# Role of microenvironment and tumor interactions in melanoma progression with special regard to the prognostic significance of immune cell infiltrate

PhD thesis

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# 1. INTRODUCTION

Malignant melanoma is tumor of cells originating from the neural crest. The great majority of melanomas arise in the skin. Their clinical appearance is variable. The prognosis is unpredictable and unfavorable. Melanomas have strong metastatic potential. The incidence of these lesions is on the rise in Europe and also all over the world.

Malignant melanoma of the skin is considered as one of the most immunogenic among human neoplasms. Though it frequently contains considerable amount of immune cells the prognosis of the disease is often very poor. Host cells representing an integral component of malignant melanoma, including elements of innate and adaptive immune system, can exert both positive and negative effects on the outcome of the disease. The presence of sufficient immune response can reduce the probability of tumor progression or occurrence of distant metastases hence reflected in better outcome of the disease. We investigate the prognostic role of tumor-infiltrating immune cells in patients with malignant melanoma.

## 1.1 Immune cell types infiltrating primary melanomas

The inflammatory and immune reactions can exert both positive and negative effects on the outcome of the disease. Increasing attention has been attracted by the local immune status, which can be described by evaluation of infiltrating immune cell types at tumor sites. In the immune infiltrate of melanomas cells of both the innate (mononuclear phagocytes, dendritic cells, granulocytes, mast cells, NK cells) and adaptive (T lymphocytes, B lymphocytes) immune system are present. Some of these cell types are considered to be dominantly immunosuppressive, like $CD4^+CD25^+FOXP3^+$ regulatory T cells, $CD4^+$ Th2 helper T cells, myeloid-derived suppressor cells (MDSC), M2 macrophages, N2 neutrophils, plasmacytoid dendritic cells, while the others mostly have anti-tumor activity, like $CD8^+$ cytotoxic T lymphocytes, Th1 helper T lymphocytes, antigen presenting dendritic cells, M1 macrophages and N1 neutrophils.

Tumor progression is frequently seen in the presence of significant lymphoid infiltrate. When assessing the prognostic impact of immune cells it is important to evaluate the lymphocyte subpopulations and characterize their activation state.

### 1.2. Immune cell profile of sentinel lymph nodes and its prognostic significance

Determining sentinel lymph node involvement is a potent prognostic indicator of survival in patients with malignant melanoma. The question whether sentinel nodes are immunologically competent or suppressed has been a matter of debate. Relatively few reports are available on the prognostic relevance of evaluation of the immune status of SLNs and the potential clinicopathologic factors correlated with immune cell density.

### 1.3. Endocrine factors

Several tumor types show different behavior in the two sexes, indicating the role of endocrine factors in the outcome of the disease. In preclinical models we observed a higher rate of colony formation by human melanoma cells injected intrasplenically in male compared to female SCID mice. According to our results the gender difference is organ specific and affects only the liver. Examining the background we failed to detect considerable amount of sex hormone receptors in human melanoma cells at mRNA and protein expression level. Sex steroids did not significantly influence their *in vitro* proliferation or matrix adhesion. The fact that steroids failed to directly influence melanoma cells may suggest that they exert their effects through their influence on the host.

## 2. AIMS

In our studies on melanoma progression we attempted to answer the following questions:

**1.** Does the degree of infiltration by different immune cell types like DC-LAMP$^+$ mature DCs, CD20 expressing B cells, FOXP3$^+$ regulatory T cells and activated T lymphocytes correlate with clinicopathological parameters or the outcome of the disease in human primary melanomas?

**2.** Is the presence of different immune cell types as OX40$^+$ activated T lymphocytes, DC-LAMP$^+$ mature DCs, FOXP3$^+$ regulatory T cells and CD123$^+$ plasmacytoid DCs in sentinel lymph nodes predictive for the progression of the tumor or the survival of the patients?

**3.** In preclinical models, is there any importance of the nonspecific host immune system, including macrophages and NK cells, in the development of gender difference observed in liver colonization of melanoma cells?

## 3. MATERIALS AND METHODS

### Tumor samples

Archival tissue samples were obtained from patients with primary melanoma who underwent surgery between 1980 and 2001 at the Institute of Dermato-Venerology, Semmelweis University and the National Institute of Oncology, Budapest. For the examination of dendritic cells, B cells, $FOXP3^+$ regulatory T cells and sentinel lymph nodes, samples from 82, 106, 97 and 60 patients were used, respectively. Patients did not receive any anticancer treatment prior to surgery. The tumors were grouped into four thickness categories (≤1.0, 1.01–2.0, 2.01–4.0, >4.0 mm), and into three categories according to disease progression (non-metastatic, lymph node metastatic, visceral metastatic within the 5-year follow-up period). All the patients had total excision of the tumor. Surviving patients had follow-up data for at least 5 years, and none of the patients died of melanoma-unrelated causes within 5 years.

### Immunohistochemical detection of infiltrating cells in melanoma samples

We performed immunohistochemistry using the following primary antibodies: mouse monoclonal anti-CD1a, anti-DC-LAMP, anti-CD45RO, anti-CD20cy, anti-FOXP3, anti-CD134, anti-CD123, anti-CD25, anti-CD21. Double staining was performed on a subset of samples, detecting dendritic cells (CD1a, DC-LAMP) or B cells (CD20) and T cells expressing the activation markers (CD25, CD134). Incubation with the primary antibodies was followed by adequate secondary antibodies and visualization with 3-amino-9-ethylcarbazole, or using Vector SG and fuchsin as chromogens in cases of double staining.

### Evaluation of the immune reactions

In primary melanomas the entire tumor area was analyzed in every case, and density of positive cells/$mm^2$ was given. The number of infiltrating cells was registered separately in intratumoral (infiltrating melanoma cell nests) and peritumoral areas (distributed in the infiltrate along the margin and the base of melanomas). The proportion of patients with significant densities of the examined cell types was calculated based on the median of the given variable in the whole patient group, with minor modifications for higher discriminative power in some

cases. We also determined the proportion of patients with significant densities of the examined cell types, using the cutoff values of each cell type separately for intra- and peritumoral localization. Density values of T cells expressing activation markers derived from our previous work.

In studies on sentinel lymph nodes, 5 areas with the highest density of positive cells (hot spots) were counted at 400x magnification. In the case of patients with more than one SLN available, the mean labeled cell densities of all SLNs studied were registered. Cutoff levels were set up for each marker.

**Experimental model: gender difference in metastasis formation**

**Tumor cells and culture conditions**

The HT168-M1 and HT199 human melanoma lines and B16 mouse melanoma cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 50 µl/ml gentamycin at 37˚C, in 5% $CO_2$ atmosphere.

**Citotoxicity test**

Spleen cells from 8 to 10 weeks old male and female SCID mice, treated with polyinosinic:polycytidylic acid (poly(I:C)) were cultured with target cells (HT168-M1 melanoma) at effector:target ratios of 100:1, 50:1, 25:1 and 12.5:1 for 22 hours. Cytotoxicity was determined based on LDH release.

**Animals**

C57BL/6, SCID (CB17/icr-Prkdc$^{scid}$) and NOD/SCID IL2R$\gamma^{null}$ (NSG; NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tmlWfi}$/SzJ) mice were bread and housed in the pathogen-free animal facility of the National Institute of Oncology. All animal studies were conducted in accordance with published guidelines on welfare of animals in cancer research. The experimental protocols were approved by the Animal Care and Use Committee of the Institute.

**Liver colonization**

Melanoma cells were injected in the spleen of the mice. The experiments were terminated 22-29 (HT168-M1) or 16 days (B16) after tumor cell inoculation. The spleen and liver of the animals were weighed and fixed in 4% formalin. Liver surface colonies were counted under stereomicroscope. In case of Kupffer or NK cell blocking experiments, $GdCl_3$ or anti-asialo GM1 antibody, respectively, was

injected intraperitoneally in physiological saline 1 day prior to melanoma cell injection, using saline as control. For determining the kinetics of liver colonization, $5x10^5$ HT168-M1 cells were injected intrasplenically in SCID mice and animals were sacrificed 1 hour, 1 day or 1 week after inoculation. The livers of mice were fixed in formalin, and the number of active, dividing melanoma cells was determined by immunohistochemical detection of Ki-67 antigen and density of melanoma cells/$mm^2$ was calculated based on counting labeled cells under light microscope.

**Lung colonization**

Melanoma cells ($10^6$) were injected into the tail vein of SCID mice. After 6 weeks the experiment was terminated, the lungs of the animals were fixed in 4% formalin, and the number of surface colonies was determined under stereomicroscope.

**Intracardiac injection**

Male and female SCID mice were surgically orchidectomized or ovariectomized, respectively. Control animals received sham operation. Two to four weeks after surgery mice were given injections of $10^6$ viable tumor cells in the left ventricle. The experiments were terminated 3-4 weeks after tumor cell inoculation. The liver, brain, heart, lung, kidneys, adrenals and bones from hind legs were removed and fixed in 4% formalin. The number of liver colonies was counted under stereomicroscope, while metastases in other organs were evaluated by light microscopy.

## 4. RESULTS

### 4.1. The significance of dendritic cells infiltrating the primary tumor

We found similar number of $CD1a^+$ dendritic cells in melanoma cell nests and in the stromal compartment, while DC-$LAMP^+$ cells were detected almost exclusively in peritumoral areas. The degree of both intratumoral and peritumoral infiltration by $CD1a^+$ DCs, as well as peritumoral density of DC-$LAMP^+$ mature dendritic cells showed strong inverse correlation with the thickness of the tumors.

In our cohort of cutaneous melanoma samples, there was no significant variation in DC densities when tumors were distinguished according to histological type, localization, patient age or sex. Ulcerated tumors contained lower numbers of infiltrating DCs, probably due to the increasing frequency of ulceration in thicker

tumors. Tumors that gave visceral metastases during the follow-up period were characterized with lower peritumoral DC density. Intense peritumoral infiltration of DCs, especially of mature ones expressing DC-LAMP, was associated with longer survival of patients.

In a previous study we analyzed the prognostic value of $CD25^+$ or $OX40^+$ activated T cells in melanoma. According to our present work density values for DCs and activated T cells showed strong correlation. The combination of high DC density and high amount of activated T cells predicted significantly better prognosis compared to other subgroups.

### 4.2. The significance of B cells infiltrating the primary tumor

In our cohort of 106 patients, we found significant amount of B cells in the peritumoral infiltrate in the majority of samples, while only a small fraction of the cells infiltrated the melanoma cell nests. The number of $CD20^+$ lymphocytes did not show correlation with the studied patient and tumor parameters, with the exception of tumor site: melanomas on the trunk or head contained a significant peritumoral B cell infiltrate more frequently than those located in the extremities.The density of B lymphocytes (either intra- or peritumoral) proved of prognostic significance.

We evaluated the prognostic effect of combination of B cell and activated T lymphocyte density values, resulting in patient subgroups with markedly different survival where combination of low B cell and low activated T lymphocyte density predicted strikingly poor prognosis compared to other subgroups.

### 4.3. The significance of $FOXP3^+$ regulatory T cells infiltrating the primary tumor

$FOXP3^+$ cells were more prevalent in the stromal, lymphocyte-rich areas than in tumor cell nests in primary tumors of patients with malignant melanoma. The amount of $FOXP3^+$ cells did not show association with any of the studied parameters: patient age or gender, tumor thickness, location, histological type, ulceration, or the outcome of the disease.

### 4.4. The significance of different immune cell types in the sentinel lymph nodes

We determined the density of several immune cell types: $OX40^+$ activated T lymphocytes, $FOXP3^+$ regulatory T cells, DC-$LAMP^+$ mature dendritic cells and $CD123^+$ plasmacytoid DCs in sentinel lymph nodes of patients with malignant melanoma. The results were evaluated with regard to association with patient and tumor parameters and the outcome of the disease.

In our patient cohort, density of DC-$LAMP^+$ mature DCs, $CD123^+$ plasmacytoid DCs and $OX40^+$ activated T cells in sentinel nodes did not show significant association with disease progression or survival. High mean density of $FOXP3^+$ T lymphocytes, on the other hand, was associated with significantly shorter progression-free and overall survival. A novel, intriguing finding of our study is that this association was observed only in cases with positive SLN status.

### 4.5. Experimental model: gender difference in metastasis formation

Corroborating the results of our previous experiments performed on two human melanoma cell lines, HT168 and HT199, we showed that intrasplenic injection of the highly metastatic HT168-M1 cells resulted in strikingly higher number of liver colonies in male than in female SCID mice.

We did not find gender difference in primary tumor weight after intrasplenic inoculation or in lung colonization after intravenous injection of HT168-M1 melanoma cells. Intracardiac injection resulted in colonies in multiple organs, however, we observed difference between the two sexes only in the case of the liver; more colonies were found in males compared to females, and orchidectomy decreased, while ovariectomy increased their number.

According to our experiments the gender difference observed in liver colonization emerged on the first day after tumor cell inoculation. The number of Ki-$67^+$ proliferating melanoma cells in the liver decreased three- and eight-fold during the first post-inoculation day in male and female mice, respectively. The number of microscopic colonies detected at day seven approximately corresponded to that of Ki-$67^+$ cells at day one, suggesting that most proliferation-competent melanoma cells were able to form microcolonies.

The fast decline of the number of proliferating melanoma cells in the liver suggests a role of host defense mechanisms. Moreover, the fact that sex steroids failed to

directly influence melanoma cells may indicate that they exert their effects indirectly, through their influence on the host. Since the elements of the natural immune system are present in SCID mice, we hypothesized that Kupffer or NK cells may be responsible for the rapid elimination of disseminated tumor cells. Both cell types have been implicated in inhibiting the formation and growth of liver metastases.

In our experiments, pretreatment of the animals with the Kupffer cell inhibitor gadolinium chloride resulted in an enhancement of liver colony formation by HT168-M1 cells in both sexes to approximately the same extent. On the other hand, a more pronounced increase was observed in the case of female mice compared to males after pretreatment with anti-asialo GM1, indicating the importance of NK cell activity in determining the fate of intrasplenically injected melanoma cells. The role of NK cells was supported by the lack of gender difference in colony formation in NSG mice, reported to be devoid of NK cell activity. To investigate the possible *in vitro* mechanism of the effect of NK cells on human melanoma cells we performed cytotoxicity assays using splenocytes from poly(I:C) treated mice, demonstrating a higher cytotoxic activity of effectors from female compared to male mice against HT168-M1 melanoma cells.

## 5. CONCLUSIONS

We investigated the prognostic value of immune cell infiltrate in melanoma patients. Our results suggest the importance of some of the studied immune markers in the primary tumors and in metastatic sentinel lymph nodes.

**1.** A prominent infiltration of DC-LAMP$^+$ mature dendritic cells in primary melanomas was found to correlate with better prognosis. High density of dendritic cells in association with high amount of activated T lymphocytes proved significant independent predictor of favorable disease outcome.

Intense infiltration by B cells showed positive correlation with melanoma prognosis. Combined analysis of peritumoral densities of B cells and activated T lymphocytes revealed very poor prognosis in the case of low amount of both cell types.

**2.** High mean density of FOXP3$^+$ regulatory T cells in sentinel lymph nodes was associated with significantly shorter progression-free and overall survival in

melanoma patients with positive SLN status, but not in those with negative SLN status or in primary melanomas.

The density of the other immune cell types studied: $OX40^+$ activated T lymphocytes, DC-$LAMP^+$ mature dendritic cells and $CD123^+$ plasmacytoid dendritic cells did not prove of prognostic significance.

**3.** In preclinical models we observed a higher rate of colony formation by human melanoma cells in male compared to female SCID mice, but only in the case of the liver and not in other organs. The gender difference could be seen at an early phase of colony formation. We observed a more prominent increase in female than in male mice in the case of NK cell inhibition. Further supporting the importance of NK cells in the lower liver colonization efficiency of melanoma cells in females, gender difference in colony formation was lost in NSG mice lacking NK activity.

## 6. PUBLICATIONS RELATED TO THE PhD THESIS

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