

# New strategies for melanoma immunotherapy

## How to overcome immunosuppression in the tumor microenvironment

Viktor Umansky

Skin Cancer Unit; German Cancer Research Center; and, University Hospital Mannheim; Heidelberg, Germany

**Keywords:** melanoma, immunotherapy, tumor microenvironment, chronic inflammation, immunosuppressive cells

**Abbreviations:** MAA, melanoma-associated antigens; BM, bone marrow; LN, lymph nodes; TRP, tyrosinase related protein; DCs, dendritic cells; MDSCs, myeloid-derived suppressor cells; Tregs, regulatory T cells; IL, interleukin; NO, nitric oxide

Using *ret* transgenic mouse model of spontaneous melanoma, we showed an accumulation of melanoma antigen-specific memory T cells. However, their antitumor effects could be blocked by myeloid-derived suppressor cells, tolerogenic dendritic cells and regulatory T cells. We suggest that effective melanoma immunotherapy should include the neutralization of immunosuppressive tumor microenvironment.

Malignant melanoma is characterized by an aggressive clinical behavior, proclivity for distant metastasis and poor response to currently applied therapies.<sup>1</sup> Melanoma immunogenicity is well-documented including an identification of large numbers of melanoma-associated antigens (MAA) and development of spontaneous tumor regressions in some patients providing direct evidence for the induction of antitumor immunity, in which T cells play a key role.<sup>2</sup> Therefore, MAA-specific memory T cells may be an ideal source for generating effector cells since they react to tumor antigens faster and stronger than naïve T cells. Tumor-specific memory T cells were reported to be accumulated in the bone marrow (BM) of cancer patients and to exert a strong antitumor reactivity in vitro and in vivo after specific re-stimulation in vitro.<sup>3</sup>

Development of new melanoma immunotherapies requires new animal models that resemble human melanoma. Conventional mouse melanoma models (e.g., B16) are based on the tumor cell transplantation, in which the natural history of the disease is not comparable with the clinical situation. In contrast, the

*ret* transgenic mouse melanoma model closely resembles human disease as to tumor genetics, histopathology, and clinical development.<sup>4,5</sup> Mice expressing the human *ret* transgene in melanocytes develop spontaneously malignant skin melanoma with metastases in lymph nodes (LN), lungs, liver, BM and brain.<sup>4,5</sup> Using this model, we found that mouse BM and tumors contained high frequencies of CD8<sup>+</sup> T cells with mostly effector memory phenotype that were specific for a MAA tyrosinase related protein (TRP)-2.<sup>5</sup> Moreover, increased numbers of BM TRP-2-specific effector memory CD8<sup>+</sup> T cells were also detected in transgenic animals without visible skin tumors. After a short-term co-incubation with BM-derived dendritic cells (DCs) pulsed with melanoma lysates, such T cells produced IFN $\gamma$  in vitro and exerted antitumor activity after adoptive transfer into melanoma bearing transgenic mice.

However, this antitumor effect was short-term and failed to lead to the tumor regression that may be due to the complex immunosuppressive network in the tumor microenvironment stimulated by chronic inflammatory conditions represented by

numerous mediators (cytokines, chemokines, growth factors, reactive oxygen and nitrogen species, prostaglandins). These factors recruit into tumor lesions and activate various immunosuppressive leukocytes such as myeloid-derived suppressor cells (MDSCs), regulatory/tolerogenic DCs, regulatory T cells (Tregs) etc.<sup>6</sup> Analyzing the production of inflammatory factors in primary melanomas and metastatic LN, we detected significant levels of interleukin (IL)-6, VEGF, and TGF- $\beta$ .<sup>7</sup> Notably, VEGF amounts positively correlated with the tumor progression.<sup>8</sup> Furthermore, concentrations of IL-6 and VEGF were significantly elevated in the serum of transgenic tumor-bearing mice compared with non-transgenic littermates.<sup>7</sup> We found also an association of elevated IL-1 $\beta$  and GM-CSF concentrations in tumors with accelerated melanoma growth.<sup>8</sup> In addition, increased levels of Ccl-2 (MCP-1) and IFN $\gamma$  have been demonstrated in mouse melanoma lesions.<sup>8,9</sup> All above-mentioned inflammatory mediators have been demonstrated to stimulate MDSC expansion, migration into tumor lesions and their activation in the tumor microenvironment.<sup>6</sup>

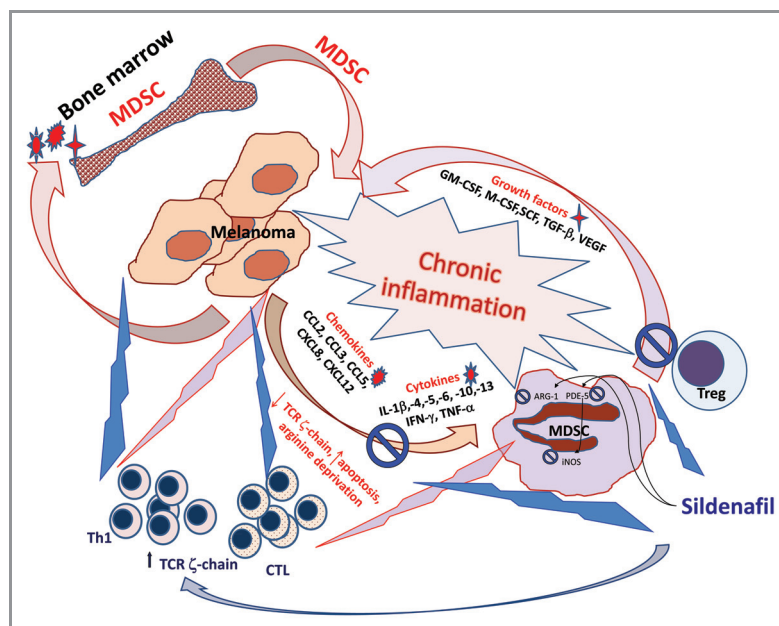
Correspondence to: Viktor Umansky; Email: V.Umansky@dkfz-heidelberg.de  
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Measurements of MDSCs revealed their remarkable accumulation among tumor infiltrating leukocytes that significantly correlated with the tumor progression.<sup>8</sup> One of the consequences of the observed enhanced MDSC proportion could be diminished numbers of mature myeloid cells like DCs. Indeed, we observed a considerable decrease in numbers of mature DCs in melanoma lesions and lymphoid organs of transgenic mice.<sup>7</sup> Tumor infiltrated MDSCs were also activated as reflected by intensive nitric oxide (NO) production and arginase-1 expression associated with their strong capacity to suppress T cell functions.<sup>8</sup> One of the major mechanisms of MDSC-mediated blocking of T-cell responses is associated with a decrease in the expression of T-cell receptor  $\zeta$ -chain, which plays a critical role in T cell signaling.<sup>10</sup> In *ret* transgenic mice, a profound downregulation of  $\zeta$ -chain expression was detected in T lymphocytes infiltrating melanoma lesions and localized in lymphoid organs.<sup>8</sup> Co-culturing normal splenocytes with tumor-derived MDSCs induced a decreased T cell proliferation and  $\zeta$ -chain expression, verifying the MDSC immunosuppressive function. Administration of the phosphodiesterase-5 inhibitor sildenafil led to reduced levels of pro-inflammatory factors in association with decreased MDSC amounts and immunosuppressive functions.<sup>8</sup> This resulted in a partial restoration of  $\zeta$ -chain expression in T cells and to a significantly increased survival of tumor bearing mice. CD8 T cell depletion mediated an abrogation of sildenafil beneficial outcome, suggesting the involvement of MDSCs and CD8 T cells in the observed therapeutic effects.

Analyzing DC subsets, we found an accumulation of immature DCs that

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**Figure 1.** Soluble mediators of chronic inflammation (like IL-1 $\beta$ , IFN $\gamma$ , TNF $\alpha$ , IL-6, CCL2, CCL3, CXCL8, GM-CSF, VEGF, TGF $\beta$ , etc.) induce the migration in tumor lesions and activation of various immunosuppressive leukocytes such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) that suppress antitumor responses mediated by effector CD4 (Th1) and CD8 (CTL) T cells via a downregulation of the  $\zeta$ -chain expression, an arginine deprivation, and apoptosis. Neutralization of chronic inflammatory melanoma microenvironment by sildenafil results in the MDSC inhibition associated with the restoration of antitumor activities of Th1 and CTL, which leads to a significant retardation of melanoma progression.

secreted more IL-10 and less IL-12 and showed a decreased capacity to activate T cells as compared with DCs from normal animals.<sup>7</sup> Observed dysfunction was linked to the activation of p38 MAPK. Blocking its activity with a specific inhibitor led to normalization of the cytokine secretion pattern and T-cell stimulation capacity of DCs from tumor bearing mice. These data demonstrate a critical role of constitutively activated p38 MAPK in the DC dysfunction during melanoma progression. Another type of immunosuppressive cells, Tregs, accumulated in melanoma lesions at early disease stages that inversely correlated with Treg

amounts in the BM suggesting a possible Treg recruitment into primary tumors.<sup>9</sup> Despite the efficient Treg depletion from lymphoid organs, melanoma development was not delayed indicating that in the autochthonous melanoma genesis, other immunosuppressive cells (like MDSCs) could play replace tumor promoting Treg functions.<sup>9</sup>

Taken together, we suggest that a key prerequisite for an effective melanoma immunotherapy should involve the neutralization of chronic inflammatory immunosuppressive microenvironment typical for melanoma before applying any immunologic treatments (Fig. 1).

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