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Dormant tumor cells in *ret* transgenic mouse melanoma model and their interaction with memory T cells

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Summary

The hypothesis of the cancer stem cell (CSC) suggests that neoplastic clones are maintained by a rare fraction of tumor cells with stem cell properties. CSCs could represent disseminated dormant tumor cells without clinical signs of tumor progression. We used a *ret* transgenic mouse spontaneous melanoma model, in which 25% of transgenic mice develop skin tumors with metastases in lymph nodes (mLN), liver, lungs and bone marrow (BM). Mice older than 20 weeks without macroscopic tumors contain in the BM tyrosinase related protein (TRP)-2-specific effector memory $CD8^+$ T cells and show no further melanoma progression. This suggests a potential role of dormant tumor cells in the maintenance of memory $CD8^+$ T cells. We found that TRP-2^+CD133^+ melanoma cells represent less than 1.5% of all cells in primary skin tumors and mLN. The majority of these cells were $Ki67^-$ suggesting thereby that these cells could remain in a dormant state. We found an increased expression of the major regulator for cell survival, self-renewal, and tumor growth, HIF-1α in TRP-2^+CD133^+ melanoma cells in large tumors in comparison with those in smaller tumors. To investigate whether TRP-2^+CD133^+ melanoma cells are disseminated in the BM of *ret* transgenic mice, we performed a triple immunofluorescence staining. We found that only 40% of mice without macroscopic tumors contained TRP-2^+CD133^+ melanoma cells in the BM. In contrast, all tumor bearing mice contained TRP-2^+CD133^+ melanoma cells. TRP-2^+CD133^+ melanoma cells were detected in 2 of 712 (0.238%) and 4 of 1285 (0.311%) disseminated melanoma cells in the BM of mice without and with macroscopic tumors, respectively. We confirmed the dormant state of TRP-2^+CD133^+ melanoma cells based on the negative expression of Ki67 and PCNA. Proteins p16 and p27, which are typically located in the nuclei of dormant cells, were found in the cytoplasmic compartment of TRP-2^+CD133^+ melanoma cells indicating their highly malignant phenotype. Investigating the interaction between memory $CD8^+$ T cells with disseminated melanoma cells in the BM, we found that TRP-2^+Ki67^- melanoma cells were co-localized with memory $CD8^+$ T cells both in mice without and with macroscopic tumors. The proportion of memory $CD8^+$ T cells interacting with TRP-2^+Ki67^- melanoma cells was lower (less than 15%) in the BM of these mice. Quantitative analyses revealed that although certain IFN-γ-producing $CD8^+$ T cells interacted either with single TRP-2^+ melanoma

cells or the smallest cluster of melanoma cells (2-5 TRP-2$^+$ cells), none of these T cells produced perforin. Only two TRP-2-specific CD8$^+$ T cells produced perforin, but none of them were co-localized either with TRP-2$^+$ melanoma cells or TRP-2$^+$CD133$^+$ melanoma cells. Furthermore, memory CD8$^+$ T cells located within the large cluster of 50 TRP-2$^+$ melanoma cells were unable to produce both perforin and IFN-γ. These findings suggest that tumor microenvironment might neutralize CD8$^+$ T cell reactivity. In conclusion, our data demonstrate the existence of a subpopulation of CD133$^+$ melanoma cells in *ret* transgenic mice. Dormant TRP-2$^+$ melanoma cells are able to interact with CD8$^+$ T cells in the BM of tumor-bearing mice.

Zusammenfassung

Nach der Hypothese der Krebsstammzellen (CSC), werden neoplastische Klone von einem seltenen Bruchteil der Tumorzellen mit Stammzell-Eigenschaften beibehalten. CSCs könnten verbreitete, ruhende Tumorzellen ohne klinische Anzeichen einer Tumorprogression darstellen. Wir verwendeten ein *ret* transgen Spontanmelanom- Mausmodell, in dem 25% der transgenen Mäuse Hauttumore mit Metastasen in den Lymphknoten (mLN), der Leber, den Lungen und im Knochenmark (BM) entwickeln. Mäuse, die älter als 20 Wochen sind und makroskopisch keine Tumore entwickeln, enthalten im BM (Knochenmark) Tyrosinase verwandtes Protein (TRP)-2-spezifische Effektorzellen $CD8^+$ T-Zellen und zeigen keine weitere Melanom Progression. Dies deutet auf eine potentielle Rolle der ruhenden Tumorzellen in der Aufrechterhaltung von Gedächtnis-$CD8^+$ T-Zellen hin. Wir fanden, dass TRP-2^+CD133^+ Melanomzellen weniger als 1,5% aller Zellen in primären Tumoren der Haut und mLN repräsentieren. Die Mehrheit dieser Zellen waren $Ki67^-$, was darauf hindeutet, daß diese Zellen dadurch in einem inaktiven Zustand bleiben könnten. Im Vergleich zu kleineren Tumoren, fanden wir in TRP-2^+CD133^+ Melanomzellen großer Tumore eine erhöhte Expression von HIF-1α, dem wichtigsten Regulator für das Überleben der Zelle, Selbsterneuerung und Tumorwachstum. Um zu untersuchen, ob TRP-2^+CD133^+ Melanomzellen im Knochenmark von *ret* transgenen Mäuse verbreitet werden, führten wir eine dreifache Immunfluoreszenzfärbung durch. Wir fanden heraus, dass nur 40% der Mäuse ohne makroskopische Tumore TRP-2^+CD133^+ Melanomzellen im BM (Knochenmark) enthalten. Im Gegenssatz dazu enthielten alle tumortragenden Mäuse TRP-2^+CD133^+ Melanomzellen. In Mäusen mit und ohne makroskopische Tumore, wurden TRP-2^+CD133^+ Melanomzellen in 2 von 712 (0,238%) und 4 von 1285 (0,311%) verbreiteten Melanomzellen im BM von Mäusen nachgewiesen. Wir bestätigten den Ruhezustand der TRP-2^+CD133^+ Melanomzellen durch die negative Expression von (oder durch eine fehlende Expression) Ki67 und PCNA. Die Proteine p16 und p27, welche sich typischerweise in den Kernen von ruhenden Zellen befinden, wurden im zytoplasmatischen Kompartiment von TRP-2^+CD133^+ Melanomzellen gefunden, was auf einen hoch-malignen Phänotyp hinweist. Durch eine Untersuchung der Wechselwirkung zwischen Gedächtnis-$CD8^+$-T-Zellen mit disseminierten

Melanomzellen im BM (Knochenmark) fanden wir heraus, dass TRP-2^{+} Ki67^{-}-Melanomzellen sowohl in Mäusen ohne, als auch in Mäusen mit makroskopischen Tumoren, mit CD8^{+} T-Zellen kolokalisieren. Der Anteil von Gedächtnis-CD8^{+}-T-Zellen, welcher mit TRP-2^{+}Ki67^{-} Melanomzellen interagiert, war im Knochenmark (weniger als 15%) dieser Mäuse geringer. Quantitative Analysen zeigten, dass obwohl bestimmte IFN-γ produzierende CD8^{+} T-Zellen, entweder mit einzelnen TRP-2^{+} Melanomzellen oder den kleinsten Melanomzell-Clustern (2-5 TRP-2^{+}-Zellen) interagierten, keine der T-Zellen Perforin produzierten. Nur zwei TRP-2-spezifische CD8^{+} T-Zellen produzierten Perforin, aber keine von ihnen war weder mit TRP-2^{+} Melanomzellen oder TRP-2^{+}CD133^{+} Melanomzellen co-lokalisiert. Außerdem konnten Gedächtnis-CD8^{+}-T-Zellen, die innerhalb des großen Clusters von 50 TRP-2^{+} Melanomzellen lokalisiert waren, weder Perforin, noch IFN-γ erzeugen. Diese Befunde legen nahe, dass das Tumor-Mikromilieu die CD8^{+} T-Zell-Reaktivität neutralisieren könnte. Zusammenfassend zeigen unsere Daten die Existenz einer Subpopulation von CD133^{+} Melanomzellen in *ret* transgenen Mäusen. Ruhende TRP-2^{+} Melanomzellen sind in der Lage, mit CD8^{+} T-Zellen im Knochenmark von tumortragenden Mäusen zu interagieren.

I. Introduction

1. Tumor initiation and progression

Current estimates regarding the global incidence of cancer predict that by year 2020, the number of new cancer cases diagnosed each year will increase to 15 million and that the disease will be responsible for more than 12 million deaths. Despite recent advances in surgical techniques, radiotherapy and the development of molecular targeted therapies, most deaths due to cancer result from the progressive growth of metastases that are resistant to current therapies (1). Malignancy is a state that emerges from a tumor microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells (2).

Malignant tumor cells recruit vasculature and stroma through production and secretion of stimulatory growth factors and cytokines (3-6). The hallmarks of cancer proposed by Douglas Hanahan and Robert A. Weinberg in 2000 and re-formulated in 2011, elegantly summarize the multistep development of human tumors (7, 8). They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Tumors exhibit another dimension of complexity: in addition to cancer cells, they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the ‘‘tumor microenvironment’’ (8)

1.1 Primary tumors

Cancer cells in primary tumors have already acquired a numbers of aggressive functions that will remain important throughout the rest of their metastatic progression. These functions generally include motility, invasiveness, resistance to hypoxia and reactive oxygen species, survival after detachment, and evasion of immune surveillance (2, 9). It has been proposed that the secondary tumor formation involves rare cell variants that have accumulated a complete set of genetic mutations in the primary tumor that enables these cells to grow in a distant organ. However, this hypothesis has been challenged by the detection of widespread gene expression patterns in primary tumors that strongly predict metastatic competence. Genetically marked transplantable tumors in mouse mammary carcinoma models were used to

demonstrate that cancer cells can disseminate at the premalignant stage (9).

1.2 Metastasis

Metastasis is responsible for approximately 90% of cancer-associated mortality, yet it remains the most poorly understood component of cancer pathogenesis. During metastatic dissemination, a cancer cell from a primary tumor executes the following sequence of steps: It locally invades the surrounding tissue, enters the microvasculature of the lymph and blood systems (intravasation), survives and translocates largely through the bloodstream to microvessels of distant tissues, exits from the bloodstream (extravasation), survives in the microenvironment of distant tissues, and finally adapts to the foreign microenvironment of these tissues in ways that facilitate cell proliferation and the formation of a macroscopic secondary tumor (colonization) (2, 10, 11).

Organ-specific colonization functions have been well documented in bone metastasis. The ability of breast cancer cells to form typical osteolytic metastases requires the production of osteoclast-activating factors, such as parathyroid hormone related protein (PTHRP), interleukin 11 (IL-11), IL-6, Tumor Necrosis Factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which act on osteoclasts to promote the secretion of receptor activator of nuclear-factor-kB ligand (RANKL), which induces osteoclast formation (12). A frequent site of distant metastasis, the skeletal system is composed of diverse cell types. When metastatic breast cancer invades bone, it frequently becomes clinically noticeable by painful fractures due to induced hyperactivation of bone-resorbing osteoclasts. Advanced prostate cancer, to the contrary, predominantly involves the stimulation of bone depositing osteoblasts, thus resulting in a net increase in bone density and eventual bone-marrow displacement (13).

Bone metastases are a frequent complication of many cancers that lead to severe symptoms such as bone fractures, hypercalcaemia, and intractable skeletal pain (14) as well as spinal cord and nerve-compression syndromes (15). It has been estimated that 70% of patients with progressive breast cancer and 84% of prostate cancer patients develop bone metastases (14, 16, 17). Bone metastasis is often classified as either osteolytic or osteoblastic and one of these effects is usually predominant. For

example, metastases from breast and lung tumors are generally osteolytic, whereas metastases from prostate cancer are generally osteoblastic (15, 17-20). In a mouse model of melanoma metastasis (B16F10 melanoma subclone), *in vivo* neutralization of RANKL by osteoprotegerin results in complete protection from paralysis and a marked reduction in tumor burden in bones but not in other organs (14).

To produce metastases via the systemic circulation, tumor cells should survive in the circulation, adhere to the microvascular wall of distal tissues and either grow locally or invade the vessel wall and grow in distant organs. Accumulating evidence suggests that tissue-specific gradients of chemokines play an important role in determining the patterns of metastasis observed in some tumors. Initial reports were focused on the role of CXCR4 (CD184) in breast cancer after it was determined that CXCL12 (stromal cell-derived factor-1, SDF-1) could be constitutively expressed by stroma fibroblasts in target organs of metastasis (i.e. bone, liver, lungs, and lymph nodes). Activation of CXCR4 on breast cancer cells has been shown to stimulate a number of cellular responses that are critical for metastasis formation, including actin polymerization, pseudopodia formation, chemotaxis, synthesis of proteolytic enzymes, and invasion. In addition, stimulation of CXCR4 on tumor cells promotes activation of integrin receptors, thereby increasing the affinity of cells for microvascular endothelial surface. Primary tumors that predominantly express CXCR1 (CD181/IL-8R) preferentially spread to the brain, whereas tumors that express CCR6 (CD196) can more likely metastasize to the pleura. Expression of CCR7 (CD197) on breast or melanoma cells has also been shown to be an important determinant in mediating skin metastasis (21).

Several studies have shown that many tumor cells mediate their adhesion to the vascular endothelium by using mechanism similar to those used by leukocytes (22-24). E-selectin is a cytokine -inducible endothelial cell glycoprotein that is responsible for directing the initial localization of neutrophils to inflammatory tissues. Vascular cell adhesion molecule-1 (VCAM-1) is an endothelial cell glycoprotein that plays an integral role in promoting the firm adhesion and transmigration of blood leukocytes. Studies examining the adhesive interactions between melanoma and endothelial cells suggest that melanoma cells use their surface very late activation antigen-4 (VLA-4) integrin to adhere to endothelial VCAM-1(25). In a spontaneous murine model of melanoma, VCAM-1 was selectively up-regulated in target organs (brain, heart, and liver) during melanoma metastasis. (21).

Clinical reports have implied that the spread of carcinomas takes place primary through the lymphatic system and that tumor of origins are more likely to disseminate through the blood vascular compartment. However, this assumption is difficult to validate because the lymphatic and vascular systems have numerous interconnections, and it is well accepted that disseminating tumor cells can pass from one system to another. Hence, the division of metastatic pathways into lymphatic spread and hematogenous spread is an arbitrary one. Invasive tumor cells can easily penetrate small lymphatic channels and then be transported in the lymph (21).

According to the classical model of metastasis, tumor cell dissemination occurs late in tumor development. Only after the primary tumor has grown, tumor cells start to invade the local tissue, enter the blood or lymphatic vessels, and colonize new sites to cause metastases (2). However, evidence increasingly indicates that single tumor cells spread to distant sites much earlier than previously believed (26). For example, single disseminated tumor cells can be found in the lymph nodes or bone marrow (BM) of women with a history of early-stage breast cancer that have no clinical evidence of metastasis or tumor recurrence. Women with such dormant cancer cells live with an increased risk of sudden metastases, which may occur more than a decade after surgical excision of the primary tumor. Similarly, single melanoma cells can be found in the lymph nodes of patients with thin melanomas. These single dormant tumor cells seem to be of prognostic relevance, due to the patients can develop metastases after more than 10 years of surgical excision of the tumor. Nonetheless, more than 90% of these patients do not develop metastases for at least 10 years after surgery (26).

Early tumor cell dissemination reminiscent of the events in human metastasis has also been analyzed by Husemann *et al.* in two distinct transgenic mouse strains that model breast cancer (27). Although these authors did not study the latency between tumor cell dissemination and metastatic outgrowth, they showed that, upon adoptive transfer, disseminated tumor cells are capable of homing to the BM and causing metastases. It is noteworthy that in these breast cancer models, the tumor cells tended to spread to the BM and the lung, whereas in the spontaneous melanoma model used by Eyles *et al*. (28) cells spread more diffusely. Importantly, in both studies apparent metastatic cells bore the genetic signature of the parental tumor. Understanding why these disseminated tumor cells fail to cause early metastasis and what events are responsible for tumor dormancy are key to determining whether new therapeutic

interventions can be developed (26).

2. Malignant skin melanoma

Melanoma is often considered as one of the most aggressive and treatment-resistant human cancers with a median overall survival of less than one year (29, 30). Melanoma originates from the malignant transformation of pigment-producing melanocytes. As in the case of most cancer types, both genetic and environmental factors are believed to contribute to melanomagenesis. Melanoma incidence is influenced by pigmentation of the population and geographical parameters such as latitude and altitude, indicating that ultraviolet (UV) light has a causal role in melanoma development (31, 32). In fact, epidemiological studies show that the major etiological melanoma risk factor is UV spectrum of solar radiation, with the highest risk associated with intermittent burning doses, especially during childhood (33-35). Noonan *et al.* have shown that UV-A but not UV-B radiation requires melanin pigment to induce melanoma (36). Other genetically determined host factors, such as fair complexion, red hair, and multiple benign or dysplastic nevi have been also associated with increased melanoma risk (31).

Melanoma progression begins with the development of either dysplastic or benign nevi (acquired or congenital). These can progress to the radial growth phase, in which the growth expands laterally but remains localized to the epidermis. At this phase, cells are still dependent on growth factors and are not anchorage independent or tumorigenic (37). Progression to the vertical growth phase is hallmarked by the invasion into the dermis, subcutaneous tissue and upper epidermis. In the vertical growth phase, cells are no longer growth factor or anchorage dependent (37). Clinical staging of melanoma progress from an *in situ* growth to one increasing in the thickness and vertical invasion, to regional lymph-node spread and, finally, to distal metastases. Vertical invasion may be representative of the degree of progression and is often measured by the Breslow thickness, a measure of the thickness of the tumor from the upper layer of the epidermis to the innermost depth of invasion (37-39).

Melanoma is widely known to be a molecularly heterogeneous disease. However, it has been possible only recently to identify patients with clinical relevant molecular signatures and assess responses to treatment in these subgroups (40, 41). A molecular classification system will replace conventional histological criteria, which divides

melanoma into four subtypes: superficial spreading, lentigo malignant, nodular, and acral lentiginous (42-44). A major impetus towards etiological molecular classification of melanoma was the identification of an oncogenic mutation in the serine/threonine-protein kinase B-raf (BRAF) gene in 60% of melanoma cell lines and short-term cultures of primary melanoma samples (42). In 2002, it was discovered that cutaneous melanoma is a molecularly heterogeneous disease bearing an activating mutation in the gene encoding for BRAF in approximately 40% to 60% of the cases with 90% of the mutations resulting in a substitution of valine for glutamate at amino acid 600 (V600E) (30, 45). Mutated BRAF leads to constitutive activation of the mitogen-activated protein kinase pathway (MAPK) (30, 46). MAPK pathway is activated in more than 80% of melanomas (47). The dysregulation of the MAPK pathway can also be caused by an activating mutation in the gene encoding for the neuroblastoma RAS viral oncogene homolog (N-Ras) which is upstream of BRAF. In addition, mutations in the oncogenes C-KIT, GNAQ, and GNA11, as well as mutations in the tumor suppressor genes PTEN (phosphatase and tensin homolog) or p53, and loss of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene products p15 and p16, have been described (29, 37, 47). Alterations of PTEN are found in up to 55% of melanoma metastases and combined MAPK pathway/PTEN alterations have been found in 20-25% of melanoma cell lines (31).

Among the most common aberrations are those affecting the CDKN2A genetic locus, which ultimately controls RB1 and tumor protein p53 networks. Approximately 10% of all melanomas are hereditary. CDKN2A is the major melanoma susceptibility locus in familial melanoma (48, 49). In addition to increased susceptibility to melanoma, inactivating germline mutations at the CDKN2A locus also increase the risk of other cancers, particularly pancreatic adenocarcinoma (49, 50). It is estimated that CDKN2A mutations contribute to 10%-40% of familiar melanoma cases. Frequent mutations affecting the CDKN2A locus in melanoma typically target $p16^{INK4A}$, suggesting an essential role for this protein in the control of cell cycle in melanocytes (49, 51). The gene is deleted in approximately 50% of melanomas and is inactivated by point mutations in approximately 9% of the tumors (51). In addition, the $p16^{INK4A}$ gene is frequently silenced by promoter methylation, a process that interferes with gene expression. Hypermethylation has been reported to occur in 20% to 75% of melanomas (49, 52, 53).

The MAPK signal transduction pathway, also known as the RAS/RAF/MEK/MAPK pathway, plays a key role in the proliferation of most solid tumors, including melanoma (49, 54). The signaling cascade is initiated by growth factor stimulation of membrane-bound receptor tyrosine kinases followed by activation of RAS, a small G protein with three isoforms (H-RAS, K-RAS, and N-RAS). Upon activation of RAS, a complex forms between RAS and one of the RAF serine/threonine protein kinase isoforms ARAF, BRAF, or RAF1. Once RAF is activated, the signal is transduced through phosphorylation of MEK leading to the MAPK phosphorylation, that induces a number of mitogenic and survival processes, including proliferation, and protection from apoptotic cell death (49).

Often called the guardian of the genome, p53 is one of the most frequently mutated genes in human cancer (49, 55-58) . It is activated by many factors, including cellular and oncogenic stress. As a transcription factor, p53 regulates a variety of genes involved in cell cycle arrest, senescence, DNA repair, and cell death (59, 60). Under conditions of substantial cellular stress, such as DNA damage caused by UV radiation, the induction and activation of p53 plays an essential role in halting cellular growth and initiating the repair of damaged DNA. An important mediator of the p53 cell cycle-inhibitory function is the cell cycle-inhibitor $p21^{WAFI/CIPI}$, which inhibits the complex of cyclin E and cyclin-dependent kinase 2 (CDK2) (49, 61, 62). Similar to the inhibition of the cyclin D-CDK4/6 complex by $p16^{INK4A}$, inhibition of cyclin E1-CDK2 by $p21^{WAFI/CIPI}$ also leads to decreased phosphorylation of RB1 and, consequently, cell cycle arrest at the G_1/S transition point (49, 61, 63).

In contrast to most other cancers, melanomas surprisingly display a low frequency of p53 mutations (9%). Although most other tumors inactivate this pathway directly at the level of p53, melanoma appears to rely on the inactivation at the level of CDKN2A and its product, $p14^{ARF}$. Most CDKN2A mutations affect $p16^{INK4A}$, either alone or in combination with $p14^{ARF}$, suggesting that it is the principal susceptibility gene at this locus (42, 49).

3. Stem cells

3.1 Normal Stem cells

Stem cells exist in many different somatic tissues and are important for their maintenance and recovery. Populations of cells that derive from stem cells are organized in a hierarchical fashion with the stem cell residing at the apex of the developmental pathway (64-66). Stem cells have three distinctive properties: self-renewal (i.e., cell division gives rise to one or two daughter cells that retain the same biologic properties as the parental cell), the capability to develop into multiple lineages, and the potential to proliferate (67). The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its dysregulation is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal in combination with the intrinsic growth potential of stem cells, may account for many features of malignant phenotype (68-70).

3.2 Melanocyte stem cells

Visible pigmentation of the skin, hair, and eyes providing protection from damage by ultraviolet (UV) (71) radiation depends primarily on the functions of melanocytes (72) which constitute 1% of skin cells (35). The skin is the main barrier to external environment and relies on melanocytes to provide photoprotection and thermoregulation by producing melanin which can absorb UV radiation affording to melanocytes to be resistant to considerable genotoxic stress (36, 73). In addition to carotenoids and hemoglobin, melanin is the main contributor to the pigmentation in the skin (74). Melanin containing granules are known as melanosomes (lysosome-like structures) and are exported from melanocytes to adjacent keratinocytes that receive and distribute melanin in the upper layers of the skin (71, 75). Melanocytic stem cells have been identified in the murine hair follicle (76). Melanocytes in the hair follicles arise from melanoblasts. Melanoblasts are derived from the embryonic pluripotent neural crest that is a migratory population that gives rise to multiple cell lineages, including neurons, glial cells of the peripheral nervous system, medullary secretory cells, cardiac cells, craniofacial tissues, smooth muscle cells, bone, and cartilage cells (71, 77, 78). Melanoblast precursors can differentiate into glial precursors and glioblasts can potentially differentiate into melanoblasts, which explains why many

markers are shared between glioma and melanoma (79, 80).

The proliferation and differentiation of melanocytes in hair follicles are closely coupled with hair regeneration cycle. The follicular melanocytes comprise a stem cell system and melanocyte stem cells reside in the upper permanent portion of the hair follicles throughout the hair cycle (78, 81). Melanocyte stem cells, which are slow-cycling cells capable of both self-renewal and differentiation into mature melanocytes, are specifically localized in the bulge area. The bulge in both human and mouse follicles is a morphological area containing quiescent cells important for hair follicle cycling (82, 83). To date, the best marker for mouse hair follicle bulge cells is CD34 (82). When melanocyte stem cells divide, at least one stem cell remains in the bulge while the other daughter stem cells migrate into the hair matrix and terminally differentiate into melanin-producing follicular melanocytes (80).

Numerous signaling pathways and transcription factors regulate all aspects of melanocyte development. These include the Wnt signaling pathway (the origin of the name Wnt comes from a portmanteau of Int and Wg [wingless] from *Drosophila melanogaster*, which is the best characterized Wnt gene) (84), the G-protein-coupled endothelin B receptor type B (EDNRB) and its ligand, endothelin 3 (ET3; EDN3), the tyrosine kinase receptor KIT and its ligand KITL (also known as stem cell factor [SCF] or mast cell growth factor [MGF]), the hepatocyte growth factor (HGF) and its ligand c-MET and the transcription factors PAX3, SOX10, and microphthalmia-associated transcription factor (MITF) which acts as a master regulator of melanocyte development (78).

Mutations in genes that are critical for melanocyte development, such as MITF, Pax3, the members of the Notch pathway, and Kit impair hair pigmentation. Similarly, mutations in enzymes involved in melanin production such as tyrosinase (Tyr), gp75 or tyrosinase-related protein 1 (TRP-1) and TRP-2 (also called dopachrome tautomerase) result in either albino mice (tyrosine mutations) or brown mice (TRP-1 and TRP-2 mutations) (71, 80). There are two main types of melanin, red/yellow pheomelanin and brown/black eumelanin (71). Both melanins derive from a common tyrosine-dependent pathway with the same precursor, tyrosine. TRP-1 and TRP-2 share 40%-45% identity with tyrosinase and are useful markers of differentiation (80, 85).

Tyrosinase, TRP-1 and TRP-2 were shown to represent enzymatic components of melanosomes (75, 86, 87). The main structural component of melanosomes is melanocyte-associated protein 17 (Pmel-17, also known as gp100 or SILV), whose sorting involves adaptor protein 1A (AP1A), AP1B, AP2 and spectrin, as well as a chaperone-like component, melanoma-associated antigen recognized by T cells 1 (MART-1) (88). During their maturation, melanosomes move from the perinuclear area towards the plasma membrane. Microtubules, dynein, kinesin, actin filaments, Rab27a, melanophilin, myosin Va and Slp2-a are involved in melanosome transport (75).

4. Cancer stem cells (CSCs)

The CSC model has suggested that only small subpopulations of cancer cells have tumorigenic potential. CSCs exhibit properties of normal stem cells such as the ability of self-renewal by symmetric division, the capacity to form cell clones with a higher rate of differentiation and proliferation by asymmetric division (89-97). In general the CSC concept suggests that many tumors are hierarchically organized with these putative CSCs being at the top of the hierarchy (89-96). CSCs are genetically identical to the rest of the malignant clone, but constitute the only cell type with tumor propagation potential within the overall tumor population (98). Simultaneously, they show significant resistance to radiation and chemotherapy due to their distinctive properties which seem to be related to their stem-like character (99). Thus, although these therapies often significantly reduce the bulk of tumor cells, resistant CSCs can be retained in the body, leading to the frequently observed relapse and metastasis formation after initially successful cancer therapies (95, 99).

Although populations of tumor cells containing CSC activity have been identified in various solid cancers including brain, breast, colon and prostate tumors, the search for reliable markers to identify CSCs is still ongoing (100, 101). It appears that marker expression on CSCs depends on tumor type and stage as well as on mutations present in each individual tumor. Moreover, the expression of certain markers on CSCs may not be stable within the tumor at any stage and time making it notoriously difficult to identify reliable general markers for CSCs in *vitro* or *in vivo* (91, 98). In addition, not all types of cancers are hierarchically organized and even in those that are, the hierarchy may flatten during tumor progression towards highly metastatic

"undifferentiated" cancers, in which tumor cells show CSCs properties (89, 98, 102, 103). Diverse CSC markers haven been reported including CD20 (104, 105), CD24 (106-109), CD34 (110), CD44 (111-114), CD166 (115), nestin (116-118), multidrug-resistance transporter ABCB5 (119-123) and CD271 (124, 125). Alternatively, JARID1B, and aldehyde dehydrogenase (ALDH) have been described as melanoma stem cell markers. JARID1B is a molecular marker for slowly cycling cells and has been associated with stem cell maintenance. In human embryonic stem cells, JARID1B blocks terminal differentiation and mediates cell cycle arrest. In melanoma, only approximately 1% of cells exhibit high expression of JARID1B (126). Aldehyde dehydrogenase (ALDH) enzyme activity has been identified as a stem cell marker in normal human hematopoietic stem cells and in multiple tumors. Boonyaratanakornkit *et al.* demonstrated that $ALDH^+$ melanoma cells displayed robust self-renewal, whereas $ALDH^-$ cells showed minimal self-renewal *in vitro*. Thus, $ALDH^+$ melanoma cells have enhanced tumorigenicity and superior self-renewal ability compared to $ALDH^-$ cells (127). On the contrary, Prasmiclaite *et al.* argued that ALDH phenotype is not associated with more-aggressive subpopulations in malignant melanoma (128). Santini *et al.* have shown that $ALDH^+$ melanoma cells express high levels of embryonic pluripotent stem cell factors Sox2, Nanog, Oct4 and Klf4 (129). CD133 has been described as the most relevant CSC marker, since it has been linked to tumor-initiating cells and implicated in tumor progression (130-133).

A number of mechanisms have been suggested to explain the exquisite resistance of CSCs to radio- and chemotherapies. For example, CSCs and normal stem cells express various ABC transporter pumps that export small molecules (drugs) out of the cell (99). They also have very efficient DNA repair mechanisms and are thought to be located in hypoxic extracellular matrix rich niches, which would mediate resistance to the radiotherapy-induced DNA damage and prevent drugs from reaching sufficient high concentrations within CSCs (134, 135). Finally, like many normal stem cells CSCs may also divide only rarely or at least are transiently in a state of deep long-term quiescence called dormancy. Such a state would make them resistant to anti-proliferative and other chemotherapeutic regimens as they are not only non-cycling, but also require little energy or oxygen making them insensitive to signaling pathway inhibitors (89).

4.1 Regulation of cancer stem activity

CXCR3 (CD183) and CXCR4 (CD184) have been described as master regulators of metastasis (136, 137), whereas vascular endothelial growth factor receptor 1 (VEGFR1) (138), and VEGFR2 (139) are essential factors to promote angiogenesis (140, 141). Approximately 60% of metastasis in malignant melanoma is found in regional lymph nodes (142). It has been demonstrated that CXCR3 plays a critical role in lymph nodes metastasis of melanoma (B16F10 cell line) (142), colon (143) and breast cancer (144). Expression of CXCR3 was found in 31 cases (33.7%) out of 92 human colon cancer specimens from cases most of which had lymph nodes metastasis (145). Importantly, the patients with $CXCR3^+$ colon cancer showed significantly shorter survivals than those without CXCR3. In addition, the patients with tumor double positive for CXCR3 and CXCR4 had significantly poorer prognosis than those with tumors positive only for CXCR4 or the double negative (144). Scala *et al.* reported that CXCR4 expression was detected in 33 out of 63 (52.4%) metastases from cutaneous melanoma (146). The metastatic potential of primary melanoma is considerably higher than other primary solid tumors when comparing the size of primary lesion (146).

Hypoxia, a condition of insufficient oxygen availability, occurs during normal development as well as tumorigenesis. Cellular responses to hypoxia are primarily mediated by hypoxia-inducible factors (HIFs) such as HIF-1α (147). Cancer stem cells are critically dependent on HIF-1α for survival, self-renewal, and tumor growth (148). HIF activity in a rare subset of hypoxic tumor cells such as CSCs, may enhance the expression or activity of specific signaling pathways such as Notch and the expression of transcription factors such as Oct4, Sox2, Klf4, c-Myc, ABCB transporters, and telomerase to promote further dedifferentiation and confer stem cell-like properties (148, 149). Hypoxia may increase metastatic homing by inducing CXCR4 expression in renal cell carcinoma (150), ovarian cancer (151), breast cancer (152) and lung cancer (153, 154). McCord *et al.* showed that hypoxia not only increased the fraction of CD133-positive cells, but also enhanced the stem-like phenotype of several cell lines (155). Soeda *et al.* demonstrated that hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α (156). Propagation of the glioma-derived CSCs in hypoxia environment also led to the expression of cells bearing CXCR4 (156). Kim *et al.* found that $CXCR4^+CD133^+$

B16/F10 melanoma cells exhibit higher metastatic activity compared to $CXCR4^{-}CD133^{+}$ cells, and blockage of CXCR4 coupled with dacarbazine efficiently inhibited both tumor growth and metastasis (157). Kumar *et al.* described that $BRAF^{V600E}$ mutation increases HIF-1α expression and melanoma cell survival under hypoxic conditions (158). It was shown that HIF-1α enhances CD39/CD73 function on epithelial cells to protect the epithelial barrier during hypoxia (159); it is well accepted today that CD39 and CD73 are potent suppressor of antitumor responses. Cancer exosomes were described to express CD39 and CD73, which suppress T cell through adenosine production (160). Thus, hypoxic microenvironments contribute to cancer progression by activating adaptive transcriptional programs that increase tumor glycolysis, promote cell survival, motility and tumor angiogenesis (148, 154).

4.2 Melanoma stem cells

A subset of melanoma cells endowed with the capacity to induce tumor growth and metastasis has been recently identified (161, 162). These melanoma-initiating cells express ABCB5, conferring resistance to chemotherapy, as well as stem cell markers CD133, CD271 and nestin, which seem to be of functional relevance for melanoma-initiating cells, since the blocking ABCB5, CD271 or CD133 reduced the capacity of melanoma cells to metastasize (161, 162). Moreover, it has been demonstrated that the frequency of melanoma-initiating cells could be dramatically higher (102, 163, 164).

4.3 CD133 as melanoma stem cell marker

CD133 (Prominin-1) was the first identified member of the Prominin family of pentaspan membrane proteins. The specific functions and ligands of the Prominins are still relatively unclear, but they are distinct in their restricted expression within plasma membrane protrusions, such as epithelial microvilli and epididymal ductal epithelial sterocilia, and hence their name derives from the Latin *prominere*, meaning “to be prominent” (165). In 1997 a novel monoclonal antibody (mAb) that recognized the AC133 antigen (human CD133), was produced, whose restricted expression in $CD34^{+}$ progenitor populations from adult blood, BM and fetal liver cells implied its function as a marker of hematopoietic progenitor cells (166). $AC133^{+}$ cells are believed to be

more primitive than $CD34^+$ cells. In the same year, Weigmann *et al.* generated a murine mAb termed 13A4 and described its corresponding antigen, Prominin (167). Prominin was enriched in the apical microvilli of mouse embryonic and adult neuroepithelium and the microvillar tips of the kidney brush border cells. Human and mouse CD133 show only 60% identity (165).

CD133 has been expressed in a number of CSCs identified to date in brain (168, 169), pancreas (170), prostate (171-173), lung (174), liver (175, 176), and colon tumors (177, 178). In brain, CD133 is highly expressed in CSCs from medulloblastoma (179) and glioblastomas (180, 181) and has been used to isolate these CSCs and characterize their highly tumorigenic potential. However, it is not clear how CD133 contributes to CSC characteristics such as resistance to the therapy (181, 182), the ability to self-renew and the potential to differentiate (183, 184). CD133-positive cervical tumor cells were described to be enriched in vaccinated mice (185). The poor clinical outcomes of vaccination are caused by the immune-resistant and stem-like phenotype within the tumor microenvironment promoted by the transcription factor Nanog which plays an important role for self-renewal of embryonic stem cells (185).

5. Dormant tumor cells

Cancer dormancy, mentioned first in 1864, re-emerged in 1934 (186) and was confirmed in 1959 (187). It has been historically defined in clinical terms, namely recurrence of the cancer systemically or locally a long time after removal of the primary tumor in a patient who has been clinically disease-free (188). Tumor dormancy is a phenomenon characterized by tumor cells persisting in the host for a long time (for months or years) as non-proliferative (189) solitary tumor cells or as micrometastases whose cellular proliferation is counterbalanced by apoptosis (i.e., tumor mass dormancy and/or angiogenic dormancy) (190). In several experimental models, the immune system controls dormancy (191). Consequently, tumor dormancy is a stage in cancer progression, in which residual disease is present but is not clinically apparent (190). The most exciting aspect of clinical cancer dormancy is resembles a chronic disease, a state of disease persistence without symptoms or signs unless this balance is disturbed and a relapse occurs (188).

In the experimental settings, such as in xenograft models a “dormant” tumor can

be defined by its microscopic size and stable non-expanding mass. In more details, dormant tumors are characterized by:

a) Tumor growth *in vivo* to ~1 mm in diameter or less, at which time further expansion ceases;
b) Inability to induce angiogenic activity due to lack of intra-tumoral microvessels, or active repulsion of extending blood vessels in the local microenvironment. These tumors are white or transparent on gross examination;
c) Expression of equal or higher amounts of anti-angiogenic (i.e. thrombospondin-1) factors compared to angiogenic (i.e. vascular endothelial growth factor, VEGF and basic fibroblast growth factor, bFGF) proteins;
d) Active tumor cell proliferation, apoptosis *in vivo,* and metabolic activity during the dormancy period;
e) Inability to spontaneously metastasize from the microscopic dormant state;
f) Harmlessness to the host until they switch to the angiogenic phenotype (192-194).

Metastatic dormancy of melanoma has not yet received sufficient attention, most likely because once detectable metastasis is almost invariably fatal and, the focus has been on finding ways to prolong life of patients with overt recurrences. Some melanomas have an ability to disseminate early during primary tumor progression and once disseminated to remain undetected (dormant) for years. Tumor dormancy mechanisms can be largely grouped in two categories: dormancy of a tumor mass and dormancy of solitary tumor cells (195).

Angiogenic metastatic dormancy applies to the dormancy of tumor mass. In this case a micro-metastatic lesion actively proliferates but it does not expand because it is avascular. The inability to recruit blood vessels is likely caused by the lack of expression of angiogenesis factors such as VEGF, and/or high expression of angiogenesis inhibitors such as thrombospondin (195, 196). It has been shown that human melanoma micrometastases are poorly vascularized and have lower rates of tumor proliferation as compared to melanoma macrometastases (195, 197-200).

Disseminated predominantly solitary tumor cells isolated from BM of patients bearing cancers of different origins are negative for markers of proliferation such as proliferating cell nuclear antigen (PCNA) or Ki67, suggesting that they can enter a state of cellular quiescence or dormancy. It was shown that solitary melanoma cells (B16F10 tumor cells) which metastasized to the lungs were dormant and did not

express markers of apoptosis (201). The experience from preclinical models suggests that tumor cells can enter dormancy if certain signaling pathways are malfunctioning and/or activated. The most common mechanism is a G_0/G_1 arrest with high expression of p21 and p27 (195, 198-200). The BM also serves as reservoir for dormant tumor cells that are resistant to chemotherapeutic treatment (15). Hence, BM might be an important reservoir that allows for disseminated tumor cells to adapt and stay hidden without clinical signs of observable metastases (202). Thus, dormant tumor cells arrested in the BM may act as a possible source of tumor cells that can be systemically released in different periods, leading to the relapse in patients that seemed to be clinically cured (203, 204). Furthermore, it has been documented that leukemic cells create BM niches that causes hematopoietic stem cell (HSC) dysfunction by usurping normal HSCs (202). Tumor derived exosomes may induce the suitable pre-metastatic niche for the attraction of tumor cells to the BM (205).

5.1 Dormant tumor cells and immunosurveillance

The original concept of tumor immunosurveillance postulated that most malignant tumors that arise are eliminated via lymphocyte-mediated responses before they become clinically detectable (26, 206). Therefore the adaptive immunity could constantly inhibit the emergence of neoplastic clones and thereby act as a major protector from the development of cancer (188, 207). However, some investigators argued that tumor cells did not possess the appropriate “danger signals” needed to alert the immune system, whereas others suggested that the immune system would ignore or tolerate a developing tumor because tumor cells were too similar to the normal cells from which they were derived (207).

The immune system plays at least three distinct roles in preventing cancer: **(i)** it protects the host against viral infection and hence suppresses virus-induced tumors; **(ii)** it prevents the establishment of an inflammatory environment that facilities tumorigenesis by eliminating pathogens and by prompt resolution of inflammation; and **(iii)** it eliminates tumor cells in certain tissues because nascent transformed cells often co-express ligands for activating receptors on innate immune cells and tumor antigens (207).

The discovery that the immune system not only controls tumor quantity but also tumor quality (immunogenicity) prompted a major revision of the cancer

immunosurveillance hypothesis. This study revealed that tumors formed in mice that lacked an intact immune system were more immunogenic than similar tumors derived from immunocompetent mice. The notion that the immune system not only protects the host against tumor formation but also shapes tumor immunogenicity is the basis of the cancer immunoediting hypothesis (207). Cancer immunoediting process, in its most complex form, proceeds sequentially through three distinct phases termed “elimination”, “equilibrium”, and “escape” (207).

In the **elimination phase**, innate and adaptive immunity work together to destroy developing tumors long before they become clinically apparent. If, a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the **equilibrium phase,** in which its outgrowth is prevented by immunologic mechanisms. T cells, IL-12 and IFN-γ are required to maintain tumor cells in a state of functional dormancy, whereas NK cells and other cells of innate immunity are not required (208, 209). Editing of tumor immunogenicity occurs in the equilibrium phase. Equilibrium may also represent an end stage of the cancer immunoediting process and may restrain outgrowth of occult cancers for the lifetime of the host. However, as a consequence of constant immune selection pressure placed on genetically (210) unstable tumor cells held in equilibrium, tumor cell variants may emerge that **(i)** are no longer recognized by adaptive immunity, **(ii)** become insensitive to immune effector mechanisms, or **(iii)** induce an immunosuppressive state within the tumor microenvironment (207-212). These tumor cells may then enter the **escape phase,** which correspond to clinically apparent disease (207-212). Nanog, which is induced as a consequence of immune selection, enhanced the stem-like features of tumor cells and protected them from killing by tumor-reactive cytotoxic T cells (CTLs) (185).

It has been described that dormant tumor cells were more resistant to apoptosis induced by specific CTLs because they express more B7-H1 (PD-L1 / CD274), and such expression was proportional to the time they had persisted in the host (191). These data indicate that tumor dormancy may result from a balance between host immune response and active mechanisms developed by tumor cells to escape from CTLs (191).

6. Melanoma-specific T cells

T cells have an essential role in protection against a variety of bacterial and viral infections (213, 214). The fundamental characteristic in describing a T-cell response is its magnitude. This is commonly represented by the frequency of antigen-specific T cells or the expression of a specific effector function, such as IFN-γ production (213). $CD8^+$ T cells are a part of the adaptive branch of the immune system and key players in mediating immunity to intracellular pathogens and tumors.

Recognition of self-antigens on cancer presents problems. First, immunity to cancer may not develop because of immune tolerance. Second, even when the immune system can recognize and respond to self-antigens, immunity may not be sufficient to reject cancers. Finally, if immunity to self-antigens develops, there are potential autoimmune sequelae (215). Therefore, it is important to keep in mind, that the antitumor immunity may be limited and often insufficient to destroy a rapidly growing neoplasma, including its CSC pool, which arises from the organism´s own tissue and therefore predominantly expresses self-antigens (as TRP-2) to which host immune cells have been tolerated (216). Identification of tumor associated antigens (TAA) that can be recognized by CTLs has been a major advancement in understanding tumor immunity. Because TAAs are self-antigens, immune responses against them are regulated by central and peripheral tolerance mechanisms (217). Generally, highly self-reactive $CD8^+$ T cells are deleted during thymic development. However, deletion of self-reactive $CD8^+$ T cells is incomplete and self-reactive $CD8^+$ T cells can escape thymic selection and persist in the periphery under the control of several regulatory mechanisms, including anergy, ignorance, suppression, and deletion (217).

The majority of the human tumor-antigens characterized to date are derived from non-mutated self-proteins (85). TRP-2 is a non-mutated, self-protein (melanoma associated self-antigen) with a low immunogenicity that is expressed by both melanocytes and melanomas. It is an epitope recognized by both human and murine CTLs and is presented by the major histocompatibility class I haplotypes HLA-A0201 and $H\text{-}2K^b$, respectively (85, 217, 218). Vaccination with recombinant TRP-2 protein-pulsed dendritic cells (DCs) induced TRP-2-specific CTLs and immunity against B16 tumor (218, 219). In addition, vaccination with TRP-2 peptide-loaded

DCs delayed B16 tumor growth and prolonged mouse survival (219, 220). Analysis of the response in treated mice revealed elevated levels of $CD8^+$ T cells specific for a peptide consisting of residues 180-188 of TRP-2 ($TRP\text{-}2_{180\text{-}188}$). There was no evidence of reactivity to the melanocyte antigens gp100, tyrosinase, MART-1/Melan-A or TRP-1 (219, 221). The development of effective antitumor responses is normally constrained by low-avidity, tumor-specific CTLs that are unable to eradicate the tumor.

In recent years, T cell receptor transgenic (TCR-Tg) mouse models have provided crucial new insights into mechanisms driving vitiligo and effective melanoma immunotherapy. The pmel-1 CD8 TCR-Tg mouse was the first of such models, possessing a TCR specific for $gp100_{25\text{-}33}$ in the context of D^b (222, 223). In addition to Pmel mice, a TRP-2 specific TCR-Tg mouse with specificity for $TRP\text{-}2_{180\text{-}188}$ (termed clone 37) was more recently generated. These mice do not develop spontaneous vitiligo, and adoptive transfer of naïve Tg T cells fails to induce rejection of established B16 tumors. The low potency of these cells may be a reflection of a lower avidity TCR, as clone 37 was originally raised by vaccination of wild-type mice with murine TRP-2 (217, 222). A third model with specificity for $Tyr_{369\text{-}277}$ in the context of HLA-A2.1 expresses the TCR from a CD8 T cell clone that was isolated following vaccination of albino (tyrosinase-deficient) HLA-A2.1 transgenic mice (224, 225). In contrast to Pmel, clone 37 mice and $Tyr_{369\text{-}277}$ TCR Tg mice develop robust vitiligo (222). A fourth model generated in 2008 represents an MHC class II-restricted, TCR transgenic mouse model in which $CD4^+$ T cells recognize a novel epitope in TRP-1. Th1 cells are the most important in tumor rejection, whereas Th17-polarized cells more effectively mediated destruction of advanced B16 melanoma. Their therapeutic effect was critically dependent on IFN-γ production (226).

6.1 Memory T cells

Memory cells have several functional properties that distinguish them from naive cells, such as (a) an ability to respond to lower antigen concentrations, (b) faster proliferation following antigenic stimulation, (c) more rapid display of effector functions, (d) the potential to release a broader spectrum of cytokine, and (e) a pattern of adhesion molecules that allows access to peripheral tissues (227, 228). Moreover,

memory T cells are less dependent on costimulation provided by antigen-presenting cells (APCs) and can divide in the periphery long after antigen stimulation, sometimes even without any evidence for the persistence of their cognate antigen (227). BM of breast cancer patients was found to contain $CD8^+$ T cells specific for peptides derived from breast cancer-associated proteins (227). These tumor-infiltrating cells had a central or effector memory phenotype and produced perforin (227). Moreover, it has been shown a selective homing of memory T cells to human tumors which suggests that tumor rejection is based on the recognition of TAA on tumor cells and dendritic cells by autologous specifically activated central and effector memory T cells (227).

It has been reported that tumor cell-vaccinated mice harbor in their BM small numbers of dormant tumor cells and also memory T cells that provide protection against further tumor cell challenge (229, 230). The BM of breast cancer patients was enriched for tumor-specific memory T lymphocytes (231). Such T cells could be stimulated *in vitro* to produce IFN-γ and to acquire antitumor cytotoxicity *in vitro* and *in vivo* (227). Thus, BM could be considered as a major site for long-term persistence of tumor-specific memory T cells and of small numbers of dormant tumor cells that are kept under active host immune T cell control (228). Using a *ret* transgenic mouse melanoma model, it has been shown BM of mice without macroscopic primary tumors contain high frequencies of $CD8^+$ T cells specific for TRP-2 and showing mostly effector memory phenotype (232). Moreover, increased numbers of BM TRP-2-specific effector memory $CD8^+$ T cells were also detected in transgenic animals older than 20 weeks with disseminated melanoma cells in the BM and lymph nodes but showing no visible skin tumors and no further melanoma progression (232). These data indicate that functionally active BM-derived melanoma-specific memory T cells are detectable at the phase of microscopic tumor load, suggesting that thereby they could control disseminated melanoma cells (232).

Memory $CD4^+$ and $CD8^+$ T cells can be divided into two subsets, T_{CM} (Central memory T lymphocytes) and T_{EM} (Effector memory T lymphocytes) are defined based on two distinct criteria: **(a)** the presence of immediate effector functions and **(b)** the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues. In mouse, central memory $CD4^+$ T cells ($CD4^+$ T_{CM}) show $CD45RB^-$ $CD62L^+$ whereas effector memory $CD4^+$ T cells ($CD4^+$ T_{EM}) show $CD45RB^-$ $CD62L^-$ phenotype. On the other hand, central memory $CD8^+$ T cells ($CD8^+$ T_{CM}) display $CD44^{high}$ $CD62L^+$ and effector memory $CD8^+$ T cells ($CD8^+$ T_{EM})

display $CD44^{high}$ $CD62L^{-}$ phenotype (233).

7. Tumor-induced immunosuppression

In earlier stages of cellular transformation, immunosurveillance can detect and eliminate tumor cells. However, with a progressing tumor growth, tumor variants with reduced immunogenicity, and/or various acquired mechanisms to corrupt the host antitumor response and to escape from the host immune system, arise, survive and grow in the host (234-236). As a result, interactions between tumor cells and host immune cells in the tumor microenvironment create an immunosuppressive network in which CSCs might actively participate in inducing the immunosuppression and promoting an evasion from immunosurveillance due to their malignant potential (236). Cancer cells display multiple immunosuppressive mechanisms to evade T cell responses either by avoiding immune recognition or by disabling effector T cells (tumor cells can develop the lack of antigen presentation leading to tolerance or T-cell anergy) (123). These include alterations of components of the antigen presentation machinery, defects in proximal TCR signaling, secretion of immunosuppressive and/or pro-apoptotic factors, activation of negative regulatory pathways, and specific recruitment of regulatory cell populations (237), such as regulatory T cells (Tregs) (238-240) and myeloid-derived suppressor cells (MDSC) which may secrete immunosuppressive factors such as IL-10, TGF-β, arginase-1 and nitric oxide (NO).

Another mechanism which is utilized by tumor cells to block antitumor immunity is the induction of the exhausted phenotype of activated $CD8^{+}$ T cells through PD-L1 (B7-H1 or CD274) expression (216, 241-243). The expression of PD-L1 has been reported on solid tumor cells including melanoma cells (123, 244, 245). Moreover, murine and human tumor cells up-regulated PD-L1 upon IFN-γ stimulation, which led to the failure of tumor eradication (246, 247). Importantly, the expression of PD-L1 on leukemia cells has been reported as a marker of tumor dormancy (248, 249). Some other molecules such as CTLA-4 (CD152) (250-252), CD39 (ectonucleoside triphosphate), CD73 (253-255), and CCR4 (256-259), expressed on human tumor cells, including melanoma cells have been documented to impair anti-tumor responses.

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a cell surface receptor that behaves as a negative regulator of the proliferation and the effector function of T cells. Therefore, the negative role of CTLA-4 on T-cell activation contributes to the physiologic termination of the immune response. CTLA-4 inhibitory function occurs upon interaction with its ligands CD80 (B7.1) and CD86 (B7.2), expressed on APCs, resulting in inhibition of IL-2, IFN-γ, IL-4 cytokines production, IL-2 receptor expression and cell cycle progression (252). It has been described that CTLA-4 is constitutively expressed on several tumor cell lines including breast, colon, renal, lung, ovarian, uterine, bladder carcinoma, neuroblastoma and melanoma and can be used to trigger apoptosis upon ligand interaction (252). The chemokine receptor CCR4 is preferentially express on certain immune cells and some hematological tumor cells (leukemia and lymphoma cells), which play pivotal roles in suppression of host immune response. The selective expression of CCR4 on Th2 and Treg cells is important in regulating immune balance (256). Tregs preferentially express CCR4 compared to conventional T cells in both mice and humans (257, 260). The binding of CCL17 and CCL22 to CCR4 helps to guide Tregs toward DC. This interaction can suppress DC-mediated immune responses (257, 261). Aberrant expression of CCR4 in human gastric cancer contributes to tumor-induced immunosuppression (256).

Another mechanism that is used by tumors to evade immunosurveillance is the generation of adenosine within the tumor microenvironment, which potently suppresses antitumor T cell responses (259). Adenosine within the tumor is generated by CD73, a membrane-bound nucleotidase that is expressed by tumor cells, suppressive immune subsets such as Tregs and MDSC and endothelial cells. Recent evidence suggests that targeted inhibition of CD73 has the potential to reduce tumorigenesis and metastasis, as well as to enhance the potency of T-cell-direct therapies (259). As CD73, CD39 is an ecto-enzyme which coordinately works with CD73 to degrade ATP leading to the extracellular adenosine generation in the tumor microenvironment. Extracellular adenosine promotes tumor growth by limiting antitumor T-cell immunity via adenosine receptor signaling (254, 255).

8. Ret transgenic mouse spontaneous melanoma model

Conventional mouse melanoma models (e.g., B16) are based on the transplantation of tumor cells, in which the natural history of the disease is not comparable with the clinical situation In contrast to transplantation models, the recently described *ret* transgenic mouse model showed similarity to human melanoma with respect to histopathology and clinical development (232, 262, 263). Mice expressing the human *ret* proto-oncogene under the control of the mouse metallothionein-I (MT) promoter-enhancer in melanocytes develop spontaneously malignant cutaneous melanoma metastasizing to lymph nodes, lungs, brain, kidney, and spleen. This metastatic profile resembles that of human malignant melanoma (262, 263). Overexpression of Ret kinase is associated with the activation of other kinases such as mitogen-activated protein kinase (MAPK) and c-Jun as well as matrix metalloproteinases located downstream of the ret kinase (262, 264-266). The activation of Ras-Raf pathway induces the degradation of p27. Because p27 cannot inhibit the activity of cyclinD1-CDK4 complexes, these complexes are able to phosphorylate pRb. Therefore, pRb is completely inactive and E2F transcription factor remains active, allowing the progression of the cell-cycle from quiescence to the proliferative state (267). Furthermore Raf activates cyclin D1-CDK4 complexes which lead to cell proliferation (268, 269).

II. Aims of the study

CSCs could represent disseminated dormant tumor cells without clinical signs of tumor progression. Disseminated dormant tumor cells in the bone marrow (BM) of mice without macroscopic primary skin tumor could be involved in the maintenance of memory $CD8^+$ T cells. The objective of this work was to investigate the interactions between dormant tumor cells and memory T cells in the bone marrow (BM) of *ret* transgenic mice and to determine whether dormant tumor cells share characteristics and properties of CSCs. The following points were addressed:

a) To examine the expression of putative cancer stem cell markers in primary tumors and lymph nodes metastasis.
b) To test the localization of TRP-$2^+$$CD133^+$ melanoma cells in primary tumors.
c) To investigate the immunosuppressive, metastatic and angiogenic profile of $CD133^+$ melanoma cells.
d) To characterize the dormant state of disseminated TRP-$2^+$$CD133^+$ melanoma in the BM.
e) To detect the frequency of dormant melanoma cells either surrounded by or co-localized with memory $CD4^+$ T cells and memory $CD8^+$ T cells in the BM.
f) To identify TRP-2-specific and perforin-producing $CD8^+$ T cells co-localized with dormant melanoma cells in the BM.

III. Materials and methods

9. Materials

9.1 Antibodies used for flow cytometry

Specificity	Conjugated	Clone	Isotype	Company
CD133	PerCp-eFluor710	134A	rat IgG1,κ	eBioscience
CD133	PE	134A	rat IgG1,κ	eBioscience
CD133	FITC	134A	rat IgG1,κ	eBioscience
CD133	Alexa Fluor 488	134A	rat IgG1,κ	eBioscience
CD31	PE-Cy7	390	rat IgG2a,κ	eBioscience
CD31	FITC	390	rat IgG2a,κ	BD
CD31	APC	MEC 13.3	rat IgG2a,κ	BD
CD45.2	APC-Cy7	104	mouse IgG2a,κ	BD
CD45.2	PerCpCy5.5	104	mouse IgG2a,κ	BD
CD45.2	FITC	104	mouse IgG2a,κ	BD
ret	PE	132507	IgG1	R & D Systems
TRP-2	purified	polyclonal	IgG	Abcam
CD20	PE	AISB12	rat IgG2a	eBioscience
CD24	PE	MI/69	rat IgG2b,κ	eBioscience
CD34	Alexa Fluor 647	RAM34	rat IgG2a,κ	BD
CD39	PE-Cy7	24DMS1	rat IgG2b,κ	eBioscience
CD44	PE	IM7	rat IgG2b,κ	BD
CD44	FITC	IM7	rat IgG2b,κ	BD
CD73	FITC	AD2	mouse IgG1,κ	eBioscience
VEGFR2/CD309	PE	Avas12α1	rat IgG2a,κ	BD
CD152/CTLA-4	PE	UC10-4F10-11	armenian hamster IgG1,κ	BD
CD166	PE	eBioALC48	rat IgG2a,κ	eBioscience
CD183/CXCR3	APC	CXCR3-173	armenian hamster IgG	Biolegend
CD184/CXCR4	Biotin	2B11/CXCR4	rat IgG2b,κ	BD
CD194 /CCR4	APC	2G12	armenian hamster IgG	Biolegend
CD271	FITC	MLR-2	IgG2a	Stem Cell Biotechnologies
CD271	Alexa Fluor 647	C40-1457	mouse IgG1,κ	BD

CD274 /PD-L1	APC	10F.9G2	rat IgG2b,κ	Biolegend
ABCB5	purified	polyclonal	rabbit anti-mouse	Rockland
Nestin	purified	--------	rabbit anti-mouse	Sigma
HIF-1α	CFS	241812	mouse IgG1	R & D Systems
HIF-1 α	PE	241812	mouse IgG1	R & D Systems
Fc block (CD16/CD32)	Purified	2.4G2	rat IgG2b,κ	BD
Anti-biotin	PE Streptavidin	--------	--------	BD
anti-rabbit	Alexa Fluor 488	--------	--------	Invitrogene

9.2 Antibodies used for immunofluorescence

Specificity	Conjugated	Specie reactivity	Clone	Isotype	Company
CD133	purified	rat anti-mouse	134A	rat IgG1,κ	eBioscience
TRP-2	purified	rabbit anti mouse	policlonal	---------	Abcam
H-2k^{b}TRP-2$_{180-188}$ Dextramer	R-PE	mouse	--------	--------	Immudex
CD31	purified	rat anti-mouse	MEC 13.3	IgG2a,κ	BD
Ki67	Alexa Fluor 647	anti-mouse	B56	IgG1	BD
p16	purified	mouse anti-mouse	--------	IgG1	Abbiotec
p27	purified	mouse anti-mouse	57/Kipl/p27	IgG1	BD
PCNA	purified	mouse anti-mouse	24/PCNA	IgG1	BD
CD4	Alexa Fluor 647	rat anti-mouse	RM4-5	IgG2a,κ	Biolegend
CD8	Alexa Fluor 647	rat anti-mouse	53-6.7	IgG2a,κ	Biolegend
CD44	Alexa Fluor	rat	IM7	IgG2b,κ	Biolegend

	488	anti-mouse			
CD45RB	FITC	rat anti-mouse	C363-16A	IgG2a,κ	Biolegend
Perforin	FITC	rat anti-mouse	CB5.4	IgG2a	Abcam
IFN-γ	purified	rat anti-mouse	R4-6A2	IgG1,κ	eBioscience
anti-rabbit	Alexa Fluor 488	goat anti-rabbit	--------	--------	Invitrogene
anti-rat	Alexa Fluor 555	goat anti-rat	--------	--------	Invitrogene
anti-rat	Alexa Fluor 594	goat anti-rat	--------	--------	Invitrogene
anti-rabbit	Alexa Fluor 405	goat anti-rabbit	--------	--------	Invitrogene

9.3 Chemicals

Component	Abbreviation	Company	Catalog number
Bovine serum albumin	BSA	Sigma	7030-50G
Dimethylsulfoxid	DMSO	Merck	109678
Ethylenediaminetetra-acetic acid	EDTA	GIBCO	15575-098
100% acetic acid	CH_3COOH	Merck	100063
Fetal bovine serum	FBS	PAN Biotech	3702-P260718
Phosphate buffered saline	PBS	PAA	H15-002
Penicillin / Streptomycin	P/S	PAA	P11-010
Trypan blue solution		Sigma	T8154
$Tween_{20}$		Sigma	P-2287
Goat serum		GIBCO	PCN5000
Collagenase II		Gibco	1701-015
Collagenase IV		Gibco	17104-019
DNAase-I		Sigma	056K7680
Gelatin		Merck	1.04078.1000
methanol		Merck	

Tissue-Tek		Sakura	116600003
acetone		Merck	1.00014.2511
Glycine	Gly, G	Roth	3908.1
Roswell Park Memorial Institute medium 1640	RPMI-1640	PAA	10 236 276 001
Fetal calf serum	FCS	PAA	
goat serum	gs	PAA	
4',6-Diamidine-2'-phenylindole dihydrochloride	DAPI	Roche	10236276001
Propidium diodide	PI	BD	51-66221E
SytoxRed		Invitrogene	S34859
Hoechst 33258 (blue dye)		Sigma	861405
Superfrost-plus	slides	Thermo scientific	J1800AMNZ
Fluoromount-G		SoutherBiotech	0100-01

9.4 Routine laboratory materials, equipment and instruments

Devices and equipment

Equipment	Model	Company
Flow Cytometry	FACS Canto II	BD
Fume hood	STA 120 1297	Prutscher
Refrigerator (-80 °C)	HeraFreeze	Heraeus
Refrigerator (-20 °C)	Premium	LiebHerr
Incubator	HeraCell	Heraeus
Inverse microscope		Leica
Fluoresce microscope	DC500	Leica
Microscope camera		Canon
Confocal microscope	DMRE	Leica
Confocal microscope	LSM-710	Carls Zeiss
Microtome	RM 2125 RT	Leica
Microtome water bath	HI 1210	Leica
Microwave oven	R-352	Sharp
pH meter	766	Calimatic

Photometer	UltroSpec 3100pro	Amersham
Laminar flow cabinet	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	Vortex Genie 2	Scientific Industries
Balance	BP 3100P	Sartorius
Water bath	DC3	HAAKE / GFL
Centrifuger	Labofuge 400R	Heraeus
Centrifuger	Biofuge pico	Heraeus
Centrifuger	Biofuge primo R	Heraeus
Centrifuger	Varifuge K	Heraeus
Centrifuger	RT 7 Plus	Thermo Electron Cooperation

Routine laboratory materials

Material / Instrument	Model	Company
Needles	0,4x19 mm Mikrolance	BD
Needles	0,3x13 mm Mikrolance	BD
Cryo tubes	Cryos	Greiner
2-20 µl, 20-200 µl, 200-1000 µl; Pipettes with adjustable volumes		Rainin
Coverglass	24 x 24 mm, Roth	
Tubes 15 ml / 50 ml	Polypropylen Falcon	Falcon
0.5, 1.5 & 2 ml microtubes		Eppendorf
1, 5 ml Syringes		BD
Cell culture plates	96-well-Platte	Greiner
100 µm cell strainer	Nylon	BD

9.5 Buffers

9.5.1 Buffers for flow cytometry

To prepare single tumor cell suspensions from mLN, we used FACS buffer-1 which contained 2mM EDTA, and 0.1% BSA in PBS without calcium and magnesium ($PBS^{without\ Ca/Mg}$). FACS buffer-2 consisted of 2mM EDTA, 0.1% BSA, in 1X permeabilization buffer (eBioscience Cat. 00-8333). Both FACS buffer-1 and -2 enable to keep single tumor cell suspensions, avoiding clump formation. We used Foxp3 Staining Buffer Set (cat number 00-5523). Fixation /Permeabilization Concentrate (Cat. 00-5123). Fixation / Permeabilization diluent (Cat. Nr. 00-5223). Permeabilization buffer (10X) (Cat. 00-8333).

9.5.2 Buffers for immunofluorescence

We prepared immunofluorescence buffer (IF-buffer) diluting 0.2% gelatin in PBS. IF-buffer-goat serum (IF-gs) consisted of 2% goat serum in IF-buffer.

9.6 *ret* transgenic spontaneous melanoma mouse model

Mice (C57BL/6 background), which express human *ret* transgene in melanocytes under the control of mouse metallothionein I promoter-enhancer were crossed and kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg, Germany). Experiments were performed in accordance with government and institute guidelines and regulations. The general performance of mice was monitored daily. Spontaneous tumor development was assessed macroscopically.

10. Methods

10.1 Single-cell suspension preparation

Fresh tumors and lymph nodes collected from *ret* transgenic tumor bearing mice were immediately transferred into PBS with calcium and magnesium (PBS with $^{Ca/Mg}$) and briefly stored on ice. After removal of necrotic tissue and fat, tumor biopsies were mechanically dissociated mincing with a scalpel into small pieces and enzymatically

digested with 1:1 (v/v) of a fresh solution of 0.25% collagenase II, 0.25% collagenase IV and 0.05% DNAase-I in $PBS^{without\ Ca/Mg}$ (this PBS reduces the collagenase activity) for 15 min at 37°C (shaking gently every 3 min). Briefly, these two specimens were washed with 10 ml of RPMI-10 FCS% and filtrated through 100 μm nylon mesh. Lysis buffer of red blood cells was not used. Lysis of red blood cells was made in the permeabilization step, in order to preserve cell surface antigens.

10.2 Flow cytometry

Single tumor cell suspensions were fixed and permeabilized with Foxp3 staining buffer set (fixation/permeabilization concentrate, and fixation/permeabilization diluent) for 30 min at 4°C. Then cell suspension was washed twice with FACS buffer-2 and incubated with 1 μL Fc block /$1x10^6$ cells for 20 min at 4°C and stained with polyclonal / monoclonal antibodies for 40 min at 4°C. Acquisition was done by six-color flow cytometry using a Canto II with FACS-Diva software (Bioscience) and dead cell and duplex exclusion based on scatter profile. FlowJo software (Tree Star) was used to analyze at least $1X10^6$ events. Data were expressed as dot plots or histograms.

10.3 Immunofluorescence

$CD133^+TRP\text{-}2^+$ melanoma cells in primary skin tumors, mLN and BM

Primary tumors express melanoma associated antigens like tyrosinase, tyrosinase related protein (TRP)-1, TRP-2 and gp100, which can be used for the melanoma detection. In this study, $CD133^+TRP\text{-}2^+$ melanoma cells were analyzed in primary skin tumors and mLN of tumor bearing mice using crysections and triple immunofluorescence (IF)-sequential protocol. The tissue specimens were surrounded and covered with Tissue-Tek and gradually frozen with isopentan up -80°C (first, samples were exposed with isopentan for 10 min at -20°C; second, samples in isopentan were placed within liquid nitrogen for 10 min at -150°C; third, samples embedded in tissue-Tek were exposed directly to liquid nitrogen for 10 min and preserved at -80°C). Consecutive cryostat sections 5 μm in thickness were fixed and permeabilized with methanol-acetone (7:3) for 10 min at -20°C, incubated with 0.1M

of glycine in PBS for 30 minutes at 4°C and first blocking step with 10% goat serum in IF-buffer was done for 30 min at 37°C. The samples were washed twice with IF-buffer and stained with first primary monoclonal rat anti-mouse antibodies against CD133 (12 μg/ml in IF-gs) overnight at 4°C, washed 5 times with IF-buffer and incubated with first secondary goat anti-rat antibodies labeled with Alexa-Fluor 594 (1:500 in IF-gs) for 1h at 4°C. The samples were washed 4 times with IF-buffer and second blocking step with 10% goat serum in IF-buffer was done.

For the identification of TRP-2, the samples were incubated with second primary polyclonal rabbit anti-mouse antibodies against TRP-2 (1:2000 in IF-gs) for 1h at 4°C, washed 4 times with IF-buffer and incubated with second secondary goat anti-rabbit antibodies labeled with Alexa-Flour-488 (1:1000 in IF-gs) for 1h at 4°C. The samples were washed 4 times with IF-buffer and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (1:3000 in PBS) for 3 min at room temperate (RT). As control we incubated the samples with first secondary goat anti-rat antibodies labeled with Alexa-Fluor 594 and second secondary goat anti-rabbit antibodies labeled with Alexa-Flour-488 as well as specimens of healthy skin and lymph nodes from wild type mice. Confocal microscopy was acquired using a confocal laser scanning microscope LSM-710 (Carl-Zeiss, Jena, Germany) under a 40 times oil-immersion objective and data were analyzed by ZEN software (Carl-Zeiss, Jena, Germany).

BM specimens were isolated from femurs by aspiration and $1x10^5$ BM cells /sample were prepared using cytospin technique. The specimens were fixed and permeabilized with methanol-acetone (7:3) for 10 min at -20°C. For CD133, TRP-2 and nuclei staining, we performed triple IF-sequential protocol as well, such as described above. We analyzed in total 10^6 BM cells per mouse from 20 mice without macroscopic skin tumors and 20 mice bearing macroscopic tumors.

Cell cycle status of $CD133^+TRP\text{-}2^+$ melanoma cells in the BM

BM samples were isolated from femurs by mechanical dissection, mounted on glasses through smear technique, fixed and permeabilized with methanol-acetone (7:3) for 10 min at -20°C. For CD133 and TRP-2 staining, we performed triple IF-sequential protocol as well, such as described above, with the exception of nuclei

which were stained with propidium iodide (1:1000 in PBS) for 10 min at the end of the whole staining. For the analysis of cyclin-dependent kinase inhibitors (CDKI) the following primary antibodies were used: mouse anti-p16 (1:50 in IF-gs) and mouse anti-p27 (1:100 in IF-gs). As indicators of cell cycle progression, the primary mouse antibodies against PCNA (1:100 in IF-gs), and mouse antibody anti-Ki67 labeled with Alexa Fluor 647 (1:10 in IF-gs). Isotype-matched irrelevant antibodies served as negative controls. For the identification of the antibodies mouse anti-p16, mouse anti-p27 and mouse anti-PCNA we used the secondary antibodies goat anti-mouse labeled with Alexa Fluor 405 (1:500 in IF-gs). The table 1 illustrates the panel of dyes used.

Table 1. Immunoflorescence staining for the determination of the dormancy status of $CD133^{+}TRP\text{-}2^{+}$ melanoma cells in the BM of *ret* transgenic mice.

A	*IF staining for p16*	
CD133	→	Alexa Fluor-555® (orange)
TRP-2	→	Alexa Fluor 488 (green)
Ki67	→	Alexa Fluor-647® (pink)
p16	→	Alexa Fluor-405® (blue)

B	*IF staining for p27*	
CD133	→	Alexa Fluor-555® (orange)
TRP-2	→	Alexa Fluor-488® (green)
Ki67	→	Alexa Fluor-647® (pink)
p27	→	Alexa Fluor-405® (blue)

C	*IF staining for PCNA*	
CD133	→	Alexa Fluor-555® (orange)
TRP-2	→	Alexa Fluor 488(green)
Ki67	→	Alexa Fluor-647® (pink)
PCNA	→	Alexa Fluor-405® (blue)

Blood vessels staining in primary tumors

To analyze the vascularization in primary skin tumors of *ret* transgenic mice, we performed cryosections and IF staining. Cryosections were prepared, fixed, permeabilized and preserved as described above. Then, the specimens were incubated with 0.1M of glycine in PBS for 30 minutes at 4°C and blocked with 10% goat serum in IF-buffer for 30 min at 37°C. The samples were washed twice with IF-buffer and stained with primary monoclonal rat anti-mouse antibodies against CD31 (1:50 in IF-gs) for 2h at 4°C, washed 5 times with IF-buffer and incubated with secondary goat anti-rat antibodies labeled with Alexa-Fluor 555 (1:250 in IF-gs) for 1.5h at 4°C. The

samples were washed 4 times with IF-buffer and nuclei were stained with DAPI (1:3000 in PBS) for 3 min at RT. As control we incubated the samples with secondary goat anti-rat antibodies labeled with Alexa-Fluor 555 as well as specimens of healthy skin from wild type mice.

Co-localization of CD133^{+} / CD133^{-} tumor cells with T cells in the BM

To study the interaction between dormant tumor cells and memory T cells in the BM of *ret* transgenic mice, we used smear technique and IF staining. For CD133 and TRP-2 staining, we performed triple IF-sequential protocol such as described above, with the exception of nuclei was not stained and the secondary goat anti-rabbit antibodies labeled with Alexa Fluor 405 were used to detect antibodies rabbit anti-TRP-2. Third blocking step with 10% goat serum in IF-buffer was included and the samples were washed 3 times with IF-buffer. We analyzed smears from BM of mice without and with macroscopic primary tumors. Then, the specimens were stained using a multicolor IF protocol for the identification of memory T cells as described below.

Memory CD4^{+} T cells

For the identification of memory CD4^{+} T cells, the samples were incubated with rat monoclonal antibodies against CD45RB labeled with FITC (1:250 in IF-gs) and rat monoclonal antibodies against CD4 labeled with Alexa-Fluor-647 (1:250 in IF-gs) for 1h at RT.

Memory CD8^{+} T cells

For the identification of memory CD8^{+} T cells, the samples were incubated with rat monoclonal antibodies against CD44 labeled with Alexa-Fluor-488 (1:250 in IF-gs) and rat monoclonal antibodies against CD8^{+} labeled with Alexa-Fluor-647 (1:250 in IF-gs) for 1h at 4°C (Table 2A).

TRP-2-specific-, perforin-producing CD8$^+$ T cells

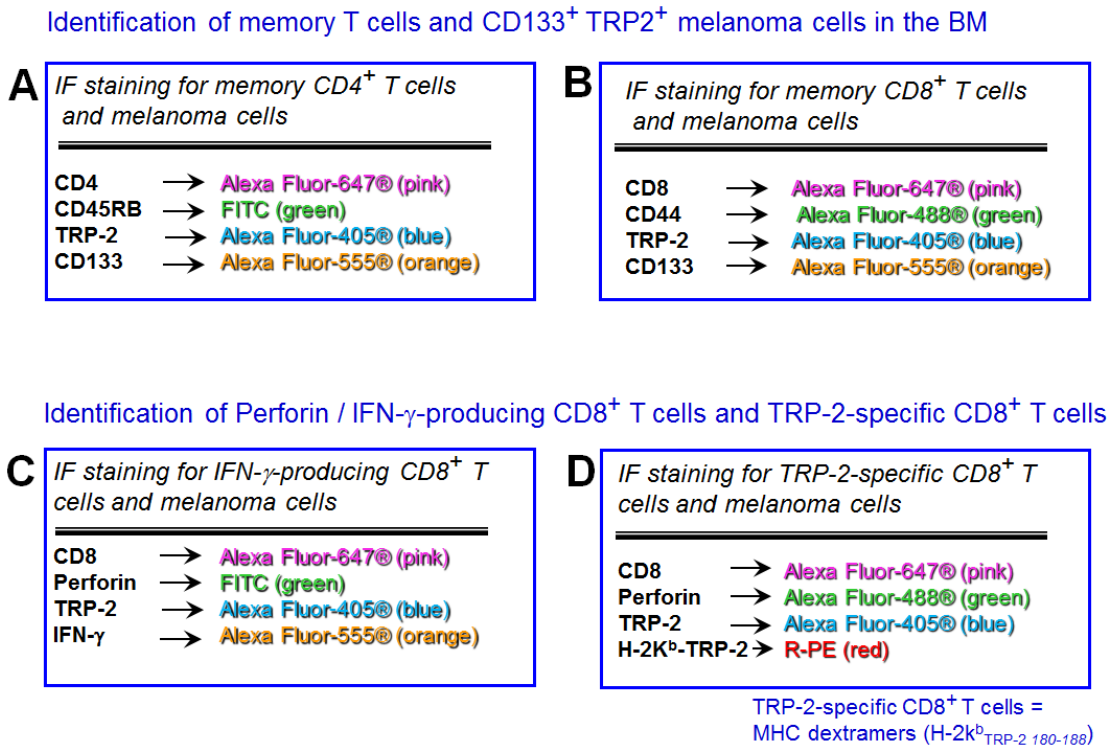
The samples were incubated with rat monoclonal antibodies against CD8$^+$ labeled with Alexa-Fluor-647 (1:250 in IF-gs), rat monoclonal antibodies against perforin labeled with FITC (1:500 in IF-gs) and H-2K^b-TRP-2$_{180-188}$ Dextramer labeled with R-PE (Table 2B).

IFN-γ-, perforin-producing CD8$^+$ T cells

The samples were incubated with rat monoclonal antibodies against IFN-γ (1:250 in IF-gs) for 1h at 4°C, washed 4 times with IF-buffer and stained with secondary goat anti-rat antibodies labeled with Alexa-Flour-555 (1:500 in IF-gs) for 1h at 4°C. Briefly, the samples were washed 4 times with IF-buffer and stained with rat monoclonal antibodies against CD8$^+$ labeled with Alexa-Fluor-647 (1:250 in IF-gs), and rat monoclonal antibodies against perforin labeled with FITC (1:250 in IF-gs) (Table 2C and 2D).

Experimental design

Table 2. Immunoflorescence staining for memory CD4$^+$ and memory CD8$^+$ T cells.



11. Statistical analysis

We used software Flowjo (Version 7.2.4), Tree Star, Inc., Ashland, USA. All data are shown as mean ± SEM for the indicated number of independent experiments. Results were assessed with a Student´s t test and Mann-Whitney U test. Statistical analyses were performed using Graph-Pad Prism software (San Diego, USA). All statistical tests were two-sided. A value of $p < 0.05$ was considered statistically significant.

IV. Results

There is an intensive debate on protocols for preparing single cell suspensions from primary tumor specimens for flow cytometric analysis of CSCs (103, 125, 270). A great majority of routine procedures consist of mechanical dissociation followed by enzymatic disaggregation where tumor specimens are incubated longer at 37°C (overnight as maximum to 0.75-2 h as minimum). Most of the enzymatic protocols use trypsin, which is tremendously aggressive because cleave proteins on the cells surface, removing the antigens that are under investigation (124, 163). Although other protocols use collagenase II and collagenase IV which are much less aggressive, tumor specimens are incubated longer (0.75-2 h) or followed by short incubation with low concentration of trypsin (124). Hence reliable methods are required for avoid the excision of antigens used as CSC markers. We applied a gentle protocol to prepare single tumor cell suspensions by incubating tumor tissues with collagenase II, collagenase IV and DNAase for a brief period of incubation at 37°C (15 minutes) shaking gently to ensure the preservation of antigens on the cell surface. Applying this protocol we obtained detection of CSCs and a higher index of live cells (more than 95%).

We investigated cancer stem-like cells in *ret* transgenic mice using CD133 (Prominin-1), which has been widely shown to be a marker of cancer stem-like cells in various solid tumors, including melanomas (168-181). Freshly isolated cells from primary skin tumors and mLN were analyzed by FACS staining: leukocytes and endothelial cells were excluded using CD45.2 and CD31 staining respectively. Duplex exclusion and dead cells were based on scatter profile. Tumor cells where stained with antibodies for TRP-2, CD133, and Ki67 (proliferation marker). Fig. 1 and 2 show the FACS staining strategy for the analysis of $CD133^{+}Ki67^{+}$ tumor cells in primary skin tumors and mLN, respectively.

The data revealed that mLN contained almost 5-fold higher numbers of $TRP\text{-}2^{+}CD133^{+}$ melanoma cells in comparison with primary skin tumors (Fig 3A). We also found that mice with larger primary tumors have more $TRP\text{-}2^{+}CD133^{+}$ melanoma cells both in tumors and mLN than mice with smaller primary tumors (Fig. 3B, C).

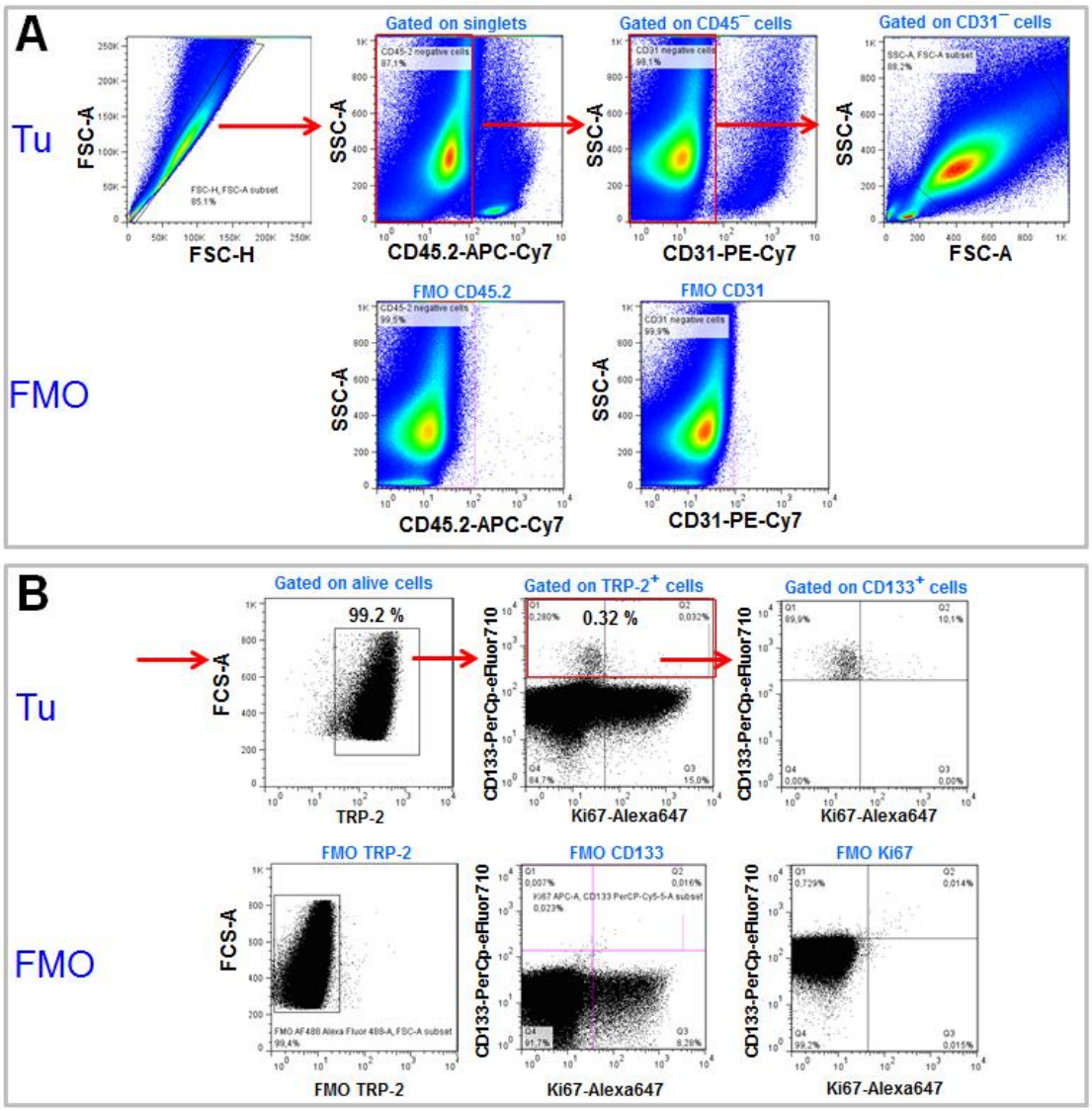


Figure 1. CD133$^+$ expression in primary tumors. Single-cell suspensions prepared from tumors of *ret* transgenic mice were stained with antibodies for CD45.2, CD31, TRP-2, CD133 and Ki67 followed by flow cytometry. **(A)** Duplex and dead cells were excluded based on scatter profile, then leukocytes and endothelial cells were excluded using CD45.2 and CD31 markers respectively. **(B)** Tumor cells were gated based on TRP-2$^+$ cells, TRP-2$^+$CD133$^+$ cells and TRP-2$^+$CD133$^+$Ki67$^+$ subpopulations. The fluorescence minus one (FMO) controls were made for all dyes. Representative dot plots are shown. Tu = primary tumor. The red arrows indicate the flow of the dot plots.

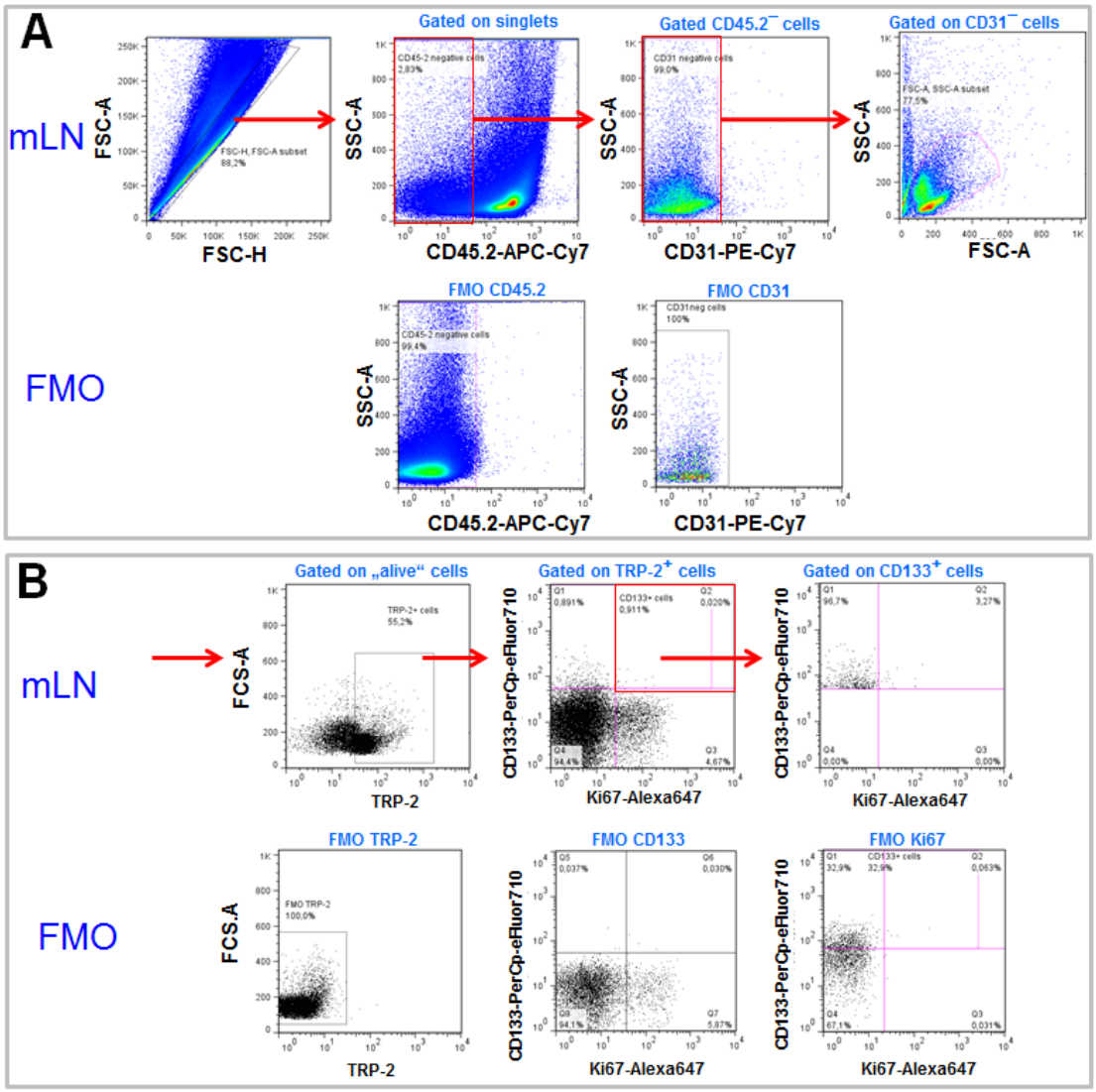


Figure 2. CD133$^+$ expression in metastatic lymph nodes. Single cell suspensions prepared from tumors of *ret* transgenic mice were stained with antibodies for CD45.2, CD31, TRP-2, CD133 and Ki67 followed by flow cytometry. **(A)** Duplex and dead cells were excluded based on scatter profile, then leukocytes and endothelial cells were excluded using CD45.2 und CD31 markers respectively. **(B)** Tumor cells were gated based on TRP-2$^+$ cells, TRP-2$^+$CD133$^+$ cells and TRP-2$^+$CD133$^+$Ki67$^+$ subpopulations. The fluorescence minus one (FMO) controls were made for all dyes. Representative dot plots are shown. mLN= metastatic lymph nodes. The red arrows indicate the flow of the dot plots.

12. CD133$^+$ melanoma cells are in a dormant state in larger primary skin tumors

It has been reported that in melanoma patients treated with chemo- and radiotherapy, the emersion of tumors from residual tumor cells, which survived long-term in a dormant state led to tumor relapse (26, 190). We assumed that dormant tumor cells may share characteristics and properties with CSCs. To investigate the dormancy status of CD133$^+$ melanoma cells, the proliferative marker Ki67 was used

to distinguish between proliferating and quiescent (dormant) melanoma cells. We found that CD133$^+$ melanoma cells from primary and mLN had an equal rate of proliferation (Fig. 3D). Interestingly, the progression of primary tumors was demonstrated to be correlated with a reduced amount of CD133$^+$ melanoma cells in proliferative state (checked by the expression of Ki67) (Fig. 3E). In contrast, CD133$^+$ melanoma cells in mLN from mice with larger primary tumors were highly proliferating (Fig. 3F). We conclude that CD133 is expressed by a fraction of TRP-2$^+$ melanoma cells (less than 1.5 %) and their dormant state is dependent on the weight of primary tumors.

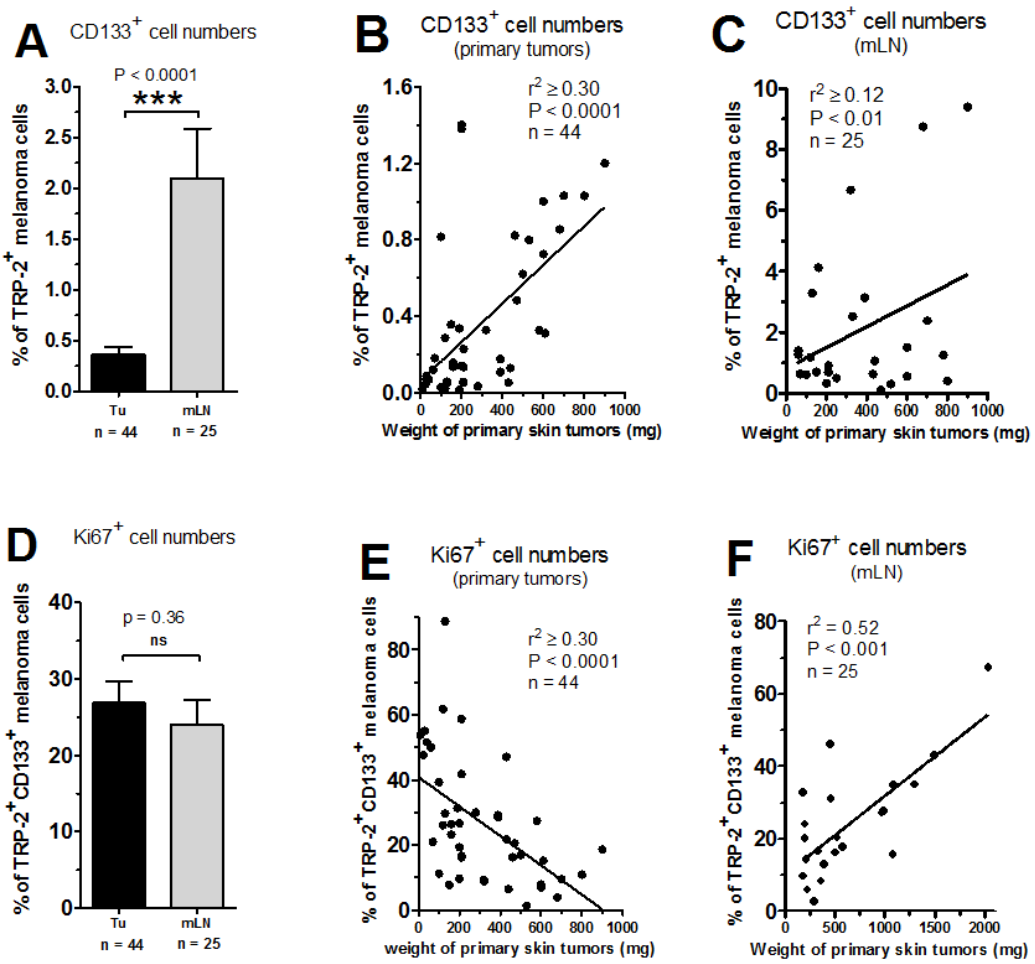


Figure 3. CD133$^+$ expression in *ret* transgenic mice. Single-cell suspensions prepared from freshly isolated primary tumors and mLN of *ret* transgenic mice were stained with antibodies for CD45.2, CD31, TRP-2, CD133, and Ki67 followed by flow cytometry. Gating strategy was done as described in figures 1 and 2. **(A)** CD133$^+$ melanoma cell numbers in primary tumors (Tu) compared to mLN. The percentage of CD133$^+$ melanoma cells was plotted against tumor weights in **(B)** primary tumors and **(C)** mLN. **(D)** CD133$^+$KI67$^+$ melanoma cell numbers in primary tumors (Tu) compared to mLN. The percentage of CD133$^+$KI67$^+$ melanoma cells was plotted against tumor weights in **(E)** primary tumors and **(F)** mLN. ns= not statistically significant.

13. CD133$^+$ melanoma cells are located in peripheral areas in primary tumors

We performed serial cryosections of primary tumors followed of immunofluorescence staining for TRP-2, CD133, Ki67 and DAPI. We observed that CD133$^+$ melanoma cells form clusters and seem to be predominantly located in the peripheral tumor areas (Fig. 4).

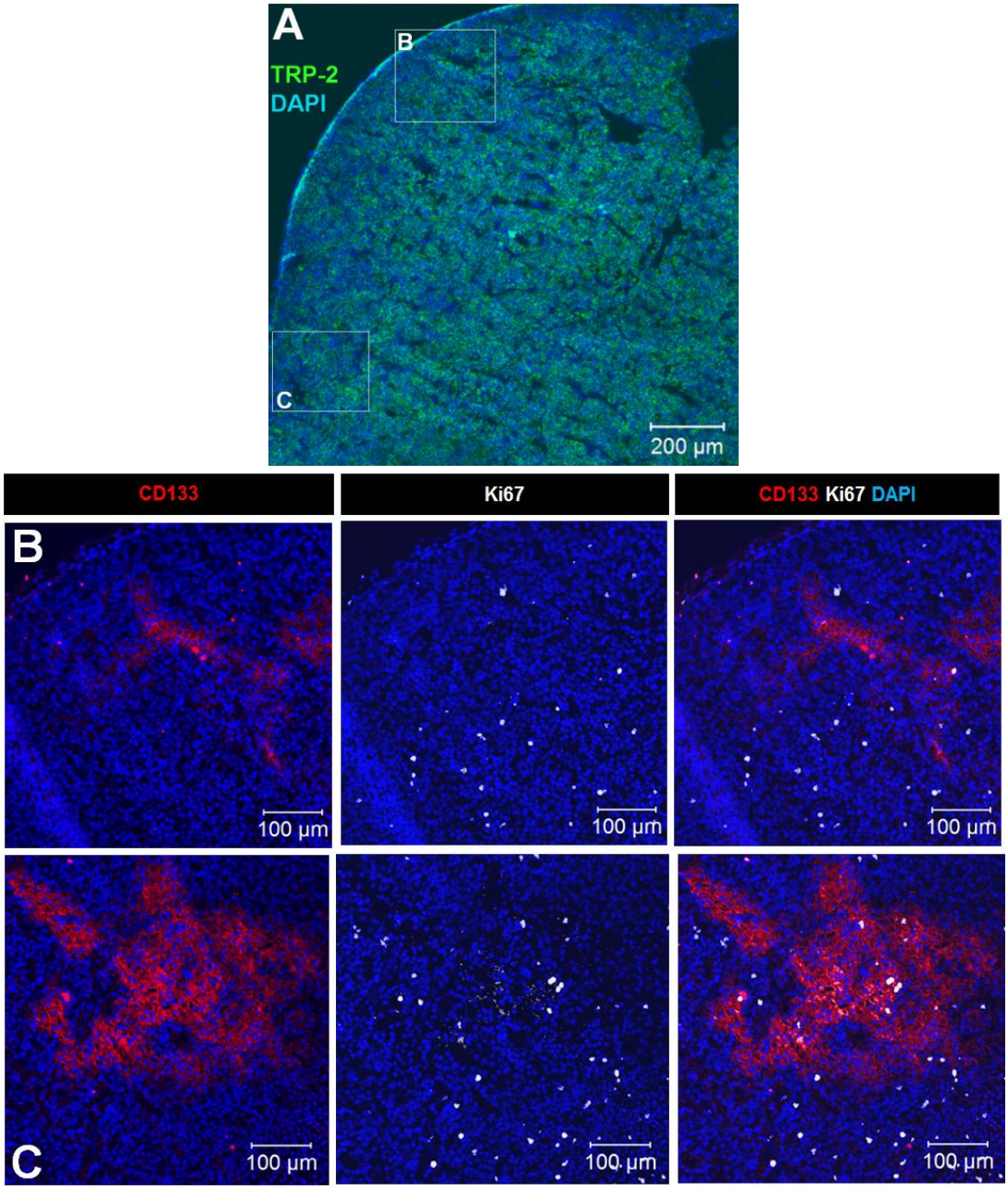


Figure 4. Localization of CD133$^+$melanoma cells in primary tumors of *ret* transgenic mice. Consecutive cryosections of tumors were stained with antibodies for TRP-2, CD133, and Ki67 and counterstained with DAPI. **(A)** Tumor section showing the areas (white squares) where CD133$^+$melanoma cells were found. **(B, C)** CD133 positive cells (red), Ki67 positive cells (gray), and nuclei (blue). Merge showing the low rate of proliferation of CD133$^+$ melanoma cells.

14. CD133$^+$ melanoma cells seem are localized in aberrant vascularized areas

Neovascularization is critical for the tumor optimal growth, cell survival and efficient tumor dissemination (271, 272). CSCs may create the niche necessary for the maintenance of tumor cells (273, 274). Aberrant architecture of blood vessels may be an indicator of tumorigenicity (271, 272). To investigate the structural distribution of blood vessels, especially in areas where CD133$^+$ melanoma cells are localized, we performed the IF staining of primary tumors using antibodies for CD31 to visualize the tumor vascularization. We found that aberrant blood vessels were variable in size, shape and branching pattern, and were not organized in conventional hierarchy of arterioles, capillaries and venules. These blood vessels have a predominant distribution in peripheral areas of tumors (Fig. 5A-C). These areas might be the niche for CD133$^+$ melanoma cells within the tumor. Further experiments are needed to confirm these observations.

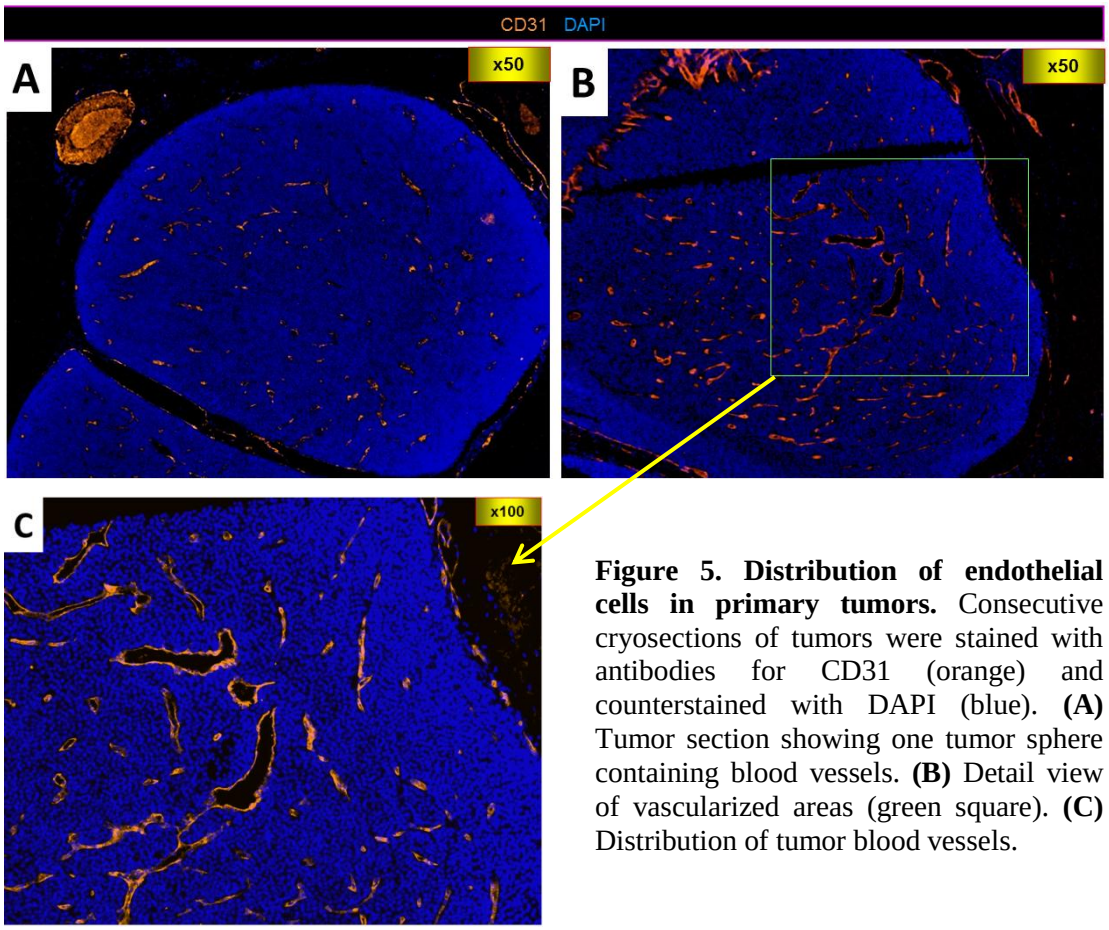


Figure 5. Distribution of endothelial cells in primary tumors. Consecutive cryosections of tumors were stained with antibodies for CD31 (orange) and counterstained with DAPI (blue). **(A)** Tumor section showing one tumor sphere containing blood vessels. **(B)** Detail view of vascularized areas (green square). **(C)** Distribution of tumor blood vessels.

15. HIF-1α and CD271 expression in CD133$^+$ melanoma cells

It has been reported that restricted oxygen conditions increase the CSC fraction and promote acquisition of a stem-like state (148). CSCs might critically be dependent on hypoxia-inducible factor 1α (HIF-1α) for survival, self-renewal and tumor growth (148, 149). To examine the expression of HIF-1α, single tumor cell suspensions isolated from primary skin tumors and mLN were analyzed by FACS staining (Fig. 6). The data revealed that mLN nodes contain elevated numbers of TRP-2$^+$CD133$^+$HIF-1α$^+$ melanoma cells in comparison with primary tumors (Fig. 7A). We observed that larger primary tumors had the tendency to contain higher numbers of TRP-2$^+$CD133$^+$HIF-1α$^+$ melanoma cells (Fig. 7B), whereas the frequency of these cells was decreased in mLN from animals with larger tumors (Fig. 7C). Interestingly, TRP-2$^+$CD133$^+$HIF-1α$^+$ melanoma cells had an elevated capacity to proliferative in primary tumors in comparison with those in mLN (Fig. 7 D). These findings suggest that HIF-1α plays a crucial role in the maintenance of CD133$^+$ melanoma cells at the beginning of the tumor formation. In fact, we observed an elevated number of TRP-2$^+$CD133$^+$HIF-1α$^+$ melanoma cells in smaller primary tumors and mLN with an increased rate of proliferation (Fig. 7 E, F).

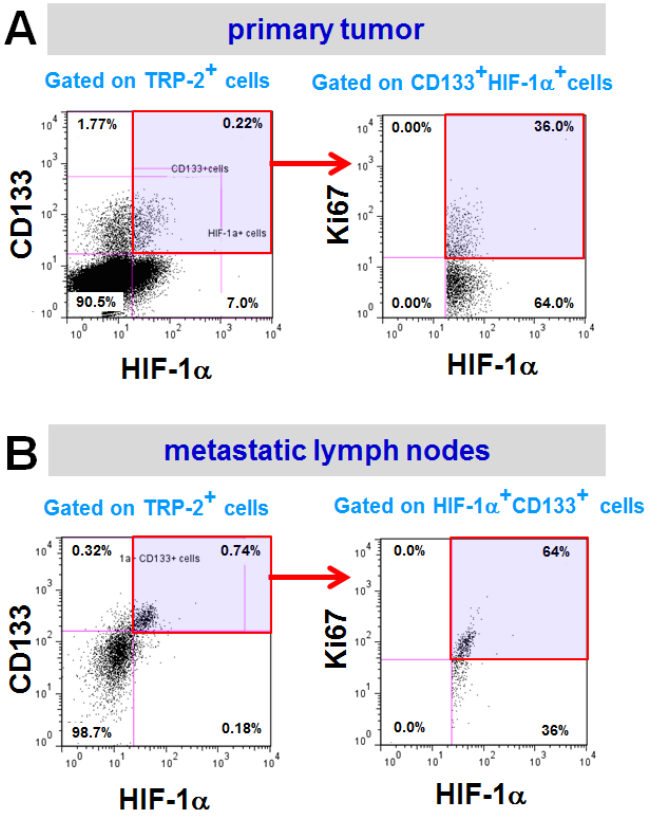


Figure 6. HIF-1α expression in CD133$^+$ melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and HIF-1α$^+$ expression on TRP-2$^+$CD133$^+$ tumor cells and Ki67 expression on TRP-2$^+$CD133$^+$HIF-1α$^+$ cells were analyzed in **(A)** primary tumors and **(B)** mLN. Representative dot plots are shown.

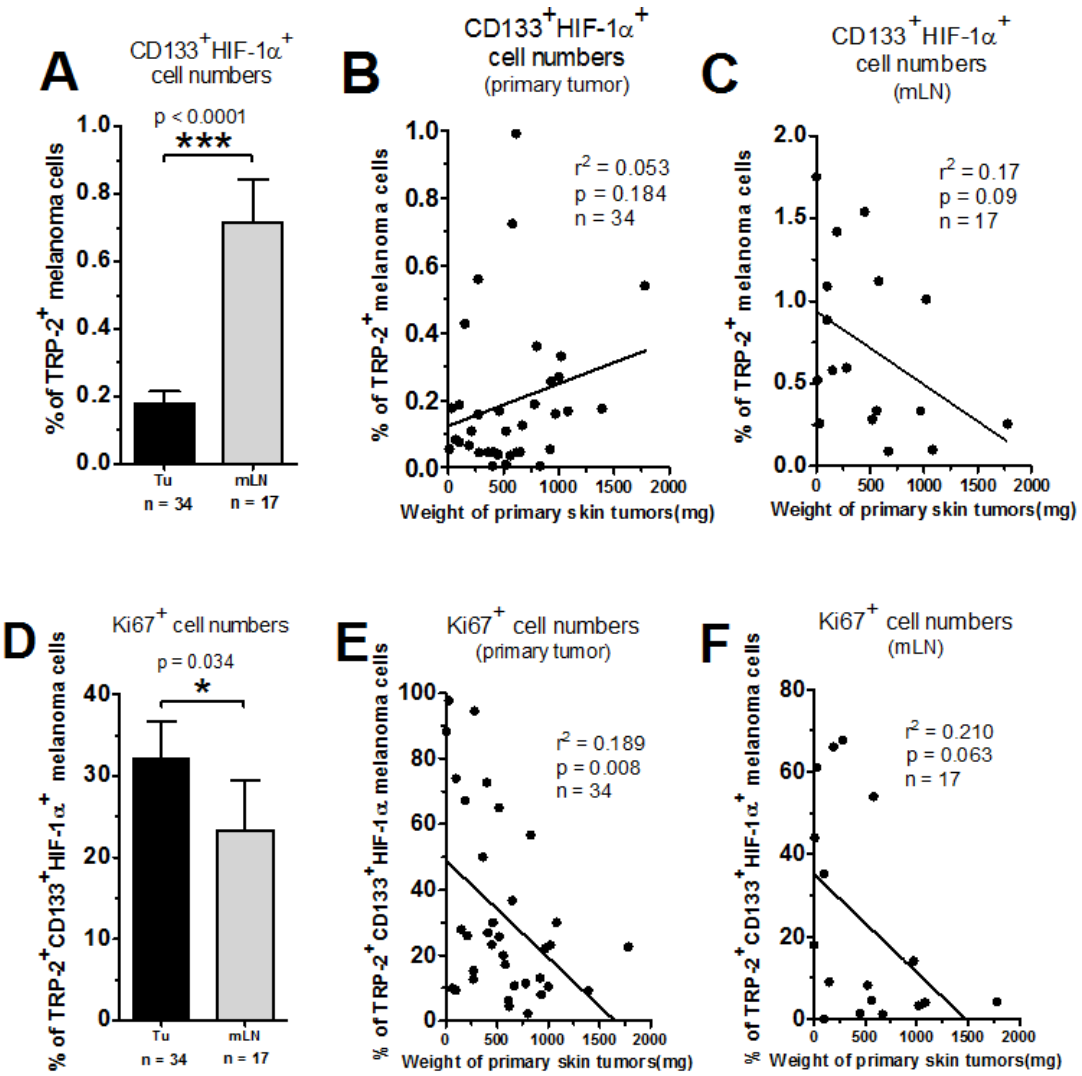


Figure 7. HIF-1α expression in CD133$^+$ melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and HIF-1α$^+$ expression on TRP-2$^+$CD133$^+$ tumor cells and Ki67 expression on TRP-2$^+$CD133$^+$HIF-1α$^+$ cells were analyzed. Gating strategy was done as described in figure 6. **(A)** CD133$^+$HIF-1α$^+$ melanoma cell numbers in primary tumors (Tu) compared to mLN. The percentage of TRP-2$^+$CD133$^+$ HIF-1α$^+$ melanoma cells was plotted against tumor weights in **(B)** primary tumors and **(C)** mLN. **(D)** TRP-2$^+$CD133$^+$HIF-1α$^+$KI67$^+$ melanoma cells in primary tumors (Tu) compared to mLN. The percentage of TRP-2$^+$CD133$^+$HIF-1α$^+$KI67$^+$ melanoma cells was plotted against tumor weights in **(E)** primary tumors and **(F)** mLN.

CD271 has been recently described as a melanoma stem cell marker with an increased tumor-initiating capacity as compared with CD271$^-$ tumor cells (124). CD271 melanoma cells have shown multipotency, self-renewal capacity, to be maintained in several rounds of serial retransplantations and higher metastatic potential (125). To address the question whether the expression of CD271 in TRP-2$^+$CD133$^+$ melanoma cells is dependent on tumor progression, melanoma cells

from primary tumors and mLN were stained with antibodies for CD271, CD133, Ki67 and TRP-2 followed by flow cytometry (Fig. 8). Lymph node metastasis displayed a tendency to elevate the numbers of $CD133^{+}CD271^{+}$ cells in comparison to primary tumors (Fig. 9A). We did not observed any correlation of tumor progression for $TRP\text{-}2^{+}CD133^{+}CD271^{+}$ cell numbers in primary tumors and mLN (Fig. 9B, C). Primary tumors were found to have an elevated number of $TRP\text{-}2^{+}CD133^{+}CD271^{+}$ melanoma cells in the proliferative state in comparison to mLN (Fig. 9D). We observed that $TRP\text{-}2^{+}CD133^{+}CD271^{+}$ melanoma cells exhibit a stable rate of proliferation in both early and advanced stages of tumor progression in primary tumors (Fig. 9E). In addition, many $TRP\text{-}2^{+}CD133^{+}CD271^{+}$ melanoma cells exhibit a pronounced proliferative capacity in early stages of tumor growth in mLN (Fig 9F).

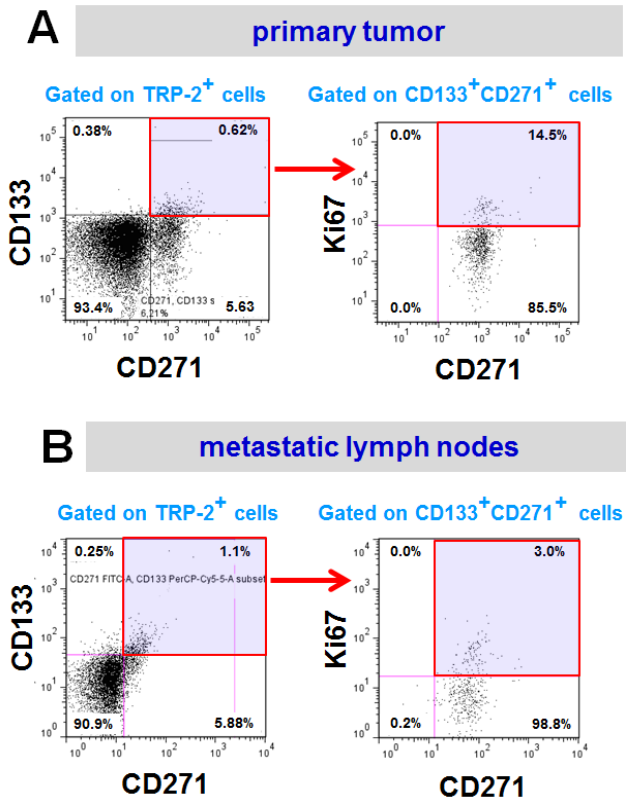


Figure 8. CD271 expression on $CD133^{+}$ melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and CD271 expression on $TRP\text{-}2^{+}CD133^{+}$ tumor cells and Ki67 expression on $TRP\text{-}2^{+}CD133^{+}CD271^{+}$ cells were analyzed in **(A)** primary tumors and **(B)** mLN. Representative dot plots are shown**.**

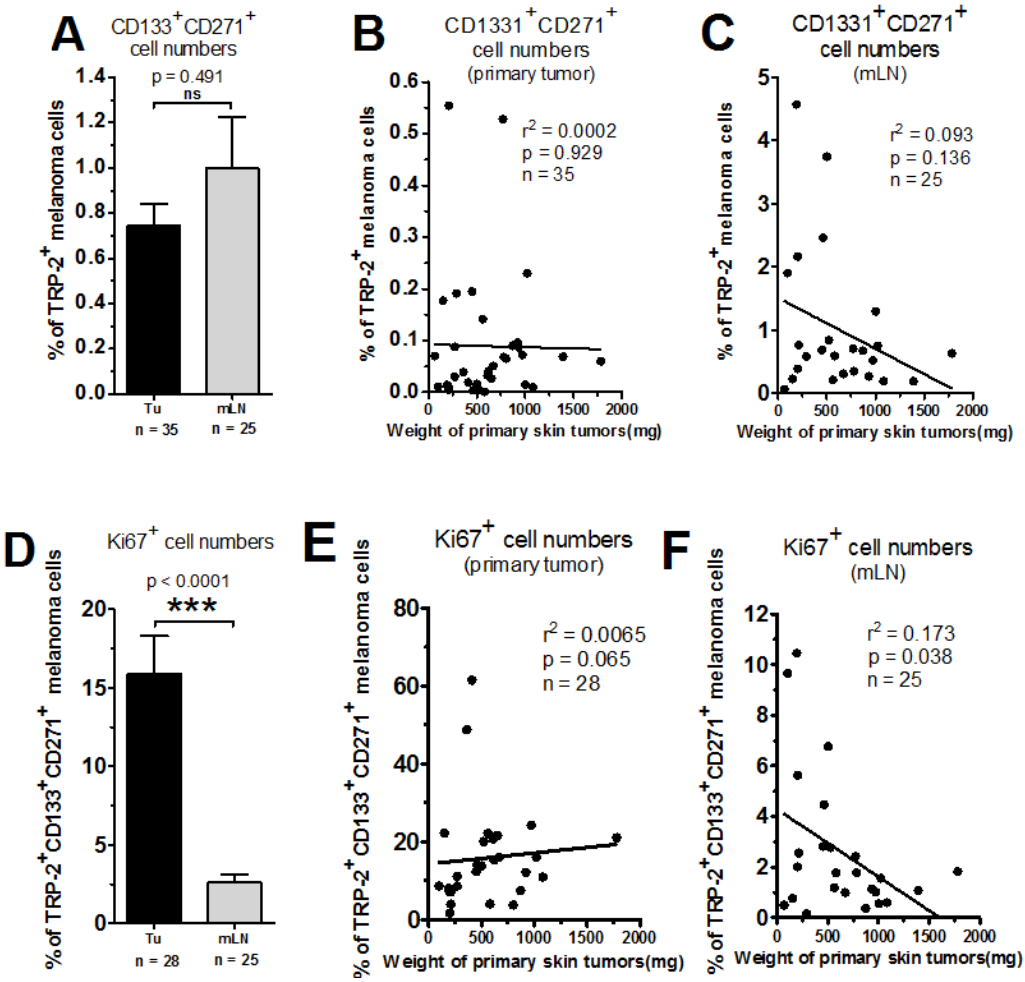


Figure 9. CD271 expression on CD133$^+$melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and CD271 expression on TRP-2$^+$CD133$^+$ tumor cells and Ki67 expression on TRP-2$^+$CD133$^+$CD271$^+$ were analyzed. Gating strategy was done as described in the figure 8. **(A)** CD133$^+$CD271$^+$ melanoma cell numbers in primary tumors (Tu) compared with mLN. The percentage of TRP-2$^+$CD133$^+$ CD271$^+$ melanoma cells was plotted against tumor weights in **(B)** primary tumors and **(C)** mLN. **(D)** TRP-2$^+$CD133$^+$CD271$^+$Ki67$^+$ melanoma cell numbers in primary tumors (Tu) compared to mLN. The percentage of TRP-2$^+$CD133$^+$CD271$^+$KI67$^+$ melanoma cells was plotted against tumor weights in **(E)** primary tumors and **(F)** mLN. ns= not statistically significant.

16. CD133$^+$ melanoma cells express PD-L1, CTLA-4, CD39, CCR4, VEGF-R2, CXCR3, CXCR4, and CD34

PD-L1 (CD274), CTLA-4 (CD152), CD39, CD73, and CCR4, have been documented as immunosuppressive factors expressed on tumor cells (123, 244, 245, 250-259), CXCR3 and CXCR4 have been described as master regulators of metastases (136, 137), whereas VEGFR is an essential factor to promote angiogenesis

(138, 139). CD34 was reported as a murine melanocyte stem cell maker (82). To examine the immunosuppressive, metastatic and angiogenic as well as the melanocyte stemness profile of CD133^{+} melanoma cells, primary tumors and mLN were stained with respective antibodies against their antigens (Fig. 10) followed by flow cytometry.

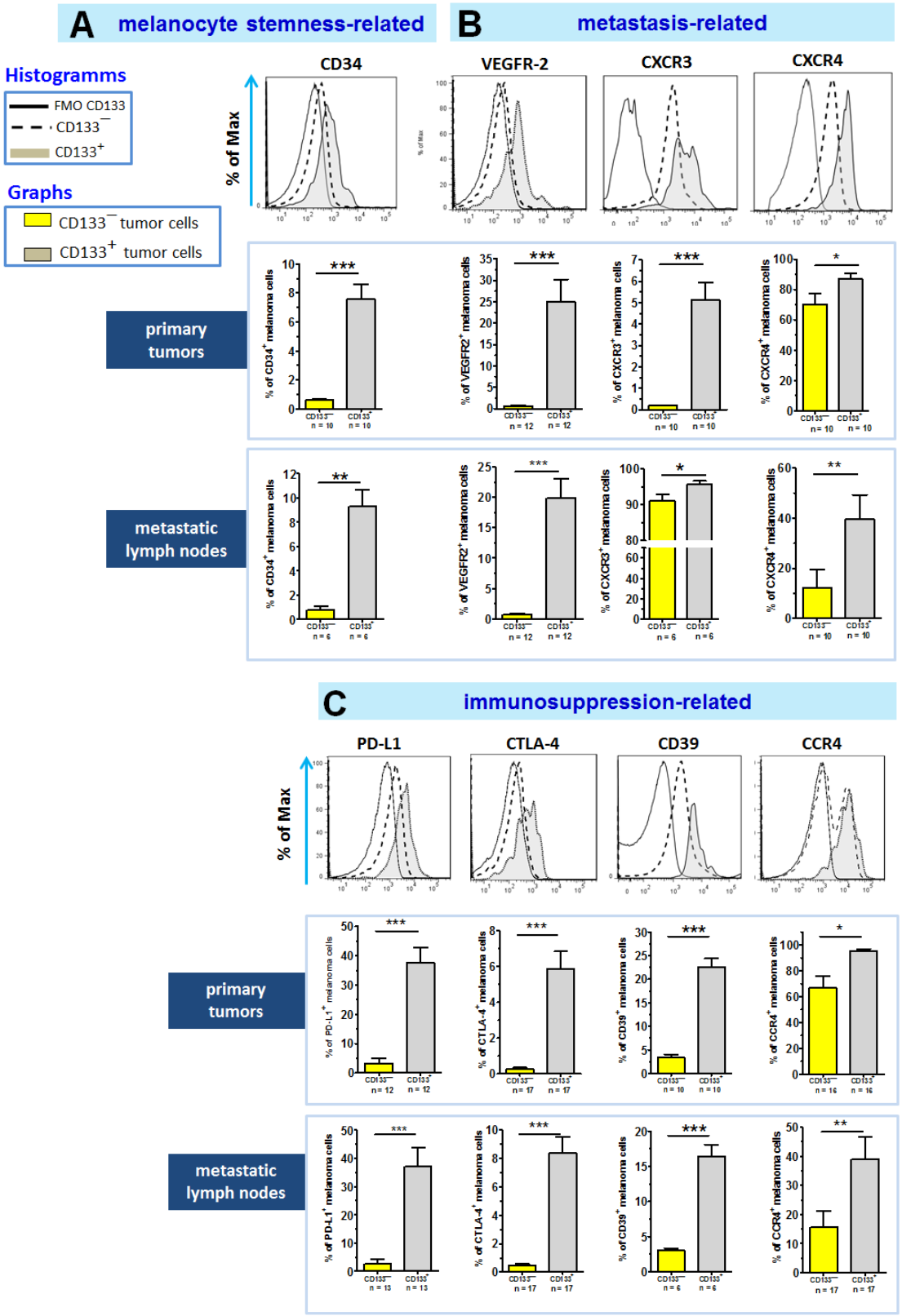


Figure 10. Phenotypic analysis of CD133$^+$ melanoma cells. Freshly isolated melanoma cells from primary tumors (Tu) and mLN were evaluated by flow cytometry. **(A)** Endothelial cells and leukocytes were excluded using antibodies for CD31 and CD45.2 respectively. Specimens were stained with antibodies for TRP-2. Then, PD-L1, CTL-4, CD39, CCR4, VEGFR2, CXCR3, CXCR4, and CD34 expression was analyzed on CD133$^+$ and CD133$^-$ melanoma cells. Representative histograms of cells from primary tumors are shown. **(A)** Melanocyte stemness marker, **(B)** metastasis-related markers and **(C)** immunosuppression-related markers. FMO CD133 = fluorescence minus one for CD133. CD133$^-$= CD133$^-$melanoma cells. CD133$^+$ = CD133$^+$ melanoma cells.

The data revealed that almost all studied markers were expressed on CD133$^+$ melanoma cells much stronger than on CD133$^-$ tumor cells (Fig. 10). CD73 expression was absent in both CD133$^+$ and CD133$^-$ tumor cells. In contrast, CXCR4 and CCR4 showed similar expression on both tumor cell subsets from primary tumors, whereas CXCR3 showed similar expression on both subsets from mLN. We concluded that CD133$^+$ melanoma cells exhibit immunosuppressive, metastatic, angiogenic and melanocyte stemness profile which might promote the establishment of tumor-initiating cells in primary tumors, their progression and metastasis to lymph nodes and distant organs.

17. Analysis of HIF-1α and CD271 expression on melanoma cells

Since HIF-1α and CD271 are important markers of CSCs and tumor dormancy, we studied their expression on melanoma cells from transgenic mice. We stained single tumor cell suspensions with antibodies for HIF-1α, CD271, CD133, TRP-2, CD31, and CD45.2 followed by flow cytometry (Fig. 11). We found that mLN contained higher frequencies of TRP-2$^+$HIF-1α$^+$ melanoma cells than primary tumors (Fig. 12A). Larger primary tumors contain a tendency to increase the numbers of TRP-2$^+$HIF-1α$^+$ melanoma cells in primary tumors (Fig. 12B), but a tendency to diminish the numbers of TRP-2$^+$HIF-1α$^+$ melanoma cells in mLN (fig. 12C). Cumulative data shown that although TRP-2$^+$HIF-1α$^+$ melanoma cells both in primary tumors and mLN had a similar rate of proliferation (Fig. 12D), we observed a higher rate of proliferation of TRP-2$^+$HIF-1α$^+$ melanoma cells in early stages of tumor development both in primary tumors and mLN (Fig. 12E, F).

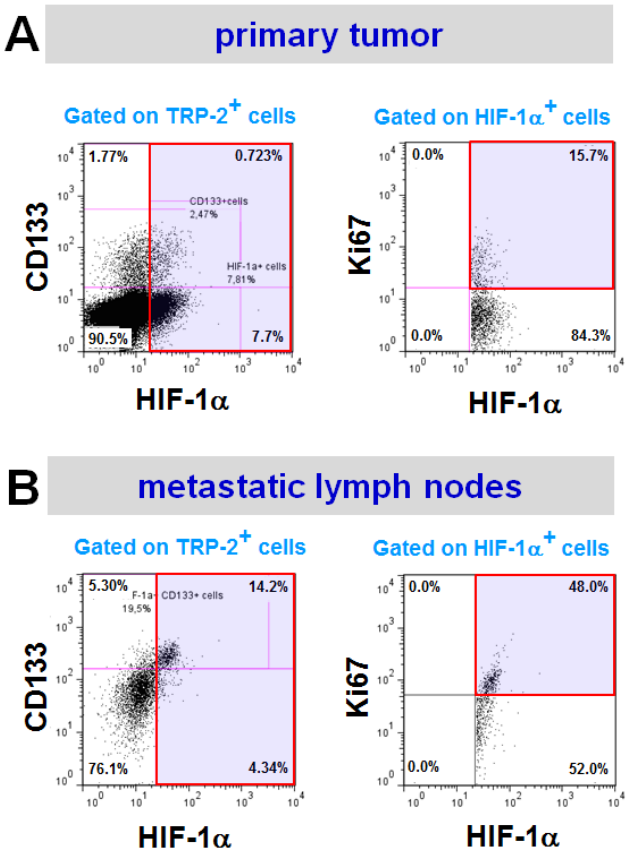


Figure 11. HIF-1α expression on melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and HIF-1α expression on TRP-2$^+$ tumor cells and Ki67 expression on TRP-2$^+$HIF-1α tumor cells were analyzed in **(A)** primary tumors and **(B)** mLN. Representative dot plots are shown**.**

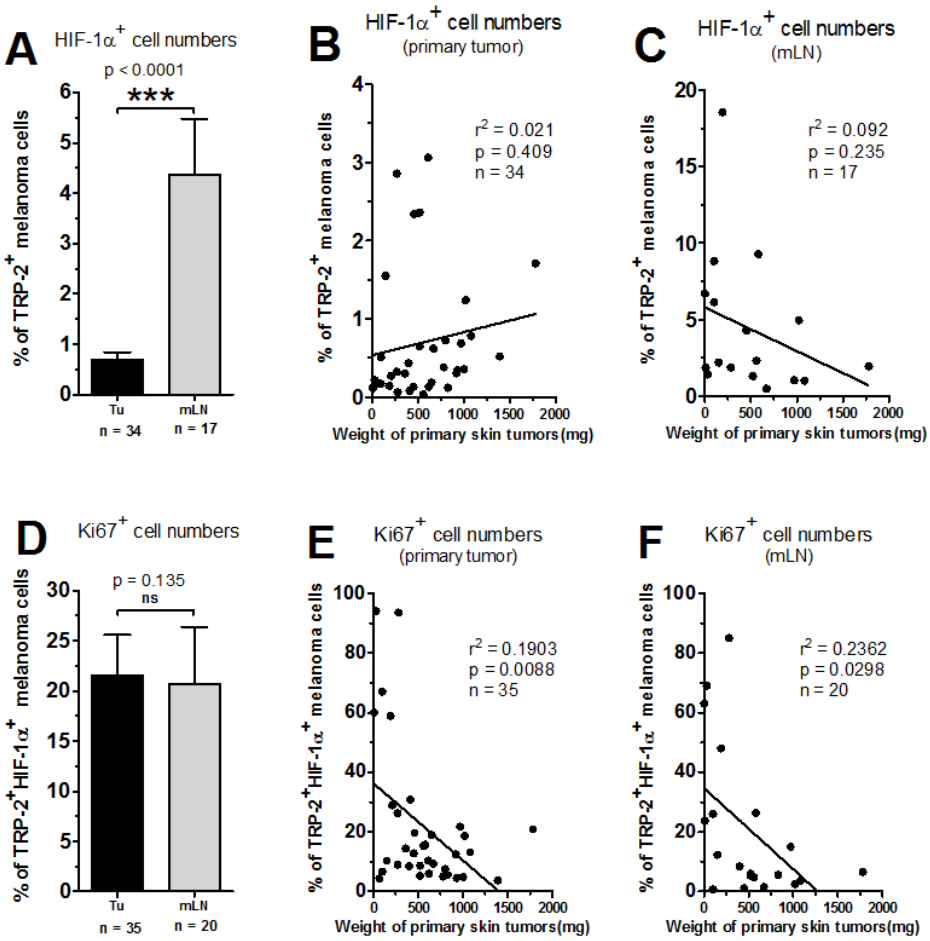


Figure 12. HIF-1α expression on melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and HIF-1α expression on TRP-2$^+$ tumor cells and Ki67 expression on TRP-2$^+$HIF-1α$^+$ were analyzed. Gating strategy was done as described in the figure 11. **(A)** HIF-1α$^+$ melanoma cell numbers in primary tumors (Tu) compared to (mLN. The percentage of TRP-2$^+$HIF-1α$^+$ melanoma cells was plotted against tumor weights in **(B)** primary tumors and **(C)** mLN. **(D)** TRP-2$^+$HIF-1α$^+$Ki67$^+$ melanoma cell numbers in primary tumors (Tu) compared to mLN. The percentage of TRP-2$^+$HIF-1α$^+$Ki67$^+$ melanoma cells was plotted against tumor weights in **(E)** primary tumors and **(F)** mLN. ns= not statistically significant.

Analyzing CD271 expression (Fig. 13A), we found that mLN have elevated numbers of TRP-2$^+$CD271$^+$ melanoma cells in comparison with primary tumors (Fig. 113B). Larger primary tumors contain elevated numbers of TRP-2$^+$CD271$^+$ melanoma cells in primary tumors (Fig. 13C), but a tendency to reduce the numbers of TRP-2$^+$CD271$^+$ melanoma cells in mLN (Fig. 13D). Similar amounts of TRP-2$^+$CD271$^+$ melanoma cells shown to be in proliferative phase both in primary tumors and mLN (Fig. 13E). Interestingly the proliferation of these cells was significantly lower in advanced primary tumors (Fig. 13F). In addition, CD271$^+$ melanoma cells in mLN displayed a strong tendency to diminish their proliferation rate in mice with larger primary tumors (Fig. 13G).

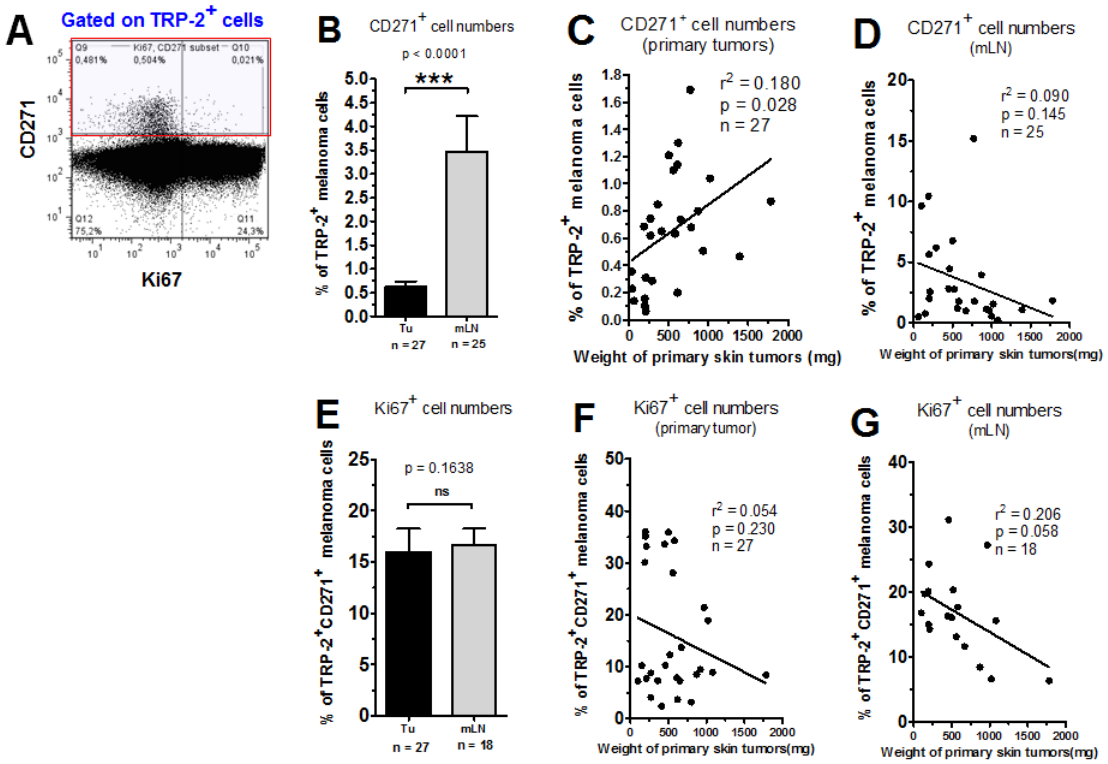


Figure 13. CD271 expression on melanoma cells. Freshly isolated melanoma cells from primary tumors and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and CD271 expression on TRP-2$^+$ tumor cells and Ki67 expression on TRP-2$^+$CD271$^+$ were analyzed in **(A)** primary tumors and mLN. Representative dot plot is shown**. (B)** CD271$^+$ melanoma cell number in primary tumors (Tu) compared to mLN. The percentage of TRP-2$^+$CD271$^+$ melanoma cells was plotted against tumor weights in **(C)** primary tumors and **(D)** mLN. **(E)** TRP-2$^+$CD271$^+$Ki67$^+$ melanoma cell numbers in primary tumors (Tu) and mLN. The percentage of TRP-2$^+$CD271$^+$KI67$^+$ melanoma cells was plotted against tumor weights in **(F)** primary tumors and **(G)** mLN.

18. Analysis of other potential cancer stem cell markers

Next we examined the expression of other potential melanoma stem cell markers such as CD20, CD24, CD34, CD44, CD166, nestin and multidrug resistant marker ABCB5, in primary skin tumors and mLN by flow cytometry (Fig. 14). We found that primary skin tumor cells had a higher proportion of CD20$^+$ tumor cells in comparison with mLN. Concerning other CSC makers, we found that nestin was constitutively expressed on most melanoma cells, whereas the multidrug resistance marker ABCB5 was expressed on around 90% melanoma cells. There were significantly more ABCB5$^+$ melanoma cells in primary tumors than those in mLN. The expression of CD166 and CD34 was detected in small amounts of cells in primary tumor and mLN (less than 2.5%).

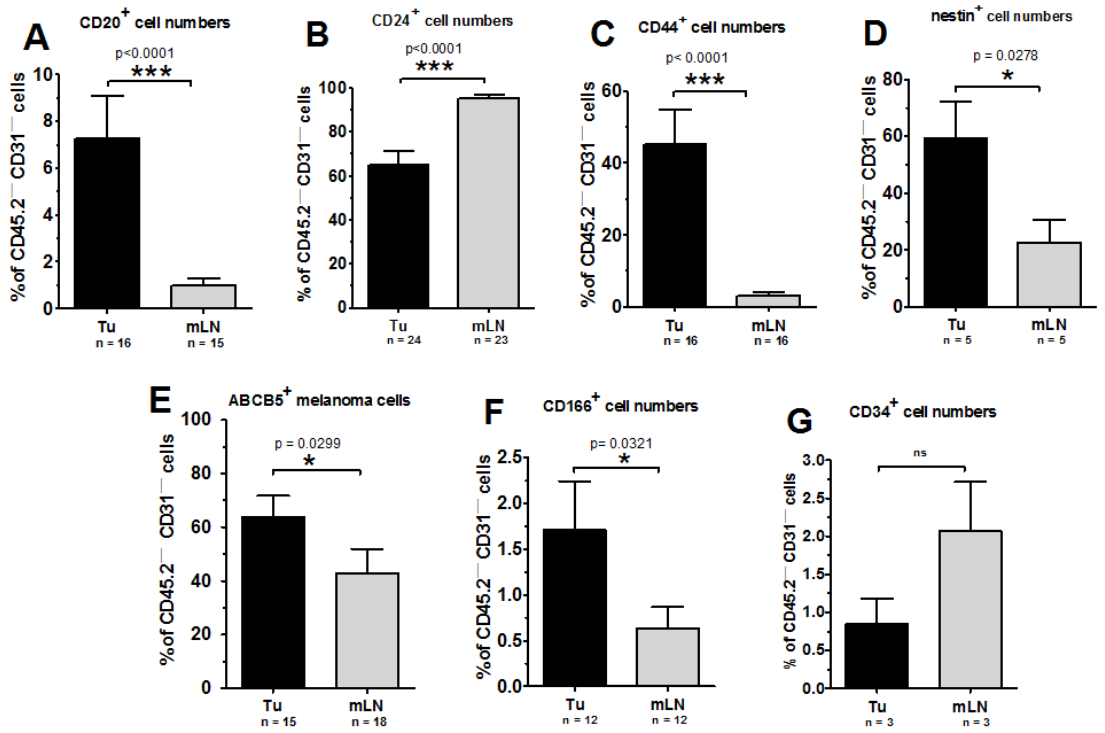


Figure 14. Analysis of other potential melanoma stem cell markers. Freshly isolated primary tumor cells (Tu) and mLN were evaluated by flow cytometry. Endothelial cells and leukocytes were excluded using CD31 and CD45.2 markers respectively and different cancer stem cell markers were analyzed. (**A**) CD20, (**B)** CD24, (**C**) CD34, (**D**) nestin, (**E**) ABCB5, (**F**) CD166, and (**G**) CD34. ns = not statistically significant.

19. BM contains disseminated melanoma cells

In this study, TRP-2^{+}CD133^{+} expressing cells were analyzed in the BM of tumor bearing mice by double and triple immunofluorescence (IF) protocol. We analyzed 10^{6} BM cells per mouse from 20 mice without macroscopic tumors (Fig. 15A, B) and 20 mice bearing macroscopic tumors (Fig. 15C-D). We found that tumor bearing mice had more TRP-2^{+} melanoma cells than mice without macroscopic primary skin tumors (Fig. 15E). The majority of TRP-2^{+} melanoma cells were in a dormant state (Ki67^{-}) (Fig. 15F). BM cells from wild type (WT) mice were used as positive control for the Ki67 staining (Fig. 15F).

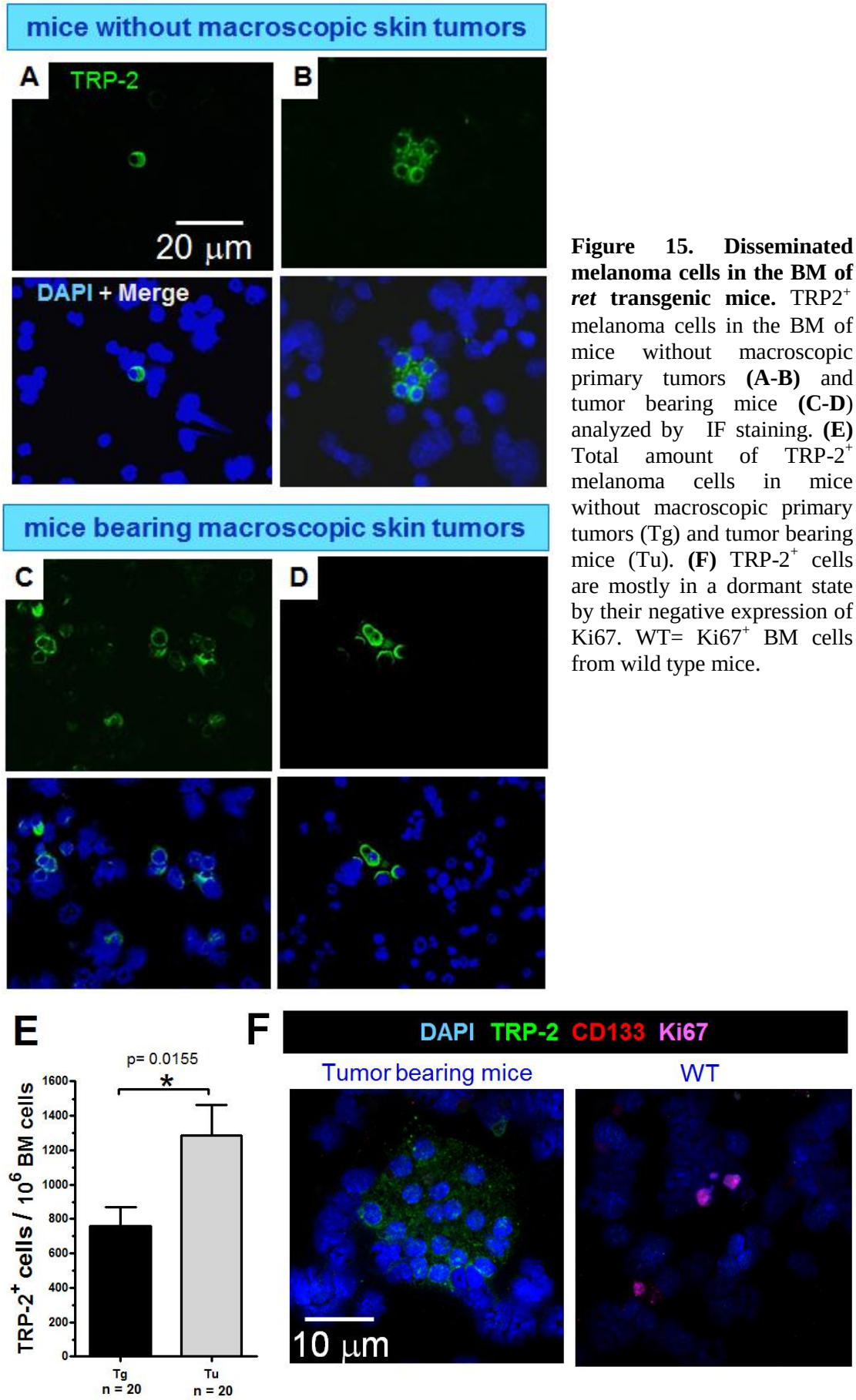
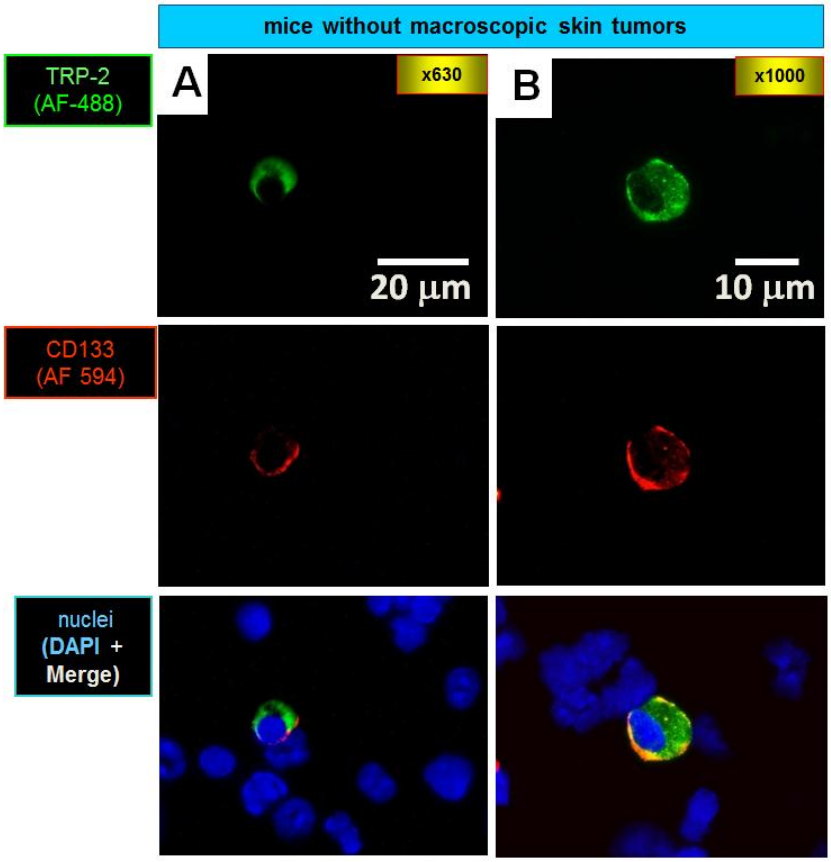


Figure 15. Disseminated melanoma cells in the BM of *ret* transgenic mice. TRP2$^+$ melanoma cells in the BM of mice without macroscopic primary tumors **(A-B)** and tumor bearing mice **(C-D)** analyzed by IF staining. **(E)** Total amount of TRP-2$^+$ melanoma cells in mice without macroscopic primary tumors (Tg) and tumor bearing mice (Tu). **(F)** TRP-2$^+$ cells are mostly in a dormant state by their negative expression of Ki67. WT= Ki67$^+$ BM cells from wild type mice.

We identified TRP-2^{+}CD133^{+} melanoma cells in mice without macroscopic primary tumors (Fig. 16A, B) and tumor bearing mice (Fig. 16C, D). We analyzed 10^{6} BM cells per mouse from 20 mice without macroscopic tumors and 20 mice bearing macroscopic tumors and found that only 40% of mice without macroscopic tumors contained TRP-2^{+}CD133^{+} melanoma cells in the BM. In contrast, all 20 tumor bearing mice contained TRP-2^{+}CD133^{+} melanoma cells. TRP-2^{+}CD133^{+} melanoma cells were detected in 2 out of 712 (0.238%) and 4 out of 1285 (0.311%) disseminated melanoma cells in the BM of transgenic mice without and with macroscopic tumors, respectively (Fig. 16E and Table 3). BM cells from wild type (WT) mice were used as positive control for the CD133 staining (Fig. 16F, G). Therefore, we did not observe any differences in the frequency of TRP-2^{+}CD133^{+} melanoma cells in the BM of *ret* transgenic mice as compared to that in mLN or primary skin tumors.



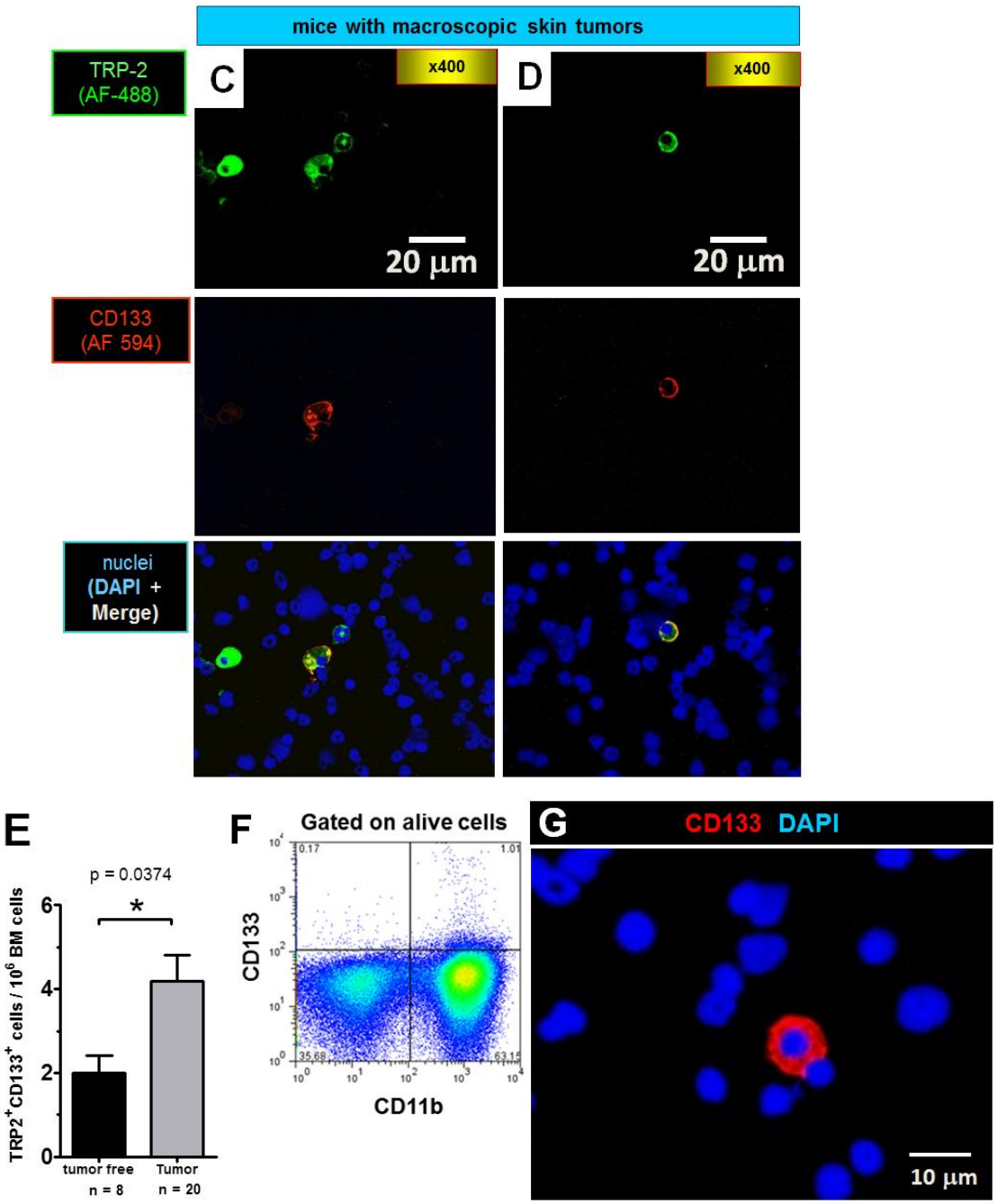


Figure 16. Disseminated melanoma cells in the BM of *ret* transgenic mice. Identification of $TRP2^{+}CD133^{+}$ melanoma cells in the BM of transgenic mice **(A-B)** without macroscopic tumors or **(C-D)** bearing macroscopic tumors by IF staining. **(E)** Frequency of $TRP2^{+}CD133^{+}$ tumor cells in 10^{6} BM cells in mice without macroscopic tumors (tumor free) and mice with macroscopic tumors (Tumor). All the pictures were done on Leica Microscope using IF lamp. **(F)** Flow cytometric analysis of CD133 expression on BM cells from wild type mouse and **(G)** using Carl-Zeiss 710 confocal microscope. CD133 (red), nuclei (blue). AF =alexa-fluor.

Table 3. Amount of TRP-2^{+}CD133^{+} melanoma cells and TRP-2^{+} melanoma cells in the BM of mice without visible primary skin tumors and mice with visible primary skin tumor.

	without macroscopic primary tumors	with macroscopic primary tumors
TRP-2^{+}CD133^{+} cells	2	4
TRP-2^{+} cells	712	1285
Percentage	0.280 %	0.311 %

To study a possible dormancy state of melanoma cells in the BM of *ret* transgenic mice, we checked nuclear Ki67 expression which is an indicator of cell proliferation, in mice with and without primary visible skin tumors (Fig. 17). We observed that disseminated TRP-2^{+}CD133^{+} melanoma stem-like cells are in a dormant state by their negative expression of Ki67 in the BM of mice without and with primary tumors (Fig. 17B). As controlled, we used BM cells from wild type mouse (WT) (Fig. 17A).

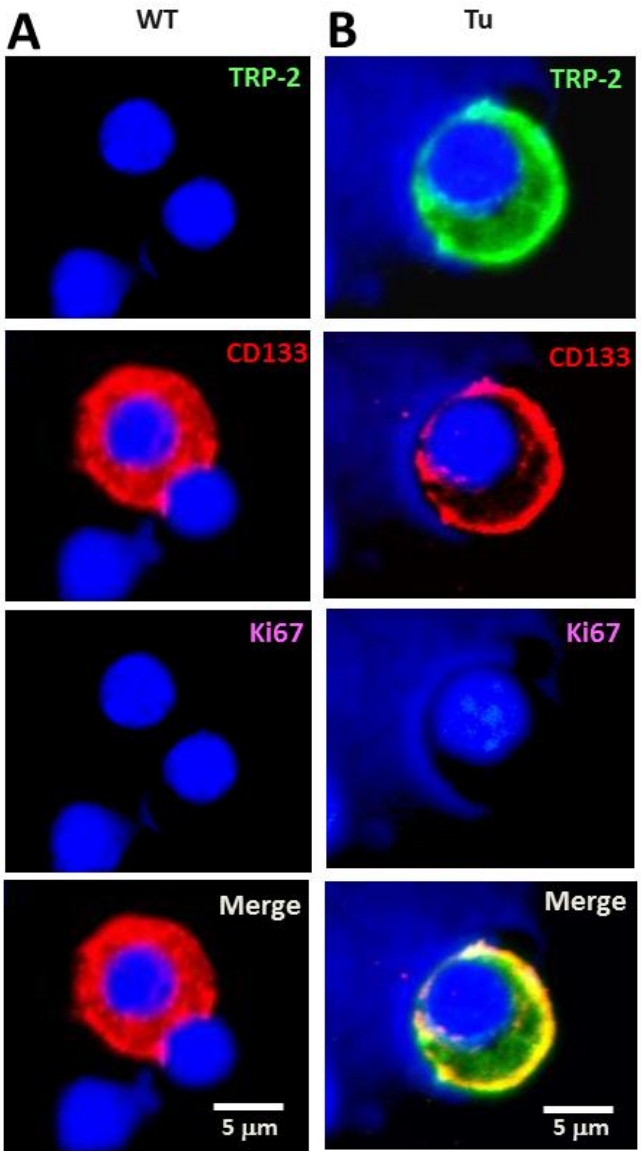


Figure 17. Disseminated dormant melanoma cells in the BM of *ret* transgenic mice. **(A)** CD133 expression on BM cells from wild type mouse (WT). **(B)** TRP-2^{+}CD133^{+} melanoma cells in the BM of tumor bearing mice (Tu). Both CD133^{+} BM cells and TRP-2^{+}CD133^{+} melanoma cell are in a dormant state by their negative expression of Ki67. BM specimens prepared using cytospin and IF staining. Samples analyzed with a Carl-Zeiss confocal microscope LSM-710. CD133 (red), TRP-2 (green), Ki67 (pink), nuclei (blue).

To corroborate the dormancy state of melanoma cells in the BM of *ret* transgenic mice, we checked several markers involved in the cell division such as p16, and p27, which have been described as cyclin-dependent kinase inhibitors (CDKI). In addition, the proliferating cell nuclear antigen (PCNA) and the Ki67 marker were analyzed (Fig. 18). Whereas melanoma cells failed to express PCNA (Fig. 18A), they were found to be positive for p16 and p27 (Fig. 18B, C). We conclude that TRP-2^{+}CD133^{+} melanoma stem-like cells are in a dormant state in the BM and present an aberrant expression of Ki67, p16 and p27.

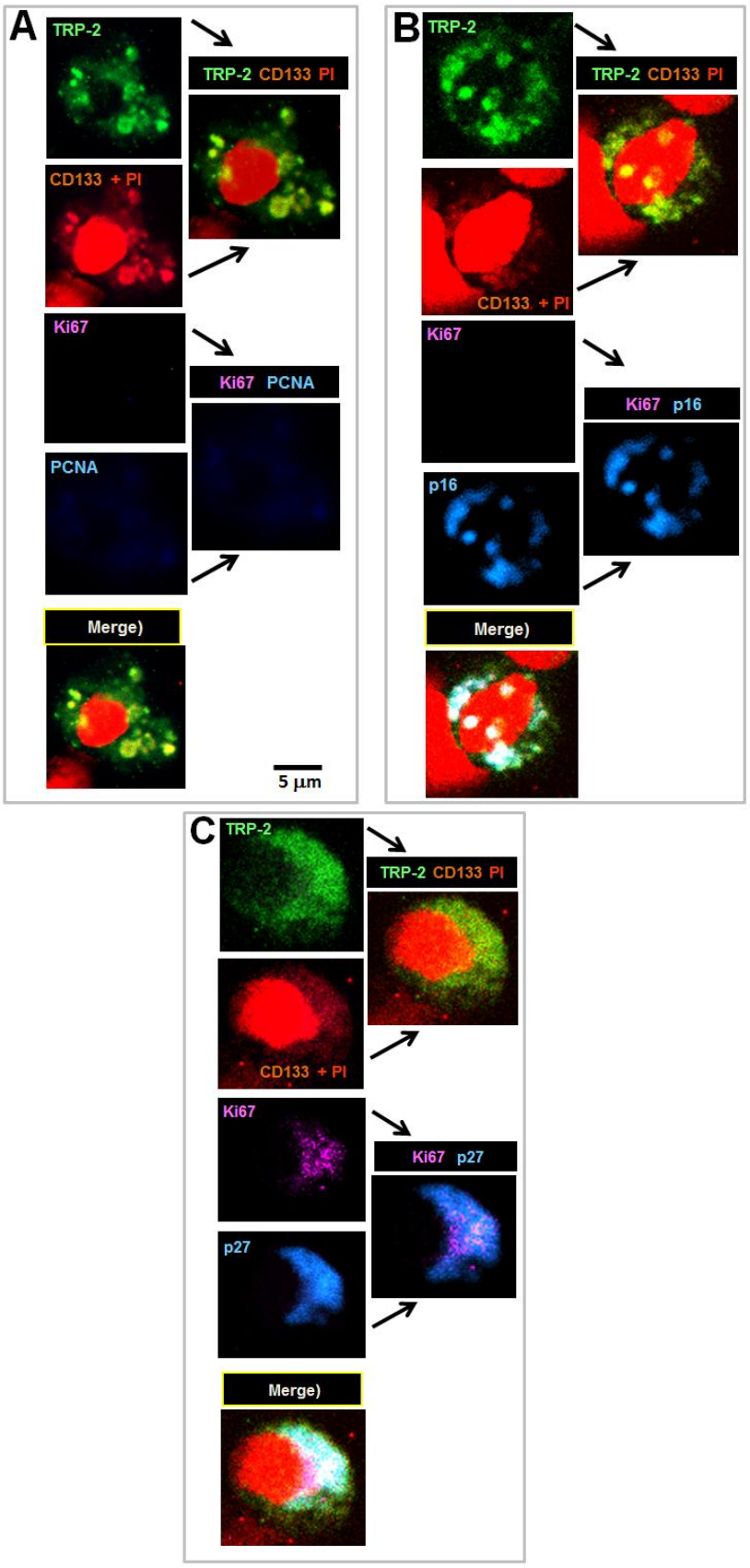


Figure 18. Expression of dormancy markers in disseminated BM tumor cells in mice without macroscopic skin tumors. Immunofluorescence staining for the analysis of **(A)** PCNA, **(B)** p16^{+}, and **(C)** p27^{+} on CD133^{+} (orange), Ki67 $^{-/cyto}$ (pink) melanoma cells. PI = propidium iodide.

To address the interaction between dormant tumor cells and memory T cells in the BM of *ret* transgenic mice, we analyzed smears from the BM of mice without visible skin tumors and with macroscopic tumors (Fig. 19 and 20).

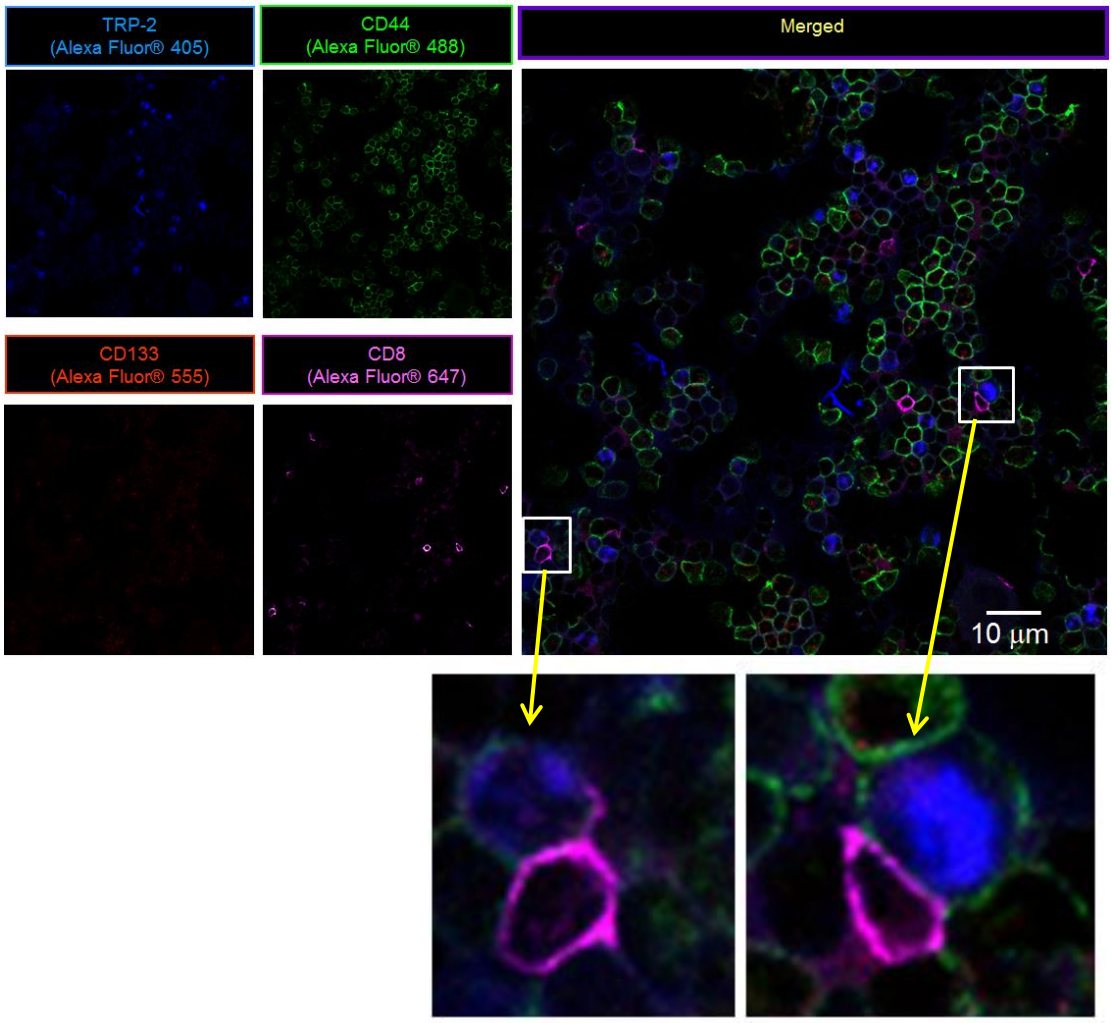


Figure 19. Memory CD8$^+$ T cells co-localized with TRP-2$^+$ cells in the BM of mice without macroscopic tumors. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), CD44 (green), CD133 (red) and CD8$^+$ (pink) molecules. The white squares show the place of interaction between CD8$^+$ T cells and TRP-2$^+$ cells.

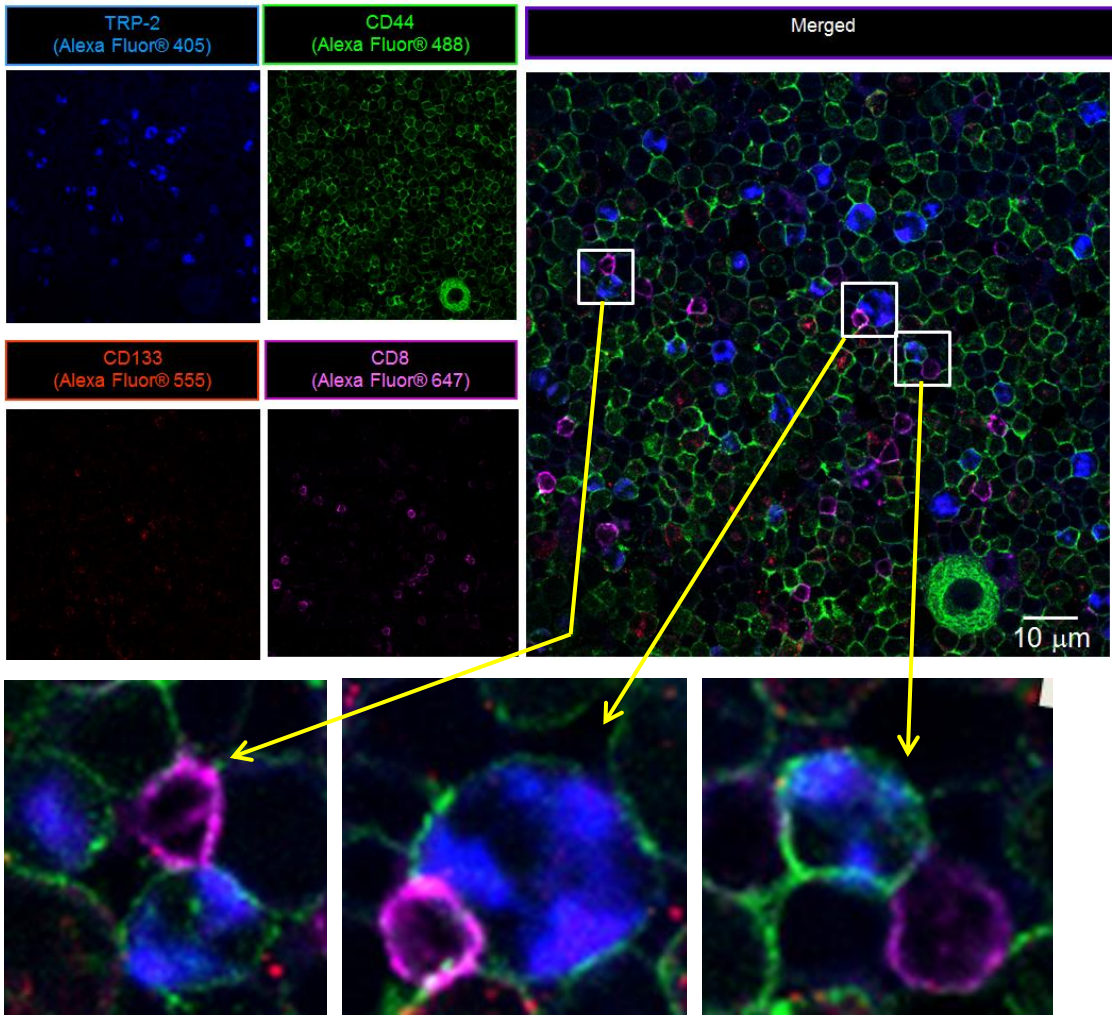


Figure 20. Memory CD8$^+$ T cells co-localized with TRP-2$^+$ cells in the BM of tumor bearing mice. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), CD44 (green), CD133 (red) and CD8 (pink) molecules. The white squares show the place of interaction between CD8$^+$ T cells and TRP-2$^+$ cells.

We found that memory CD8$^+$T cells (CD8$^+$CD44^{high}) were co-localized with TRP-2$^+$ melanoma cells not only in tumor bearing mice (Fig. 20) but also in mice without macroscopic tumor (Fig. 19). Importantly, the proportion of memory CD8$^+$ T cells interacting with TRP-2$^+$ melanoma cells was around 15% out of total memory CD8$^+$ T cells. However, we did not detect any co-localization of memory CD8$^+$ T cells with disseminated TRP-2$^+$CD133$^+$ melanoma cells in the BM of both mice without visible primary tumors and tumor bearing mice (n=8). Moreover, we found that also memory CD4$^+$ T cells (CD4$^+$CD45RB$^-$) had a direct contact with TRP-2$^+$ melanoma cells but not with TRP-2$^+$CD133$^+$ melanoma cells in the BM of tumor bearing mice (Fig. 22) as well in this organ from mice without visible skin tumors (Fig. 21).

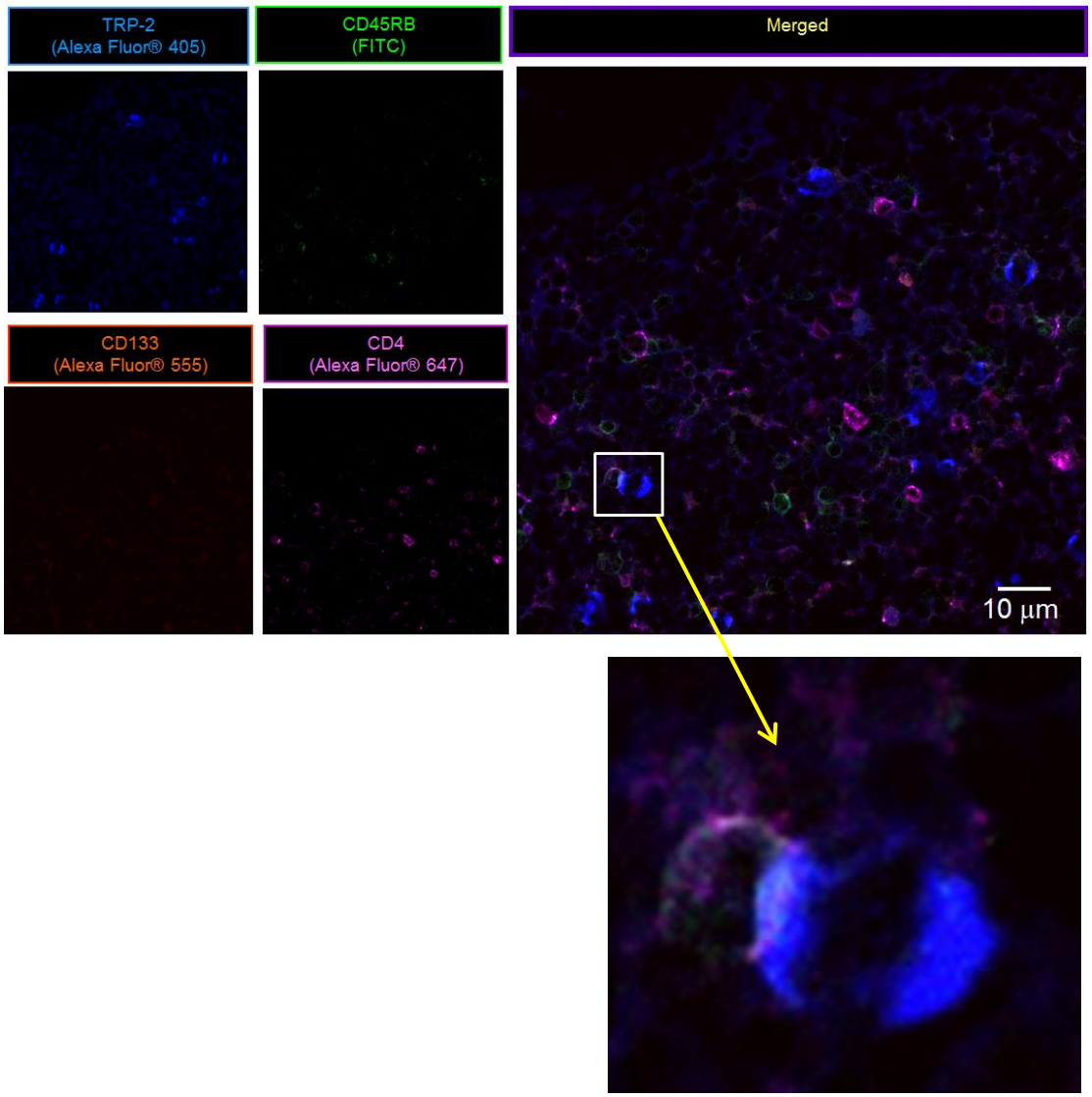


Figure 21. Memory CD4$^+$ T cells co-localized with TRP-2$^+$ cells in the BM of mice without macroscopic tumors. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), CD45RB (green), CD133 (red) and CD8$^+$ (pink). On the right picture is represented the merged of all four dyes. CD45RB$^-$CD8$^+$ T cells were associated with TRP-2$^+$ single melanoma cells. The white square shows the place of interaction between CD4$^+$ T cells and TRP-2$^+$ cells.

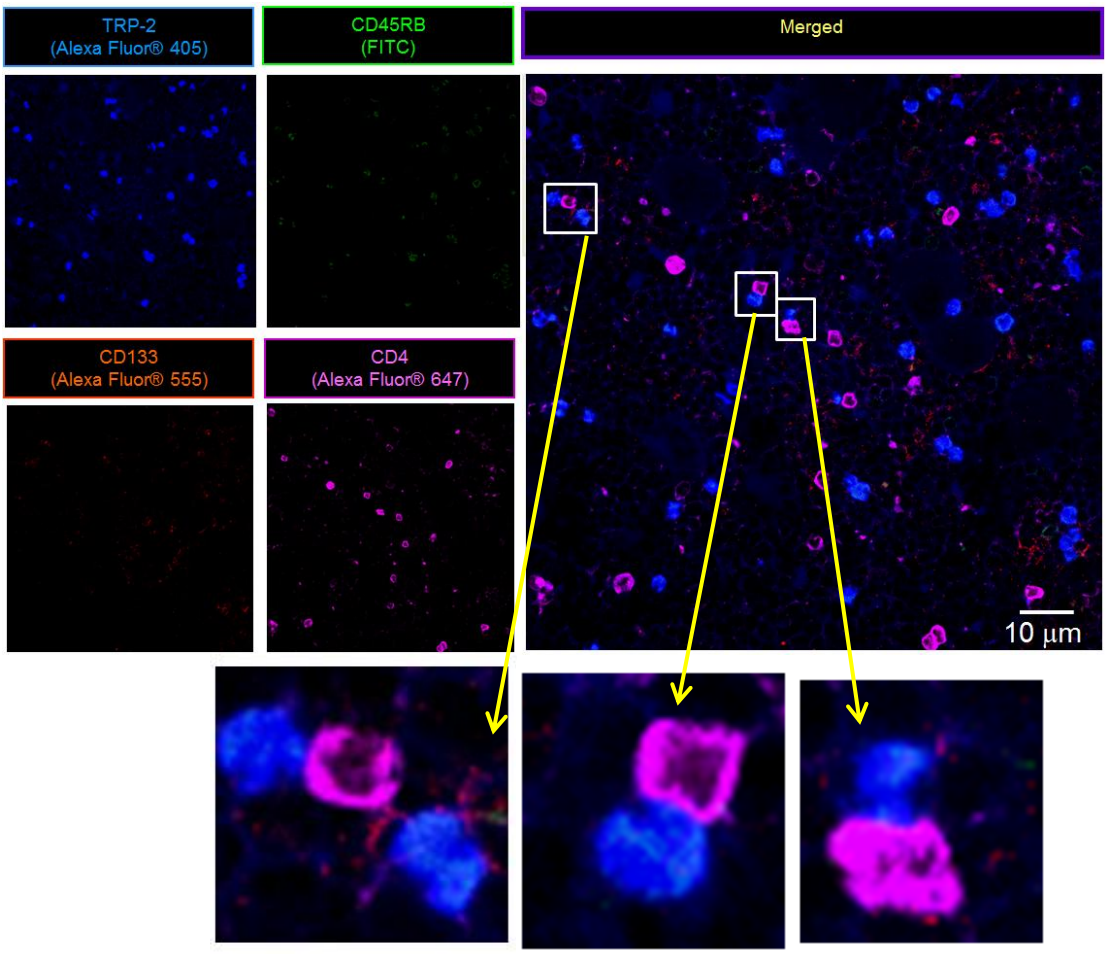


Figure 22. Memory CD4$^+$ T cells co-localized with TRP-2$^+$ cells in the BM of mice with macroscopic tumors. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), CD45RB (green), CD133 (red) and CD4 (pink). The white squares show the place of interaction between CD4$^+$ T cells and TRP-2$^+$ cells.

20. Perforin and IFN-γ-producing CD8$^+$ T cells in the BM

To investigate whether CD8$^+$ T cells associated with melanoma cells can possess effector function producing IFN-γ or perforin, we analyzed smears from the BM of *ret* transgenic mice. We found IFN-γ-producing CD8$^+$ T cells co-localized with TRP-2 melanoma cells (Fig. 23) but not with TRP-2$^+$CD133$^+$ cells. Interestingly, analyzing the BM of mice without visible skin tumors, we found that CD8$^+$ T cells located within the large cluster of 50 TRP-2 melanoma cells were unable to produce both perforin and IFN-γ (Fig. 24). Moreover, evaluating TRP-2 specific CD8$^+$ T cells by dextramer staining, we detected these cells in the BM failed to observe their co-localization with TRP-2$^+$ melanoma cells (Fig. 25). These findings suggest that tumor microenvironment might neutralize CD8$^+$ T cell reactivity.

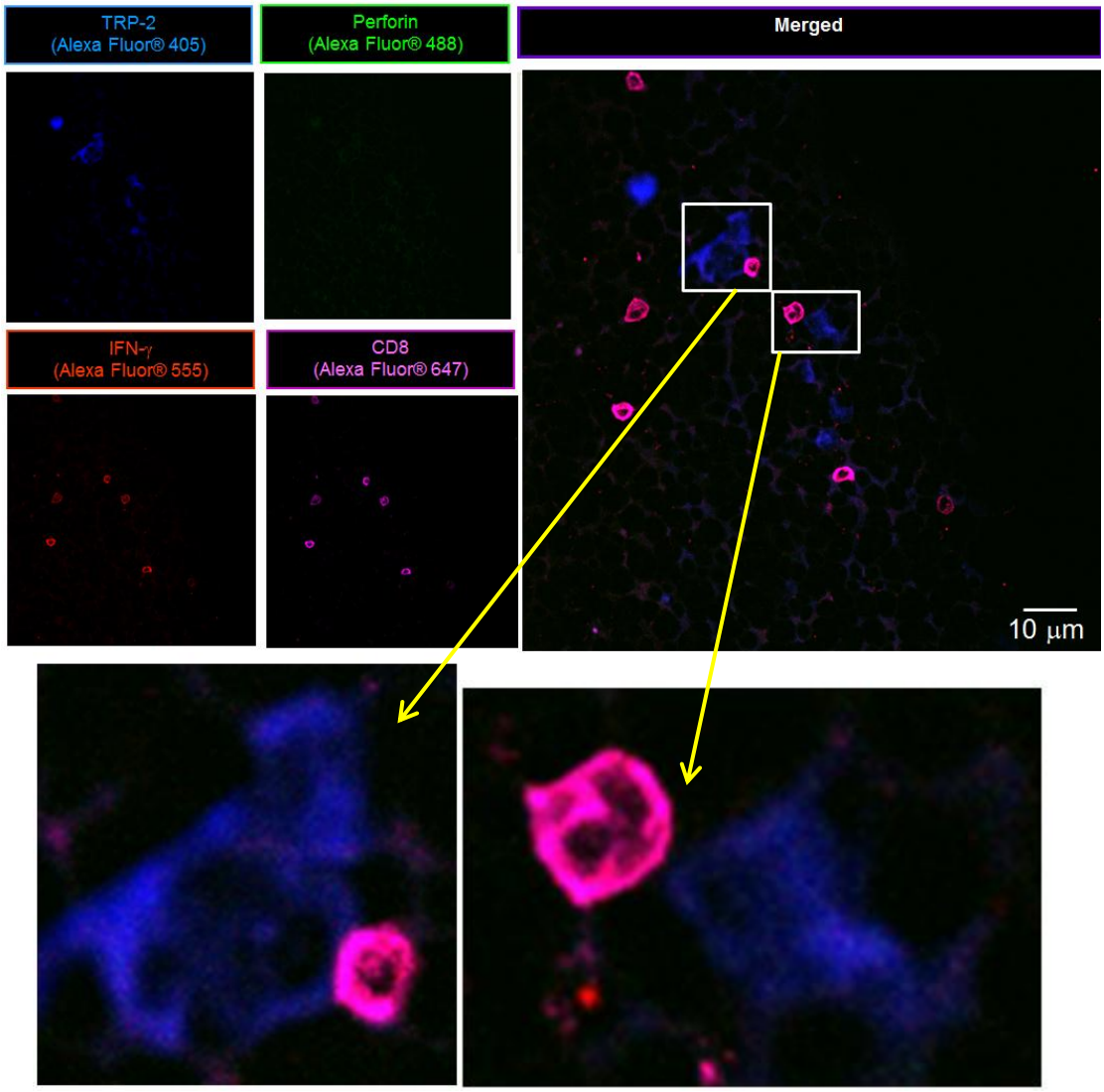


Figure 23. Evaluation of perforin and IFN-γ-producing CD8$^+$ T cells in the BM of tumor bearing mice. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), CD45RB (green), CD133 (red) and CD8 (pink). The white squares show the place of interaction between CD8$^+$ T cells and TRP-2$^+$ cells.

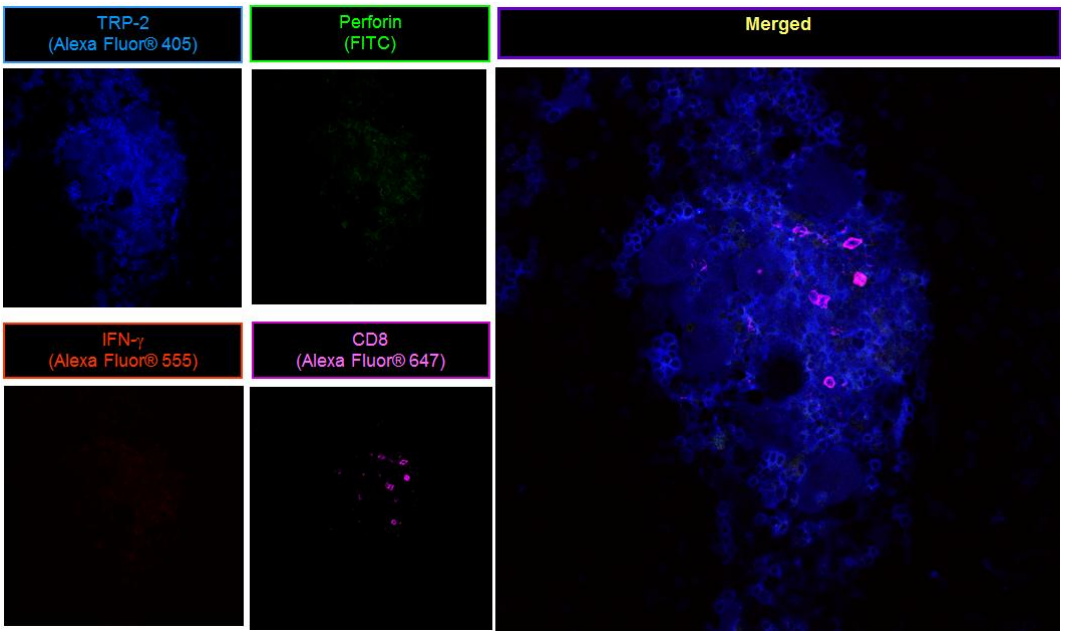


Figure 24. Evaluation of perforin and IFN-γ production in CD8$^+$ T cells in mice without macroscopic primary tumors. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), perforin (green), IFN-γ (red) and CD8 (pink). CD8$^+$ T cells located within a cluster of melanoma cells were unable to produce both perforin and IFN-γ.

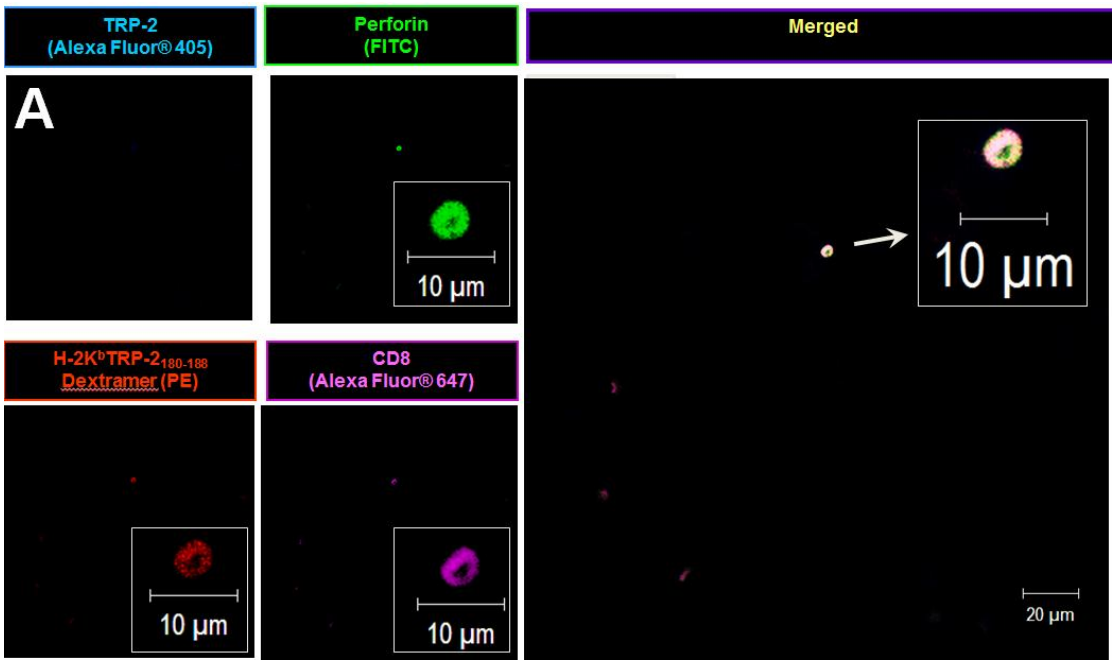


Figure 25. TRP-2-specific CD8$^+$ T cells in the BM of mice without macroscopic tumor. Smear technique and multicolor IF staining was performed to analyze TRP-2 (blue), Perforin (green), H-2k^b-TRP-2$_{180-188}$ dextramer (red) and CD8 (pink). Perforin and IFN-γ-producing CD8$^+$ T cells localized as single T cells.

Next we analyzed the proportion of CD8$^+$ T cells interacting with TRP-2$^+$Ki67$^-$ melanoma cells and found that about 15% of memory CD8$^+$ T cells interacted with

TRP-2^{+}Ki67^{-} melanoma cells in the BM of mice without and with primary tumors (Fig. 26, Table 4). Quantitative analyses revealed that although certain memory CD8^{+} T cells interacted either with single TRP-2^{+} melanoma cells or the smallest cluster of melanoma cells (2-5 TRP-2^{+} melanoma cells), none display staining for perforin. Only two TRP-2-specif CD8^{+} T cells were able to produce perforin, but none of them were co-localized either with TRP-2^{+} melanoma cells or TRP-2^{+}CD133^{+} cells in the BM of *ret* transgenic mice (Fig. 25).

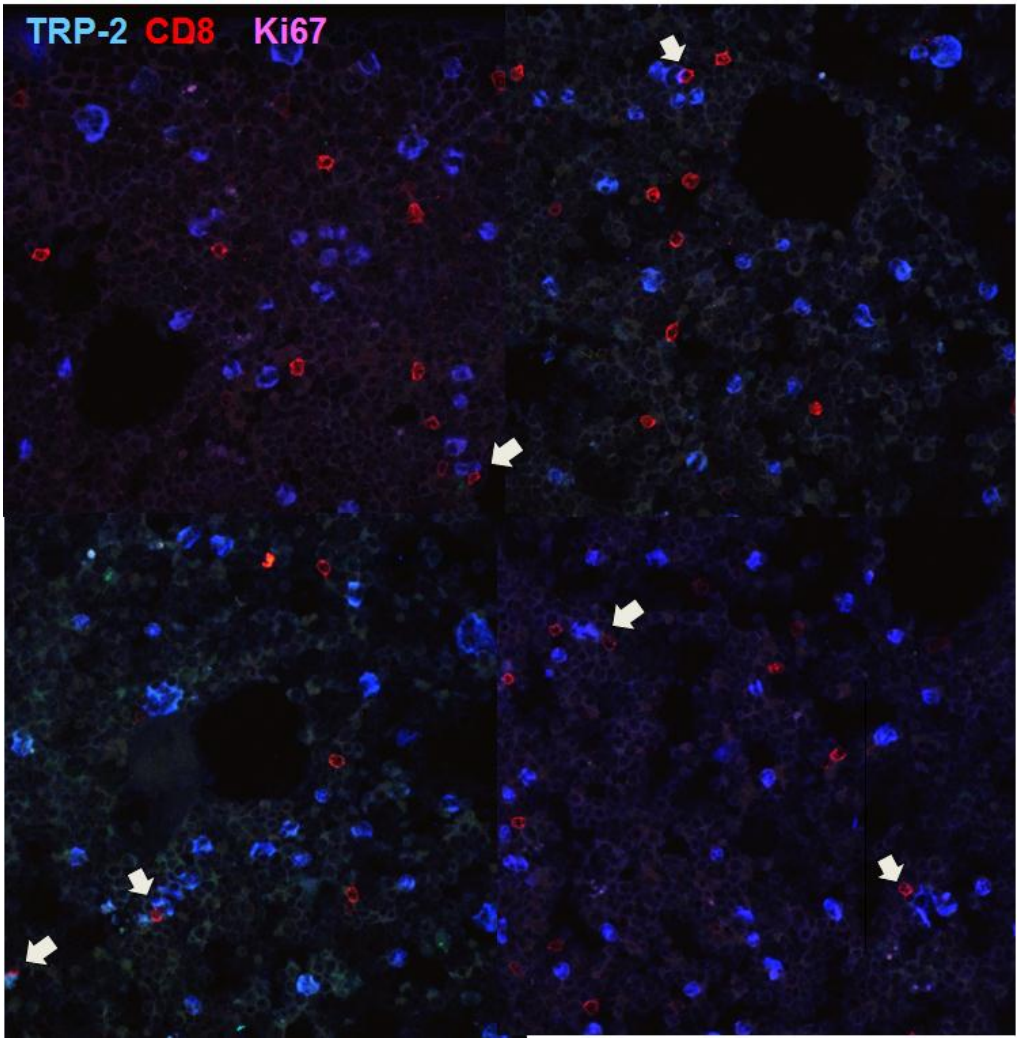


Figure 26. CD8^{+} T cells in the BM interacting and non-interacting with TRP-2^{+} melanoma cells. Smear technique and multicolor IF staining was performed to analyze BM cells of tumor bearing mice. TRP-2 (blue), CD8^{+} T cells (red) and Ki67 (pink). The white arrows show the place of interaction between CD8^{+} T cells and TRP-2^{+} cells.

Table 4. Interaction between TRP-2$^+$ melanoma cells with CD8$^+$ T cells in the BM of tumor bearing mice. The quantification was done from the fig. 25.

TRP-2$^+$cells	114 cells
CD8$^+$ cells	41 cells
TRP-2$^+$ cells with CD8$^+$ cells	6 CD8$^+$ cells (14.6%)

Taken together, our data demonstrate the existence of a fraction of dormant CD133$^+$ melanoma cells in *ret* transgenic mice. HIF-1α plays a central role in the maintenance of CD133$^+$ melanoma cells at the beginning of the tumor formation. A subpopulation of CD133$^+$ tumor cells co-expressed CD271, considered as another melanoma stem cell marker. CD133$^+$ melanoma cells shown stronger immunosuppressive, metastatic, angiogenic, and melanocyte stemness profile in comparison with CD133$^-$ melanoma cells, suggesting a greater biological advantage to evade antitumor immunity, to grow and metastasize. Disseminated TRP-2$^+$CD133$^+$ melanoma cells in the BM were in a dormant state by the absence of Ki67 and PCNA expression and shown an aberrant expression of p16 and p27. None of the characterized CD8$^+$ T cells (TRP-2-specific CD8$^+$ T cells, perforin and IFN-γ-producing CD8$^+$ T cells) were co-localized with disseminated BM TRP-2$^+$CD133$^+$ melanoma cells in mice with and without primary skin tumors. TRP-2$^+$CD133$^+$ melanoma cells may not be responsible to maintain memory CD8$^+$ T cells in the BM.

V. Discussion

Malignant melanoma is a very aggressive metastatic tumor originating from melanocytes. The metastatic melanoma displays a marked resistance to conventional therapies resulting in disappointing clinical outcomes when currently available radio- and chemotherapeutic treatment strategies are applied. Furthermore, immunotherapies based on antibodies, adoptive T cell transfers, and vaccinations shown promising but still poor clinical outcomes to eradicate tumors. These results from early clinical trials persuaded investigators to elucidate mechanisms responsible for the failure of tumor eradication. The resistance to chemo- radio- and immunotherapy has been attributed to the presence of either CSCs or dormant tumor cells. In the present study, we investigated CSCs in *ret* transgenic mouse model of spontaneous melanoma using CD133 (Prominin-1), which has been used as a marker of CSCs in various solid tumors (130-133).

The analysis of CSCs markers has been an extensive debate in the current protocols for preparing single tumor cell suspensions from primary tumor specimens for flow cytometry measurements due to CSC markers are expressed on very few tumor cells (103, 125, 270). It was stressed that aggressive protocols may remove antigens on the cell surface giving erroneously the impression that only very few tumor cells can express CSCs markers (91, 163, 275). Thus, the inclusion of aggressive enzymes in the dissociation procedure will result in contamination of the negative fraction with cells that actually express the antigen (91). Most of the enzymatic protocols use trypsin, which is tremendously aggressive because cleave several proteins on the cell surface (124, 163); although other protocols use collagenase which are much less aggressive, tumor specimens are also incubated longer (0.75-2 h) or followed by short incubation with trypsin (124). Instead of these aggressive methods, we used a reliable method to avoid the excision of antigens considered as CSC markers. Tumor cells were treated with collagenase II and collagenase IV for 15 minutes at 37°C to ensure the preservation of CSC markers on the cell surface that resulted in a high yield of live cells (more than 95%).

Using this optimized protocol we found that primary tumors contain approximately 1.5% of $CD133^+$ melanoma cells. Indeed, we found that the dormant state of $CD133^+$ melanoma cells in primary tumors and mLN was dependent on the weight of primary skin tumors. CSCs may be responsible to initiate and propagate

tumors, so called tumor-initiating cells. CSCs exhibit a higher resistant to chemo- and radiotherapy regimens due that they remain mostly arrested in G_0/G_1 of the cell cycle. CSCs and dormant tumor cells have been distinctly defined; however both share the particular feature that are mostly arrested in G_0/G_1 of the cell cycle (189). Therefore, we proposed that dormant tumor cells share characteristics and properties of CSCs.

Interestingly, immunofluorescence staining has shown that $CD133^+$ melanoma cells formed smaller clusters predominantly located in peripheral tumor areas where these cells could reside for their maintenance (273, 274). Blood vessels are essential to supply the nutrients and oxygen, so tumor growth is angiogenesis-dependent (271, 272). We found an aberrant architecture of blood vessels in peripheral areas of primary tumors. This finding is in agreement with other publication showing that vascularity of tumor varies tending to be greatest in regions of active growth, regularly at the periphery (276). The abnormal nature of tumors results in abnormal blood vessels. Tumor vessels are variable in size, shape and branching pattern and are not organized in conventional hierarchy of arterioles, capillaries and venules (277). Compared with quiescent established blood vessels, endothelial cells in angiogenic blood vessels can strongly proliferate (278, 279). Furthermore, although blood vessels within the tumors are built by existing blood vessels or by incorporation of bone marrow progenitor cells (277, 280), it is possible that $CD133^+$ melanoma cells in *ret* transgenic mice may differentiate into endothelial cells (vasculogenesis) to support the angiogenesis giving rise to aberrant blood vessels (281-283). This could be supported by our finding that $CD133^+$ melanoma cells express VEGFR2, a receptor that recognizes VEGF (282, 283). Moreover, we observed that large primary tumor collected in *ret* transgenic tumor bearing mice were consistently bloody. Rapid endothelial cell proliferation contributes to the instability of tumor vessel walls leading to hemorrhage described as tumors particularly bloody (277). In addition, it has been recently shown that sequencing of tumor genomes from different areas of the same tumor reveal striking intratumoral genetic diversification which may lead to functional specialization among cancer cells (284, 285). The presence of $CD133^+$ melanoma cells in peripheral tumor areas, where aberrant blood vessels were localized, may indicate that these zones might be the niche for $CD133^+$ melanoma cells (279, 281, 286), at least in *ret* transgenic melanoma model. Nevertheless, further experiments are needed to confirm these observations.

Interestingly, we found a higher rate of proliferation of CD133^{+} melanoma cells in mLN compared to those in primary tumors from mice with advanced tumors. These findings suggest that melanoma cells in mLN probably may also colonize their tumor of origin in the process called “tumor self-seeding” (287). Self-seeding of melanoma cells is mediated by aggressive circulating tumor cells and can accelerate primary tumor growth and angiogenesis, and could explain relationship between anaplasia, tumor size, vascularity, and local recurrence (12). It would be interesting to investigate whether CD133^{+} melanoma cells possess these self-seeding properties. We do not discard other mechanisms that may promote the growth of primary tumors.

We also found that HIF-1α might play an important role in the maintenance of CD133^{+} melanoma cells, mainly in early stages of tumor formation where tumor cells are poorly vascularized and in advanced tumors where hypoxia (1% oxygen) occurred due to the inevitable outcome of the rapidly growing tumors (156). Restricted oxygen conditions increase the CSC fraction and promote acquisition of a stem-like state (288). Here we detected CD133^{+}HIF-1α^{+} melanoma cells which could be maintained by hypoxic conditions. In addition, we found that CD133^{+} melanoma cells displayed higher expression of CXCR4 than CD133^{-} melanoma cells. CXCR4 is considered to be a master regulator of metastases (144). Furthermore, our findings are in agreement with data of Soeda *et al.* who showed that the propagation of the glioma-derived CSCs in a hypoxic environment led to the expansion of cells bearing CXCR4 (156). We suggest that HIF-1α might contribute the maintenance of CD133^{+} melanoma cells in *ret* transgenic mice by activating adaptive transcriptional programs that promote cell survival, motility, tumor angiogenesis and self-renewal capacity. Indeed, we demonstrated that CD133^{+}HIF-1α^{+} melanoma cells had a higher rate of proliferation at earliest stages of tumor progression both in primary tumors and mLN. Moreover, it has been shown that hypoxia induces an embryonic stem cell (ESC)-like transcriptional program including Oct4, Nanog, Sox2, Klf4, c-Myc in cancer cell lines from brain, kidney, cervix, lung, colon, liver and breast tumors (289). In hypoxic glioma cell line U251, Mathiel *et al.* observed an upregulation of CD133, a glioma stem cell marker accompanied by a consistent increase of Nanog and c-Myc expression (289). It is known that glioma (originated from glial cells) and melanoma (originated from melanocytes) share many cell surface markers due to both have arisen from melanoblasts, those precursor derived from the embryonic neural crest (71,

77, 78).

It is important to note that CD271 has been recently described as an important CSC marker, especially in melanoma with an increased tumor-initiating capacity as compared with $CD271^{-}$ tumor cells (124). We found that primary tumor had an elevated number of TRP-$2^{+}CD133^{+}CD271^{+}$ melanoma stem-like cell fraction in proliferative state compared to mLN. However, mLN showed elevated numbers of TRP-$2^{+}CD133^{+}CD271^{+}$ melanoma cells in proliferative state in early stages of tumor progression in comparison to primary tumors. The proliferative capacity of TRP-$2^{+}CD133^{+}CD271^{+}$ cells is restricted in advanced tumors, where the tumor bulk is already well-established. In this respect, it has been reported that $CD271^{+}$ melanoma cells can be multipotent and capable to establish the heterogeneity of parental tumor, retaining the self-renewal property. Furthermore, $CD271^{+}$ melanoma cells could be passaged more than 5 retransplantations and $CD271^{+}SOX10^{+}$ melanoma cells correlated with higher metastatic potential (125). We believe that $CD133^{+}CD271^{+}$ melanoma cells might possess CSCs properties in *ret* transgenic tumor-bearing mice. Analyzing other potential cancer stem cell markers, we found that high amounts of tumor cells expressed CD20, nestin and ABCB5, whereas CD34 and CD166 were presented only on a small tumor cell subset. It has been shown that ABCB5-expressing melanoma cells exhibit multidrug resistance (MDR) features (119, 123). The most common cause of MDR in human cancers is the functional expression of one or more ATP-binding cassette (ABC) transporters that efflux (extrusion of toxic substances outside the cell) anticancer drugs from the tumor cells (122, 216). Thus, $ABCB5^{+}$ melanoma cells in *ret* transgenic mice may display MDR features.

Since primary tumors and mLN contain $CD133^{+}$-expressing melanoma cells, we addressed the question of the biologic function of these cells. It was found that immunosuppression-related markers (PD-L1, CTLA-4, CD39 and CCR4), angiogenic-related marker (VEGFR2), metastatic-related markers (CXCR3 and CXCR4) and melanocyte stemness-related maker (CD34) were strongly expressed on $CD133^{+}$ cells as compared to $CD133^{-}$ counterparts. These findings demonstrate that interactions between tumor cells and host immune cells in the tumor microenvironment create an immunosuppressive state in which $CD133^{+}$ melanoma cells might actively participate inducing the immunosuppression and promoting an evasion from immunosurveillance due to their malignant potential (234-236). Thus,

dormant CD133^{+} melanoma cells may then enter the escape phase, which correspond to clinically apparent disease. In the escape phase, CD133^{+} melanoma cells might contribute to the formation of tumor bulk.

Once dormant / slow-cycling CD133^{+} cells evaded antitumor immunity, they might acquire the capacity to produce tumor cell clones to proliferate rapidly. For an efficient proliferation, these tumor cells require of blood vessels. Dormant termination of tumor cells has been reported by the overexpression of VEGF (291). Thus, the expression of VEGFR2 on CD133^{+} melanoma cells could promote angiogenesis. Furthermore, CD133^{+} melanoma cells may metastasize to distant organs through the expression of CXCR3 and CXCR4. We observed that CD133^{+} melanoma cells in primary tumors and mLN possessed a higher expression of VEGFR2, CXCR3 and CXR4 than CD133^{-} melanoma cells. These findings explain, at least in part, why the metastatic potential of primary melanoma is considerably higher than that of other solid tumors (146). Around 60% of metastasis was reported to be located in regional lymph nodes in malignant melanoma (142). It was demonstrated that CXCR3 plays a critical role in lymph node metastasis of melanoma (B16F10 cell line) (142), and that CXCR4^{+}CD133^{+} B16/F10 melanoma cells exhibited higher metastatic capacity than CXCR4^{-}CD133^{+} counterparts (157). Moreover, patients with tumor double positive for CXCR3 and CXCR4 were reported to have significantly poorer prognosis than those with tumors positive only for CXCR4 or the double negative (144).

Furthermore, CD133^{+} melanoma cells have a pronounced expression of the murine melanocytic stem cell marker CD34, providing an additional biological advantage to retain the melanocyte stemness phenotype and probably the machinery necessary to maintenance the stem-like cell character. In this regard, it would be interesting to investigate the expression of the typical stem cell makers such as Oct-4 Sox-2, Kfl-4, Nanog on CD133^{+} melanoma cells in *ret* transgenic mice.

In *ret* transgenic mouse melanoma model, a majority of mice older than 20 weeks without macroscopic tumors contained TRP-2-positive disseminated melanoma cells both in the BM and lymph nodes and showed no further melanoma progression. These reports argue for a potential role of dormant tumor cells in the maintenance of a long-term persistence of tumor antigen-specific memory T cells, which could restrict tumor growth *in vivo* (228, 232). In this study, TRP-2^{+}CD133^{+}-expressing cells were analyzed in the BM of tumor bearing mice. We found that TRP-2^{+}CD133^{+} melanoma

stem-like cells represented only 0.238% and 0.31% from all disseminated melanoma cells in mice without and with macroscopic tumors, respectively.

To study the dormancy state of TRP-2$^+$CD133$^+$ melanoma stem-like cells in the BM of *ret* transgenic mice, we analyzed several proteins evolved in the progression of the cell cycle including p16 and p27 expression which have been described as cyclin-dependent kinase inhibitors (CDKI) (292). We also analyzed the proliferating cell nuclear antigen (PCNA) and the Ki67 antigen. We observed cytoplasmic staining of p16. It is well-established that p16 protein acts as cell cycle inhibitor in the nucleus. However in breast cancer, cytoplasmic accumulation of p16 identifies a subset of tumor cells with accelerated proliferation (293). In addition, nuclear and cytoplasmic p16 overexpression in breast cancer was associated with a highly malignant phenotype (293). Moreover, we found membranous and cytoplasmic staining of Ki67 in same BM single tumor cells. Cell membrane and cytoplasmic staining of Ki67 has been described in invasive breast carcinoma and adenoma of the thyroid gland associated with tumors that are high grade (294). Cytoplasmic localization of p27 was identified as well, providing additional evidences that CD133$^+$ melanoma cells may be highly tumorigenic (295). These data demonstrated that TRP-2$^+$CD133$^+$ melanoma stem-like cells are in a dormant state and possess an aberrant expression of Ki67, p16 and p27 in the BM.

It has been reported that the BM could be a major site for long-term persistence of tumor-specific memory T cells that could interact with disseminated tumor cells (228). Here we have demonstrated direct cell-cell contact between BM TRP-2$^+$ tumor cells and memory CD8$^+$ T cells. It has been proposed that disseminated BM TRP-2$^+$ tumor cells which probably might be in a dormant state, could be responsible in the maintenance of TRP-2-specific memory CD8$^+$ T cells and functionally active TRP-2-specific, effector memory CD8$^+$ T cells in *ret* transgenic mice without visible primary tumors but with disseminated tumor cells. Therefore, these cells might stimulate the formation of melanoma-reactive CD8$^+$ T cells which restrict the growth of macroscopic primary tumors (232) and block the progression of metastases, keeping the animals in the equilibrium phase, in agreement with the immunosurveillance theory (207).

Memory CD8$^+$T cells (CD8$^+$CD44^{high}) were co-localized with TRP2$^+$ melanoma cells, where the proportion of memory CD8$^+$ T cells interacting with TRP-2$^+$

melanoma cells was lower (less than 15%). These findings suggest that memory phenotype of $CD8^+$ T cells might be induced by TRP-2^+ melanoma cells. However we did not find memory $CD8^+$ T cells co-localized with disseminated TRP-2^+CD133^+ melanoma stem-like cells in the BM of mice with and without macroscopic primary skin tumors. In addition, we found that memory $CD4^+$ T cells ($CD4^+$CD45RB^-) had direct contact with TRP-2^+ melanoma cells but not with TRP-2^+CD133^+ melanoma stem-like cells in the BM of mice with and without macroscopic primary skin tumors. Moreover, we found that none of the TRP-2-specific, perforin-producing $CD8^+$ T cells were co-localized either with TRP-2^+ melanoma cells or TRP-2^+CD133^+ melanoma stem-like cells in the BM of mice with and without macroscopic primary skin tumors. Furthermore, we could only find IFN-γ-producing $CD8^+$ T cells co-localized with TRP-2^+ melanoma cells but not with TRP-2^+CD133^+melanoma stem-like cells. Interestingly, analyzing the BM of mice without macroscopic primary skin tumors, we found $CD8^+$ T cells co-localized within one large cluster of TRP-2^+ melanoma cells (50 TRP-2^+ tumor cells). Nevertheless these $CD8^+$ T cells were unable to produce intracellularly both perforin and IFN-γ. These findings suggest that tumor microenvironment might neutralize $CD8^+$ T cell reactivity.

Therefore, these data indicated that none of the characterized $CD8^+$ T cells (TRP-2-specific $CD8^+$ T cells, perforin and IFN-γ-producing $CD8^+$ T cells) were co-localized with disseminated BM TRP-2^+CD133^+ melanoma stem-like cells in mice without and with primary skin tumors. We suppose that memory $CD8^+$ T cells in the BM might be maintained by both single melanoma cells and the smallest cluster of melanoma cells. Large melanoma cell cluster may induce inactivation of $CD8^+$ T cells, as was shown by the lack of intracellular production of IFN-γ. The indeterminate dormant phenotype of TRP-2^+CD133^+ melanoma stem-like cells may be a crucial mechanism to the maintenance of memory $CD8^+$ T cells for a long period of time.

It has been shown that BM displays structural and functional features of a secondary lymphoid organ, contains follicle-like structures similar to lymph nodes or spleen and can initiate not only primary, but also secondary T-cell responses (296). Thus, cell-cell contacts between TRP-2^+ melanoma cells and memory $CD8^+$ T cells may stimulate secondary immune responses (which is faster and stronger than primary immune responses) and subsequently protective anti-tumor immunity by promoting melanoma-specific effector memory $CD8^+$ T cells. The effector reactivity

of these $CD8^+$ T cells may occur systemically and/or in the tumor microenvironment (296) and not within the BM, where they fail to produce perforin.

Taken together, we provide evidence that $CD133^+$ melanoma stem-like cells might modulate immune responses in primary tumors and lymph node metastasis. The data also suggests that HIF-1α plays a central role in the maintenance of $CD133^+$ melanoma cells. Disseminated TRP-2^+CD133^+ melanoma in the BM are in the dormant state having an aberrant expression of p16 and p27 due to their cytoplasmic localization. Finally, none of the characterized $CD8^+$ T cells (TRP-2-specific $CD8^+$ T cells, perforin and IFN-γ-producing $CD8^+$ T cells) were co-localized with disseminated BM TRP-2^+CD133^+ melanoma stem-like cells in *ret* transgenic mice. These results suggest that TRP-2^+CD133^+ melanoma cells may not be responsible to maintain memory $CD8^+$ T cells in the BM.

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VII. Abbreviations:

A

APC	Antigen-presenting cell
ALDH	Aldehyde dehydrogenase. Putatite cancer stem cell marker.
ABCB5	ATP-binding cassette sub-family B member 5 also known as P-glycoprotein. Putative melanoma stem cell marker
Ab	antibody

B

BM	bone marrow
BSA	bovine serum albumin
B-RAF	B-Raf proto-oncogene serine/threonine-protein kinase
B16F10	melanoma cell line from a mouse model of melanoma metastasis
bFGF	basic fibroblast growth factor

C

CSCs	cancer stem cells
CCL	chemokine ligand
CTLA-4	cytotoxic T lymphocyte antigen- 4
CCR	chemokine receptor
CD	cluster of differentiation
cm	centimeter
CFS	carboxyfluorescein (similar to FITC)
CTL	cytotoxic T lymphocyte
°C	degree Celsius
CD4	It serves as a co-receptor for the TCR (on T cells). Together with the TCR, CD4 binds to a class II MHC protein (on APCs).
CD8	It serves as a co-receptor for the TCR (on T cells. Together with the TCR, CD8 binds to a class I MHC protein (on APCs).
CD45RB	It plays a critical role in TCR and BCR signaling. As T cells become activated and progress from naïve to memory cells, CD45RB expression is downregulated.
CD62L	L-selectin. It is a cell adhesion molecule found on lymphocytes.
CXCR3	C-X-C chemokine receptor type 3. CXCR3 is able to regulate leukocyte trafficking.
CXCR4	C-X-C chemokine receptor type 4. alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1 also called CXCL12)
CD20	Expressed on the surface of all B-cells beginning at the pro-B phase. It is considered as putative CSC marker
CD24	Expressed at the surface of most B lymphocytes and differentiating neuroblasts. It is considered as putative CSC marker.
CD31	Endothelial cell marker. It is used as indicator of vasculatization.
CD34	Melanocyte stem cell marker (in mouse)
CD39	ectonucleoside triphosphate
CD44	It is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. CD44 expression is an indicative marker for effector-memory T-cells.

CD73	ecto-5´-nucleotidase is a glycosyl-phosphatidylinositol-linked cell surface enzyme
CD80	B7.1 expressed on antigen-presenting cells (APCs)
CD86	B7.2 expressed on antigen-presenting cells (APCs)
CD133	Prominin-1, putative CSCs marker
CD152	CTLA-4 (cytotoxic T lymphocyte antigen- 4)
CD166	Activated leukocyte cell adhesion molecule. It is considered as putative CSC marker
CD271	Nerve growth factor receptor (NGFR). It is considered as putative melanoma stem cell marker.
CD274	PD-L1 (programmed cell death-1).
CDK4	cyclin dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDK4/6	cyclin-dependent kinase 4/6 (important for cell cycle regulation)
CDKI	cyclin-dependent kinase inhibitors

D

DAPI	4,6-diamidino-2-phenylindole (blue dye)
DC	dendritic cell
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
DNAase-I	Deoxyribonuclease-I

H

HSC	Hematopoietic stem cell (s)
H-2k^{b}TRP-2$_{180-188}$	MHC dextramers H-2K^{b}TRP-2$_{180-188}$ which recognize TRP-2-specific CD8^{+} T cells

E

EDTA	Ethylenediaminetetraacetic acid
E2F	Elongation Factor 2 (a family of transcription factors)

F

FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FITC	fluorescein-5-isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter
Fc	Fragment crystallizable region. It is the tail region of an antibody that interacts with cell surface receptors called Fc receptors and some proteins of the complement system.

G

g	gram
g	acceleration due to gravity, g = 9,81m/s2
GM-CSF	granulocyte/macrophage colony stimulating factor
gp100	glycoprotein 100, a melanoma-associated antigen
G1/S	Gap / Synthesis (cell cycle phase)

H

h	hour
HIF-1α	hypoxia-inducible factor 1α
HSCs	hematopoietic stem cells
Hoechst	solution for the cell nuclear staining
H-2K^b	murine MHC antigen. H-2K^b is involved in antigen presentation to T cells expressing CD3/TCR and CD8 molecules.

I

IF	immunofluorescence
IF-buffer	immunofluorescence buffer
IF-gs	IF-buffer plus 2% of goat serum in IF-buffer
IFN	interferon
IFN-α	Interferon alpha
IFN-γ	interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin isotype G
IgG,κ	Immunoglobulin isotype G with a kappa light chain
IL	Interleukin
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-11	Interleukin-1
Ipilimumab	Therapeutic antibody against CTLA-4
iPS	induced pluripotent stem cell

K

Ki67	proliferation marker

L

l	litter

M

MAA	melanoma-associated antigen
MAPK	mitogen activated protein kinase
mAb	monoclonal antibody
MDSCs	myeloid-derived suppressor cells
MEK	MAP-ERK kinase
MART-1	Melanoma-associated antigen recognized by T cells (also known as Melan-A).
MHC	major histocompatibility complex
MITF	microphthalmia-associated transcription factor
mLN	metastatic lymph nodes
MDR	Multidrug resistance
MT	mouse metallothionein-I (MT) promoter-enhancer
M	molar (mol/l)
ml	milliliter
mg	milligramm
mm	millimeter

µm	micrometer
µg	microgramm
µl	microliter
ml	milliliter
µg	microgram
mg	milligram
min	minute

N

nevi	Moles (Moles, also known as melanocytic nevus, plural: nevi)
NK	natural killer cells
NO	nitric oxide
nm	nanometer

O

Ova	ovalbumin

P

Pen/Strep	penicillin/streptomycin
%	percent
pg	picogram
PBS	phosphate buffered saline
PE	phycoerythrin
PD-1	programmed cell death-1
PD-L1	Programmed cell death 1 ligand 1 (CD274 or B7-H1)
PCNA	proliferating cell nuclear antigen
PTEN	Phosphatase und Tensin homolog
PTHRP	parathyroid hormone related protein

R

RANKL	receptor activator of nuclear-factor-kB ligand
ret	human ret proto-oncogene
rpm	revolutions per minutes
RNA	ribonucleic acid
RT	room temperature

S

SSC	side scatter

T

TCR	T-cell receptor
TILs	tumor-infiltrating lymphocytes
TGF	transforming growth factor
TNF	tumor necrosis factor
Treg	regulatory T cell
TRP	tyrosinase related protein
$TRP\text{-}2_{180\text{-}188}$	TRP-2 sequence / TRP-2 peptide
TRP-1	Tyrosinase-related protein 1 gp75)
TRP-2	tyrosinase-related protein 2 (dopachrome tautomerase)
TLR	Toll-like receptor

Tu	tumor (specimenes collected from primary tumors)
Tyr	tyrosinase

V

VEGF	vascular endothelial growth factor
VEGFR1, 2	vascular endothelial growth factor receptor type 1 or 2
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late activation antigen-4
V600E	Mutations resulting in a substitution of valine for glutamate at amino acid 600
V	volume
v/v	volume per volume

U

UV	ultraviolet

W

WT	wild type
Wnt	Portmanteau of Int and Wg [wingless] in Drosophila melanogaster, which is the best characterized Wnt gene

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