200  $\mu$ l medium) were cocultured with peptide-loaded DCs at 1:5 ratio in U-bottom 96-well plate for 3 days at 37 °C. Supernatants were collected and stored at -20 °C in aliquots. IFN- $\gamma$  level in supernatant was detected by ELISA kit.

## **12.8. ELISA**

Mouse IL-12p70, IL-10, and IFN- $\gamma$  ELISA assays were performed according to manufacturer's instructions. ELISA plates were precoated with capture antibodies overnight at 4 °C, washed 3 times with the washing buffer (PBS with 0.05% Tween-20) and blocked with assay diluent (PBS with 10% FCS) at room temperature for 1 h. After next washing, standard and samples were incubated at room temperature for 2 h followed by 5 times washing with the wash buffer. Afterwards, detection antibodies and streptavidin-HRP reagent were added to each well, and incubated at room temperature for 1 h followed by 7 times washing with wash buffer. Then substrate solution was added to each well, and plates were incubated in dark around 10-30 min. To stop the reaction, a stop solution (1 M H<sub>3</sub>PO<sub>4</sub>) was added to each well. Extension was measured using spectrophotometer at 450 nm.

## **12.9. IFN- $\gamma$ ELISPOT**

ELISPOT assays employ the sandwich ELISA technique. Either monoclonal or polyclonal antibodies specific for the particular cytokine was pre-coated onto a polyvinylidene difluoride (PVDF)-backed microplate. Appropriately stimulated cells were pipetted into the wells and the microplate was placed into a humidified 37 °C CO<sub>2</sub> incubator for a certain period of time. During this incubation period, the immobilized antibody binds secreted cytokines in the immediate vicinity of the secreting cells. After washing away cells and unbound substances, biotinylated polyclonal antibodies specific for the chosen cytokine was added to the wells. After washing, alkaline-phosphatase conjugated to streptavidin was added. Unbound enzyme was subsequently removed by washing, and a substrate solution (AEC) was

added. A red colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual analyte-secreting cell. The spots can be counted with an automated ELISPOT reader system.

In the present work, IFN- $\gamma$  ELISPOT assay was performed as following. First, ELISPOT plates were coated with 50  $\mu$ l diluted anti-IFN- $\gamma$  antibody (10  $\mu$ g/ml) overnight at 4 °C. After 5 times washing with sterile PBS, plates were blocked with complete culture medium (200  $\mu$ l/well) at 37 °C for 1 h. Then, block medium was removed and 100  $\mu$ l cell suspension containing  $1 \times 10^5$  naive OT-I CD8 T cells or  $1 \times 10^4$  OVA-specific T-cell line was pipetted to each well followed by adding peptide loaded DCs in 1:5 or 1:10 DC/T ratio. Cells were cultured for another 3-4 days (naive OT-I T cells) or 40 h (OVA-specific CD8<sup>+</sup> T cell line established upon peptide immunization of C57BL/6 mice via SIINFEKL together with T-helper peptide (aa128-140) derived from HBV core antigen). After co-culture, supernatants were removed and plates were washed 3 times with the wash buffer (PBS with 0.05% Tween-20). 50  $\mu$ l biotinylated anti-mouse IFN- $\gamma$  antibody was added to each well and plates were incubated at room temperature for 2 h followed by 3 times washing with wash buffer. Then, 50  $\mu$ l 1:100 diluted streptavidin-horseradish peroxidase was added to each well and plates were incubated at room temperature for 2 h followed by 3 times washing with wash buffer and another 3 times washing with PBS. 100  $\mu$ l AEC buffer was added to each well. Plates were incubated in dark for 5-20 minutes until clear red spots can be seen. To stop the reaction, plates were washed with tap water. After air dried, plates were read in Bioreader 3000. Spots measured in the presence of unpulsed DCs were considered as non-specific background (negative control). Samples were considered to contain OVA-reactive T cells producing IFN- $\gamma$  when spot numbers in experimental triplicates were significantly higher than the numbers in negative control triplicates ( $p < 0.05$ ).

### **12.10. Reverse transcription (RT)-PCR**

RT-PCR is a process of converting RNA to DNA followed by PCR amplification of the reversely-transcribed DNA. First, complementary DNA (cDNA) is made from an mRNA template using dNTPs and reverse transcriptase. After the reverse transcriptase

reaction is complete, cDNA has been generated from the original single strand mRNA and standard PCR is initiated.

In the current work, total RNA from investigated cells (freshly isolated spleen DCs, bone marrow derived DCs, and tumor cell lines) was extracted by using TRIZOL reagent according to the manufacturer's instruction. RNA was reverse-transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase and random hexamers for 2 h at 42 °C. 3 µl cDNA was then amplified with 2.5 U of Tag polymerase in PCR reaction buffer using specific primer pairs. PCRs for  $\beta$ -actin were run to normalize the levels of mRNA in the samples. Primers used were: IL-10, 5'-TCA AAC AAA GGA CCA GCT GGA CAA CAT ACT G-3', 5'-CTG TCT AGG TCC TGG AGT CCA GCA GAC TCA-3'; TGF- $\beta$ 1, 5'-CTC CCA CTC CCG TGG CTT CTA G-3', 5'-GTT CCA CAT GTT GCT CCA CAC TTG-3'; TLR4, 5'-AGT GGG TCA AGG AAC AGA AGC A-3', 5'-CTT TAC CAG CTC ATT TCT CAC C-3'; IL-6, 5'-CCC AAC AGA CCT GTC TAT ACC-3', 5'-CAG CTT ATC TGT TAG GAG AGC-3'; VEGF, 5'-TTA CTG CTG TAT CTC CAC C-3', 5'-ACA GGA CGG CTT GAA GAT G-3';  $\beta$ -actin, 5'-CAC CGG AGA ATG GGA AGC CGA A-3', 5'-TCC ACA CAG ATG GAG CGT CCA G-3'.

### III. Results

#### 13. Genotypization and tumor development of *ret* transgenic mice

As shown in Fig. 13.1, the sample from mouse, which expressed *ret* gene showed a clear band around 600kb (line S2), whereas the sample from non-transgenic littermate showed no band at this position (line S1).

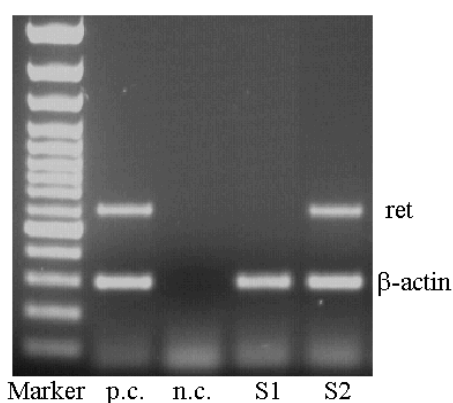


Figure 13.1. A typical genotypization result after agarose gel electrophoresis. Lines from left to right: DNA marker (Marker), positive control (p.c., cDNA from *ret* transgenic mouse), negative control (n. c., PCR product without adding cDNA template), sample 1 (S1), sample 2 (S2).

After genotypization, tumor development of *ret* transgenic mice was monitored macroscopically twice a week. After a short latency (20-70 days of age), around 25% of all transgenic mice develop skin tumors subcutaneously on the face (nose, ears, eyes and neck), back or on the tail (Fig.13.2 A, B). Tumor bearing mice developed metastases in the lymph nodes (Fig. 13.2 C), lungs, liver and brain (data not shown).

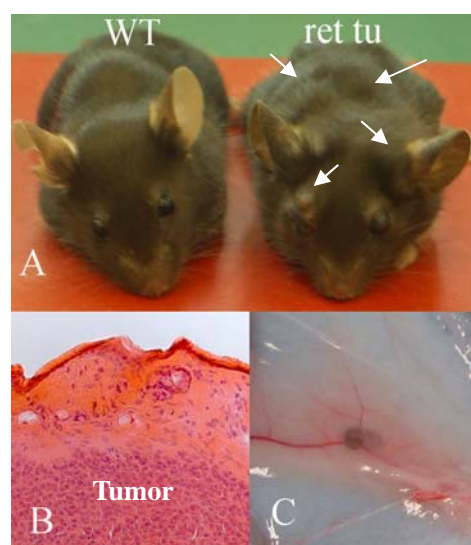


Figure 13.2. Melanoma development in *ret* transgenic mice. (A) Tumor localization in *ret* transgenic mouse (*ret tu*, right) is indicated with arrows. (B) Hematoxylin and eosin staining of the subcutaneous primary tumor. Magnification x 200. (C) Metastatic lymph nodes in *ret* transgenic mouse with macroscopical tumors.

## **14. Phenotype analysis of dendritic cells in lymphoid organs of *ret* transgenic mice**

Phenotype of DCs from the spleen, bone marrow and lymph nodes of *ret* transgenic mice were analyzed using flow cytometry. MHC class II<sup>+</sup> CD11c<sup>+</sup> cells from above mentioned organs were considered as DCs. Expression of CD40, CD80 and CD86 costimulatory molecules were used to investigate DC maturation status.

### **14.1. Analysis of total DC numbers**

Gating strategy used for the DC analysis is shown in Fig. 14.1 A. A significant reduction of DC number was found in both spleen and bone marrow (BM) of *ret* transgenic mice with macroscopical tumors (tumor bearing mice) as compared to non-transgenic littermates (wild type) or *ret* transgenic mice without macroscopical tumors (tumor free mice) (Fig. 14.1 B).

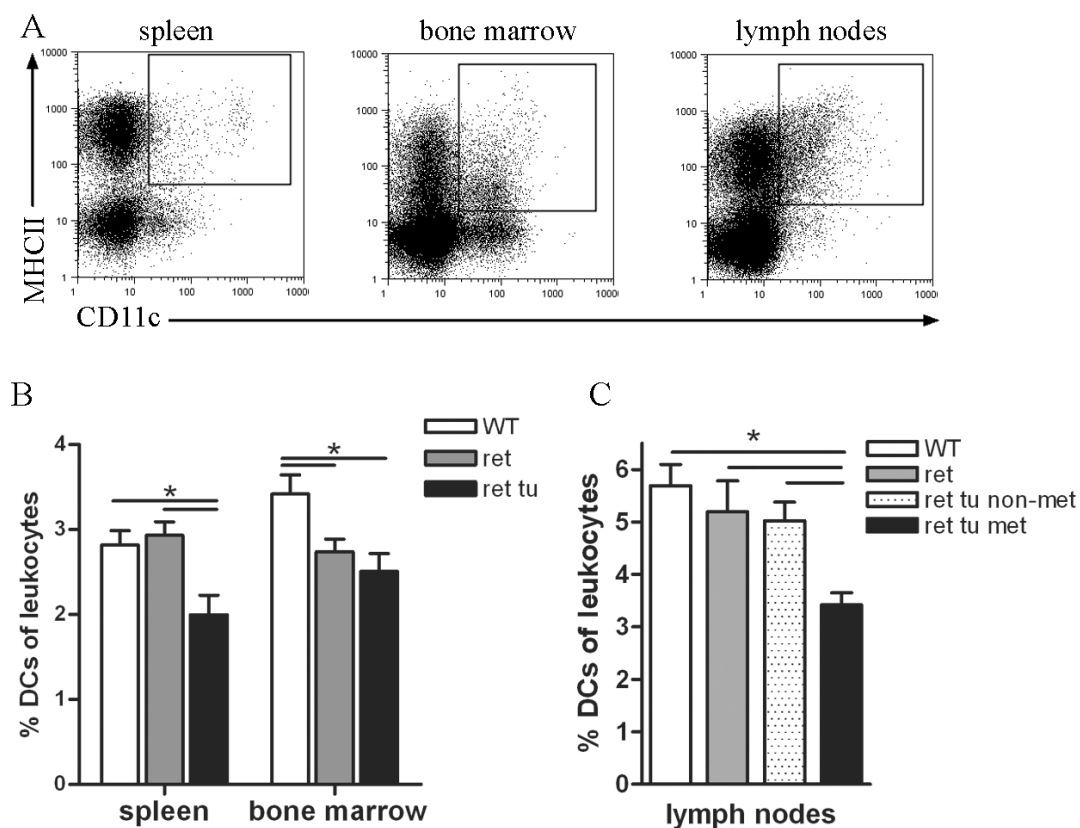


Figure 14.1. Analysis of DCs in the lymphoid organs of *ret* transgenic mice. Cells from *ret* transgenic mice with macroscopical tumors (*ret tu*) or without them (*ret*) as well as from non-transgenic littermates (WT) were stained with mAbs for CD11c, MHC class II, and leukocyte marker CD45.2 followed by flow cytometry. (A) Dot plots are representative of tumor bearing mice. CD11c<sup>+</sup> MHC class II<sup>+</sup> cells are defined as DCs. (B) Accumulative data for DCs in spleens and BM are expressed as the percentage within leukocytes. (C) 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. Data are means  $\pm$  SEM from 5-20 mice. \*  $P < 0.05$ , significant differences between groups indicated with the lines.

Similar to the spleen and BM, there was a significant decrease in total DC numbers in lymph nodes with metastasis (*ret tu met*) as compared to lymph nodes without visible metastases from the same tumor bearing mice (*ret tu non-met*), lymph nodes from tumor free or from wild type mice (Fig. 14.1 C).

## 14.2. Analysis of DC maturation status in spleen, BM and lymph nodes of *ret* transgenic mice

Next, we investigated the numbers of mature DCs in the spleen, BM and lymph nodes. A profound reduction in numbers of DCs expressing CD40, CD80 or CD86 molecule was observed in all investigated lymphoid organs as compared to control groups ( $P < 0.05$ , Fig. 14.2 A-C).

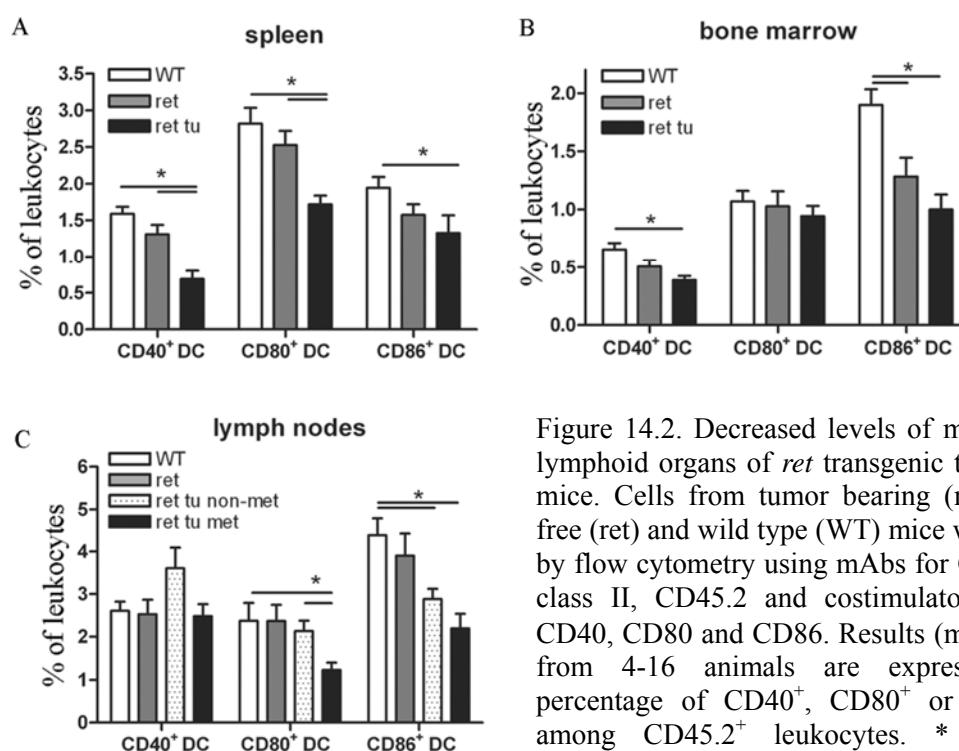


Figure 14.2. Decreased levels of mature DCs in lymphoid organs of *ret* transgenic tumor bearing mice. Cells from tumor bearing (*ret tu*), tumor free (*ret*) and wild type (WT) mice were analyzed by flow cytometry using mAbs for CD11c, MHC class II, CD45.2 and costimulatory molecules CD40, CD80 and CD86. Results (means  $\pm$  SEM) from 4-16 animals are expressed as the percentage of CD40<sup>+</sup>, CD80<sup>+</sup> or CD86<sup>+</sup> DCs among CD45.2<sup>+</sup> leukocytes. \*  $P < 0.05$ , differences between indicated groups.

These data indicated a systemic decrease in numbers of total and mature DCs in lymphatic organs from *ret* transgenic mice with macroscopical tumors. No statistical correlation between observed alterations and mouse age, tumor weight or the dynamics of tumor growth was found (data not shown).

## 15. Phenotype analysis of tumor infiltrating DCs

Furthermore, phenotype of tumor infiltrating dendritic cells (TIDCs) was investigated in primary tumors. There are two ways to get a single cell suspension of

tumor tissue: 1) to destroy tumor tissue mechanically; 2) to digest tissue with enzymes, such as collagenase and DNase. In our experiments, single cell suspension from tumor samples was prepared mechanically to exclude the possibility to damage the expression of surface markers by the enzyme treatment. TIDC numbers were examined within alive  $CD45.2^+$  cells.  $CD45.2^+$  was used as a marker of tumor infiltrating leukocytes (TILs, Fig. 15 A). Tumor progression in *ret* transgenic mice with macroscopical tumors was evaluated by the tumor weight measurement after mice have been sacrificed.

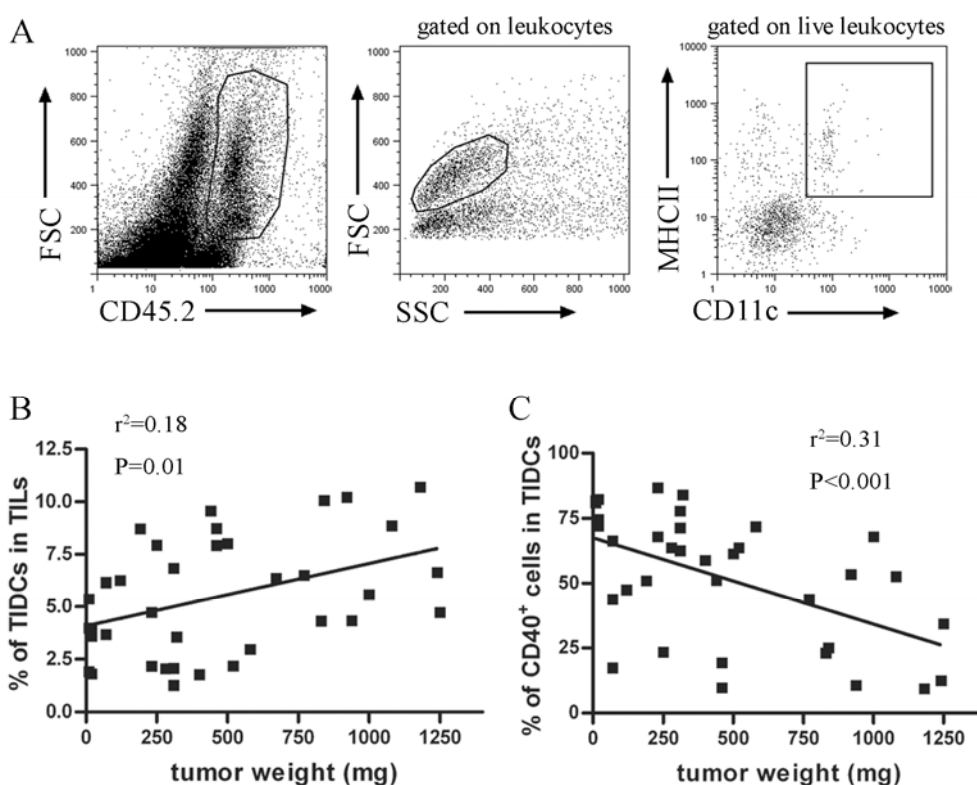


Figure 15. Tumor infiltration with DCs is dependent on tumor progression. Single cell suspensions prepared from tumors of *ret* transgenic mice were stained with mAbs for CD11c, MHC class II, CD45.2, and CD40. (A) Representative dot plots are shown. (B and C) The weight of each tumor sample are plotted against the percentage of tumor infiltrating DCs (TIDCs) within  $CD45.2^+$  tumor infiltrating leukocytes (TILs) (B) or against the percentage of  $CD40^+$  mature DCs within TIDCs (C). The correlation between the two variables was calculated using a linear regression analysis.

Elevating proportions of TIDCs among TILs were found to correlate with increasing weight of primary melanomas ( $r^2=0.18$ ;  $P < 0.05$ ; Fig. 15 B). Although more DCs infiltrated into larger tumors, obviously they did not correlate with a better

prognosis in *ret* transgenic mice. Therefore, we further investigated the relationship between the maturation status of TIDCs and tumor progression. Notably, in larger tumors, significantly higher amounts of TIDCs displayed immature phenotype according to the CD40 expression profile as compared to DCs infiltrating smaller tumors ( $r^2=0.31$ ;  $P < 0.05$ ; Fig. 15 C).

This data implied that tumor attracted DC but blocked DC maturation at a more immature stage. The outcome of such effect was an accumulation of immature DCs in tumors leading thereby to the failure in the initiation of effective anti-tumor T-cell responses.

## 16. Functional assay of dendritic cells of *ret* transgenic mice

To determine if DCs in *ret* transgenic mice with macroscopical tumors showed the functional impairment, spleen DCs were investigated for their cytokine production and the capacity to activate T cells.

### 16.1. Cytokine profile of DCs

First, cytokine profile of DCs was tested at the mRNA level. Spleen DCs were isolated using CD11c<sup>+</sup> magnetic positive selection kit and mRNA was extracted immediately after cell sorting. Expressions of TGF- $\beta$ 1 and IL-10 mRNAs in spleen DCs were shown in Fig. 16.1 A. Compared to *ret* transgenic mice without macroscopical tumors or non-transgenic littermates, DCs from *ret* transgenic mice with macroscopical tumors showed a tendency to express higher levels of TGF- $\beta$ 1 and IL-10 mRNAs.

Next, we analyzed the cytokine production profile of DCs at the protein level. Two cytokines were investigated in these experiments: the pro-inflammatory cytokine IL-12p70 and the anti-inflammatory cytokine IL-10. However, due to very low production of cytokines in the steady state *ex vivo*, we did not detect IL-12 or IL-10 producing DCs in the spleen, BM or lymph nodes by intracellular FACS staining (data not shown). Therefore, *ex vivo* isolated spleen DCs were stimulated with CpG 1668 (a TLR9 ligand) for 24 h. Supernatants were then collected and the production of

IL-12p70 and IL-10 was detected by ELISA in supernatants. 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. 16.1 B). Moreover, the amount of IL-10 produced by tumor DCs was significantly increased as compared to DCs from non-transgenic littermates ( $P < 0.05$ ; Fig. 16.1 C). In addition, the ratio between IL-12p70 and IL-10 produced by DCs from tumor bearing animals was found to be higher than by DCs isolated from wild type mice ( $P < 0.05$ ; Fig. 16.1 D).

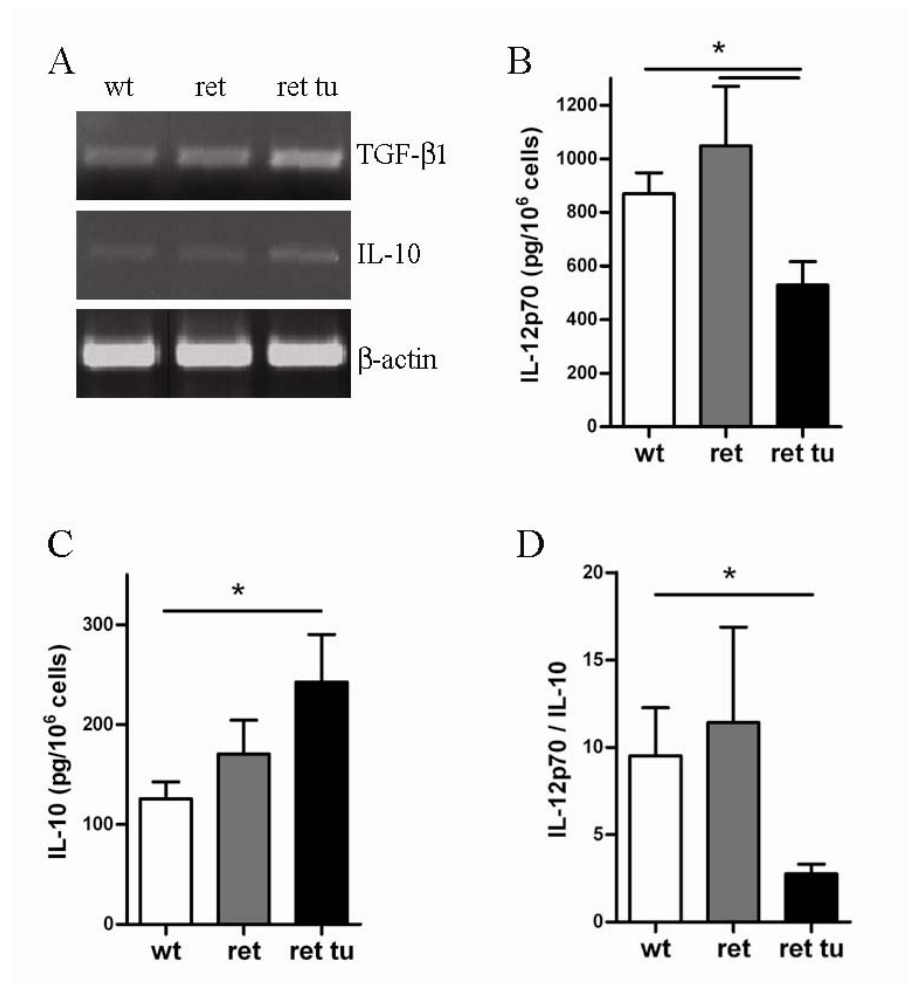


Figure 16.1. DCs from tumor bearing mice (ret tu) display tolerogenic pattern of cytokine production as compared to tumor free (ret), and non-transgenic mice (WT). (A) TGF-β1 and IL-10 mRNA in freshly isolated spleen DCs was tested by RT-PCR. Results are representative of two independent experiments. Spleen DCs were stimulated with CpG1668 for 24h *ex vivo* followed by detection of IL-12p70 (B) and IL-10 (C) in supernatants using ELISA as well as calculation of IL-12p70/IL-10 ratio (D). Data are mean  $\pm$  SEM from 10 mice per experimental group.

## 16.2. T cell stimulation capacity of DCs

To address the question whether the T cell stimulating capacity of DCs from *ret* transgenic mice with macroscopical tumors was also impaired, *ex vivo* isolated spleen DCs were loaded with ovalbumin (OVA) derived peptide, SIINFEKL, and cocultured with naive CD8<sup>+</sup> T cells isolated from spleen of OT-I mice. These T cells can be specifically activated by SIINFEKL. After 3 days of co-culture, numbers of IFN- $\gamma$  producing cells in ELISPOT assay were measured to evaluate capacity of DCs for T cell activation. As shown in Fig. 16.2 A, we detected significantly lower amounts of IFN- $\gamma$  producing T cells in the presence of DCs from transgenic mice with macroscopical tumors than in samples with DCs from wild type mice ( $P < 0.05$ ).

T-cell stimulation capacity of DCs from *ret* transgenic mice with macroscopical tumors was also investigated using an OVA-specific CD8<sup>+</sup> T-cell line, which is established from OVA-immunized C57BL/6 mice. Data in Fig. 16.2 B indicated that DCs from tumor bearing mice are less potent for T cell stimulation when compared to tumor free or non-transgenic littermates.

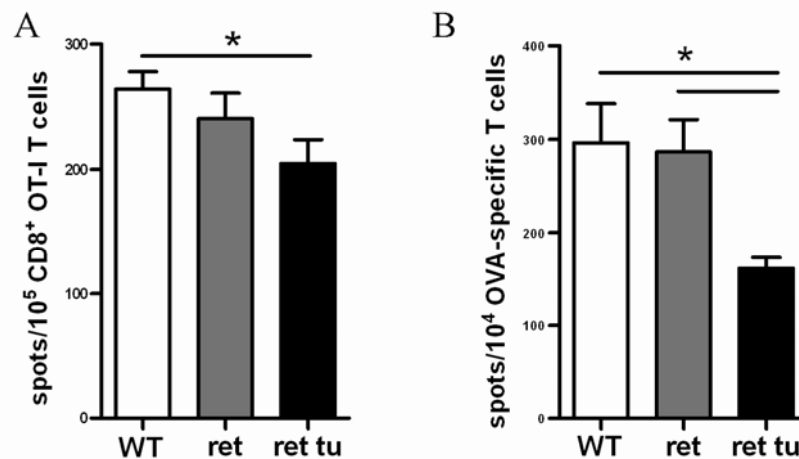


Figure 16.2. DCs from tumor bearing mice fail to promote a strong T cell stimulation. CD11c<sup>+</sup> DCs were isolated using positive selection, loaded with the peptide SIINFEKL at 37°C for 1h and co-cultured for 3 days with CD8<sup>+</sup> T cells isolated from spleens of OT-I mice (A), or co-cultured with an OVA-specific T cell line for 40h (B). T cell activation was evaluated by spot numbers in the IFN- $\gamma$  ELISPOT assay. Means  $\pm$  SEM from 4 animals per experimental group are shown. \*  $P < 0.05$ , differences between indicated groups.

Taken together, the data on phenotype analysis showed that tumor bearing mice contained reduced numbers of total DCs as well as mature DCs in the lymphoid organs. Accumulation of immature DCs was found in the tumors, suggesting that in the process of tumor progression the suppressive tumor microenvironment blocked DC development at the immature stage. Moreover, DCs from tumor bearing mice showed their functional impairment. Such DCs secreted a less IL-12 and more IL-10 after stimulation, and were less potent to activate T cells, suggesting that DCs from *ret* transgenic mice with macroscopical tumors displayed a tolerogenic pattern.

## **17. Mechanisms of tolerogenic DC generation in *ret* transgenic mice**

DCs from *ret* transgenic mice with macroscopical tumors were shown to display a tolerogenic pattern. It has been previously reported that tumor cells or tumor-derived factors could induce dysfunctional DCs in both carcinoma patients and tumor animal models (68, 70, 80, 88, 97).

Therefore, we aimed to further study 1) at which level of DC differentiation can tumor-derived factors influence DC function, 2) which tumor-derived factors may be particularly important in tolerogenic DC generation during melanoma progression in *ret* transgenic mouse model, and 3) which transcription factors could play a crucial role in the turning of DCs to acquire tolerogenic functions during melanoma progression in *ret* transgenic mouse model.

### **17.1. Generation of tolerogenic DCs from BM precursors of *ret* transgenic mice *in vitro***

DCs were generated from precursor cells derived from the BM of *ret* transgenic mice with macroscopical tumors in the presence of recombinant mouse IL-4 and GM-CSF *in vitro*. As shown in Fig. 17.1.1, DCs generated from precursor cells derived from *ret* transgenic mice with macroscopical tumors showed significantly lower expression of MHC class II molecule as compared to non-transgenic littermates, although the total number of generated CD11c<sup>+</sup> cells was not changed. This indicated that the tumor microenvironment *in vivo* changed the ability of precursor cells to differentiate into mature DCs.

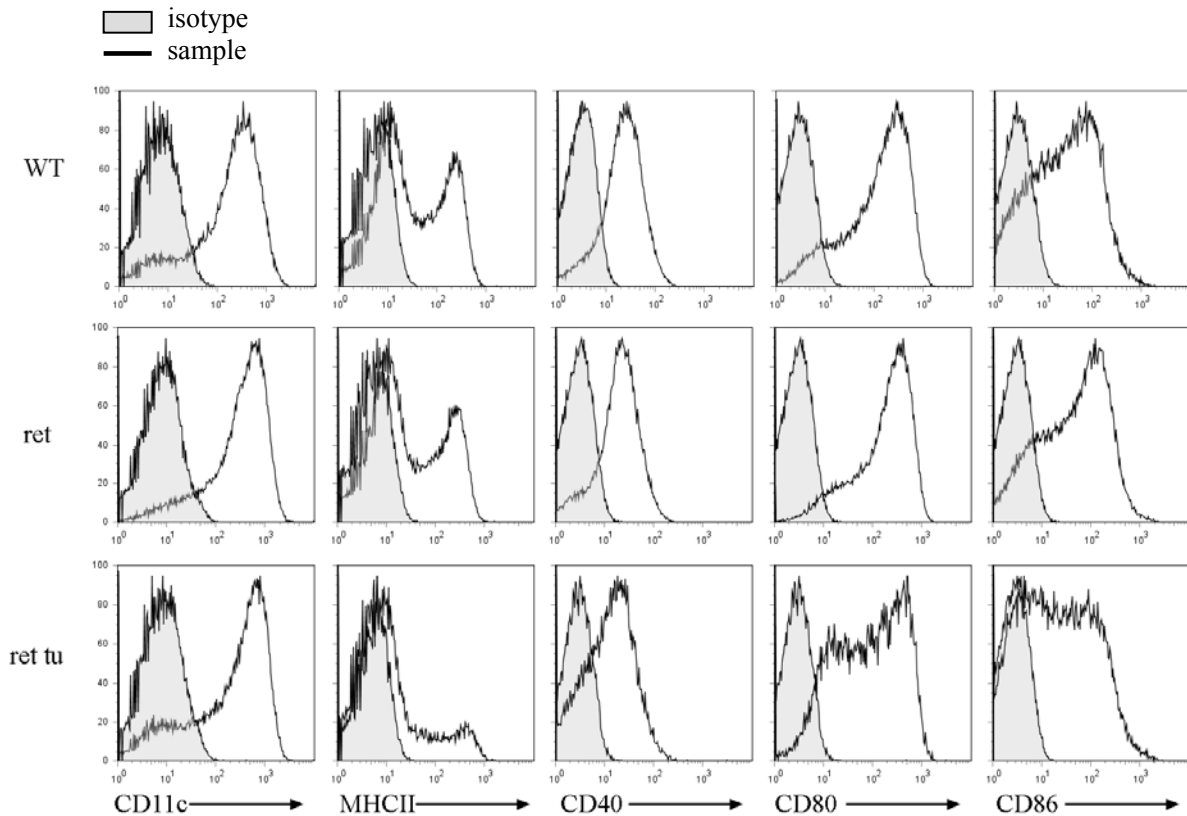


Figure 17.1.1. Phenotype analysis of DCs generated from precursors derived from bone marrow of *ret* transgenic mice with macroscopical tumors (*ret tu*). Immature DCs were stimulated 1 $\mu$ g/ml LPS for maturation. After 24h, phenotypes of mature DCs were tested by antibodies against MHC classII, CD40, CD80, and CD86. CD11c is used as DC marker. Data are representative for five experiments.

Furthermore, we observed a stronger expression of IL-10 and TGF- $\beta$ 1 mRNA after LPS stimulation in DCs generated from BM precursors from *ret* transgenic mice with macroscopical tumors than from non-transgenic littermates. This may not be due to the different expression level of LPS receptor, TLR4, on DCs, because the expression of TLR4 mRNA was found to be comparable between the groups (Fig. 17.1.2A).

Testing cytokine secretion by *in vitro* generated DCs after stimulated with LPS, we also revealed a significant decreased IL-12p70 production by DCs from tumor bearing group as compared to wild type group ( $P < 0.05$ ; Fig. 17.1.2 B). In this experimental setting, IL-10 production in supernatant was under the detection level (data not shown).

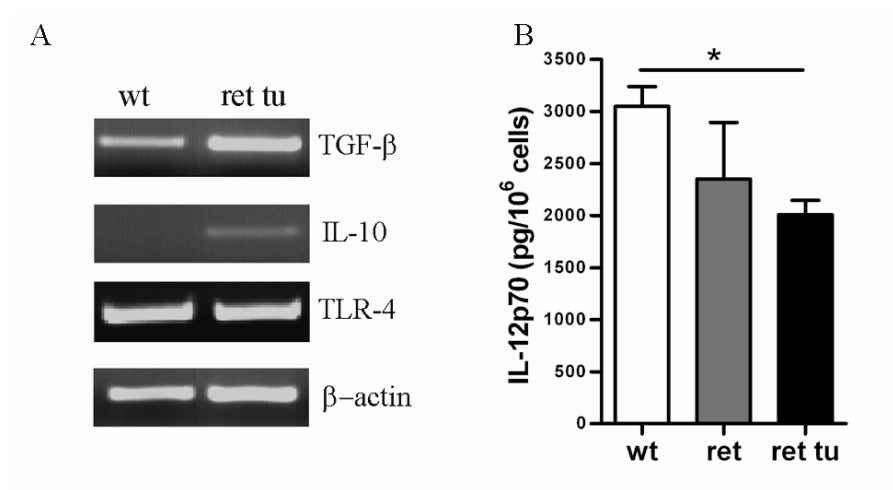


Figure 17.1.2. The profile of cytokine and growth factor expression in DCs generated from bone marrow precursors of *ret* transgenic mice with macroscopical tumors (ret tu). Generated DCs were stimulated 1  $\mu$ g/ml LPS for 24h. (A) mRNA was extracted from DCs and expression of IL-10 and TGF- $\beta$ 1 were examined by RT-PCR. Data are representative for five experiments. (B) Supernatants were collected and IL-12p70 expression level was tested by ELISA. Data are expressed as means  $\pm$  SEM from four experiments (\*,  $P < 0.05$ ).

Our data indicated that tumors suppressed the ability of BM precursor cells to differentiate into mature DCs, which led to the generation of DCs with more immature phenotype and suppressed IL-12 production *in vitro*.

## 17.2. Production of immunosuppressive cytokines and growth factors in tumor bearing mice *in vivo* and by Ret melanoma cells *in vitro*

To investigate which tumor-derived factors could be involved in the development of DCs with a tolerogenic profile, we first examined the expression of some cytokines and growth factors like IL-6, IL-10, VEGF, and TGF- $\beta$ 1 by RT-PCR at the mRNA level in the melanoma cell line, which was established from primary skin melanomas in *ret* transgenic mice (Ret melanoma cells), and in B16F10 melanoma cells used as a control. As shown in Fig. 17.2 A, IL-6, VEGF and TGF- $\beta$ 1 mRNAs were detected in both cell lines, whereas the IL-10 mRNA expression was not detectable (data not shown). Considerable amounts of VEGF and TGF- $\beta$ 1 proteins were demonstrated in supernatants from cultured Ret melanoma cells by the single-plex technology (data not shown).

Next we analyzed the expression of all four above mentioned factors in primary tumors freshly isolated from *ret* transgenic mice both at mRNA and protein levels. Similar to above mentioned cell lines, primary cutaneous melanomas expressed IL-6, VEGF, and TGF- $\beta$ 1 mRNAs; however IL-10 mRNA was not found (Fig. 17.2 A). At the protein level, we observed IL-6, VEGF and TGF- $\beta$ 1 production in primary tumors using multiplex and single-plex technology. Notably, level of VEGF in primary tumors were found to correlate with the tumor weight ( $r^2=0.54$ ;  $P < 0.05$ ; Fig. 17.2 B). Moreover, concentrations of IL-6 and VEGF were significantly elevated in the serum of *ret* transgenic mice with macroscopical tumors as compared to non-transgenic littermates ( $P < 0.05$ ; Fig. 17.2 C).

To investigate a direct effect of these tumor-derived factors on DCs, supernatants from cultured Ret melanoma cell (Ret conditioned medium, Ret-CM) was mixed with the culture medium (50%, v/v) and used for the DC generation from the BM precursors of wild type mice. As shown in Fig. 17.2 D, DCs generated under these conditions secreted significantly lower amounts of IL-12p70 ( $P < 0.05$ ) upon LPS stimulation than DCs generated in the presence of normal DC medium. Furthermore, after adding neutralizing antibodies for IL-6 or VEGF or TGF- $\beta$ 1 to the DC medium supplemented with Ret-CM, we found a significant increase in IL-12p70 production by LPS-stimulated DCs as compared to DCs generated in the absence of these antibodies ( $P < 0.05$ ; Fig. 17.2 D).

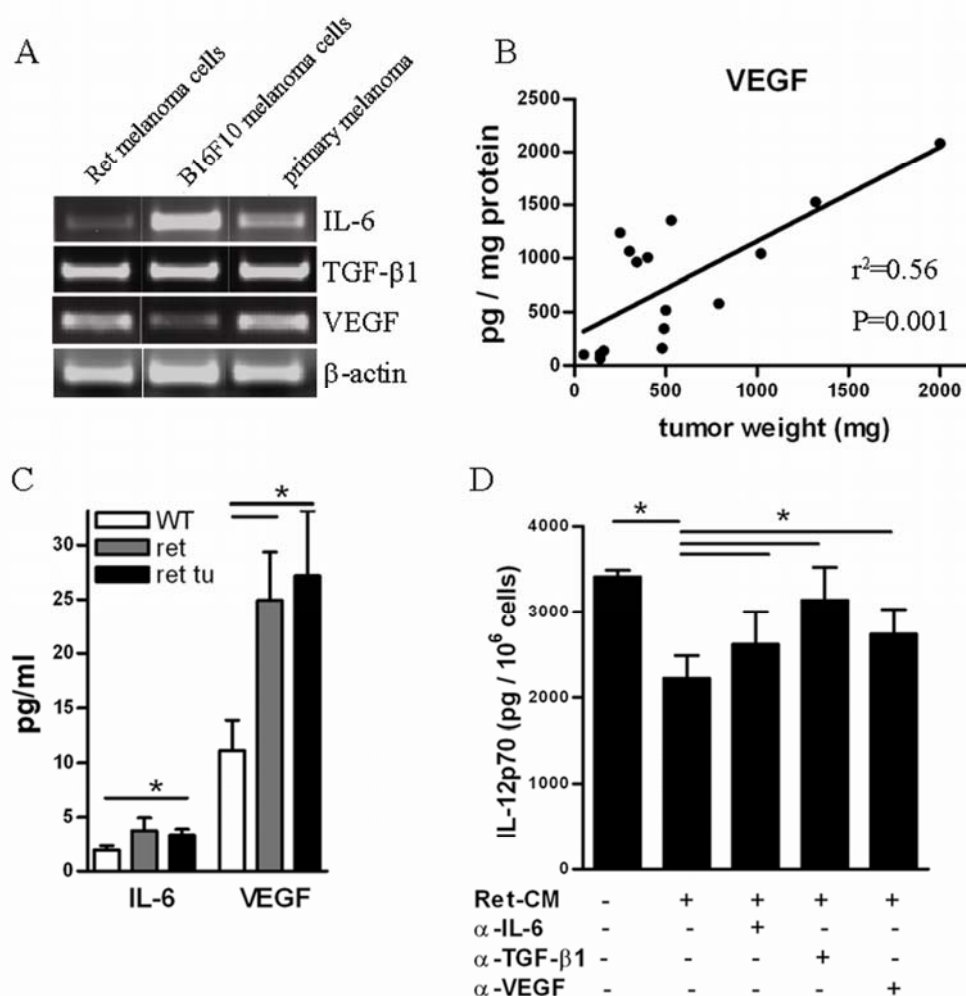
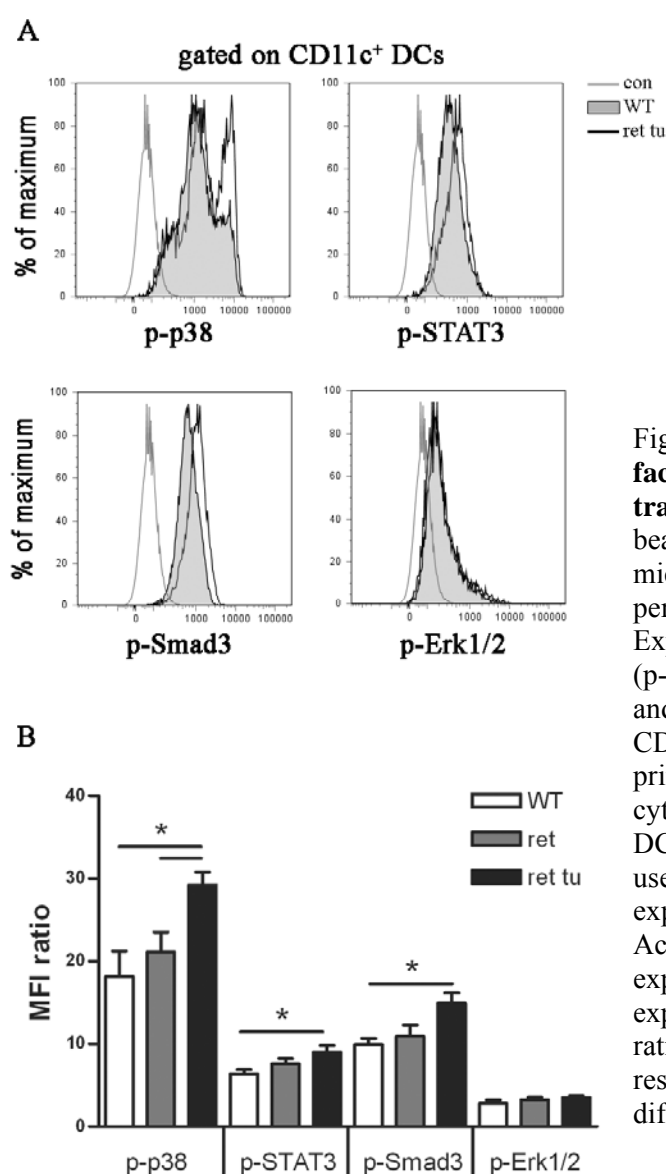


Figure 17.2. Tumor-derived cytokines and growth factors promote downregulation of IL-12 production by DCs. (A) Total RNA was extracted from primary tumors, Ret melanoma cells and B16F10 melanoma cells. IL-6, TGF-β1 and VEGF expression was determined via RT-PCR using corresponding specific primers. Results from one representative experiment of three are shown. (B-D) Concentrations of IL-6 and VEGF at the protein level in tumor lysates and murine serum were measured using multiplex technology. Values in tumor lysates (B and C) expressed as pg/mg protein are plotted against the weight of each tumor sample. The correlation between the two variables was calculated using a linear regression analysis. Data in serum (D) are expressed as pg/ml and means  $\pm$  SEM from 6-12 mice per group are depicted. \*  $P < 0.05$ , differences between indicated groups. (E) DCs were generated from BM precursors of non-transgenic mice in the presence of supernatants from cultured Ret melanoma cells (Ret conditioned medium, Ret-CM; 50%, v/v) and of neutralizing antibodies for IL-6 (α-IL-6) or VEGF (α-VEGF) or TGF-β1 (α-TGF-β1) followed by LPS stimulation for 24h. IL-12p70 levels were tested in supernatants via ELISA. Results (means  $\pm$  SEM) of 4 independent experiments are expressed as pg/ $10^6$  cells. \*  $P < 0.05$ , differences between indicated groups.

### 17.3. p38 MAPK is strongly activated in DCs of *ret* transgenic mice with macroscopical tumors

Having demonstrated a critical importance of IL-6, VEGF and TGF- $\beta$ 1 for the acquirement of tolerogenic properties by DCs from tumor bearing mice, we then addressed the question which transcription factors regulating functions of these factors are responsible for the observed impairments. Phosphorylation levels of p38 MAPK, STAT3, Smad3 and Erk1/2 MAPK were examined in freshly isolated spleen DCs by flow cytometry (Fig. 17.3 A). To prevent any artificial effect introduced during preparation, spleen cells were immediately fixed in PFA and permeabilized in ice-cold methanol after single cell suspension was made.



**Figure 17.3. Expression of transcription factors in DCs from melanoma bearing transgenic mice.** Spleen cells from tumor bearing (*ret tu*), tumor free (*ret*) or wild type mice (WT) 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<sup>+</sup> DCs after staining with respective primary and secondary mAbs using flow cytometry. As a negative control (con), CD11c<sup>+</sup> DCs stained only with secondary mAbs were used. Results from one representative experiment of three are shown. (B) Accumulative data for the transcription factor expression in spleen DCs (mean  $\pm$  SEM) are expressed as mean fluorescence intensity (MFI) ratio (MFI of experimental samples/MFI of respective negative controls). \*, P < 0.05, differences between indicated groups.

We found that DCs from mice with macroscopical tumors displayed a significant upregulation in the expression of phosphorylated p38 MAPK, STAT3 and Smad3 as compared to those in DCs from wild type mice ( $P < 0.05$ ), whereas the level of phosphorylated Erk1/2 MAPK remained unchanged (Fig. 17.3 A, B). Notably, p38 MAPK activity shows the highest upregulation among studied factors. Therefore, we suspected that p38 MAPK might be a key regulator of the DC dysfunction in *ret* transgenic mice with macroscopical tumors.

#### **17.4. Inhibition of p38 MAPK activity normalized functions of DCs from tumor bearing *ret* transgenic mice**

We next assessed whether the inhibition of p38 MAPK activity could reverse the altered pattern of cytokine production shown by DCs from *ret* transgenic mice with macroscopical tumors. *Ex vivo* isolated spleen DCs were incubated in the presence of the p38 MAPK inhibitor SB203580 followed by stimulation with CpG. Suppression of the p38 MAPK activity was found to lead to the profound decrease of IL-10 production ( $P < 0.05$ ) as compared to untreated cells (Fig. 17.4 B). Furthermore, the IL-12/IL-10 ratio was also markedly increased ( $P < 0.01$ ; Fig. 17.4 C). Thus, the cytokine secretion profile observed after treatment of DCs from tumor bearing mice with the p38 MAPK inhibitor was comparable to that for DCs from non-transgenic littermates.

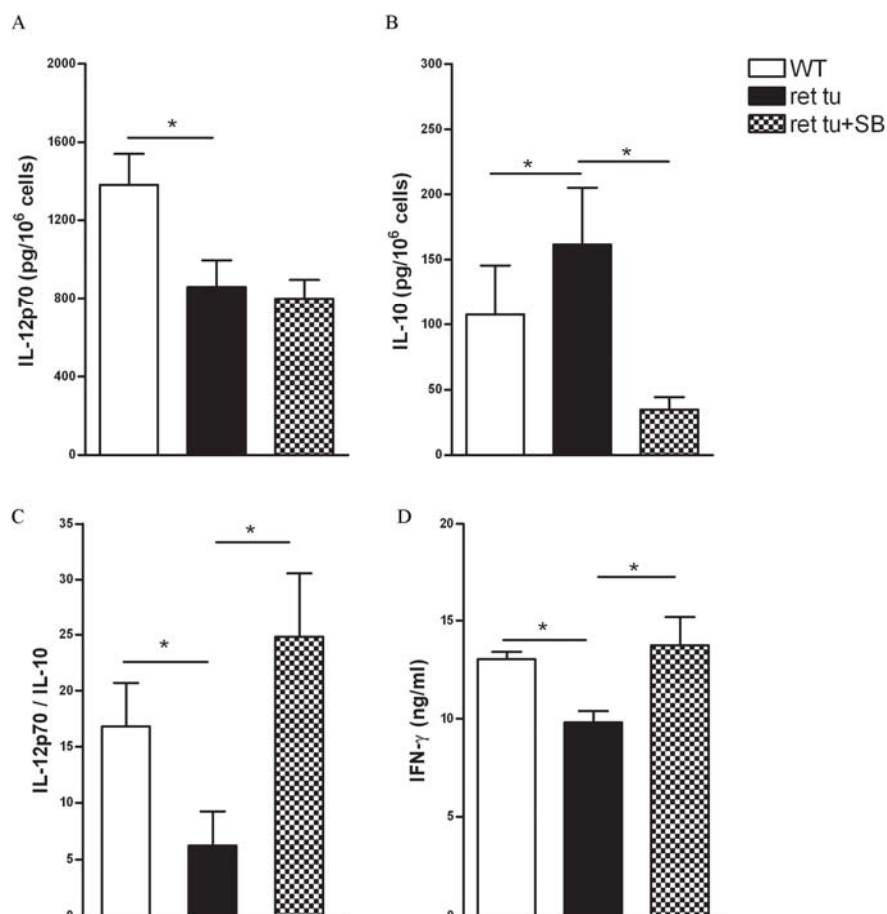


Figure 17.4. Inhibition of p38 MAPK in DCs from tumor bearing mice restores their pattern of cytokine production and ability to stimulate T cells. (A-C) Spleen CD11c<sup>+</sup> DCs were *ex vivo* isolated from tumor bearing (ret tu) or wild type mice (WT) using MicroBeads isolation kit, incubated with or without an inhibitor of p38 MAPK SB203850 (10  $\mu$ M) for 1h and stimulated with CpG1668 for another 24h followed by measurement of IL-12p70 (A) and IL-10 (B) in supernatants using ELISA as well as calculation of IL-12p70/IL-10 ratio (C). Data are mean  $\pm$  SEM from five independent experiments. \*P < 0.05, differences between indicated groups. (D) *Ex vivo* isolated spleen DCs were incubated with or without SB203580 during the loading with the OVA-peptide SIINFEKL for 1h. After extensive washing, DCs were co-cultured with CD8<sup>+</sup> T cells isolated from OT-I mice for 3 days followed by measurement of IFN- $\gamma$  levels in supernatants by ELISA. Means  $\pm$  SEM from three independent experiments are depicted. \*P < 0.05, differences between indicated groups.

We further examined if the p38 MAPK inhibitor could restore impaired capacity of DCs from *ret* transgenic mice with macroscopical tumors to stimulate T cells. Freshly isolated spleen DCs were loaded with the peptide SIINFEKL in the presence of SB203580. After washing out the inhibitor, tumor DCs were co-cultured for 3 days with CD8<sup>+</sup> T cells isolated from spleens of OT-I mice followed by the measurement of IFN- $\gamma$  in the supernatant by ELISA. As shown in Fig. 17.4 D, DC pretreatment with

the p38 MAPK inhibitor was found to reconstitute IFN- $\gamma$  production by T cells to the level detected after stimulation with normal DCs. Therefore, inhibition of p38 MAPK in DCs from tumor bearing mice resulted in the normalization of IL-12 and IL-10 production as well as in the restoration of DC ability to stimulate OT-I CD8<sup>+</sup> T cells.

In conclusion, our findings provide evidence that DC dysfunction in *ret* transgenic spontaneous melanoma mouse model can be induced by tumor-derived factors such as VEGF, IL-6 and TGF- $\beta$ . Constitutive activation of p38 MAPK by these factors may result in a tolerogenic cytokine profile of DCs with low IL-12 and high IL-10 secretion levels, which led to the impairment of antitumor T-cell mediated responses. Suppression of the p38 MAPK activity in DCs from tumor bearing mice can reconstitute their impaired cytokine pattern and capability to stimulate T cells suggesting a crucial role of p38 MAPK activity in the generation of tolerogenic DCs in *ret* transgenic tumor bearing mice.

## IV. Discussion

### 18. Reduction of DCs in lymphoid organs and accumulation of immature DCs in primary tumors of *ret* transgenic mice

A significant decrease in total DC numbers was observed in the spleen and bone marrow of *ret* transgenic mice during tumor progression as compared to wild type mice or tumor free *ret* transgenic mice. The earliest report demonstrating a decline in Langerhans cells (skin DCs) during human melanoma progression is given by Stene et al. in 1988 (198). Later on, numerous publications showed that the number of DCs could be dramatically reduced in the periphery blood of patients with different types of tumors, such as lung cancer, squamous cell carcinoma of the head and neck, myeloma, invasive breast cancer, leukemia and hepatocellular carcinoma (40, 41, 43, 46, 199, 200). Moreover, the presence of metastases resulted in a more profound decrease in numbers of circulated peripheral blood DCs in cancer patients (46, 47). In agreement with these reports, we demonstrated a significant decrease in DC amounts in metastatic lymph nodes from *ret* transgenic tumor bearing mice as compared to those in non-metastatic lymph nodes from the same mice or animals of control groups.

A decrease of DC numbers in cancer patients can be induced by several mechanisms. One of them is the induction of apoptosis in DCs and their precursors. *In vitro* and *in vivo* studies demonstrate that DCs undergo apoptosis after interacting with cancer cells or soluble tumor-derived factors (49-52). Tumor induced DC apoptosis has been reported to be mediated by cytochrome c release, which further leads to cytoplasm shrinking, caspase-3 activation, upregulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic proteins Bcl-x<sub>L</sub> and Bcl-2 (49-51). Furthermore, growth of RM-1 prostate cancer cells could be significantly inhibited in mice treated intratumorally with DCs which were transduced with murine Bcl-x<sub>L</sub> gene (50). Other anti-apoptotic molecules are also shown to be able to inhibit tumor-mediated DC apoptosis, such as Fas associated death domain (FADD)-like ICE inhibitory proteins (FLIP) which blocks binding of procaspase-8 to FADD (52). In addition to the

cell-cell contact mechanism, induction of DC apoptosis can be induced by some tumor-derived factors, like IL-10, nitric oxide (NO), TGF- $\beta$ , and gangliosides. For example, tumor-derived TGF- $\beta$ 1 can induce apoptosis of DCs in sentinel lymph nodes without evidence of metastasis in patients with non-small cell lung carcinoma and thereby facilitate metastasis within those lymph nodes (201, 202). In our experiments with co-incubation of normal spleen DCs with Ret melanoma cell conditioned medium from these cells, we were not able to demonstrate a substantial apoptosis rate in DCs (data not shown).

Decreased numbers of total DCs could also be attributed to the inhibition of their generation from precursors. Impairment of differentiation from hematopoietic progenitor cells can be induced by tumor-derived factors. It has been reported that growth factors and cytokines like VEGF, M-CSF, TGF- $\beta$ , IL-6 and IL-10 produced by human and mouse tumor cells could suppress maturation of DCs from CD34<sup>+</sup> precursors *in vitro* (66, 67, 75, 85, 95). In particular, tumor-derived VEGF affected early stage of DC maturation in the bone marrow *in vivo* (85). IL-10, IL-6 and TGF- $\beta$  profoundly affected phenotype and function of DCs in patients with advanced pancreatic carcinoma (203).

Therefore, DC differentiation could be blocked 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 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 TIDCs with the immature phenotype. Blocking of normal DC differentiation and maturation may lead to the accumulation of cells with properties of myeloid derived suppressor cells (MDSCs). This heterogeneous population of myeloid cells consisting of monocytes/macrophages, granulocytes and DCs at different stages of differentiation and expressing various surface markers has been recently found to induce a dramatic suppression of T cell functions in mouse tumor models and in cancer patients (204-207). However, it still remains to be determined if the enrichment of MDSCs can directly lead to the loss of DCs in lymphoid organs of *ret* transgenic tumor bearing mice.

Another explanation of decreased DC numbers in lymphoid organs could be their

migration to tumors. After antigen uptake, immature DCs are activated and gain the mature phenotype and function. In addition to the DC elimination by apoptosis, tumor cells utilize other mechanisms to escape from DC-mediated immunosurveillance. For example, tumor-derived VEGF and  $\beta$ -defensins recruited immature DCs from BM to the tumor microenvironment and transformed them into endothelial-like cells that engaged in vasculogenesis and functioned as promoters of tumor progression (208). TGF- $\beta$  and IL-10 secreted by tumor-derived CD4<sup>+</sup> CD25<sup>+</sup> Tregs could block the maturation of tumor infiltrating DCs by markedly down-regulating their costimulatory molecule expressions (113). In *ret* transgenic mice with macroscopical tumors, it was found using flow cytometry that the percentage of TIDCs showed a positive linear correlation with the tumor weight ( $r^2=0.18$ ,  $P < 0.05$ ), indicating an accumulation of DCs in tumors during melanoma progression. However, in agreement with the above mentioned publications TIDC maturation was blocked at the immature stage, because the percentage of CD40 expressing DCs in TIDCs decreased with the elevation of tumor weight ( $r^2=0.31$ ,  $P < 0.05$ ). Further immunohistochemistry study needs to be done to investigate the localization of TIDCs and their interaction with other host cells.

In *ret* transgenic mice with macroscopical tumors, numbers of DCs in metastatic lymph nodes decreased significantly compared to non-metastatic ones. One explanation of these findings is that tumor microenvironment in metastatic lymph nodes induces DC apoptosis *in situ*. Another reason could be the link to the disability of DC homing to lymph nodes. After taking up tumor associated antigen from tumor regions, homing of mature DCs to tumor draining lymph nodes depends on the surface expression of certain chemokines and chemokine receptors (209). Therefore, further work needs to be done to investigate whether chemokine receptors, such as CCR7, are differently expressed on DCs from metastatic lymph nodes as compared to non-metastatic lymph nodes from *ret* transgenic mice with macroscopical tumors.

## 19. Tolerogenic cytokine profile and impaired T cell stimulation

DCs from *ret* transgenic mice with macroscopic tumors were found to be impaired not only quantitatively but also qualitatively. When stimulated *ex vivo*, DCs from *ret*

transgenic mice with macroscopic tumors showed higher expression of IL-10 and lower expression of IL-12p70. IL-12 has been described during last years to be a critical cytokine for T-cell stimulation and to promote DC maturation and survival (210). However, production of IL-12 by DCs could be downregulated by different tumor-derived factors. Bombesin-like peptide (BLP) secreted by human lung cancer cells downregulated IL-12 production by DCs (211). Gangliosides produced by various human tumor cells suppressed DC maturation and function by reducing IL-12 secretion (212-214). Moreover, tumor-derived IL-10, IL-6 and TGF- $\beta$  profoundly affected the function of DCs in patients with advanced pancreatic carcinoma in favor of DCs showing immature phenotype and impaired IL-12 production (203).

Function of DC precursors in *ret* transgenic mice with macroscopical tumors has also been affected by tumor cells or tumor-derived factors. Even being incubated in the medium for DC generation *in vitro*, these cells differentiated into DCs which expressed much lower levels of MHC class II molecule and produced significantly less IL-12 after LPS stimulation as compared to non-transgenic littermates. Similar data has also been shown in multiple myeloma patients (60).

Consistent with the data from cancer patients (48, 201, 211), DCs from *ret* transgenic mice with macroscopic tumors showed impaired capacity to stimulate naïve CD8<sup>+</sup> cells from OT-I mice. Mechanisms of the suppressed T-cell activation by DCs from *ret* tumor bearing mice are still unclear and need to be further investigated. Zou et al. (143) reported that some DC subsets suppressed T-cell function by delivering inhibitory signals through binding of B7-H1, B7-H2 or B7-H3 molecules to their receptors on T cells. In addition, several research groups showed that the IDO<sup>+</sup> DCs could suppress T-cell activation. For example, mouse PDCs downregulated T-cell responses through the upregulation of IDO (141). Moreover, Baban et al. (142) characterized the IDO<sup>+</sup> CD19<sup>+</sup> DCs suppressed T-cell function following CD80/CD86 ligation by CTLA4 or TLR9 ligation.

## 20. Activation of p38 MAPK drives DCs to display tolerogenic pattern during melanoma development

Having established that DCs in *ret* transgenic mice with macroscopic tumors were functionally tolerogenic, we focused on studying the mechanisms of the generation of tolerogenic DCs in *ret* transgenic mice. Using a multiplex technology, we found that primary tumors from *ret* transgenic mice produced several immunosuppressive cytokines and growth factors like IL-6, IL-10, VEGF, and TGF- $\beta$ 1 and all of them were detectable in cell lysate of tumor tissues. Moreover, level of VEGF in primary tumors showed a positive linear correlation with the tumor weight ( $r^2=0.56$ ,  $P < 0.05$ ). In addition, VEGF and IL-6 were found to be expressed at the mRNA level both in primary tumors isolated from *ret* transgenic mice and Ret melanoma cells, and both factors were detected in the culture medium of Ret melanoma cells. We observed also significantly upregulated serum levels of IL-6 and VEGF in transgenic tumor bearing mice. von Felbert et al. showed that IL-6 knockout in *ret* transgenic mice induced decrease of melanoma incidence as well as tumor sizes, indicating the role of IL-6 in tumor progression in *ret* transgenic mouse model (197). In our experiments, the application of neutralizing antibodies against IL-6, VEGF or TGF- $\beta$ 1 significantly reconstituted IL-12 production by DCs generated under Ret melanoma cell conditioned medium.

Although a 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 their signaling pathways. Therefore, we investigated some key transcription factors involved in IL-6, VEGF and TGF- $\beta$  signaling pathways in DCs. Compared to non-transgenic littermates or *ret* transgenic mice without macroscopic tumors, DCs from tumor bearing mice showed significantly higher levels of phosphorylated-p38 MAPK, STAT3 and Smad3. In agreement with our data, Wang et al. showed that tumor-derived VEGF and IL-10 inhibited DC maturation through activation of STAT3 in DCs (116). Moreover, inhibiting STAT3 signaling in DC progenitors has been shown to reduce accumulation of immature DCs by tumor-derived factors *in vitro* (117). Finally, ablation of STAT3 in hematopoietic cells enhances DC maturation in tumor-bearing mice and results in a multicomponent antitumor immunity (118). Erk1/2 MAPK has also been shown to play an important role in the upregulation of

IL-10 and the suppression of IL-12 production by DCs (124, 125). In addition, inhibition of Erk function restored the impaired function of DCs (125, 126). However, in DCs from *ret* transgenic mice, the expression of phosphorylated Erk1/2 MAPK were not changed significantly, suggesting the involvement of mechanisms other than Erk1/2 activation in the tolerogenic function of DCs in this mouse model.

Constitutively activated p38 MAPK contributed to the high IL-10 production of *ex vivo* activated DCs from *ret* transgenic mice with macroscopical tumors, which resulted in impaired T-cell responses. Inhibition of p38 MAPK activity by its inhibitor in DCs from *ret* transgenic mice with macroscopical tumors significantly down-regulated IL-10 expression and normalized the imbalanced IL-12/IL-10 ratio. Furthermore, p38 MAPK inactivation in DCs from *ret* transgenic mice with macroscopical tumors up-regulated IFN- $\gamma$  production by primed OT-I T cells. A role of p38 MAPK in DCs during tumor progression has been controversially discussed in recent publications. Escors et al. (215) demonstrated that constitutive activation of p38 MAPK in mouse DCs resulted in their maturation and in the stimulation of T-cell responses against clinically relevant tumor antigens. On the other side, in agreement with the findings reported here, Wang et al. (60) found that the phenotype and T-cell stimulatory capacity of 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 enhanced their ability to produce IL-12, attenuated regulatory T cell induction and stimulated the antitumor therapeutic efficacy of DCs pulsed with tumor-specific antigens (134).

Application of p38 MAPK inhibitors for melanoma immunotherapy *in vivo* will require a thorough examination of their effects on host T-cell anti-tumor reactions, since the role of p38 MAPK in these cells is not completely clear. While the p38 activation was shown to be important for T-cell development and effector functions (), other publications demonstrated that its activity was a prerequisite for the induction of Treg suppressor functions (217) or for apoptotic CD8<sup>+</sup> T-cell death (218). Anti-tumor effects of the p38 MAPK inhibitor in our melanoma murine model *in vivo* are currently under investigation.

## 21. Summary

In the present work, we investigated the mechanisms of DC dysfunction during spontaneous melanoma development in *ret* transgenic mice. Animals with macroscopical tumors showed significantly reduced numbers of total DCs and mature DCs in the lymphoid organs, which may lead to insufficient stimulation of anti-tumor T-cell responses. An accumulation of immature DCs in tumors suggests that tumor microenvironment could block DC maturation and their migration to draining lymph nodes. Not only the phenotype but also DC functions were affected during tumor progression. DCs from tumor bearing mice displayed a tolerogenic cytokine profile (higher level of IL-12 and lower level of IL-10) and showed the impaired capacity of T-cell stimulation. Moreover, function of DC precursors in BM of tumor bearing mice was affected by tumors, and DCs generated *in vitro* from these precursors showed a tolerogenic pattern.

Changes of DC functions in tumor bearing mice could be induced by tumor-derived factors such as IL-6, VEGF and TGF- $\beta$  through activation of p38 MAPK. Suppression of p38 MAPK activity by a specific inhibitor could down-regulate IL-10 production and reconstitute T-cell stimulation capacity of DCs from *ret* transgenic mice with macroscopical tumors.

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

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## **VI. Abbreviations:**

### **A**

APC     antigen-presenting cell

### **B**

BSA     bovine serum albumin

bp       base pair

### **C**

CCL     chemokine ligand

CCR     chemokine receptor

CD       cluster of differentiation

cm       centimeter

Con A   Concanavalin A

CTL      cytotoxic T lymphocyte

°C       degree Celsius

### **D**

DC       dendritic cell

DNA      deoxyribonucleic acid

### **E**

EDTA    ethylenediaminetetraacetic acid

ELISA    enzyme-linked immunosorbent assay

ELISPOT enzyme-linked immunosorbent spot

### **F**

FACS     fluorescent activated cell sorting

FCS       fetal calf serum

FITC      fluorescein-5-isothiocyanate

FSC       forward scatter

### **G**

g           gramm

g           acceleration due to gravity,  
g = 9,81m/s<sup>2</sup>

GM-CSF   granulocyte/macrophage colony stimulating factor

### **H**

h           hour

HRP       horseradish peroxidase

H<sub>2</sub>O       water

### **I**

IDO       indoleamine 2,3-dioxygenase

IFA       incomplete Freund's adjuvant

IFN       interferon

Ig          immunoglobulin

IL        interleukin

**L**

l        liter

**M**

MAA    melanoma-associated antigen  
MDCs   myeloid dendritic cells  
MHC    major histocompatibility complex  
min     minute  
M       molar (mol/l)  
mM     millimolar  
ml      milliliter  
mg      milligramm  
mm     millimeter  
μl      microliter  
μg      microgramm  
mRNA   messenger RNA

**N**

NK cells    natural killer cells  
nm        nanometer  
N        number

**O**

Ova        ovalbumin

**P**

%        procent  
PBS      Phosphate Buffered Saline  
PCR      Polymerase chain reaction  
PE        Phycoerythrin  
Pen/Strep    Penicillin/Streptomycin  
PDCs    plasmacytoid dendritic cells  
pg        pikogramm

**R**

RNA      ribonucleic acid  
rpm      rounds per minutes  
RT        room temperature

**S**

SSC      side scatter

**T**

TCR      T-cell receptor  
TGF      transforming growth factor  
TNF      tumor necrosis factor  
Treg      regulatory T cell  
TRP      tyrosinase related protein

**V**

VEGF    vascular endothelial growth factor

V        volt

v/v      volume per volume

**W**

WT      wild type

w/v      weight per volume

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