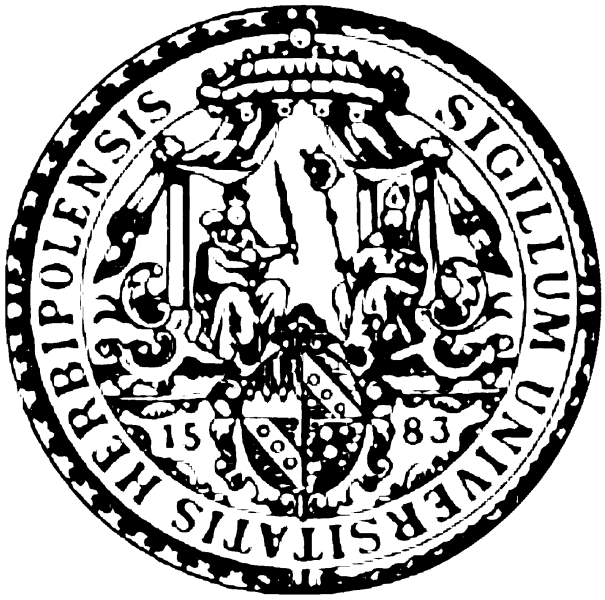
Scientific basics for new immunotherapeutic approaches towards Merkel cell carcinoma

Grundlagen neuer immuntherapeutischer Ansätze gegen das Merkelzellkarzinom



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Preface

The herein presented thesis consists of a selection of three original articles that are published or submitted for publication.

The work presented in this thesis was carried out between February 2010 and May 2015 and was supervised by principal supervisor Prof. Dr. Dr. Jürgen C. Becker and project supervisor Dr. *rer. nat.* David Schrama.

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(III) The Center for Cancer Immune Therapy (CCIT) at the Herlev Hospital, Denmark

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Summary

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer that has been associated with the Merkel cell polyomavirus (MCPyV). Indeed, MCC is one of the cancers with the best-established viral carcinogenesis. Despite persistence of the virus in MCC cells and the subsequent expression of viral antigens, the majority of MCC tumors are able to escape the surveillance of the immune system. Therefore the aim of the here presented thesis was to scrutinize immune escape mechanisms operative in MCC. A better understanding of their underlying molecular processes should allow to improve immunotherapeutic treatment strategies for MCC patients. The manuscripts included in this thesis characterize three novel immune evasion strategies of MCC.

I) the epigenetic silencing of the NKG2D ligands MICA and MICB via histone H3 hypoacetylation
II) reduced HLA class I surface expression via epigenetic silencing of the antigen processing machinery (APM)
III) the activation of the PI3K-AKT pathway in a mutation independent manner as potential immune escape strategy

MCC tumors and MCC cell lines were analyzed for their expression of MICA/B, HLA and components of the antigen processing machinery as well as for the activation of the PI3K-AKT pathway *in situ* and *in vitro*. These analysis reviled MICA and MICB, as well as HLA class I were not expressed or at least markedly reduced in ~80% of MCCs *in situ*. The PI3K-AKT pathway, that had only recently been demonstrated to play a significant role in tumor immune escape, was activated in almost 90% of MCCs *in situ*. To determine the underlying molecular mechanisms of these aberrations well characterized MCC cell lines were further analyzed *in vitro*. The fact that the PI3K-AKT pathway activation was due to oncogenic mutations in the *PIK3CA* or *AKT1* gene in only 10% of MCCs, suggested an epigenetic regulation of this pathway in MCC. In line with this MICA/B as well as components of the

APM were indeed silenced epigenetically via histone hypoacetylation in their respective promoter region. Notably MICA/B and HLA class I expression on the cell surface of MCC cells could be restored after treatment with HDAC inhibitors in combination with the Sp1 inhibitor Mithramycin A in all analyzed MCC cell lines *in vitro* and in a xenotransplantation mouse model *in vivo*. Moreover inhibition of HDACs increased immune recognition of MCC cell lines in a MICA/B and HLA class I dependent manner.

Several studies have accumulated evidence that immunotherapy is a promising treatment option for MCC patients due to the exquisite immunogenicity of this malignancy. However, current immunotherapeutic interventions towards solid tumors like MCC have to account for the plentitude of tumor immune escape strategies, in order to increase response rates. The immune escape mechanisms of MCC described in this thesis can be reverted by HDAC inhibition, thus providing the rationale to combine ‘epigenetic priming’ with currently tested immunotherapeutic regimens.

Zusammenfassung

Das Merkelzellkarzinom (MCC) ist ein aggressiver neuroendokriner Hautkrebs, der mit dem Merkelzell-Polyomavirus (MCPyV) assoziiert ist. Das MCC ist eine der Krebserkrankungen mit der am besten etablierten viralen Karzinogenese. Trotz der Anwesenheit des MCPyV in MCC-Zellen und der daraus einhergehenden Expression viraler Antigene sind die meisten MCC-Tumoren in der Lage der Überwachung durch das Immunsystem zu entgehen. Aus diesem Grund war das Ziel der hier vorliegenden Arbeit, neue im MCC operative „*immune escape*" Mechanismen zu ermitteln. Ein besseres Verständnis der hierbei zugrunde liegenden Mechanismen, sollte es ermöglichen, immuntherapeutische Behandlungsstrategien für MCC-Patienten zu verbessern. Die vorgestellten Manuskripte beschreiben drei neuartige „*immune evasion*" Strategien des MCC:

I) die epigenetische Inaktivierung der NKG2D-Liganden MICA und MICB mittels Histone-H3-Hypoacetylierung
II) eine reduzierte HLA Klasse I-Oberflächenexpression aufgrund epigenetischer Inaktivierung der Antigenprozessierungsmaschinerie (APM)
III) die mutationsunabhängige Aktivierung des PI3K-AKT-Signalweges, als potentieller „*immune escape*" Mechanismus

MCC-Tumoren und MCC-Zelllinien wurden sowohl bezüglich der Expression von MICA/B, HLA Klasse I und Komponenten der APM als auch auf die Aktivierung des PI3K Signalweges *in situ* und *in vitro* untersucht. Diese Analysen zeigten, dass sowohl MICA und MICB als auch HLA Klasse I in ca. 80% der MCC-Tumoren *in situ* nicht, oder nur sehr reduziert, exprimiert wurden. Der PI3K-AKT-Signalweg, welcher erst kürzlich mit Tumor „*immune escape*" in Verbindung gebracht wurde, war in fast 90% aller MCC-Tumoren *in situ* aktiviert. Um die zugrunde liegenden molekularen Mechanismen dieser Aberrationen zu entschlüsseln, wurden gut charakterisierte MCC-Zelllinien *in vitro* untersucht. Die Tatsache, dass der PI3K-AKT-Signalweg in nur 10% der MCCs auf Mutationen im *PI3KA*- oder *AKT1*-Gen zurückzuführen war,

suggeriert eine epigenetische Regulation dieses Signalwegs. In Übereinstimmung hiermit waren sowohl MICA/B als auch Gene der APM epigenetisch mittels Histon-Hypoacetylierung in ihren jeweiligen Promoterregionen inaktiviert. Bemerkenswerterweise konnten *in vitro* und in einem Xenotransplantations-Mausmodell *in vivo* sowohl die MICA/B als auch die HLA Klasse I-Oberflächenexpression aller untersuchter MCC-Zelllinien durch die Behandlung mit HDAC-Inhibitoren in Kombination mit dem Sp1-Inhibitor Mithramycin A wieder hergestellt werden. Des Weiteren erhöhte die Inhibition von HDACs die MCC-Immunerkennung auf eine MICA/B und HLA Klasse I-abhängige Weise.

Zahlreiche aktuelle Studien bestärken die Annahme, dass aufgrund der besonderen Immunogenität des MCC, die Immuntherapie eine aussichtsreiche Behandlungsoption für MCC-Patienten darstellt. Nichtsdestotrotz müssen die derzeitigen immuntherapeutischen Methoden zur Behandlung solider Tumore die Vielzahl von Tumor „*immune escape“* Mechanismen mitberücksichtigen, um die Ansprechrate zu erhöhen. Die Tatsache, dass die hier beschriebenen „*immune escape*“ Mechanismen durch HDAC-Inhibition aufgehoben werden können, spricht für die Hypothese, dass eine Kombination von „epigenetischem Priming“ mit derzeitig untersuchten immuntherapeutischen Ansätzen sinnvoll ist.

Introduction

Merkel cell carcinoma

Merkel cell carcinoma (MCC) is a neuroendocrine skin cancer. It was first described in 1972 by Cyril Toker as trabecular carcinoma of the skin [1]. MCC is a very aggressive malignancy with local recurrences and regional and distant metastasis making it one of the deadliest skin cancers with a disease associated mortality rate of 46% at five years, i.e. more than double as high as for melanoma [2]. Although MCC is an uncommon tumor, with an incidence of only 0.6 in 100.000 (in 2006), recorded cases of MCC have tripled over the last 20 years [3] and its incidences continues rising about 8% per year [4]. On the one hand this might be due to increased awareness and improved diagnostic methods, on the other hand MCC risk factors are increasing. Those risk factors include an age over 50, immunosuppression, UV exposure and fair skin [5]. The average age of MCC onset is at 75 [6] and it mostly arises in Caucasian individuals [5]. Historically, MCC was named after its presumed cell of origin, the Merkel cell (MC) [7]. MCs are sensory neuroendocrine cells in the basal layer of the skin that were first describe by Friedrich Sigmund Merkel in 1875. For years it was believed that MCs originate from neural crest cells [8], yet more recent findings indicate that MCs in fact arise from epidermal progenitor cells [9]. MCs are considered a part of a diffuse neuroendocrine system and are defined by the expression of a set of neuroendocrine markers like chromogranin A, synaptophysin, and neuron-specific enolase as well as cytokeratins 8, 18, 19, and 20 [10]. Since MCC marker expression widely overlappes with this expression pattern of MCs it was tempting to suggest Merkel cells as cells of origin for this malignancy. Yet, during the last decade, controversy sparked about the cell that MCC arises of. Currently discussed cells of origin of MCC are Merkel cell progenitors, epidermal stem cells, dermal stem cells, skin derived precursor cells [11] and early B cells [12]. Much like the cell of origin, also the processes underlying malignant transformation of MCC cells had been a mysterious for

decades. This changed in 2008 when a new polyomavirus was discovered in MCC cells [13].

Merkel cell polyomavirus

In 2008, Merkel cell polyomavirus (MCPyV) was described as an oncogenic virus [13]. Other viruses associated with cancer are for example the Epstein Barr virus (EBV), which is associated with Hodgkin's and non-Hodgkin's lymphoma [14], Hepatitis B and C virus that cause liver cancer [15, 16], human papilloma virus, which is associated with cervical cancer [17] and human herpes virus 8 (HHV8), which is involved in carcinogenesis of Karposi sarcoma [18-20]. MCPyV, is a typical, non-enveloped, double stranded DNA polyomavirus (Figure 1a).

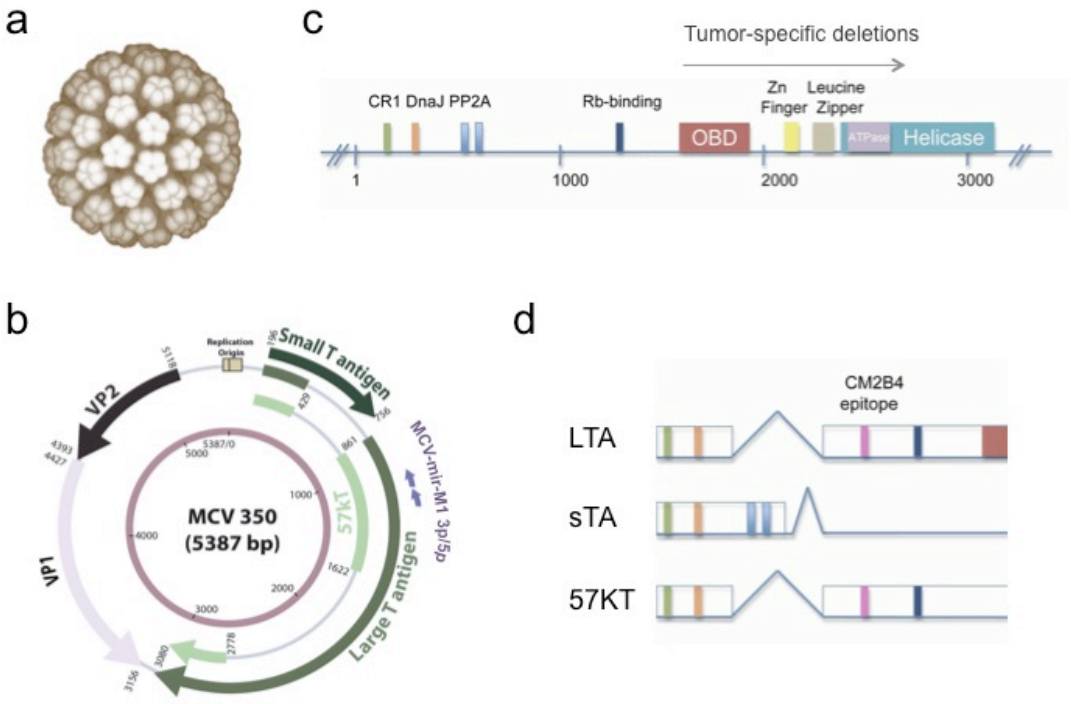

Figure 1: The Merkel cell polyomavirus (MCPyV); adapted from [21] and David Schrama. a) Capsid of MCPyV. b) The MCPyV circular Genome encoding for the T antigens (sTA, LTA) and viral capsid proteins (VP1, VP2, VP3) genome and LTA/STA/57KT. c) Within the T antigen T encoded are a CR1 and DNAJ domain, as well as PP2A and RB bining site, regions further downstream are tumor specific deleted. d) LTA, sTA and the 57KT polypeptides are processes via alternative splicing from the MCPyV T antigen locus, and depending on splicing and truncation exhibit varying functional domains.

It is part of the normal skin viriome, as it could be detected in skin swaps of 60-80% of healthy volunteers and in 90% of MCC patients [22, 23]. A current model suggests that in the case of MCC, MCPyV integrates into a single target cell, which is then clonally expanding [13], [24]. The site of MCPyV integration seems to be random, since no integration hot spots have been identified so far [25-27]. While most of the early studies state that around 80% of MCC tumors harbor the MCPyV [13], [28-31], this frequency has been discussed recently after new detection methods suggest that most, if not all, MCCs are $MCPyV^+$ [32]. The MCPyV genome is around 4.5 k, circular and encodes for an early and a late gene region which is divided by a non- coding regulatory region [13] (Figure 1b). The early region encodes for the MCPyV Large T (LTA) and small TA (sTA) and 57kT antigen (Figure 1c). LTA, sTA, and 57kT are encoded overlapping and can be processed by alternative RNA splicing [33] (Fig. 1d). The MCPyV genome also encodes for two microRNAs MCV-mir-M1-3p and MCV-mir-M1-5p [34] (Figure 1b). The non-coding region of the MCPyV genome contains the origin of replication (ori), promoters, a glucocorticoid response element (GRE Element) and an enhancer region. An important feature in the case of MCC is that the integrated MCPyV genome contains individual truncating mutations in the T antigen region [33] (Figure 1c) and deletions in the *VP1* gene [28] leaving it unable to replicate. MCPyV's oncogenic potential, as far as it is known today, is mainly mediated by LTA and sTA. While LTA's p53 binding side is lost due to the truncation of LTA, it still encodes for a LxCxE domain, which can bind to cellular pocket proteins, and a DnaJ domain, which can bind heat shock proteins [33]. It has been demonstrated *in vitro* and in a xenotransplantation study *in vivo*, that binding of retinoblastoma protein (pRb) to LTA's LxCxE motive is critical for MCC cell survival [35, 36]. When pRb is bound to LTA, it can not bind and suppress the transcription factor E2F, enabling MCC cells to enter into S-phase and to proliferate [33] (Figure 2a). Despite the loss of the p53 binding site, there are discussions whether MCPyV LTA might still be able to indirectly inactivate p53 or not [37-39]. Like LTA, sTA possesses the heat shock protein binding site and an additional PP2A binding site [40]. It is still controversially discussed whether MCC cells are dependent on the expression of MCPyV sTA to proliferate [41-43]. Yet, it has been demonstrated that MCPyV sTA acts

downstream of the mTOR pathway by maintaining 4E-BP1 hyperphosphorylation, and thus increases cap dependent translation via an yet unknown mechanism [44] (Figure 2b). Further more it was recently demonstrated that MCPyV sTA also mediates microtubule destabilization and thus promotes cell motility and migration of MCC cells [45]. Knight *et al.* also implicated that the PP2A binding site of the MCPyV sTA might be involved in this process via the binding of PP4C [45].

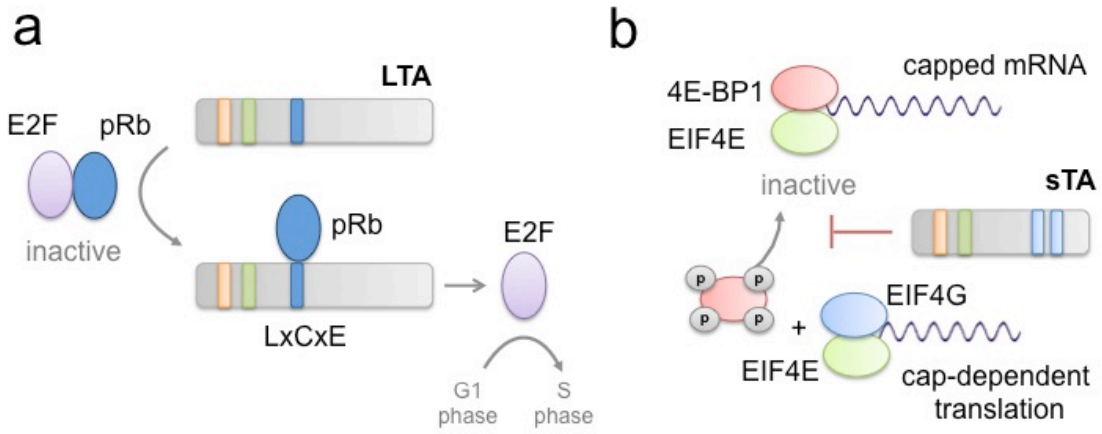

Figure 2: MCPyV LTA and sTA mode of action. a) MCPyV LTAs LxCxE motive can bind pR. As a result the transcription factor E2F is released and MCC cells can enter into S-phase and proliferation. b) MCPyV sTA acts downstream of the mTOR pathway via 4E-BP1 hyperphosphorylation, resulting in an increased cap dependent translation.

Current therapy options for MCC

Particularly for the advanced stages of MCC, there is currently no consensus, how to treat MCC patients most effectively [46]. Due to the rareness of MCC prospective randomized clinical trials are lacking and therefore, therapeutic options are limited. At early tumor stages the national Comprehensive Cancer Network (NCCN) guidelines recommend surgical excision with a wide margin, biopsy of the sentinel lymph node and if necessary adjuvant therapy [47]. The necessary excision margin between 1 and 3 cm as well as the need of adjuvant radiation therapy after, are still controversial discussed [48-50]. Metastatic or advanced locoregional tumors that are not accessible to surgery are treated with chemotherapeutics like doxorubicin, vincristin, etopsid and cisplatin, alone or in combination [51]. MCC is considered a chemosensitive

tumor, based on *in vitro* observations [52, 53]. But, despite the fact that about 60% of patients respond to the first treatment with chemotherapeutic drugs, after the second to third cycle those tumors become resistances towards this treatment, and thus no study so far has shown any survival benefit for MCC patients treated with chemotherapy [48, 49] [54-57]. Moreover, chemotherapy is accompanied by severe side effects e.g. sepsis, fever, skin toxicity, neutropenia and even death, particularly in the elderly. Hence, especially for the main group of MCC patients with an age over 75 [6], chemotherapy is particularly grueling [58]. Conspicuously there is an urgent need for more efficient and better-tolerated therapy options for MCC patients. A very promising new therapeutic approach is the so-called *cancer immunotherapy*, which will be discussed in the next chapters.

Cancer Immunology

The human immune system consists of the fast but not specific acting innate immune response and the slow and specific adaptive immune response, which are in an extensive crosstalk with each other. The innate immune system is the first barrier against infection and consists of soluble factors, granolcytes, mastcells, dentritic cells (DCs), macrophages and natural killer (NK) cells that work together to control inflammatory responses [59]. The adaptive immune response specifically targets in an antibody and antigen dependent manner and consist of B cells and T cells [59]. Natural killer T cells (NKT) cells and γδ T cells function at the interface between the innate and the adaptive immune response, combining features of both systems [59] (Figure 3). It is well known, that components of the innate and adaptive immune system work together in a complex process to protect the host against pathogens like viruses and bacteria, but during the last century it also became evident that the immune system plays an important role in the surveillance of cancer.

In 1909 Paul Ehrlich hypothesized, that the immune system besides the protection against pathogens like viruses and bacteria also plays an important role in the surveillance of cancer cells. He supposed that malignant cells

constantly arise in the body, but that an intact immune system can protect the host against those transformed cells [60]. In the 1950s first experimental evidence was provided in transplantations studies supporting his theory [61], but due to controversial results obtained with the available methods at the time the concept of cancer immune surveillance was debated for decades among scientist. It was not until the 1990s, after convincing observations in human cancer patients and experimental evidence in knock-out mice, that the concept of cancer immune surveillance gained general acceptance [59].

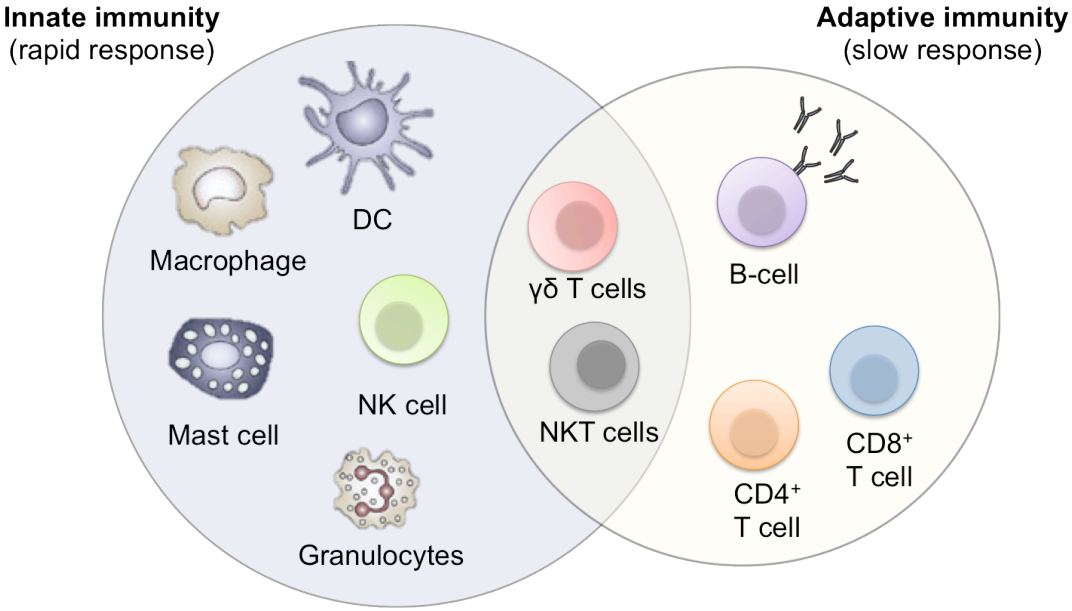

Figure 3: Elements of the innate and adaptive immune system; adapted from [59] The fast responding innate immune system consists of mast cells, macrophages, natural killer (NK) cells, dendritic cells (DCs) and granulocytes. γδ T cells and natural killer T (NKT) cells build the bridge between the adaptive and the innate immune system. Antibody producing B cells, $CD8^+$ T cells and $CD4^+$ T cells are part of the slow responding adaptive immune system.

For example it was demonstrated that patients receiving immunosuppressive drugs after solid organ transplantation were at higher risk to develop a cancer [62, 63]. Also Patients with T-cell deficiencies are at a severely elevated cancer risk. For example, the HIV-1 virus selectively targets and kills $CD4^+$ T cells and thus ultimately leads to the acquired immunodeficiency syndrome (AIDS) in HIV infected individuals. Due to this, the incidence of cancers caused by oncogenic viruses, like Kaposi sarcoma, non-Hodgkin's lymphoma and cervical cancer [64], but also other types of cancer is extremely elevated

in AIDS patients [65]. In children with an congenital deficiencies of the humoral immune response, like the common variable immunodeficiency or X –linked immunodeficiency with hyper-IgM, cancer incidence is higher than average [66, 67]. Further evidence for the cancer surveillance theory is that circulating tumor reactive T cells and antibodies do exist in a variety of cancers [68, 69]. Additionally tumor infiltrating lymphocytes (TILs) are associated with a better prognosis in several solid tumors like melanoma [70], non small lung cancer [71] and ovarian cancer [72]. Selective knock out experiments with mice delivered new insights into the concept of tumor immune surveillance. Immune deficient knock out mice were more prone for cancer formation and their tumors grew faster than in immune competent control mice. In particular $CD8^+$ T cell, $CD4^+$ T cell and NK cell deficiencies led to an increased tumor incidence [73, 74]. Also γδ T cell knock out mice were more susceptible to multiple regimens of cutaneous carcinogenesis [75]. Notably mice with a combined T cell and NK cell dysfunction were at even higher risk to develop tumors, this fact demonstrate that the innate as well as the adaptive immune system are necessary for tumor immune surveillance [76, 77]. Based on these findings the current opinion on cancer immune surveillance is, that cells of the innate and adaptive immune system are in a complex crosstalk with each other to detected and eliminate cancer cells.

Effector cells against tumors and their mechanisms of action

The immune recognition of cancer cells is a complex process, requiring the precise interplay of all components of the innate and adaptive immune system. The main effector cells against tumors however are $CD4^+$ helper T cell, $CD8^+$ cytotoxic T cells, γδ T cells, and NK cells (Figure 4). $CD4^+$ T cells, also called T helper cells, recognize antigens presented on MHC class II complexes presented on the cell surface of professional antigen presenting cells like, macrophages and DCs [78]. In contrast $CD8^+$ T cells detect antigens that are processed of intracellular proteins and presented on MHC class I molecules on the cell surface of almost all nucleated cells [78]. MHC class I molecules are heterodimers composed of constant light chain, called

β2-microglobulin (β2m) and a varying heavy α chain. In humans the different MHC class I alleles are referred to as human leukocyte antigens (HLA) A, B and C. A complex machinery, the antigen processing machinery (APM) is required to process and present peptides on MHC class I as well as on MHC class II molecules on the surface of cells. The APM is composed of proteasome like subunits (LMP2, LMP7), peptide transporter subunits (TAP1, TAP2) and chaperone proteins (Tapasin) [79]. Another T cell subset are γδ T cells. They posses an alternative T cell receptor (TCR) that is in contrast to the TCR of $CD4^+$ and $CD8^+$ T cells not composed of an α and β subunit, but of a γ and δ subunit, hence the name. They express T cell receptors, but do not require antigen processing and presentation via MHC class I or II to be activated. Their exact mechanism of action however is still controversially discussed [80, 81].

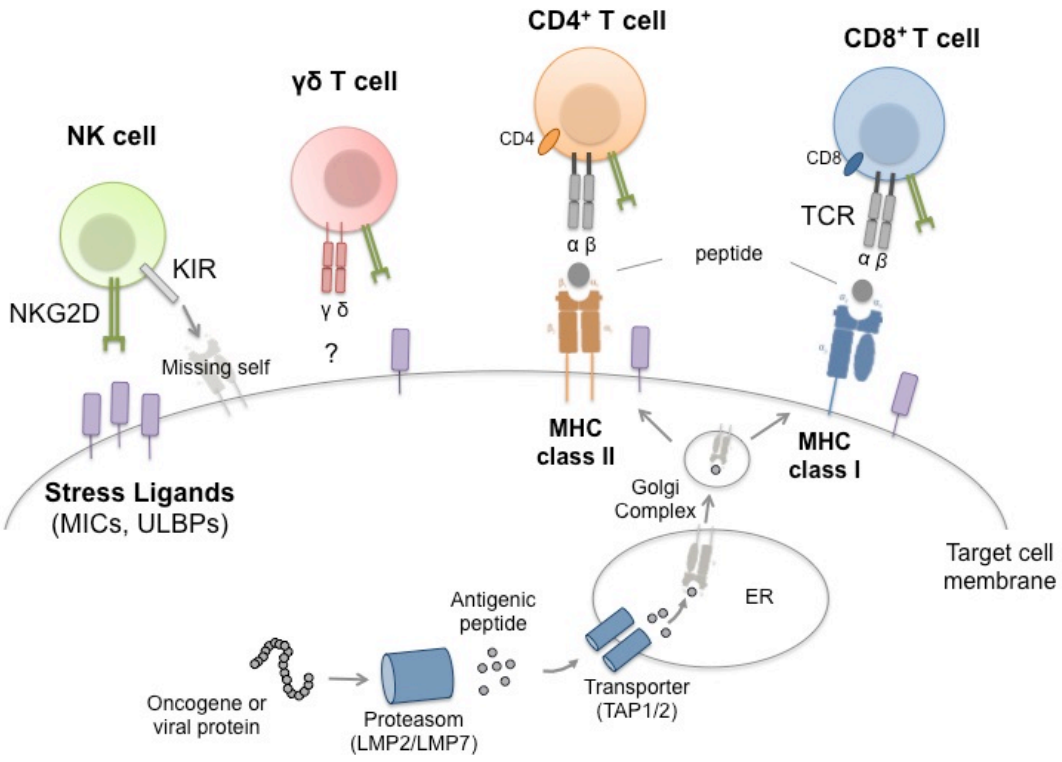

Figure 4: Tumor targeting cells. The key effector cells on anti tumor immune responses are $CD4^+$ helper T cell, $CD8^+$ cytotoxic T cells, γδ T cells, and natural killer (NK) cells. $CD4^+$ and $CD8^+$ cells are dependent on antigen presentation on the surface of the target cell via MHC class II or MHC class I complexes respectively. Antigenic peptides are processes from intracellular proteins via the antigen processing machinery (APM), which is composed of proteasome subunits (LMP2 and LMP7), transporters (TAP1 and TAP2) and chaperon proteins. γδ T cells are activated via a yet unknown MHC independent mechanism. NK cells are activated via ligand binding to activating receptors (e.g. NKG2D) or missing ligands of inhibitory receptors (e.g. KIR).

Also NK cells can detect and eliminate infected, stressed and abnormal cells [82]. They contain characteristic cytoplasmatic granulas that are released upon triggering to lyse target cells by perforating their cell membrane [83]. They are activated via two main mechanisms, (i) the lack of MHC class I molecules on the cell surface of a target cell, the so called *missing self* signal or (ii) via activating ligands like MHC class I polypeptide-related sequence genes (MICs) or ULPBs, which are expressed on virus infected or stressed cell, the so called *induced self* signal [84]. Examples for inhibitory receptors are killer-cell immunoglobulin-like receptors (KIRs) and leucocyte inhibitory receptors (LIRs)[85]. Activating receptors of NK cells are for example, CD16 (FcγIIIA) and NKG2D receptors [85]. NKG2D receptors are expressed not only on NK cells, but also on $CD4^+$ helper T cell, $CD8^+$ cytotoxic T cells and γδ T cells as activating or co-activating receptors.

Immunogenicity of MCC

Discoveries made during the last decades accumulated evidence that MCC *per se,* much like other virus-associated malignancies, is a highly immunogenic tumor. Approximately 8% of MCC patients are severely immunosuppressed [5]. Accordingly among immune suppressed individuals the incidence of MCC is significantly higher with an onset at a younger age than in the general population. High risk groups here are patients with chronic lymphocytic leukemia and other hematologic malignancies with a 34 to 48-fold increased risk [5], solid organ transplant recipients with a 10-fold increased risk [86], HIV infected patients with an 8-fold higher risk [87] and also patients with auto-immune diseases, treated with immunosuppressive medication are at higher risk to develop a MCC [88]. Additionally immune suppression is associated with a poor prognosis at a 50% reduced three year survival rate [89]. Notably, over the years there have been reports of cases of spontaneous remission of MCC tumors especially after the immunosuppressive state of patients had been abrogated [90, 91]. Moreover viral antigens like LTA and sTA, which are exclusively expressed in tumor cells make MCC cells an ideal target for T cell mediated tumor immunotherapeutic approaches. Indeed MCPyV specific T cell responses have been reported [92, 93]. Next to viral

antigens, MCCs also overexpress tumor antigens like survivin, MUC-1, Ep-cam, bcl-2 and p63 that would make ideal targets for T cell mediated immunotherapies [94-97]. Accordingly, some MCC tumors are heavily infiltrated by immunogenic cells, and intra-tumoral infiltration of $CD8^+$ T cells is associated with an improved prognosis [98, 99]. All these findings lead to the conclusion that in theory the immune system should be able to detect and destroy MCC cells. But in reality, except for the few described cases of spontaneous regressions of MCC, this is not the case. This fact suggests that MCC possess superior capabilities to evade immune responses.

MCC immune evasion – current state of knowledge

In the year 2000 Hanahan and Weinberg postulated the six hallmarks of cancer [100]. In 2011 two additional important features of cancer cells were added to the „next generation“ of cancer hallmarks [101]: one of which was the immune evasion of cancer cells. It had become apparent that despite immune surveillance, tumors are still able to develop, even in hosts with a fully functioning immune system. *Cancer immunoediting* describes a process, were in a heterogeneous population of tumor cell, variant cells with a reduced immunogenicity are enriched, due to their survival benefit [102]. The cancer immunoediting process is devided into three phases: 1) *elimination* (immunesurveillance), during this phase the immune system reacts to the tumor and tumor cells are eliminated; 2) the *equilibrium* is the phase in which the immune system does react, but can not eliminates the tumor cells to a full extent. It is in this phase when certain cancer cells acquire features to protect them from immune recognition; 3) the *escape* phase in which surviving immune resistant cancer cells can form tumors that are evading the immune response. For a virus-associated malignancy like MCC immune evasion is particularly difficult to accomplish. But despite the continuous expression of viral and tumor antigens MCC cell still can escape immune responses. While MCPyV specific T cells are present in the majority of MCC patients, they rarely infiltrate into MCC tumors [98]. This fact suggests that MCC cells and cells of the MCC microenvironment create an immunosuppressive

atmosphere that inactivates MCC specific T cells and prevents them from infiltrating, thus facilitating the immune escape of MCCs. Indeed during the last years several studies accumulated evidences that MCC cells employ a variety of immune escape strategies. For example two independent studies highlighted the importance of the PD1/PD-L1 Axis in the immune escape of MCC. Programmed cell death 1 (PD1) is a member of the CTLA-4 family and is expressed mainly on activated T cells, but also on early B cells and NK cells [103]. Upon binding to its ligand PD-L1, an inhibitory signal is send to the T cell, leading to their inactivation [103]. In the case of MCC, PD-L1 is expressed on 50% of $MCPyV^+$ MCCs [104]. At the same time PD-1 is highly expressed on the circulating and infiltrating MCPyV specific T lymphocytes, resulting in an exhausted state of those cells, marked by the expression of Tim-3 [105]. Additionally Dowlatshahi *et al.* reported that tumor specific T cells of MCC patients might be inhibited by regulatory T cells (T_{regs}) [106]. They demonstrated an increased number of CD4 and CD8 T_{regs} in MCC tumors. Unlike Lipson *et al.* they found PD-1L and PD-2L only expressed in the tumor microenvironment but not on MCC cells [106]. Most recently Paulson *et al.* described a reduced MHC class I expression on the majority of MCCs [107]. They also demonstrated that MHC class I expression in $MCPyV^+$ MCCs was significantly lower than that of $MCPyV^-$ tumors and that MHC class I reduction was reversible. Those findings demonstrated that especially $MCPyV^+$ T cells are under a high immunoselective pressure and therefor exploit multiple immune evasion strategies at the same time. In order to successfully implement immunotherapy options in the treatment of MCC, it is of great importance to uncover all those immune escape strategies to accordingly adapt immunotherapy.

Cancer immunotherapy

The development of cancer immunotherapy was lengthy, full of obstacles and defeats, but also crowned with great success (Figure 5). Cancer Immunotherapy was first performed in 1891 when surgeon William Coley had heard of a case of complete tumor remission after a severe infection [59]. He

injected bacteria into patients' tumors to provoke immune responses against the infected tissue, and as he hoped also the tumor cells. Unfortunately, although he had some success, it shouldn't be that simple and with the invention of radiation therapy and the improvements in chemotherapy his ideas were almost forgotten. Until almost a 100 years later, when the first immunotherapeutic cytokines, interferon alpha (IFN-α) and interleukin 2 (Il-2, Aldesleukin) were approved by the FDA for the treatment of melanoma. Unfortunately response rates were low, e.g. IL-2 alone led to complete remission in only 6% of melanoma patients, while it's side effects killed 2% [108]. IFN did reduces the risk of recurrence by 10% in melanoma patients, but did not have any effect on overall survival [109]. After this discouraging results immunotherapy did not get much attention for decades, until 2013, when cancer immunotherapy was named the scientific break through of the year by the *Science* magazine [110]. The introduction of a new class of immunotherapeutic drugs, so-called checkpoint blocking antibodies, significantly contributed to the acceptance of cancer immunotherapy (Figure 5).

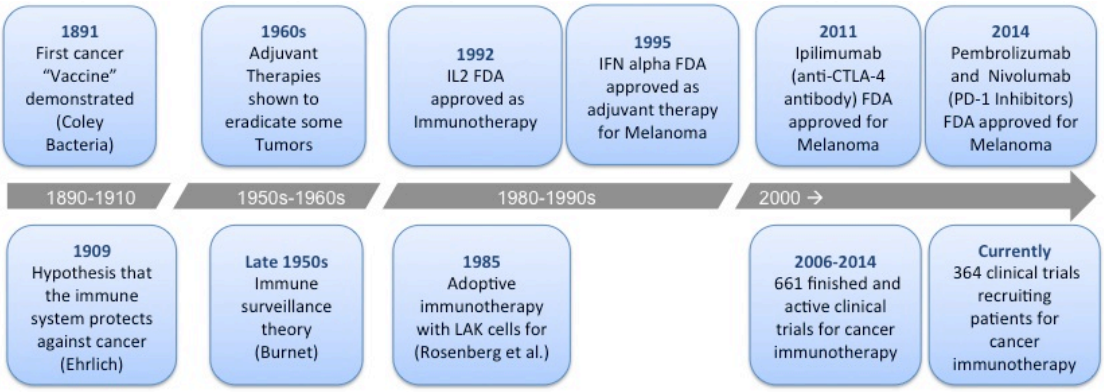

Figure 5: Milestones of cancer immunotherapy. The foundations of cancer immunotherapy go back to the late 1800 and early 1900. Further understanding about the immune surveillance of tumors was gained in the 1950s and 60s. In the 1980s and 90s the first immunotherapeutic trial with adoptive LAK cell transfer were conducted and the cytokines IL2 and IFNα were approved by the FDA for the treatment of cancer. After 2000 checkpoint blocking antibodies Ipilimumab, Pembrolizumab and Nivolumab were approved by the FDA for the treatment of Melanoma.

The checkpoint blocking antibody, Ipilimumab was approved by the FDA in March 2011 [111]. With Ipilimumab, it was possible for the first time to prolong the lives of late stage melanoma patients [112, 113]. FDA approval of two PD-

1 inhibitors, Pembrolizumab and Nivolumab, followed in 2014 after they had archived durable response rates in clinical trials on melanoma patients [114]. On the basis of this success stories, numerous new immunotherapeutic drugs are developed and tested alone or in combination in clinical trials, also on MCC patients.

Immunotherapeutic clinical trials on MCC

Due to the strong rational for immunotherapeutic approaches towards MCC a number of clinical trials are currently ongoing in this regard (Table 1). Based on the realization that the PD-1/PD-L1 axis was blocked in MCC, drugs reverting this state are currently tested for the treatment of MCC. Phase II clinical trials are recruiting MCC patients for the treatment with the anti-PD-L1 antibody MSB0010718C (NCT02155647) and the anti-PD1 antibody Pembrolizumab (NCT02267603). The cytotoxic T-lymphocyte antigen 4 (CTLA-4) blocking antibody Ipilimumab is currently tested in a phase II clinical trial for adjuvant treatment of MCC (NCT02196961). CTLA-4 is expressed on $CD4^+$ cytotoxic T lymphocytes and mediates a inhibitory signal that can be augmented with ipilimumab, resulting in an intensified immune response [113]. Also cytokine-based approaches against MCC are currently explored, but since it became obvious that systemic treatment with cytokines is not effective enough for the treatment of solid tumors this treatment options had to be improved. Therefor, clinical trials employing cytokines for the treatment of MCC are focusing on the delivery of interleukins directly to the MCC tumor site. A phase I/II clinical trial utilizes an F16-IL-2 immunocytokine in combination with paclitaxel for targeted delivery of IL-2 to the tumor microenvironment. IL-2 is a cytokine mainly produced by activated T cells and NK cells and it stimulates the proliferation and expansion of other $CD4^+$ and $CD8^+$ T cells, NK cells and B cells [59]. The F16 antibody part targets tenascin C expressed on the stroma cells in the MCC microenvironment and thus directs IL-2 to the tumor site (NCT02054884). Another phase II clinical trial that is currently ongoing uses electroimmunotherapy to deliver plasmids coding for Interleukin 12 (IL-12) with short electric pulses into MCC cells, leading to a local increase in IL-12 production (NCT01440816). IL-12 is a pro-

inflammatory cytokine produced mainly by phagocytotic cells like macrophages and induces cytokine, mainly Interferon gamma (INF-γ), production of NK and T cells [115].

Table 1: Currently ongoing immunotherapeutic clinical trials against MCC. Obtained from www.clinicaltrials.gov, May 2015.

Trial number	Drug	Treatment	Combination	Indication	Mechanism
NCT02054884	F16-IL-2	Antibody IL-2 fusion protein	Paclitaxel	adjuvant	IL-2 delivery to tumor stroma
NCT01440816	IL-12-Plasmid	IL-12-DNA electroporation	-	palliative	Promotes TH1 response, increases IFN-γ and cytolytic activity
NCT02267603	Pembrolizumab	Anti-PD-1	-	palliative	Blocks inhibitor/exhaustion signal to $CD8^+$ T cells
NCT02155647	MSB0010718C	Anti-PD-L1	-	palliative	Blocks inhibitor/exhaustion signal to $CD8^+$ T cells
NCT02196961	Ipilimumab	CTLA-4 Blocking Antibody	-	adjuvant	Blocks CTLA-4 mediated inhibition of immune activation
NCT01758458	$CD8^+$ T cells	Adoptive T cell therapy	Aldesleukin	adjuvant	Expansion and activation of tumor targeting lymphocytes
NCT02035657	GLA-SE	Toll-like Receptor-4 Agonist	-	palliative	Immune stimulatory

After encouraging response rates in malignant melanoma [116] adoptive immunotherapy is currently tested for the treatment of MCC patients. In this approach MCPyV specific tumor infiltrating lymphokines (TILs) are isolated from MCC tumors, expanded, activated *in vitro* and re-administered to the patient. This method is particularly attractive for the treatment of MCC, due to the fact that MCPyV encoded foreign proteins are only expressed in MCC tumor cells and not in healthy tissue. A phase I/II feasibility study with MCPyV T antigen-specific polyclonal autologous $CD8^+$ T cells in combination with aldesleukin (IL-2) (NCT01758458) is currently ongoing for MCC patients. In a

phase I proof of principal study 10 MCC patients will be treated with the toll like receptor (TLR) 4 agonist GLA-SE (NCT02035657). Toll like receptor agonists can stimulate Th1 immune responses via increasing co-stimulatory molecules like CD40, CD80 and CD86 on myeloid dendritic cells and their secretion of pro-inflammatory cytokines like interleukine 2, 6 and 12 and tumor necrosis factor alpha (TNF-α) [117, 118].

Limitations of immunotherapy

While clinical trials for the immunotherapy of MCC are still ongoing, there are finished trials on other malignancies, like e.g. on malignant melanoma (MM) that have taught some valuable lessons. While for the first time immunotherapy resulted in long lasting complete response rates in advanced melanomas, this was only the case in a minority of patients [114, 119]. For example treatment with high-dose IL-2 alone induced objective clinical responses in 15–20% of patients, but this responses were only complete and durable in around 6% of these patients [120]. With the CTLA-4 antibody Ipilimumab alone, overall response rates were 10% but the response was only long lasting for about half of those initially responding patients [112]. It became apparent that cancer patients respond very different to immunotherapy and so far there are almost no predictive markers, which patient will benefit from immunotherapy. Also it became obvious that combination of different drugs will be the key to success for immunotherapy. This is not surprising since in most malignancies and especially in virus-associated cancers like the MCC, multiple immune escape strategies are exploited in parallel. Indeed combination of IL-2 with Ipilimumab resulted in a complete response of 17% of melanoma patients, that was long lasting in all cases [119]. Additionally one of the major future challenges in the immunotherapy of solid tumors will be, not only to activate cytotoxic lymphocytes, but also to guide them towards the tumor microenvironment and to stimulate them to infiltrate and destroy the tumor [121]. To achieve this goal in MCC it is necessary to unravel further immune escape strategies that are exploited by tumor cells and MCPyV, and to decrypt the underlying mechanisms in order to utilize new treatment options that can be combined with established immunotherapy.

Manuscripts

I) Activation of the PI3K/AKT pathway in Merkel cell carcinoma

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Published in *PLoS One*, February 2012

II) Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

Cathrin Ritter, Kaiji Fan, Kelly G. Paulson, Paul Nghiem, David Schrama, Jürgen C. Becker

Submitted to *Science Translational Medicine*, March 2015

III) HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

Cathrin Ritter, Kaiji Fan, Annette Paschen, Sine Reker Hardrup, Soldano Ferrone, Paul Nghiem, David Schrama, Jürgen C. Becker

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Article I

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Activation of the PI3K/AKT Pathway in Merkel Cell Carcinoma

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Abstract

Merkel cell carcinoma (MCC) is a highly aggressive skin cancer with an increasing incidence. The understanding of the molecular carcinogenesis of MCC is limited. Here, we scrutinized the PI3K/AKT pathway, one of the major pathways activated in human cancer, in MCC. Immunohistochemical analysis of 41 tumor tissues and 9 MCC cell lines revealed high levels of AKT phosphorylation at threonine 308 in 88% of samples. Notably, the AKT phosphorylation was not correlated with the presence or absence of the Merkel cell polyoma virus (MCV). Accordingly, knock-down of the large and small T antigen by shRNA in MCV positive MCC cells did not affect phosphorylation of AKT. We also analyzed 46 MCC samples for activating *PIK3CA* and *AKT1* mutations. Oncogenic *PIK3CA* mutations were found in 2/46 (4%) MCCs whereas mutations in exon 4 of *AKT1* were absent. MCC cell lines demonstrated a high sensitivity towards the PI3K inhibitor LY-294002. This finding together with our observation that the PI3K/AKT pathway is activated in the majority of human MCCs identifies PI3K/AKT as a potential new therapeutic target for MCC patients.

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Introduction

Merkel cell carcinoma (MCC) is a very aggressive malignant skin tumor. The disease typically affects elderly patients. It is preferentially localized in the chronically UV-exposed skin. The correlation between UV light and MCC is probably due to the immunosuppressive rather than the mutagenic effect of UV irradiation. In patients with immunosuppression, MCC may occur at a significantly younger age.

The pathogenesis of MCC is as yet not completely understood [1], but the recent demonstration that the Merkel cell polyoma virus (MCV) DNA is frequently present in MCC suggests a viral induced carcinogenesis [2,3]. Despite the recent demonstration that MCV infected MCC cells require expression of the MCV encoded T antigens for proliferation and survival [4], little is known on cooperating oncogenic events. Previous studies found no evidence for mutations in classical oncogenes [5].

Still, high resolution comparative genomic hybridization revealed a number of chromosomal regions with gains and losses in MCC; the frequent loss of chromosome 10 where the tumor suppressor gene phosphatase and tensin homologue (PTEN) is encoded, suggests that aberrations of the PI3K/AKT pathway may be involved in the pathogenesis of MCC [6]. Moreover, while inactivating *PTEN* mutations are rare in MCC, the lack of PTEN protein expression is frequent observed in MCC [7].

The PI3K/AKT (phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homologue) pathway is a major signaling pathway downstream of many growth factor receptors and possibly the most frequently activated signaling pathway in human cancer [8]. Indeed, it has an important impact on apoptosis, proliferation, cell growth and malignant transformation. PI3K contributes to the signaling from receptor tyrosine kinases upon growth factor binding and generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PTEN reverses this step. PIP_3 induces downstream phosphorylation and activation of the survival kinase AKT1. Besides loss of PTEN, the PI3K/AKT pathway can be activated by oncogenic mutations. Somatic mutations in the *PIK3CA* gene, encoding for the α isoform of the p110 subunit of PI3K, have been identified in a wide variety of human tumors including benign skin tumors [9,10]. Furthermore, an oncogenic hotspot mutation in the pleckstrin homology domain (PHD) of *AKT1* is present in several tumor entities, albeit at a lower frequency than *PIK3CA* mutations [11].

Here, we demonstrate PI3K/AKT pathway activation, which is independent of the presence of MCV, and oncogenic *PIK3CA* mutations in human MCC. Activating *PIK3CA* mutations appear to occur at a low frequency, indicating that additional mechanisms contribute to PI3K/AKT pathway activation in MCC.

Materials and Methods

Sample acquisition

Formalin-fixed paraffin embedded histologically proven MCC samples (primary tumors and metastases) were retrieved from

histological files for the generation of a tissue microarray and for DNA isolation. Written, informed consent had been obtained from all patients to use tumor material not needed for histopathological diagnosis for further scientific workup; the study was performed according to the guidelines of the local ethics committee (Ethikkommission der Medizinischen Fakultät der Universität Würzburg; sequential study number 124/05) and the declaration of Helsinki. In addition, MCC cell lines were used. The cell lines WaGa, BroLi, HeRo and LoKe were derived from MCC patients of the Department of Dermatology, University of Würzburg [4], while UISO, [12] MCC13, [13] MCC26 [14], MKL-1 and MKL-2 [15] have been established in other laboratories. DNA was isolated from cell lines and formalin-fixed paraffin-embedded tissues containing at least 60–80% of tumor cells using standard protocols.

Immunohistochemistry

Immunohistochemistry was performed using a tissue microarray for MCC, malignant melanoma and basal cell carcinoma. The staining followed standard protocols. The antibody was directed against phosphorylated AKT at threonine 308 (rabbit polyclonal (#38449), Abcam, Cambridge, UK) and was used at a dilution of 1:200. The overall pAKT T308 staining intensity (not the frequency of positive tumor cells) was scored from 0 (negative), 1+ (weak), 2+ (strong), and 3+ (very strong) by two individual investigators (R.H. and J.C.B.). Each sample was represented in triplicate on the tissue microarray. In total, 41 samples (most of them not identical with the MCC used for genetic analyses; 14 primary tumors and 27 metastases) were evaluated on the MCC tissue microarray, as well as 67 melanomas (17 nodular, 17 acrolentiginous, 16 lentigo maligna melanoma, 17 melanoma metastases) and 45 basal cell carcinomas (20 nodular, 17 nodular and ulcerated, 2 nodular and pigmented, 6 not available).

Knock down of the MCV LT antigen

The MCV positive MCC cell lines WaGa, BroLi, MKL-1 and MKL-2 were infected with the lentiviral shRNA vector KH1 encoding either a scrambled shRNA or a shRNA targeting the MCV T antigen mRNAs. Successful knock down of large and small T antigen in MCV positive MCC cells using this construct has been recently described [4]. Total cell lysates were harvested on day 5 following infection and analyzed by immunoblotting. T antigen knock down was confirmed using the Large T antigen (LT) specific antibody CM2B4 [16]. α-AKT and α-phospho-AKT (T308 and S473) antibodies were purchased from Cell Signaling (Danvers, MA, USA).

PI3K/AKT inhibition

Cells were seeded in 96 well plates and the PI3K inhibitor was added at varying concentrations. After an incubation period of 24, 48 and 72 hours cellular metabolic activity was assessed by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA). To this end, 10 µl of CellTiter 96® AQueous One Solution Reagent containing a tetrazolium compound (MTS) were added to each well and the cells were incubated for approximately 5 hours at 37°C. Metabolically active, viable cells convert MTS into a colored formazan product that was measured in a spectrophotometric microplate reader (Perkin-Elmer Inc., MA, USA) at 493 nm. Furthermore, the cellular DNA content was measured in ethanol fixed, propidium iodide stained cells by flow cytometry as described previously [4].

Genetic analyses

Exons 9 and 20 of the *PIK3CA* gene were sequenced directly. These exons contain the majority of *PIK3CA* mutations yet found in human cancer. Samples which could not be sequenced successfully were analyzed by a recently described, more sensitive *PIK3CA* SNaPshot® assay in combination with a nested-PCR approach [17]. This assay covers the most important hotspot mutations at codons 542, 545 and 1047 of the *PIK3CA* gene. Furthermore, exon 4 of *AKT1* was sequenced directly, as this exon harbors the E17K hotspot mutation. In 8 MCC cell lines, exon 3 of *AKT3* was sequenced directly. Primer sequences and PCR conditions can be obtained from the authors. The presence of MCV DNA was assessed as described previously [2].

Statistical analysis

After the data passed the Shapiro-Wilk normality test, statistical differences between two groups were evaluated by the t-test, and between more than two groups by the repeated measures ANOVA followed by the Dunnett's multiple post test. A p value$\leq$0.05 was considered as significant.

Results

AKT activation in MCC

To investigate a possible activation of the PI3K/AKT pathway in MCC, immunohistochemical staining was performed for phospho-AKT threonine 308 (pAKT T308) taking advantage of a MCC tissue microarray. In total, 41 MCC samples (14 primary tumors and 27 metastases from a total of 26 patients) were evaluated. Expression of pAKT T308 was scored from 0 (negative) to 3+ (strongly positive).

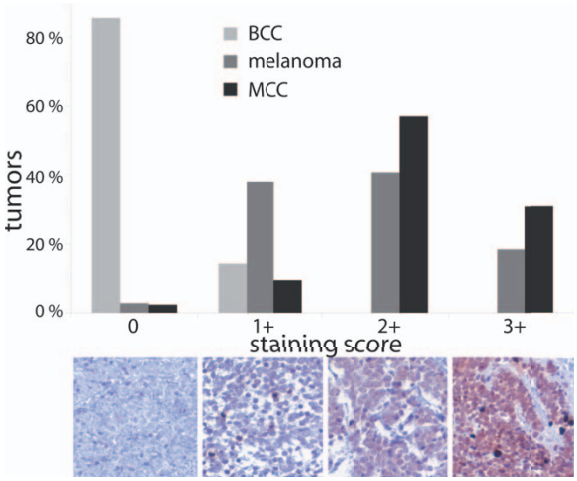

Figure 1. Activating phosphorylation of the AKT protein at position threonine 308 in Merkel cell carcinoma, malignant melanoma and basal cell carcinoma. The presence of AKT phosphorylated at T308 was analyzed by immunohistochemistry using a phospho-specific antibody on tissue micro arrays representing 41 MCCs (each in triplicates), 45 basal cell carcinomas (BCC) and 67 melanomas, respectively. Samples were scored from 0 (negative) to 3+ (strongly positive). The percentage of the samples for each expression score is indicated as bar graph and examples for the staining intensity in MCC are depicted below. In total, 88% of MCC samples showed strong (+2) or very strong (+3) staining for phospho-AKT T308, while 59% of melanoma samples were observed in these categories. Basal cell carcinoma showed only negative or weak AKT phosphorylation.
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Table 1. Analysis of *PIK3CA*, *AKT1* and pAKT T308 in Merkel cell carcinoma.

					PIK3CA		*AKT1*	IHC
no.	**sex**	**age**	**type**	**MCV**	**Exon 9**	**Exon 20**	**Exon 4**	**pAKT T308**
1	f	46	cell line (UISO)	−	wt	wt*	wt	2
2	f	80	cell line (MCC13)	−	wt	wt	wt	2
3	m	64	cell line (MCC26)	−	wt	wt	wt	2
4	m	26	cell line (MKL-1)	+	wt	wt	wt	3
5	m	55	cell line (BroLi)	+	wt	wt	wt	3
6	m	67	cell line (WaGa)	+	wt	wt	wt	2
7	f	73	cell line (HeRo)	+	wt	wt	wt	3
8	m	64	cell line (LoKe)	+	wt	wt	wt	3
9	m	83	metastasis	+	wt*	−	−	2
10	−	70	metastasis	−	wt*	−	−	−
11	−	44	metastasis	+	wt	wt	−	−
12	−	77	metastasis	+	wt*	−	−	−
13	−	45	metastasis	+	wt	wt	wt	−
14	f	80	metastasis	+	wt	wt	wt	−
15	m	66	metastasis	+	wt*	−	−	−
16	m	68	metastasis	+	wt*	−	−	3
17	m	−	metastasis	−	wt*	wt*	−	−
18	f	77	metastasis	+	wt*	wt*	−	2
19	f	72	metastasis	+	wt*	wt*	−	2
20	f	56	metastasis	+	wt*	wt*	−	−
21	f	68	primary tumor	+	wt	wt*	wt	−
22	m	46	metastasis	−	wt	wt*	wt	2
23	m	83	metastasis	+	**E545Q**	wt	wt	3
24	m	64	metastasis	+	wt	wt*	wt	−
25	m	77	metastasis	+	wt	wt	−	2
26	m	75	primary tumor	+	**E542K**	wt	wt	2
27	m	75	metastasis	+	wt*	wt*	−	−
28	m	79	primary tumor	+	wt*	−	−	2
29	−	58	metastasis	+	wt	wt*	−	−
30	−	73	metastasis	−	wt*	wt*	wt	−
31	f	80	metastasis	+	wt*	wt*	−	−
32	m	93	primary tumor	−	wt*	wt*	−	−
33	m	77	primary tumor	+	wt*	−	wt	−
34	f	65	primary tumor	+	wt	wt	wt	−
35	f	72	metastasis	+	wt*	wt*	−	−
36	m	82	primary tumor	+	wt	wt*	wt	−
37	m	80	primary tumor	+	wt*	wt*	−	−
38	f	89	primary tumor	+	wt	wt	wt	−
39	m	62	metastasis	+	wt	wt*	wt	−
40	m	55	primary tumor	+	wt	wt	wt	−
41	f	93	primary tumor	+	wt	wt	−	−
42	m	88	metastasis	+	wt	wt*	−	−
43	m	84	primary tumor	+	wt*	wt*	−	−
44	m	53	primary tumor	+	wt	wt*	−	−
45	m	49	primary tumor	+	wt*	wt*	−	−
46	m	82	metastasis	+	wt*	wt*	wt	−

Age, age at the time of diagnosis; m, male; f, female; MCV, Merkel cell polyoma virus (the MCV status was assessed as described previously [2]); wt, wild-type; wt*, these samples could not be sequenced directly, but were analyzed by a modified SNaPshot® assay; −, not available; IHC pAKT T308, immunohistochemistry for phospho-AKT threonine 308 scored from 0 (negative) to 3+ (very strong) staining intensity; it has to be noted that only 9 samples used for mutation analysis were also present on the tissue microarray.
doi:10.1371/journal.pone.0031255.t001

One MCC (2%) was categorized as negative (0), 4 (10%) as weak (1+), 23 (56%) as strong (2+) and 13 (32%) as very strong (3+) for their respective pAKT T308 expression (**Figure 1**). There were no significant differences between primary tumors and metastases regarding pAKT expression (mean expression scores of 2.36 (primary tumors) and 2.07 (metastases); $p = 0.23$). In addition, 8 MCC cell lines were stained for pAKT T308 (**Table 1**). Four cell lines revealed a strong (2+) and four MCC cell lines a very strong (3+) expression of pAKT T308. The immunohistochemical results obtained by analyzing both MCC tumor tissue and cell lines indicate that in the majority of MCC the PI3K/AKT pathway is activated.

To compare the level of AKT activation in MCC with other skin cancers, we additionally performed immunohistochemical staining for pAKT T308 in 67 malignant melanomas, a tumor with established frequent AKT pathway activation [18,19], and 45 basal cell carcinomas which have been shown to be characterized by only low levels of phospho-AKT [20,21]. In line with these pre-published data, basal cell carcinomas stained negative or very weakly for pAKT, while melanomas revealed a higher phosphorylation (**Figure 1**). Malignant melanoma showed lower levels of AKT phosphorylation than MCC with 59% of samples categorized as strong (+2) or very strong (+3), compared with 88% of MCC samples. However, the comparability of the different tumor entities might be diminished by different proportions of primary versus metastatic tissues.

AKT activation is independent of MCV

Given the recent discovery that most MCCs are characterized by the integration of a polyoma virus, i.e. the Merkel cell polyomavirus (MCV), it is important to note that the oncogenic proteins encoded by polyomaviruses have been implicated in the activation of the PI3K/AKT pathway. For example, SV40 small

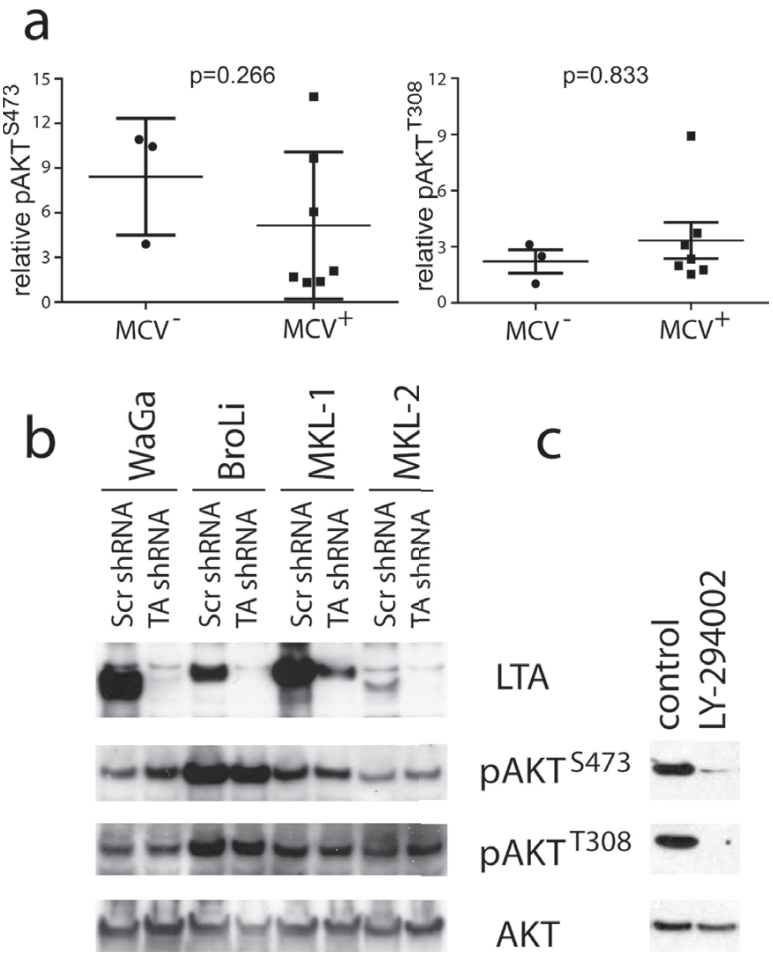

Figure 2. Merkel cell polyomavirus T antigens do not affect AKT phosphorylation in Merkel cell carcinoma. a) Total cell lysates of 7 MCV positive and 3 MCV negative MCC cell lines were subjected to Western blot analysis applying antibodies to pAKT^{T308}, pAKT^{S473} and tubulin. Signal intensity was quantified using the imageJ software and the values normalized p-values according to the Mann-Whitney are indicated. **b)** The indicated cell lines were infected with the lentiviral shRNA vector KH1 encoding GFP and either a shRNA targeting all MCV TA mRNAs or a scrambled shRNA; Infection rates as determined by GFP flow cytometry analysis were 98% for WaGa, 94% for BroLi, 90% for MKL-1 and 96% for MKL-2. Total cell lysates harvested on day 5 following infection were then analyzed by immunoblotting for expression of large T antigen (LTA) and AKT and for the presence of AKT phosphorylated at T308 or S473. The variations in molecular size of the LTA proteins in the different cell lines are due to different stop codon mutations truncating the C-terminal part of the protein. **c)** Treatment with the PI-3 kinase inhibitor LY-294002 demonstrated inhibition of AKT phosphorylation at the phosphorylation sites T308 and S473 by blocking the upstream kinase.
doi:10.1371/journal.pone.0031255.g002

T antigen inhibits the protein phosphatase 2A (PP2A) which dephosphorylates AKT on both activating phosphorylation sites [22]. Consequently, we addressed the possible contribution of MCV T antigens to AKT activation in MCC cells. To this end, AKT phosphorylation was measured by Western blot analysis in seven MCV positive and three MCV negative cell lines. Quantification of the western blot signals, however, demonstrated lack of any significant correlation between pAKT and MCV status (**Figure 2 a**). Furthermore, a possible functional role of the MCV T antigens on AKT activation in MCC was tested by silencing T antigen expression in four MCV positive MCC cell lines by infection with a lentiviral shRNA construct targeting Large as well as small T antigen (LT and sT) mRNA; LT and sT mRNA are derived by alternative splicing from the same genomic locus and share common sequences in exon 1 [3]. T antigen knock down did not affect phosphorylation of AKT at T308 and S473 in any of the tested cell lines (**Figure 2 b**). In contrast, treatment with the PI-3 kinase inhibitor LY-294002 demonstrated that AKT phosphorylation at these two sides can be inhibited by blocking the upstream kinase (**Figure 2 c**).

In accordance with the observations in the cell lines, the expression level of pAKT T308 *in situ* did not correlate with the MCV status (i.e. immunohistochemical mean scores of 2.11 and 2.21 for MCV negative (n = 9) and positive (n = 29) samples, respectively; $p = 0.74$). These lines of evidence strongly suggest that the MCV T antigens are not critical for AKT pathway activation in MCC cells, although it cannot be excluded that the difference between MCV negative and positive samples would reach significance with a higher number of samples.

PIK3CA mutations in MCC

In many cancers, PI3K/AKT pathway activation is mediated by oncogenic mutations in *PIK3CA* and *AKT1* genes. Thus, we tested both the MCC tissue samples as well as the MCC cell lines for *PIK3CA* and *AKT1* hotspot mutations. The characteristics of the patients and samples are given in **Table 1**. In total, 46 MCC samples (14 primary tumors, 24 metastases and 8 cell lines) were analyzed. Heterozygous *PIK3CA* mutations were identified in two out of 46 samples (4%) (**Figure 3**). Both mutations (E542K and E545Q) are localized in exon 9 of *PIK3CA*, which encodes the helical domain, and in both cases the tumors harbored MCV DNA. Both mutations were tested by an independent second PCR and additionally by a modified *PIK3CA* SNaPshot® assay. The detected mutations were independently confirmed by these alternative methods. DNA isolated from peripheral blood lymphocytes revealed a wildtype status in both cases, thus confirming the somatic nature of the mutations. Subsequently, additional samples of the two patients were tested. The patient with the E542K mutation in the primary tumor revealed the same mutation in two metastases of the temple and the parotid gland. The E545Q mutation found in a metastasis of the second patient was detected in two of three further metastases on the head. These results suggest that in both cases the mutation occurred before metastatic tumor spread.

The two MCC samples harboring a *PIK3CA* mutation were also present on the tissue microarray. The sample with the E542K mutation revealed a strong (2+) and the one with the E545Q mutation a very strong (3+) pAKT T308 protein expression (**Table 1**). Increased AKT phosphorylation can also be caused by defined *AKT* mutations. The E17K *AKT1* mutation causes a pathological localization of AKT1 to the plasma membrane and thereby stimulates downstream signaling proteins [23]. Sequencing of exon 4 of *AKT1*, containing the E17K hotspot locus, was possible for 24 samples, but did not reveal any *AKT1* mutation in MCC. In addition, exon 3 of *AKT3* harbouring the E17 hotspot locus was sequenced in 8 MCC cell lines (UiSo, MCC13, MCC26, MKL-1, LoKe, WaGa, HeRo, MaTi, BroLi), but did not reveal any mutations.

MCC cells are sensitive to PI3K inhibition

We analyzed whether inhibition of the PI3K/AKT pathway would impact the viability and growth of MCC cells. To this end, we incubated 5 MCC cell lines (WaGa, MKI-1, MKI-2, MCC13, UISO) for 24, 48 and 72 hours with the PI3K inhibitor LY-294002 at different concentrations (range 12.5–50 μM) and subsequently analyzed the cells using the MTS assay. In addition, since inhibition of the AKT pathway is regarded as a reasonable therapeutic option in melanoma [24,25], two melanoma cell lines carrying known PI3K/AKT pathway activating mutations served as positive controls; Skmel-28 was described to have a *PTEN* mutation [26] while sequencing of the *NRAS* gene in BLM cells revealed a Q61R mutation (data not shown). LY-294002 reduced the metabolic activity for all 6 MCC cell lines in a time and dose dependent manner between 10–95% (**Figure 4**). Indeed, at all time points analyzed the means were significantly different ($p<0.0001$; repeated measures ANOVA); Dunnett's multiple post test revealed significantly inhibition by each inhibitor concentration compared to control ($p<0.05$). Interestingly, the MCC cell lines demonstrated in response to the PI3K inhibitor at least an equal reduction in MTS signal as the melanoma cell lines, which are proliferating much faster than most of the MCC cell lines (**Table 2**). The MTS assay does not distinguish between apoptosis and cell cycle arrest. Therefore, we performed DNA staining after 40 hours of LY-294002 (25 μM) treatment. Only WaGa cells displayed a strong increase in sub-G1 cells at this time point while all other cell lines - although dead cells were increased in all cases in the presence of LY-294002 – showed only a moderate apoptotic response (**Table 2**). Cell cycle arrest induced by the PI-3 kinase

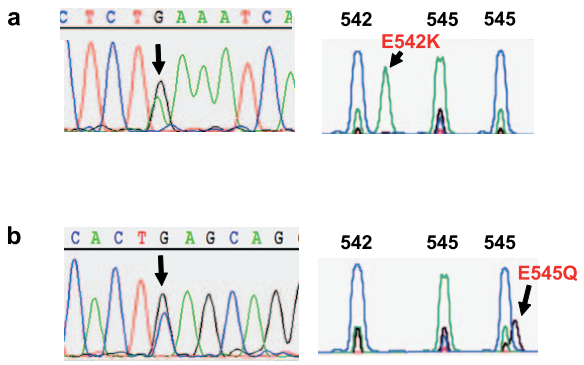

Figure 3. *PIK3CA* hotspot mutations in Merkel cell carcinoma. (a) The heterozygous p.E542K (c.G1624A) *PIK3CA* hotspot mutation was detected in sample no. 27 by direct sequencing (left) and a *PIK3CA* SNaPshot® assay (right) covering the most frequent hotspot *PIK3CA* mutations. The number of the wildtype codons is indicated above the peaks in the SNaPshot® assay. In brief, the SNaPshot® assay comprises a multiplex PCR for exons 9 and 20 of *PIK3CA*, followed by extension of 4 primers specific for the most frequent *PIK3CA* hotspot mutation loci. Because fluorescent dideoxynucleotides are used for this primer extension step, only one peak appears at the base position with the potential mutation. The color of the peak allows discrimination of wildtype and mutated alleles. (b) The heterozygous p.E545Q (c.G1633C) *PIK3CA* hotspot mutation was detected in sample no. 24 by direct sequencing (left) and a *PIK3CA* SNaPshot® assay (right) covering the most frequent hotspot *PIK3CA* mutations. The number of the respective wildtype codon is indicated above the peaks in the SNaPshot® assay.
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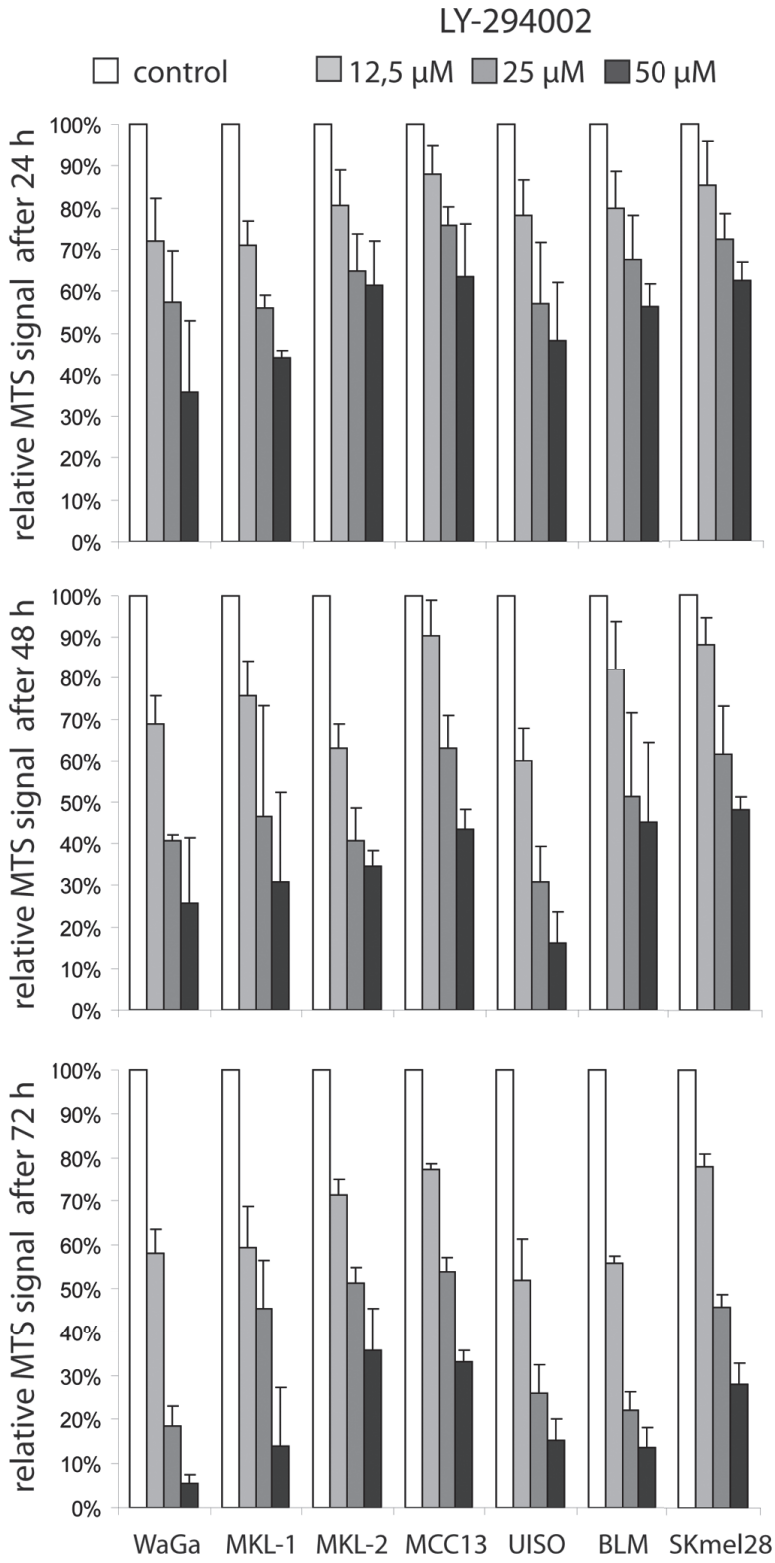

Figure 4. MCC cell lines are sensitive to the PI3K inhibitor LY-294002. The indicated MCC and melanoma cell lines were incubated with LY-294002 at three different concentrations. After the indicated time period the cells were subjected to the MTS assay in triplicates. The reduction in extinction relative to the DMSO (solvent of LY-294002) controls is depicted. The graphs represent mean values (± standard deviation) of at least three independent experiments.
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Table 2. Response of Merkel cell carcinoma and melanoma cell lines to LY-294002.

	doubling time (days)	IC $50_{LY\text{-}294002}$ [μM]			cells in S-phase [%]		sub-G1 cells [%]	
		24 hours	48 hours	72 hours	control	LY-294002	control	LY-294002
WaGa*	3	32,5	23,1	12,0	6,8	4,8	4,5	31,1
MKL-1*	3	38,3	27,6	18,4	9,4	5,8	2,1	4,8
MKL-2*	4	64,3	26,1	30,1	9,3	3,9	19,5	27,8
MCC13	1	74,3	41,6	30,7	12,5	10,3	1	4,3
UISO	2	43,0	16,8	14,4	14,7	8	5,2	8,8
BLM	1	57,2	38,8	13,8	11	5,8	1,7	2,9
Skmel28	1	70,9	45,7	26,4	15,1	3,6	0,6	1,3

Doubling times were roughly estimated from the necessary split ratios during culture. IC50 values for the inhibition by LY-294002 were calculated from the MTS assay date depicted in Figure 4 assuming an exponential relation. The percentage of S-phase and sub-G1 cells were estimated by flow cytometry analysis of propidium iodide stained cells following 40 hours in the presence of 25 μM LY-294002.
*MCV positive cell lines.
doi:10.1371/journal.pone.0031255.t002

inhibitor was also observed in all cell lines but the measured decrease in S-phase cells was quite moderate in many cases (**Table 2**). Although we know from previous experiments that a cell cycle arrest is difficult to demonstrate in the slowly cycling MCC cells [4] it is likely that not only reduced cell numbers but also reduced metabolic activity per cell may contribute to the effects in the MTS assay. Nevertheless, analysis of the cellular DNA content suggests that both, cell cycle arrest as well as apoptosis induction contribute to inhibition by LY294002 in MCC cells.

Discussion

PIK3CA mutations have been already identified in a broad range of human cancers at varying frequencies, including liver (36%), breast (26%), colon (25%), urothelial (13%), ovarian (9%), gastric (7%), brain (6%), and lung cancer (2%) as well as leukaemia (1%) [9,27]. The present study adds Merkel cell carcinoma to this list of human cancers harboring *PIK3CA* mutations, although in MCC these mutations occur obviously at a low frequency. Both mutations are localized in the helical domain, whereas in most other cancers, mutations in the kinase domain, i.e. in exon 20, are more common (www.sanger.ac.uk/genetics/CGP/cosmic/). Nevertheless, the E542K mutation is one of the most frequent *PIK3CA* hotspot mutations and results in a strong activation of PI3K [28]. The E545Q missense mutation is less frequent but has been described in breast, anaplastic thyroid, ovary, and esophageal cancer [29].

To the best of our knowledge, this is the first report of oncogenic mutations in human MCC. Notably, the analysis of several other oncogenes including *HRAS*, *KRAS*, *NRAS*, *BRAF*, *C-KIT*, and genes of the Wnt pathway did not show any mutations in human MCC [5,30,31,32,33]. Indeed, all of the previously reported mutations in MCC were restricted to tumor suppressor genes. *PTEN* mutations were observed at a very low frequency, although loss of heterozygosity at the *PTEN* locus at chromosome 10q seems to be a frequent event [34]. Similarly, mutations in other tumor suppressor genes such as *p53*, *p73* and *CDKN2A* have been reported, however, only in a very small fraction of MCCs.

Immunohistochemical analysis of the two MCC tumors carrying activating *PIK3CA* mutations demonstrated a strong AKT phosphorylation; this observation is in line with the fact that these mutations contribute to an activation of the PI3K/AKT signaling pathway. However, the two tumors harboring the activating *PIK3CA* mutations were not exceptional with respect to AKT pathway activity. In fact, 88% of a series of 41 MCC tissues were classified in the same categories (strong or very strong staining for pAKT T308) suggesting alternative mechanisms (e.g., PTEN alterations) of AKT pathway activation in the majority of cases.

Merkel cell polyomavirus has been recently identified as a widespread virus which upon integration into the genome of MCC precursor cells and acquisition of truncating mutations in the viral large T antigen is likely to contribute to MCC development and progression [3,16,35]. In this respect we recently demonstrated that MCV infected MCC cells require expression of the MCV T antigens for proliferation and survival [4]. MCV genomes encode for the presumably oncogenic large and small T antigens; for the respective homologs encoded by SV40 it has been demonstrated that they can activate the AKT pathway. While for small T this happens via inhibition of the protein phosphatase 2A [22], large T antigen activates the AKT pathway through its interaction with the insulin receptor substrate 1 [36]. Surprisingly, however, shRNA knock down of both MCV T antigens in infected MCC cell lines did not affect AKT phosphorylation and the lack of correlation between MCV status and AKT phosphorylation in the tumor samples also suggests that the MCV viral T antigens do not contribute to AKT pathway activation in MCC cells, although the significance of the latter observation is impaired by the low number of MCV negative samples. Thus, the presence of the viral proteins seems neither to be sufficient nor necessary for AKT pathway activation in MCC. A very recent report demonstrating that transformation of rodent fibroblasts by MCV small T antigen is independent of PP2A binding further supports that MCV T antigens function different than the related SV40 oncoproteins and that the AKT pathway is not a critical target of MCV T antigens [37]. Future studies are warranted to elucidate the molecular mechanisms for AKT pathway activation in MCC.

Since activation of the PI3K/AKT signaling pathway represents one of the most frequent events in human cancer, specific inhibitors of PI3K, AKT and additional components of the PI3K/AKT signaling pathway are currently tested in preclinical and clinical trials [8]. Notably, MCC showed a significantly higher AKT phosphorylation than malignant melanoma in our study. In melanoma, phosphorylation of AKT and activation of the PI3K/AKT signaling pathway is a well known feature. A previous study identified AKT phosphorylation in 66% of melanoma samples [18], congruent with the results observed in this study. Consequently, inhibition of the PI3K/AKT pathway by specific

inhibitors has evolved as a promising treatment strategy for malignant melanoma [24,25]. In our study, MCC cells showing strong activation of the PI3K/AKT pathway were sensitive to the PI3K inhibitor LY-294002 *in vitro* although we cannot exclude that off target effects may contribute to the observed inhibition.

Since metastasized MCC is a very aggressive tumor with poor prognosis and very limited therapeutic options, the presented observations are opening the avenue for targeting the activated PI3K/AKT pathway as an interesting new option for patients suffering from advanced MCC.

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Author Contributions

Conceived and designed the experiments: CH RH JB. Performed the experiments: AB CR. Analyzed the data: CH RH AB CR DS ML JB. Contributed reagents/materials/analysis tools: CH RH JB. Wrote the paper: CH RH DS ML JB.

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Manuscript II

Submitted Manuscript

Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

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One Sentence Summary: With the advent of effective cancer immunotherapy emphasizing the need for a detailed understanding of immune escape mechanisms of cancer cells, we demonstrate that the epigenetic silencing of the NKG2D ligands MICA and MICB in Merkel cell carcinoma cells impairs their susceptibility to immune mediated lysis, which, however, can be reverted by inhibition of histone deactylases.

Abstract: Merkel cell carcinoma (MCC) is a virally associated cancer, which despite its strong immunogenicity is not sufficiently controlled by the immune system, thus indicating effective immune escape mechanisms. In particular, both viral infection and malignant transformation induce expression of MHC class I chain-related protein (MIC) A and B, which signal stress to cells of the immune system via Natural Killer group 2D (NKG2D) resulting in elimination of target cells. However, despite transformation and the continued presence of virally-encoded proteins, MICs are only expressed in a minority of MCC tumors in situ and are completely absent on MCC cell lines in vitro. Here we demonstrate that this lack of MIC expression is due to epigenetic silencing via MIC promoter hypoacetylation; indeed, MIC expression was re-induced by pharmacological inhibition of histone deacetylases (HDACs) both in vitro and in vivo. Re-induction of MICs rendered MCC cells more sensitive to immune-mediated lysis suggesting this intervention as a potential therapeutic approach for advanced MCC, particularly in combination with currently tested immune modulating therapies such as immune checkpoint blocking antibodies or tumor-targeted cytokines.

Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer with neuroendocrine features. The histogenesis of MCC remains controversial, with either epidermal stem cells or pre/pro-B cells as possible cells of origin *(1)*. Currently, there are no approved therapies for advanced MCC that is not surgically resectable, thus almost half of the patients diagnosed with MCC will die from the disease *(2)*. This situation is compounded because the reported incidence of MCC has more than tripled over the last few decades *(3)*. This increase in incidence is true across all age groups, indicating that the aging general population is not the sole reason for the increased incidence. Nevertheless, advanced age is still one of the relevant risk factors among UV exposure and immune suppression *(4)*.

The strong correlation of MCC and immune suppression prompted the discovery of a polyomavirus associated with MCC, termed Merkel cell polyomavirus (MCPyV)*(5)*. MCPyV is present and clonally integrated in at least 80% of MCCs *(6)*. Most MCPyV positive MCC cell lines critically dependent on virally encoded transforming early genes, i.e. small and large T-antigen, in order to maintain the oncogenic phenotype *(7, 8)*. The continuous expression of these viral proteins helps explain the exquisite immunogenicity of MCC. Notably, despite the highly aggressive phenotype of MCC, spontaneous regression or regression after cessation of immune suppressive measures are well established *(9)*. Moreover, we and others have recently demonstrated the presence of spontaneous adaptive cellular immune responses specific for epitopes derived from the MCPyV early genes in peripheral blood of MCC patients *(10, 11)*. However, despite the continuous expression of the relevant antigens and the presence of respective specific cytotoxic T-cell responses, it is obvious that clinically manifest MCCs are able to escape destruction by the immune system. While this fact can be readily explained by a general immune suppression in approximately 10% of the patients *(12)*, the immune escape mechanisms of MCC are less clear for the remaining 90% *(13)*.

Natural Killer group 2D (NKG2D) is a lectin-like type 2 transmembrane receptor encoded by the gene Klrk1 (killer cell lectin-like receptor subfamily member 1) and is part of a critical pathway signaling cellular stresses to the innate and adaptive immune system. Charged residues in the transmembrane region enable NKG2D to pair with the signaling adaptor protein DAP10, which is essential for NKG2D surface expression and downstream signaling to PI3K and GRB2 *(14)*. These signaling molecules then stimulate proliferation, cytokine production, immune cell activation, and cytotoxic potential of NK and T cells *(14)*. A recent study suggested a link between the Natural Killer group 2D (NKG2D) receptor system and up-regulation of immune responses to MCC. Specifically, transcriptional analyses of MCC tumors revealed that NKG2D was among the highest expressed mRNAs in tumors obtained from patients with a good prognosis *(15)*. However, these tumors represented a minority of patients, suggesting most MCCs evade NKG2D signaling as a means of immune escape.

The NKG2D ligands include UL16-binding proteins (ULBPs) as well as the MHC class I chain-related protein (MIC) A and B family *(16)*. MICA and MICB are present at low to undetectable levels in normal cells, but are induced by cellular stresses including infectious agents and neoplastic transformation. Indeed, MICA and MICB are highly expressed in a number of solid tumors like carcinomas of the breast, colon, kidney, ovary, or prostate *(17)*, as well as in melanoma *(18)*. However, NKG2D

expression renders tumor cells more susceptible to elimination by the immune system *(19)*. The importance of MICA and MICB induced NKG2D-signaling for immune surveillance of virally infected and transformed cells is highlighted by the fact that viruses and cancer cells have developed mechanisms to interfere with this interaction *(14)*. These mechanisms include shedding of surface expressed molecules, binding and retaining of MICA and MICB proteins in the cytoplasm, over-expression of MICA and MICB mRNA-targeting microRNAs, as well as other epigenetic mechanisms such as chromatin remodeling (*14, 20*).

Viral carcinogenesis should predispose MCC for induction of MICA and MICB expression; however, when screening for the respective mRNA expression using publicly available data from the Gene Expression Omnibus (GEO), we observed that both MICA and MICB mRNA were rarely present in MCC. Prompted by this observation, in the present study we confirmed these data in an independent set, and extended this notion to the protein level. Furthermore, we demonstrate that this lack of MICA and MICB expression in MCC is due to epigenetic silencing by promoter hypoacetylation. Notably, MICA and MICB expression, particularly MICB expression, can be induced by histone deacetylase inhibitors, which in turn rendered the MCC cells more susceptible to lysis by cytotoxic lymphocytes. These findings open new avenues for therapy of advanced MCC particularly in combination with immune modulating molecules such as immune checkpoint blocking antibodies.

Results

MCC tumors express low levels of MICA and MICB mRNAs

Since both viral infection and malignant transformation induce expression of the immune activating NKG2D ligands MICA and MICB, we screened for the respective mRNA expression among 75 MCC tumors from 61 patients and a number of MCC cell lines. For this, we employed 2 publicly available gene expression arrays obtained online from GEO (accession numbers GSE22396 *(15)* and GSE 39612 *(21)*; Supplementary Fig. 1). Somewhat unexpectedly, MICA mRNA was expressed only at very low levels in the MCC tumors and cell lines when compared to genes commonly expressed in MCCs such as RB1, E2F2, ENO2 or RPLP0. The MICB mRNA expression level was also low compared to those genes, but generally higher than for MICA. On the GSE22396 Array a subset of tumors (24%) were characterized by moderate to high levels of MICB mRNA. Notably, patients with higher levels of MICB mRNA in their tumors where characterized by better outcomes. In line with this, in MCPyV positive tumors, MICB mRNA expression correlated with the gene expression signature for infiltrating immune competent cells, a feature that had been associated with a good prognosis previously *(15)* (Supplementary Fig. 2).

MCC tumors largely lack MICA and MICB expression in situ

Next, we analyzed tissue microarrays (TMA) comprising 134 FFPE fixed paraffin embedded MCC tumors of 99 patients by immunohistochemistry (IHC) using an antibody reacting with both MICA and MICB to determine MICA/B protein expression *(18)* (Fig 1A). MICA/B protein was present in only 18% of MCC tumors, while 82% were negative (Supplementary Fig. 3).

The samples used in the cDNA microarray and the TMA were partly overlapping. Thus, to confirm the lack of MICA and MICB expression in a completely independent set of samples, we analyzed 50 additional MCC tumor samples of 34 patients for their MICA and MICB expression by IHC in conventional tumor sections. The use of sections of the whole tumor also allowed us to determine the expression pattern of MICA and MICB. A heterogeneous expression pattern of other immune modulatory molecules such as CD274 (aka PD-L1) had recently been described for MCC *(22)*.

Tumors classified as negative or positive for MICA and MICB expression are exemplarily depicted in Fig. 1a; notably, the strongest staining observed for the respective antibody is depicted. In line with higher MICB mRNA expression, MICB staining intensity was stronger than that of MICA. Overall we observed that more than half (54%) of the lesions expressed neither MICA nor MICB protein, 20% were weakly positive for only MICA, and 12% were classified positive for only MICB; and only 14% of the tumors stained positive for both MICA and MICB (Fig. 1B). The effective frequency of MICA and MICB expression remained essentially the same when calculated for each patient instead of the individual lesions (Supplementary Fig. 4). Indeed, when examining multiple lesions, i.e. primary tumors and metastatic lesions obtained from the same patients, the expression pattern of the MICA and MICB was concordant (data not shown). Similarly, with respect to a possible heterogeneity of MICA and MICB expression within the tumor, our analyses revealed that both the expression as well as the lack of it was homogenous throughout the tumor. This homogenous intralesional and intraindividual expression pattern suggests a rather robust mechanism for suppressed protein expression, such as epigenetic silencing.

MICA and MICB expression in MCC cell lines

To explore the underlying mechanism, we determined whether the suppression of MICA and MICB expression was robust enough to be maintained when cells were propagated in vitro, in cell culture conditions. This question is of particular relevance since in vitro culture of cells is a well-established factor known to induce MICA and MICB expression *(23, 24)*. We therefore performed qRT-PCR with MICA and MICB mRNA specific primers using the melanoma cells FM79, FM82 and IF6 as positive controls (Fig. 2A). These analyzes demonstrated that MICA mRNA in MCC cell lines was only expressed in very low amounts, i.e. close to or below the detection threshold; indeed, relative expression values calibrated to IF6 ranged close to zero. Similar to the results from the analyses of the MCC tumor samples, MICB mRNA expression was low, but still higher than MICA mRNA, with relative expression ranging from approximately 0.02 to 0.48 when calibrated to the positive control melanoma cell line IF6's mRNA expression. Inconsistencies between mRNA and protein expression for MICB have been repeatedly described *(25)*, thus, to determine whether the detected MICB mRNA was indeed translated into protein we performed immunoblots of total cell lysates demonstrating that MICB protein was below the detection limit even for the AlDo MCC cell line characterized by the highest MICB mRNA expression (Fig. 2B). Accordingly, flow cytometric analysis for MICA/B membrane expression demonstrated no expression in any of the analyzed MCC cell lines (Fig. 2C). To determine how broadly NKG2D mRNA expression was suppressed, we subjected MCC cells to a number of stress factors known to induce NKG2D ligand expression *(24, 26)*. These stressors included serum starvation, high concentrations of DMSO, heat shock at 41.5°C, 1000 U/ml interferon α, and 1000 U/ml interferon γ. These interventions had no or only negligible effects on MICA/B surface expression (Supplementary Fig. 5 A-F). Finally, we took advantage of a recently established inducible knock down system for viral T-antigens to stress the cells by withdrawal of the oncogene to which they are addicted; this knock down had no effect on MICB expression (Supplementary Fig. 5 G).

The complete lack of MICA and MICB protein expression in MCC cell lines even under diverse cell stress conditions suggests that MICA and MICB silencing in MCC is an active and robust process.

MICA and MICB promoters are silenced by histone hypoacetylation.

The robust silencing of MICA and MICB expression in MCC observed in vivo and in vitro suggests that this regulation takes place on a transcriptional or epigenetic level rather than post-transcriptionally *(27)*. We therefore determined histone H3K9 acetylation at the MICA and MICB promoter region of WaGa cells as a known indicator for transcriptional activity. Chromatin immune precipitation (ChIP) assays clearly demonstrated histone hypoacetylation at both the MICA and MICB promoter: Only ~20% of histones in the MICA promoter and ~50% of histones in the MICB promoter were acetylated in WaGa cells (Fig. 3A). The lower acetylation levels of histone H3 at the MICA promoter compared to the MICB promoter in WaGa cells is in accordance with a lower expression of MICA compared to MICB mRNA as described above.

MICA and MICB promoter acetylation is inducible.

Apparently, the expression of MICA and MICB in MCC cell lines is silenced via histone H3 hypoacetylation at their promoter region. To test whether the silencing of these NKG2D ligands is indeed due to histone hypoacetylation or if additional

mechanisms for MICA and MICB suppression are operative *(14)*, we next tested if their expression could be induced by reversal of histone hypoacetylation. WaGa cells were subjected to a clinically relevant concentration of 1.25 μM (the plasma concentration reached by currently applied treatment regimens) of the histone deacteylase (HDAC) inhibitor vorinostat *(28)*. When WaGa cells were cultured for 24 hours in the presence of vorinostat, there was an induction of histone acetylation at the MICA and MICB promoter regions (Fig. 3a). However, with ~56% of histone acetylation in the MICA and ~70% of histone acetylation in the MICB promoter, this induction was rather modest. We therefore combined vorinostat with mithramycin A, a drug that has synergistic effects with HDAC inhibitors by (i) transcriptionally inhibiting the compensatory inductions of certain HDACs *(29)* and (ii) by preventing the formation of SP1/HDAC inhibitory complexes at the promoters' GC box *(30)*. We confirmed these synergistic effects in the MCC cell lines: Treatment with mithramycin A alone already reduced transcription of most class I and II HDAC genes in MCC cell lines and, most importantly, mithramycin A prevented the regulatory induction of HDACs by vorinostat (Supplementary Fig. 6). Subsequently, ChIP assays of accordingly treated MCC cells revealed that the addition of mithramycin A boosted vorinostat induced histone acetylation resulting in an acetylation of ~66% of histones at the MICA promoter and almost complete histone acetylation at the MICB promoter. Treatment with mithramycin A alone had negligible effects on histone acetylation levels in the MICA and MICB promoter of WaGa cells.

To extend this observation to a larger series of MCC cell lines, we performed an immunoblot for global histone acetylation using the same anti-AcH3K9 antibody we employed for the ChIP assay. This analysis confirmed the increased acetylation of global histones upon combined vorinostat and mithramycin A treatment in 4 out of 6 cell lines (Fig. 3B). In MKL-2 and, surprisingly, in WaGa cells we did not observe a further increase in global histone H3 acetylation by adding mithramycin A. This observation was unanticipated, since we observed a synergistic effect of the combined drugs on histone H3 acetylation at the MICA and MICB promoter regions of WaGa cells (Fig. 3A). A possible explanation is that both the MICA and MICB promoter regions may be more sensitive to mithramycin A induced histone acetylation due to the presence of a SP1 binding site (GC Box) *(30)*.

MICA and MICB expression can be re-induced on MCC cells in vitro.

Since the combined treatment of MCC cells with vorinostat and mithramycin A induced strong histone acetylation at the MICA and MICB promoter, we next tested whether this also leads to an increased transcription of MICA and MICB genes. First, we determined the respective mRNA expression with or without treatment by quantitative RT-PCR (Fig. 4A). The combination of vorinostat and mithramycin A led to increased MICA and MICB mRNA expression in all tested MCC cell lines, with the exception of MKL-2 in which no MICA mRNA was detected. Notably, the synergistic effects of vorinostat and mithramycin A were so strong that the relative expression to the respective untreated cell line in figure 4a is depicted on a logarithmic (log10) scale. Next, immunoblot and flow cytometry assays confirmed that the increased mRNA expression translated into MICB protein expression in general (Fig. 4B) and more importantly membrane expression (Fig. 4C and D). The degree of induction of membrane bound MICA/B was comparable to that of total MICB protein expression, suggesting that most of the induced MICA/B proteins are transported to the cell surface and no additional post-translational mechanisms

interfere with MICA/B surface expression in MCC cells. It should be noted, however, that the combined treatment did not affect all cells of the individual cell lines to the same extent. For a small but distinct subpopulation encompassing between 10 to 15% of the total population, MICA/B surface expression was almost unaltered by this treatment (Fig. 4C).
When we subjected MCC cells to increasing concentrations of vorinostat starting at 1.25 µM used throughout the previous experiments to 10 µM we observed that vorinostat alone is capable of inducing strong MICA/B surface expression in the majority of AlDo, BroLi and WaGa cells if present at high concentrations, whereas in LoKe, MKL-1 and MKL-2 cells the magnitude of vorinostat inducible MICA/B expression is lower and the plateau of expression is reached already at intermediate concentrations (Supplementary Fig. 7). This observation together with the observation that a subpopulation of cells did not respond to the synergistic effect of mithramycin A suggests that histone hypoacetylation at the MICA and MICB promoter is maintained by different mechanisms depending on both the cell line per se as well as the functional state of the cell.
An alternative HDAC inhibitor, i.e. the "classical" HDAC inhibitor trichostatin A (TSA), alone or in combination with mithramycin A produced essentially the same results as observed with the "second-generation" HDAC inhibitor vorinostat suggesting that only class I and II HDACs are involved in silencing MICA and MICB (Supplementary Fig. 8).

Induction of MICA/B enhances LAK cell mediated lysis of MCC cells.
The expression of NKG2D ligands such as MICA and MICB render tumor cells more susceptible to being killed by NK and T cells. Notably, T-cell activation can be mediated by NKG2D even without contribution of TCR-recognition*(31)*. Thus, we addressed whether induction of MICA and MICB on the surface of MCC cells results in increased killing by cytotoxic cells. The circumstance that MCC cell lines grow in spheroids necessitated the use of a flow cytometry based cytotoxic assay. Lymphokine- activated killer (LAK) cells were chosen as effector cells because they are a clinically applicable heterogeneous population of $NKG2D^+$, interleukin 2 (IL-2) activated NK, NKT and T cells *(32)* and we are currently conducting a clinical trial based on the antibody targeted delivery of IL-2 to the MCC tumor microenvironment (www.immomec.com). The gating strategy to differentiate dead from living cells is illustrated in figure 5A for untreated or treated MCC cells (BroLi) at an effector to target ratio of 40:1. In line with the magnitude of the induced membrane expression of MICA/B, the highest LAK cell mediated cytotoxicity was observed for MCC cells treated with the combination of vorinostat and mithramycin A, whereas mithramycin A alone had no and vorinostat alone only an intermediate effect (Fig. 5B). Notably, the LAK cell mediated cytotoxicity correlated with the surface expression of MICA/B. A blocking experiment using saturating amounts of an anti-MICA/B antibody confirmed that increased lysis of MCC cells was indeed dependent on the induction of MICA/B molecules (Fig. 5C). Hence, vorinostat and mithramycin A mediated induction of MICA/B molecules is responsible for the augmented sensitivity of treated MCC cells towards LAK cell mediated lysis.

MICA/B expression can be re-induced on MCC cells in vivo.
To translate these in vitro observations into a preclinical in vivo setting, we took advantage of a recently established MCC xenotransplantation model in which MCC tumors are induced by subcutaneous injection of WaGa cells in NOD/SCID mice

(33). Once tumors reached a volume of approximately 100 mm^3, mice were treated for two weeks by intraperitoneal injections of placebo, vorinostat, mithramycin A or the combination thereof at concentrations resulting in serum levels corresponding to those in the clinical setting in humans. After the last dosage animals were sacrificed, and the xenotransplants subjected to detailed characterization. By means of immunohistochemistry, we confirmed both the induction of histone H3 acetylation as well as MICB protein expression (Fig. 6A). In xenotransplanted tumors of mice treated with placebo, histones were hypo-acetylated and inhibition of HDACs induced histone H3K9 acetylation in vivo. A substantial induction of histone acetylation, however, was already achieved by vorinostat or mithramycin A alone; still this induced histone acetylation was further enhanced by the combined therapy. The more pronounced effects of the single agents may be due to either the prolonged exposure of either drug in the in vivo experiments (14 days with 10 days of drug administration versus 24h) and/or by other mechanisms such as inflammatory responses, interaction of MCC cells with components of the microenvironment or hypoxia. In accordance with the higher level of histone acetylation in MCC xenotransplants, MICB expression was already present in tumors of untreated mice. Still, the induced histone H3K9 acetylation also resulted in an increased expression of MICB protein (Fig. 6B). Quantification of MICB mRNA expression clearly demonstrated the synergism of the drug combination ($p < 0.05$) (Fig. 6C); however, it was not as evident as in the in vitro experiments.

Discussion

Virally associated cancers are characterized by a pronounced immunogenicity. To this end, the higher prevalence of Merkel cell carcinoma (MCC) in immune compromised patients, the high rate of spontaneous regression and the improved prognosis for patients whose tumor is infiltrated by T lymphocytes prompted the discovery of the Merkel cell polyomavirus (MCPyV). It has been shown that most MCC patients mount specific T-cell responses against epitopes derived from proteins encoded by the transforming MCPyV early genes *(10, 11)*, which are persistently expressed in MCC lesions in patients and are required for ongoing growth of MCC cell lines *(7, 8)*. Despite this immunogenicity, MCC is a very aggressive cancer causing disease-specific death in more than 40% of the patients after primary diagnosis and of almost all patients once distant metastases occur *(3)*. It should be further noted, that more than 90% of MCCs arise in fully immune competent patients *(12)*, suggesting tumor specific immune escape mechanisms must be employed. The two main pathways that allow tumors to escape the immune system are loss of immunogenic determinants and the tumor-driven suppression or desensitization of the immune response. Here, we present strong evidence that epigenetic silencing of the stress induced non-classical MHC class I molecules MICA and MICB is one of the major immune escape mechanisms of MCC. These molecules are ligands of the immune regulatory receptor NKG2D expressed on a variety of cytotoxic effector cells of both the innate and adaptive immune system and, most importantly, the NKG2D-NKG2D ligand system has been identified as being essential for the immune surveillance of cancer (*41*).

MICA and MICB are not or only slightly expressed by MCC tumors in situ and completely absent on MCC cell lines in vitro. We demonstrate that the lack of MICA and MICB mRNA and protein expression in MCC is largely due to epigenetic silencing via histone hypoacetylation in their promoter region. This epigenetic silencing is very robust even in the presence of several well-established stress factors known to induce their expression. However, this silencing can be abrogated by

treatment with HDAC inhibitors both in vitro and in vivo. Since the ultimate goal of our studies was to establish a therapeutic approach for MCC, we used a clinically relevant concentration, i.e. concentrations attained in patients treated with the FDA approved HDAC inhibitor vorinostat (SAHA, Zolinza^{TM}) *(28)*. Although this concentration of vorinostat increased histone acetylation at the MICA and MICB promoter as well as subsequent mRNA and protein surface expression in MCC cell lines, the effects were not very strong. This outcome was not entirely surprising, given the fact that HDAC inhibitors are very potent treating hematological malignancies, but have so far failed to achieve significant effects as mono-therapy for solid tumors in vivo *(34)*. Classical MCC cell lines grow as 3D-cultures in large spheroids and therefore represent the in vivo situation of a solid tumor much closer than other cancer cell lines *(35)*. To increase the susceptibility of cancers to HDAC inhibitors, they are frequently combined with other drugs *(36)*. Mithramycin A is a gene selective Sp1 inhibitor, which has been reported to potentiate HDAC inhibitor induced transcriptional activation *(37)*. To this end, promoter acetylation of MICA and MICB genes and subsequent mRNA and protein expression were markedly enhanced in MCC cells upon this combined treatment. This, however, is not in line with observations in other cell types where Sp1 appears to be necessary for MICA and MICB transcription and thus mithramycin A inhibits their expression *(38, 39)*. Treatment of MCC cell lines with vorinostat or mithramycin A markedly reduced (and the combination almost fully eliminated) Sp1 protein expression. This finding was in line with our observation of decreased Sp1 binding at the MICA and MICB promoter (Supplementary Fig. 9). Thus, we assume that in MCC cells, Sp1 is not necessary (or when in a complex with certain HDAC molecules even inhibitory) for the transcription of MICA and MICB. This altered Sp1 function may be caused by the presence of MCPyV. Likewise, Venkataraman et al. *(38)* reported that in cytomegalovirus-infected cells, unlike in non-infected cells, MICA and MICB expression was independent of Sp1.

In a small sub-population of MCC cells, induction of MICA/B surface expression by treatment with HDAC inhibitors in combination with mithramycin A was not as pronounced as in the main population (Fig. 4C and Supplementary Fig. 8A). These cells may exploit resistance mechanisms such as increased drug efflux or represent a slow cycling subpopulation. Since we could detect this subpopulation only by flow cytometry analysis for MICA/B surface expression, other mechanisms to counteract MICA and MICB surface expression, e.g. cytoplasmic retention or shedding from the cell surface, may be operative *(14, 20)*.

The principal effect of NKG2D signaling is an enhanced cytotoxic activity of lymphocytes towards the NKG2D ligand-expressing cells. In line with this notion, we observed that HDAC inhibitor treatment of MCC cells resulted in an increased susceptibility to LAK cell-mediated killing. Importantly, this increased cytotoxicity was decreased by a MICA/B blocking antibody. However, this blockade only partially diminished LAK cell mediated cytotoxicity, suggesting that besides MICA and MICB, other immunogenic molecules are induced by the combined treatment with vorinostat and mithramycin A. Indeed, HDAC inhibitors are known to induce the expression of other NKG2D ligands such as ULBPs *(40)*. It should be further noted that LAK cells include several possible effector cells. NKG2D is expressed as an activating or co-activating receptor not only on NK cells and $CD8^+$ T cells, but also on γδ T cells which are important for surveillance of virally associated cancers *(19, 41)*. In murine models, γδ T cells are strongly protective against polyomavirus

induced tumors; this protection critically depends on their activation via NKG2D *(42)*.

We recently reported that MCPyV-specific $CD8^+$ T cells are present in peripheral blood of more than half of MCC patients *(10)*, and that intra-tumoral infiltration of $CD8^+$ lymphocytes is a positive prognostic marker for these patients *(15)*. Unfortunately, MCPyV-reactive $CD8^+$ T cells are not fully functional in most MCC patients *(43)*. This exhausted phenotype was associated with expression of PD-1 on the MCPyV-reactive T cells; notably, PD-L1 expression has been reported for both MCC cells and myeloid cells infiltrating the tumor microenvironment *(10, 22)*. Signaling via NKG2D may prevent the exhaustion of MCPyV-reactive $CD8^+$ T cells. In addition to restoring preexisting T-cell responses, induction of NKG2D ligand expression on MCC cells is likely to trigger new T-cell responses. Activation of NK and γδ T cells via NKG2D increases tumor cell killing and thus cross-presentation of antigens, as well as production of chemokines and cytokines attracting and activating $CD8^+$ T cells *(44, 45)*. Furthermore, naïve $CD8^+$ T cells express NKG2D as a co-activating receptor and binding to NKG2D ligands boosts their activation *(46)*. In preclinical models it is well established that NKG2D ligand over-expression on tumor cells results in an increased priming and activation of tumor-specific $CD8^+$ T cells and long lasting T-cell memory responses even against NKG2D-negative tumor cells*(47)*: (i) Induction of NKG2D ligands on carcinoma cells boosts anti-tumor effects of CTLA-4 blockade *(48)*, and (ii) treatment with immune stimulating cytokines such as IL-2 and IL-12 is more effective against NKG2D ligand expressing tumors *(49)*.

Thus, HDAC inhibitor mediated MICA and MICB induction in MCC is likely to enhance the effects of immune therapeutic approaches currently tested in the clinic: (i) autologous MCPyV specific CD8+ T cell transfer (NCT01758458), (ii) CTLA-4 blocking antibody ipilimumab (NCT02196961), (iii) PD-L1 blocking antibody MSB0010718C (NCT02155647), or cytokine based therapies using (iv) tumor-stroma targeting antibody-IL2 fusion proteins (NCT02054884) or (v) IL12-encoding plasmids delivered by electroporation (NCT01440816).

A limitation of our study is the use of allogeneic LAK cells as effector cells in the cytotoxicity experiments. Unfortunately, autologous peripheral blood or tumor infiltrating lymphocytes from the same patients the respective MCC cell lines were derived from, are not available. LAK cells are a heterogeneous population of highly activated T, NK and NKT cells; hence, it was not possible to further scrutinize the detailed mechanisms by which HDAC inhibition in MCC cells boosts their susceptibility to immune recognition. Furthermore, we focused in this study on the transcription and membrane expression of only MICA and MICB. The expression of other NKG2D ligands is likely to be regulated by histone acetylation as well; however, the fact that the increased susceptibility of MCC cells after inhibition of histone acetylation to LAK cell mediated cytotoxicity is abrogated by an MICA/B blocking antibody strongly argues that induced MICA and MICB expression is the dominating effect.

Recently, many exciting developments have led to new, effective cancer immunotherapies *(50)*. Immune checkpoint blockade, cytokines with and without tumor targeting, as well as adoptive T cell transfer with and without chimeric antigen receptors results in objective, long lasting clinical responses with response rates, speed and depth even in advanced tumor stages *(51)*. However, a majority of patients still do not benefit from therapy. Predictive biomarkers for response to immunotherapy are immune response gene signatures or the presence of clonally expanded CD8+ T cells within the tumor *(52)*. Unfortunately, only 20% of the

patients' MCC lesions are characterized by such a favorable immune signature *(15)*. The lack of MICA and MICB expression of MCC cells is likely to contribute to this immunological state as re-induction of these NKG2D ligands by HDAC inhibition restores the susceptibility of MCC cells to cytotoxic lymphocytes. Thus, "epigenetic priming" of cancer cells for immune recognition appears to be a valuable addition to current immune therapeutic interventions for MCC *(53)*.

Material and Methods

Patients
A total of 50 archived paraffin-embedded tumor samples from 34 MCC patients were selected from the Department of Dermatology, Medical University of Graz. All tumor samples were histologically confirmed MCC lesions, i.e. primary tumors, local recurrences as well as skin and nodal metastasis. Utilization of the tumor specimens for this study was approved by the institutional review board of the Medical University of Graz (24-295 ex 11/12) and the methods were carried out in accordance with the approved guidelines.

Immunohistochemistry (IHC)
IHC was performed on formalin fixed and paraffin embedded (FFPE) tissue using the Autostainer Link 48 (Dako, Glostrup, Denmark). After deparaffinization in xylene, sections were rehydrated with 100%, 96%, 70%, and 50% ethanol for 5 min each and finally rinsed with demineralized water. Antigen retrieval for the anti-MICA antibody was performed with EDTA (1mM EDTA, 0.05% Tween 20, pH 8.0) and for the anti-MICB antibody with citrate (Dako retrival solution, cat. no. S1699, pH 6) in a steamer at 100°C for 30 minutes. After cooling for 20 minutes and two additional washing steps, sections were blocked with peroxidase blocking solution (Dako) followed by incubation over night at 4°C with antibodies to MICA (AF130, R&D Systems, MN, USA) or MICB (orb 1241, Biorbyte, Cambridge, UK) diluted in antibody diluent (Dako) to 1:200 or 1:100, respectively. After washing steps, incubation with a biotinylated secondary antibody, further washing steps, addition of streptavidin peroxidase, detection was obtained using ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. After counterstaining of nuclei with haematoxylin (Dako), sections were dehydrated and mounted in Tissue Tek glass mounting medium (Sakura Finetek, Torrance, CA, USA). Three independent investigators (CR, DS, JCB) classified the tumors as positive or negative for expression of MICA and MICB.

Cell culture
The MCC cell lines WaGa, MKL-1, MKL-2, BroLi, AlDo, LoKe[55] and melanoma cell lines FM79, FM82, IF6[56] have been described before. All cell lines were maintained in RPMI-1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Biochrome, Berlin, Germany). For the cell line AlDo the medium was additionally supplemented with 30% fibroblast conditioned medium. For treatment with specific inhibitors, cells were cultured at a concentration of $1x10^6$ cells/ml in 6 well plates. Inhibitors were dissolved according to the manufacturers' guidelines and used at 1.25 µM vorinostat (Selleckchem, Munich, Germany), 0.3 µM mithramycin A (Sigma) and 0.3 µM trichostatin A (Selleckchem) for 24 hours if not otherwise stated.

Quantitative real time-PCR
RNA of in vitro propagated cells or cryopreserved xenotransplants was isolated using PeqGOLD total RNA Kit (Peqlab, Erlangen, Germany) and transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Quantitative real time polymerase chain reactions (qRT-PCR) were performed using SYBR green or TaqMan PCR master mix

(Sigma) on the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). RPLP0 was used as endogenous control, and detected with the sense-primer: CCA TCA GCA CCA CAG CCT TA, the antisense-primer: GGC GAC CTG GAA GTC CAA CT, and the probe ATC TGC TGC ATC TGC TTG GAG CCC A. MICA and MICB mRNA was detected using the SYBR green primers: MICA/B-sense: CAC CTG CTA CAT GGA ACA CAG C, MICA-antisense: TAT GGA AAG TCT GTC CGT TGA CTC T, and MICB-antisense: ACA TGG AAT GTC TGC CAA TGA TC. Relative quantification was calculated by the $\Delta\Delta C_T$ method using the melanoma cell line IF6 as calibrator.

Immunoblotting
Cell lysates were generated by lysing $3x10^6$ cells per sample in protein extraction buffer supplemented with a proteinase inhibitor cocktail as described before[57]. Lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page), samples were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), blocked for 1 hour in a blocking buffer according to the respective antibody's data sheet and then incubated overnight at 4°C with primary antibodies diluted in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBST) or tris buffered saline with 0.1% Tween-20 (TBST) according to data sheet: MICB (R&D Systems, Minneapolis, MN, USA) 1:1000 in PBST, acetyl-histone H3 (Lys9) (Cell Signaling Technology, Danvers, MA, USA) 1:1000 in TBST, Sp1 (Cell Signaling) 1:1000 in TBST or β-tubulin (Sigma) 1:1000 in PBST. After membranes were washed trice for 10 minutes each in the respective buffer, they were incubated for 1h with the appropriate peroxidase-coupled secondary antibodies (Dako), followed visualization using the ECL Western Blotting Substrate (Pierce, Rockford, IL, USA).

Flow Cytometry
Cell surface expression of MICA and MICB was determined by flow cytometry. $1x10^6$ cells were washed with ice cold PBS and incubated with the PE-conjugated anti-human MICA/B antibody (6D4; Biolegend, San Diego, CA, USA) in PBS with 0.1% bovine serum albumin (BSA) for 90 minutes at 4°C in the dark. After washing steps, cells were stained with 10 µg/ml 7-aminoactinomycin (7AAD, Sigma) to exclude non-viable and measured on a FC500 Flow Cytometer (Beckman Coulter, Brea, CA, USA). Flow cytometry data were analyzed with FlowJo Version 8.7 software (TreeStar, Sunnyvale, CA, USA).

Chromatin immune precipitation (ChIP)
ChIP assays were performed using the SimpleChiP® Enzymatic Chromatin IP Kit with Agarose beads (Cell Signaling). In brief, proteins were cross-linked to DNA with 1.5 % formaldehyde for 10 minutes. Nuclear membranes were broken up using the UP50H Sonicator (Hielscher, Teltow, Germany) set to 100%, 0.9 output for 20 seconds, 6 times in a row with incubation on ice for 30 seconds between sonication pulses. Afterwards, antibodies against histone H3 (cat. no. 6420), acetyl-histone H3 (Lys9) (AcH3K9) (cat. no. 9671), or normal rabbit IgG (Cell Signaling) were used for immune precipitation. The immune precipitated DNA was subsequently analyzed by qRT-PCR, using SYBR green primers specific to the MICA or MICB promoter region: MICA promoter sense CGG ATC CTG GAA TAC GTG GG, antisense ACT CAC ACC TGC CCG TTA TG; MICB promoter sense GCG ACA GGG TCC AGG TCG TGC TC, antisense CCC TAC GTC GCC ACC TTC TCA GCT. The percentage of acetylated histones (AcH3K9) was normalized to total Histones H3 and calculated using following equation:

$$\% \text{ AcH3K9 over total H3} = 100*2\text{^}(C_T \text{ H3- } C_T \text{ AcH3K9})$$

Flow cytometry based cytotoxicity assay
Peripheral blood mononuclear cells (PBMCs) where isolated via gradient centrifugation with Lymphoprep™ (Stemcell Technologies, Vancouver, BC, Canada), and cultured for 3 days in CellGro®SCGM (CellGenix, Freiburg, Germany) supplemented with 10% FBS (Invitrogen, Grand Island, NY, USA) and 500 IU interleukin 2 (IL-2) per ml (Miltenyi Biotec, Bergisch

Gladbach, Germany) to generate lymphokine-activated killer (LAK) cells. MCC cell lines served as target cells either without or with inhibitor (Vorinostat, mithramycin A, or combination thereof) treatment for 12 hours at the concentrations described above; the shortened incubation time was chosen to assure that cells are indeed vital and target cell membranes are fully intact for the cytotoxicity assay. After target cells were washed 3 times in RPMI with 10% FBS, they were labeled by incubation in 3 µM CFSE (Sigma) in pre-warmed RPMI with 10% FBS for 10 minutes at 37°C, followed by another round of 3 washing steps to remove any excessive CFSE.
$2x10^4$ CFSE labeled target cells were incubated alone to establish spontaneous cell death, or co-incubated at varying effector:target ratios, i.e. 40:1, 20:1, 10:1, for 4h at 37°C in a total volume of 100 µl. Before flow cytometry, cells incubated in 10 µg/ml 7AAD (Sigma). Dead target cells were defined as $CFSE^+/7AAD^+$, and the percentage of cytotoxicity was calculated as following:

% cytotoxicity = (% dead target cells experimental - % dead target cells spontaneous)/(100-% dead target cells spontaneous)*100

For blocking experiments target cells were incubated with saturating concentrations of blocking antibodies against MICA/B (clone 6D4; Biolegend) or isotype control for 2h at 37°C. before incubation with LAK cells. Pre incubation with F(ab')2 fragments for 30 minutes (Life Technologies) was performed to avoid Fc-receptor mediated antibody-dependent cell-mediated cytotoxicity.

Xenotransplantation experiments
Six-week-old female NOD.CB17/Prkdc^{scid} mice were obtained from Charles River Laboratories, and housed under specific pathogen-free conditions. Tumors were induced by s.c. injection as described before[30]. Twenty-four days after tumor cell inoculation, when the tumors reached a volume of approximately 100 mm^3, treatment was started. Mice were divided into four groups of six mice each ensuring an equal overall tumor burden. Immediately before injection a 1 M (264.3 mg/ml) vorinostat stock solution was diluted in 45% Polyethylene glycol (PEG-400, Sigma) to 10 mg/ml and 60 mg/kg bodyweight were administered i.p. per mouse. For mithramycin A, a 5 mg/ml stock solution was diluted in H_2O to 0.33 mg/ml and 0.2 mg/kg bodyweight were injected i.p. per mouse. The placebo group received the same volume of the respective solvents. Mice were treated five consecutive days a week for two weeks. Afterwards tumor tissue was formalin fixed and paraffin embedded for IHC or cryo-preserved for RNA isolation. All animal studies had been approved by the Austrian ministry of education and science according to the regulations for animal experimentation (BMWF-66.010/0151-II/3b/2012).

Statistical Analyses
Statistical analyses were performed using Graphpad Prism 6.0 Software (Graphpad Software Inc., San Diego, CA, USA). Cell culture experiments were analyzed using Friedman test, a paired non-parametric ANOVA. The xenotransplantation experiments were analyzed using Kruskal-Wallis test, an unpaired non-parametric ANOVA. A p-value smaller than 0.05 was considered significant; the respective p-values are indicated in the figures as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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Author contributions CR, DS and JCB designed the study, wrote the manuscript and evaluated the immunohistochemistry stainings; CR and KF performed the experiments and analyzed the data. KGP and PN provided tissue microarrays, cDNA array and clinical data and reviewed the manuscript. **Competing financial interests** None of the authors declares any competing financial conflict.

Figures:

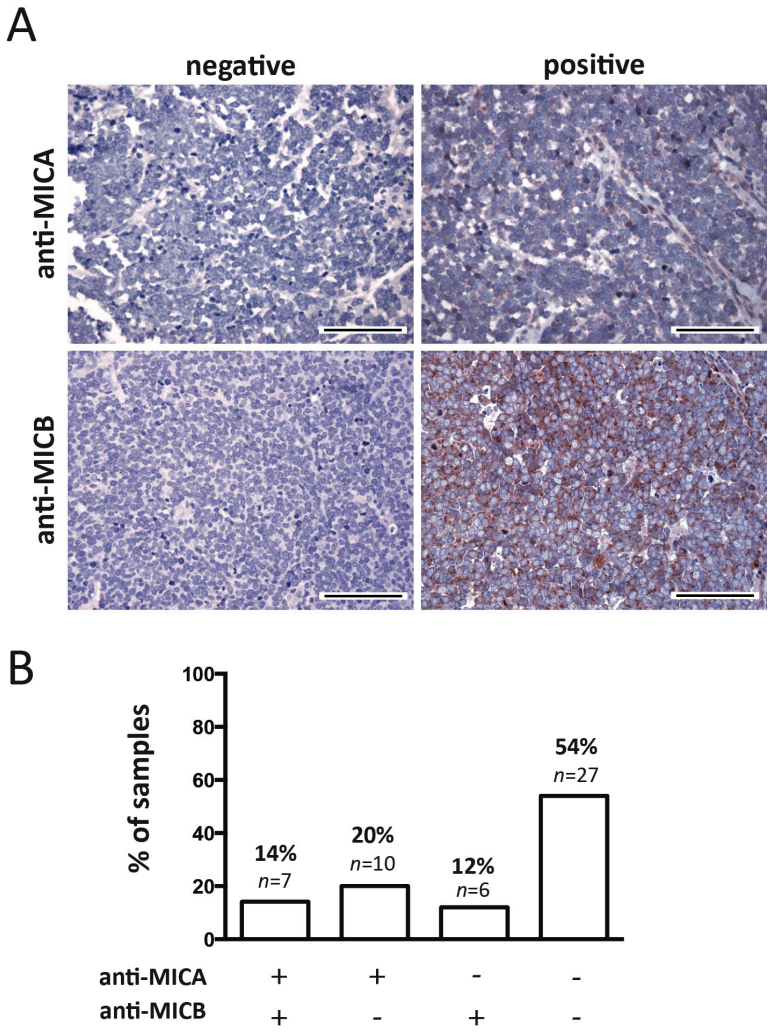

Fig. 1. MICA and MICB expression of MCC tumors in situ.
50 MCC tumor samples from 34 patients were analyzed by immunohistochemistry for expression of MICA and MICB. (**A**) Samples were classified as positive or negative. Representative negative and positive samples are depicted at 40x magnification, scale bars are 100µm. The positive examples represent the strongest obtained signal with the respective antibody. (**B**) Tumor samples were stratified into 4 groups: Double positive for MICA and MICB (+/+, 14%, n=7), only positive for MICA (+/-, 20%, n=10), only positive for MICB (-/+, 12%, n=6), or double negative (-/-, 54%, n=27).

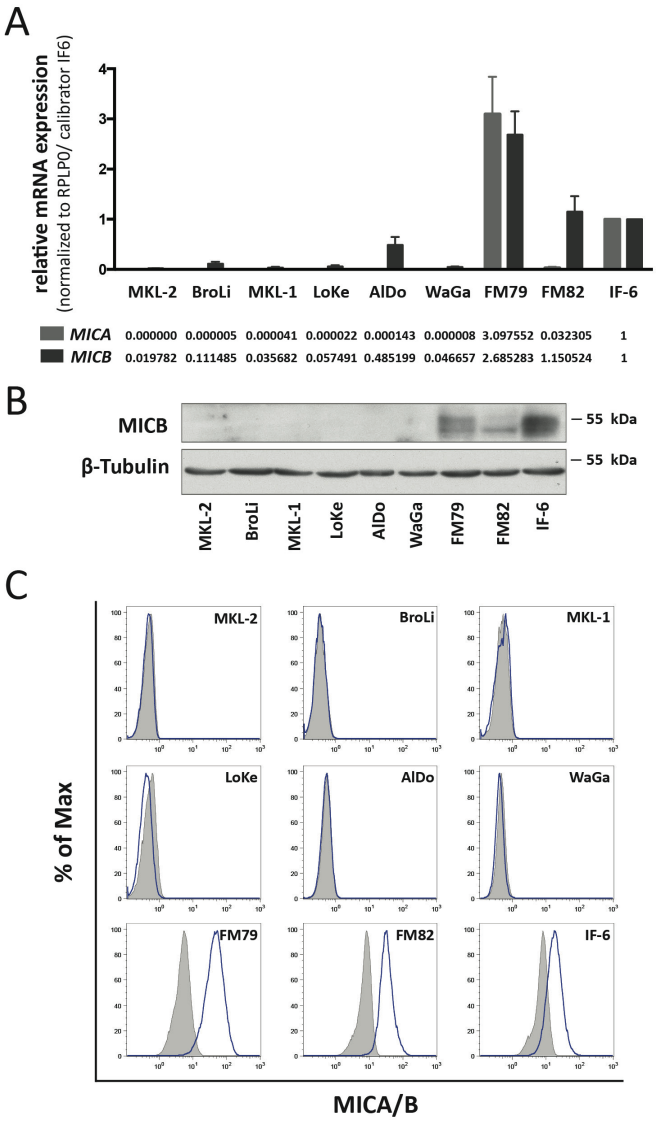

Fig. 2. MCC cell lines do not express MICA and MICB protein despite low levels of MICA and MICB mRNA.

A) MICA (light grey) and MICB (dark grey) mRNA expression was determined by qRT-PCR in MCC cells. Relative expression levels were calculated by normalization of C_T values to RPLP0 and calibration to the melanoma cell line IF6. (**B**) MICB protein expression of whole cell lysates was determined by immunoblot; β-tubulin served as a loading control. (**C**) MICA/B cell surface expression was determined by flow cytometry using an antibody recognizing both MICA and MICB (clone 6D4; blue line); matched isotype control is depicted as grey filled area. Melanoma cell lines FM79, FM82 and IF6, served as positive control for MICA and MICB expression in all assays illustrated in this figure.

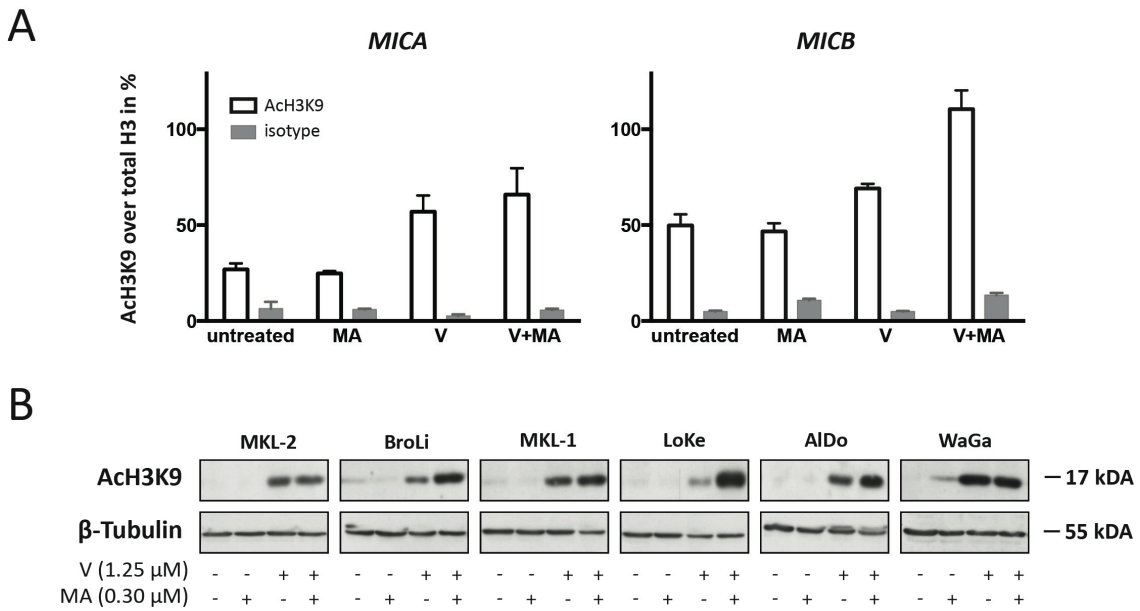

Fig. 3. Vorinostat alone or in combination with mithramycin A increases global as well as *MICA* and *MICB* promoter-specific histone H3 Lysine 9 (H3K9) acetylation in MCC cell lines.

(**A**) Chromatin immunoprecipitation (ChIP) assay was performed with untreated, mithramycin A (MA), vorinostat (V), or the combination thereof (V+MA) treated WaGa cells followed by a qRT-PCR using *MICA* or *MICB* promoter specific primers. C_T values of anti-acetyl-H3K9 (AcH3K9) antibody or rabbit IgG isotype control precipitated DNA were normalized to total histone H3 antibody as described in materials and methods. White bars represent the percentage of acetylated H3K9, grey bars the respective control. Experiments were performed in duplicates and results are expressed as mean ± SEM. (**B**) Global H3K9 acetylation of untreated, V, MA, or V+MA treated MCC cell lines was determined by immunoblot with the same AcH3K9 antibody used in the ChIP assay; β-tubulin served as loading control.

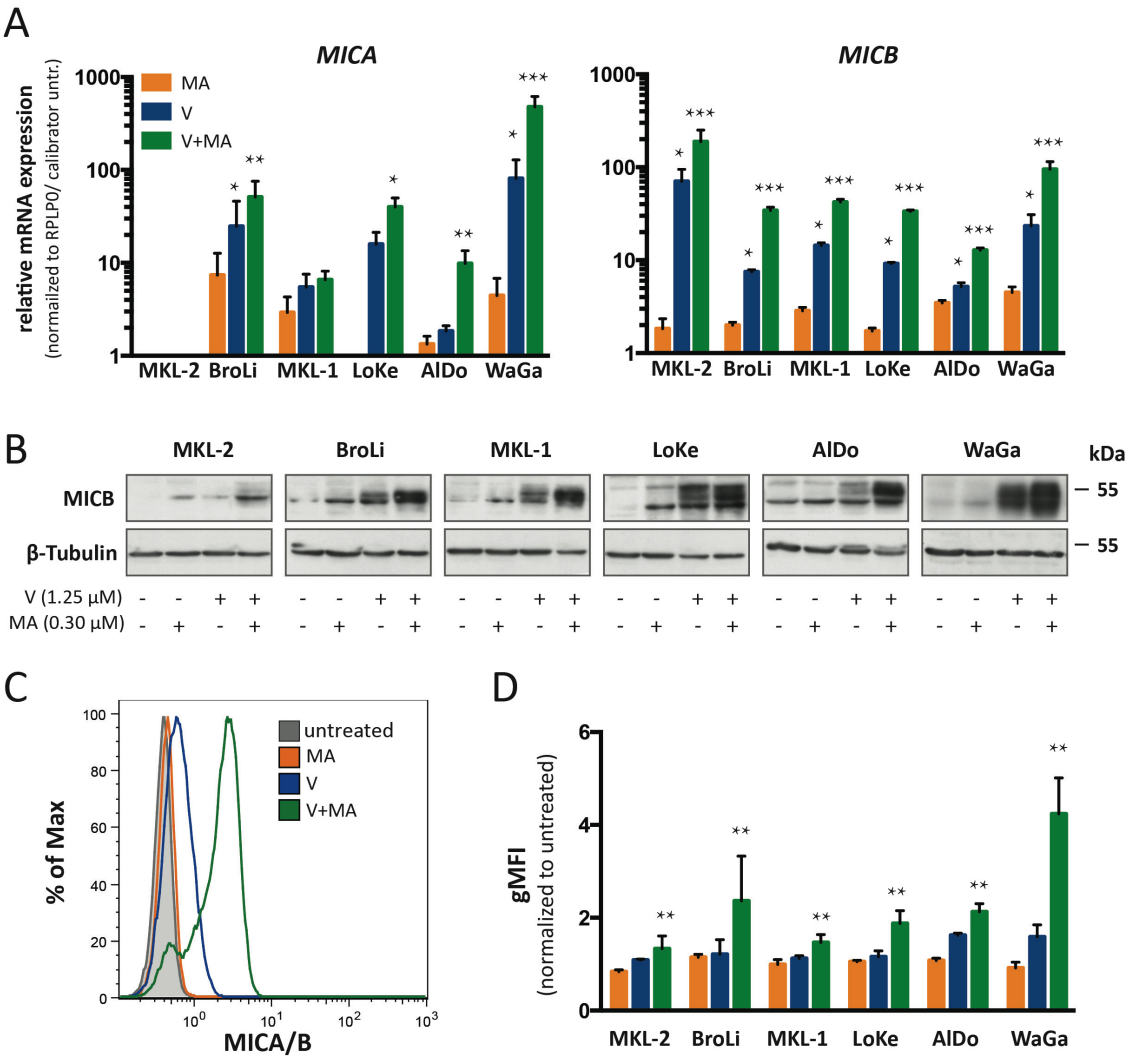

Fig. 4. Induction of MICA and MICB expression by vorinostat alone or in combination with mithramycin A.

MICA and MICB mRNA and protein expression of untreated MCC cell lines were compared to the respective expression after treatment with mithramycin A (MA, orange), vorinostat (V, blue), or the combination thereof (V+MA, green). (**A**) mRNA expression of MICA and MICB was determined by qRT-PCR in duplicates in three independent experiments; C_T values were normalized to RPLP0 and calibrated to the ΔC_T value of the respective untreated cell line; relative mRNA expression is depicted on a logarithmic scale (log 10) ± SEM. (**B**) MICB expression in whole cell lysates of MCC cell lines was detected by immunoblot using a MICB specific antibody; β-tubulin served as loading control. (**C**, **D**) MICA/B cell surface expression was determined by flow cytometry using an antibody recognizing both MICA and MICB (clone 6D4), which is exemplified for WaGa (**C**); the results for all cell lines are depicted as the geometric mean fluorescence intensity (gMFI) of MICA/B staining, normalized to the respective untreated cell lines ± SEM in three independent experiments (**D**). Statistical analysis was performed using the Friedman test as indicated.

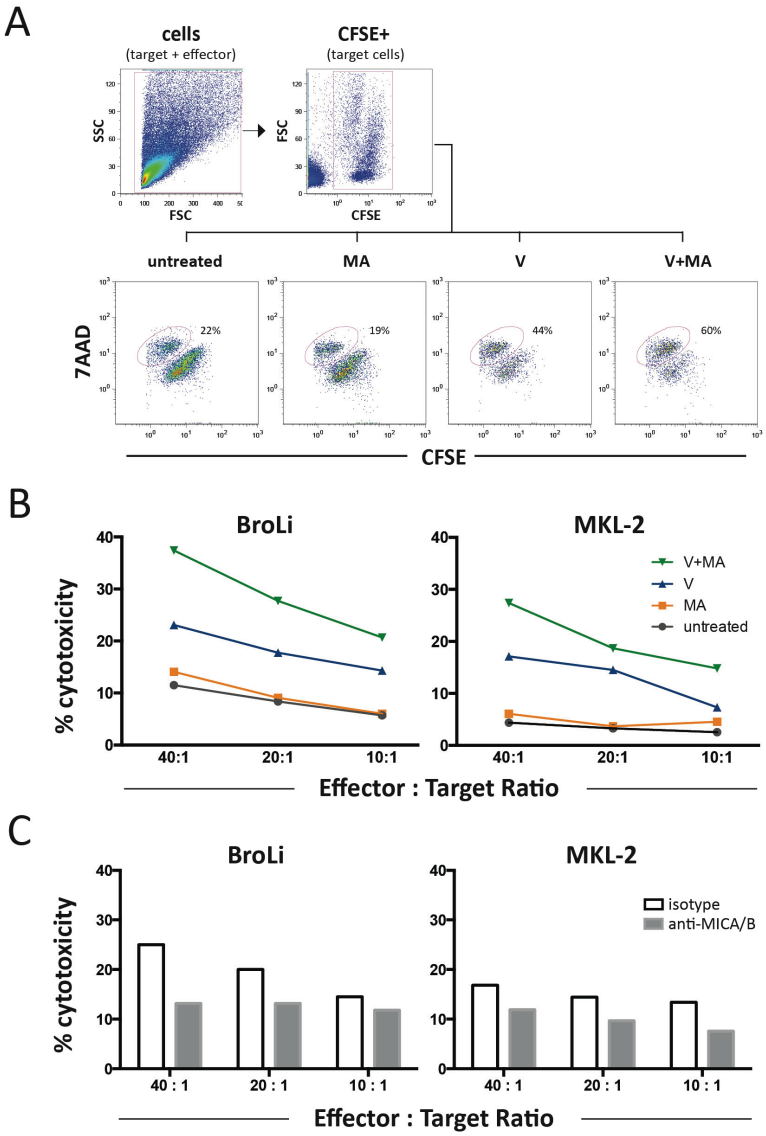

Fig. 5. Inhibition of HDACs in MCC cell lines increased their susceptibility to LAK cell mediated lysis, which is subdued by MICA/B blockade.
The flow cytometry based cytotoxicity assay was performed as described in Material and Methods. (**A**) The gating strategy is illustrated for untreated and treated BroLi cells used at an effector to target ratio of 40:1; target cells were gated as CFSE positive cells in an FSC/CFSE plot, lysed target cells were defined as 7AAD/CFSE double positive cells and are quantified as percentage of all target cells. (**B**) Untreated (grey), mithramycin A (MA, orange), vorinostat (V, blue), or the combination thereof (V+MA, green) treated BroLi and MKL-2 cells served as target cells for LAK cells at the indicated effector to target ratios in a 4h cytotoxicity assay. The lysis of the respective target is given as average of three independent experiments. (**C**) Vorinostat plus mithramycin A treated BroLi and MKL-2 cells served as target cells for LAK cells at the indicated effector to target ratios in a 4h cytotoxicity assay in the presence of saturating amounts of a MICA/B specific blocking antibody (grey bars) or an isotype control antibody (white bars); Fc receptors of effector cells were blocked by saturating amounts of F(ab)2 fragments.

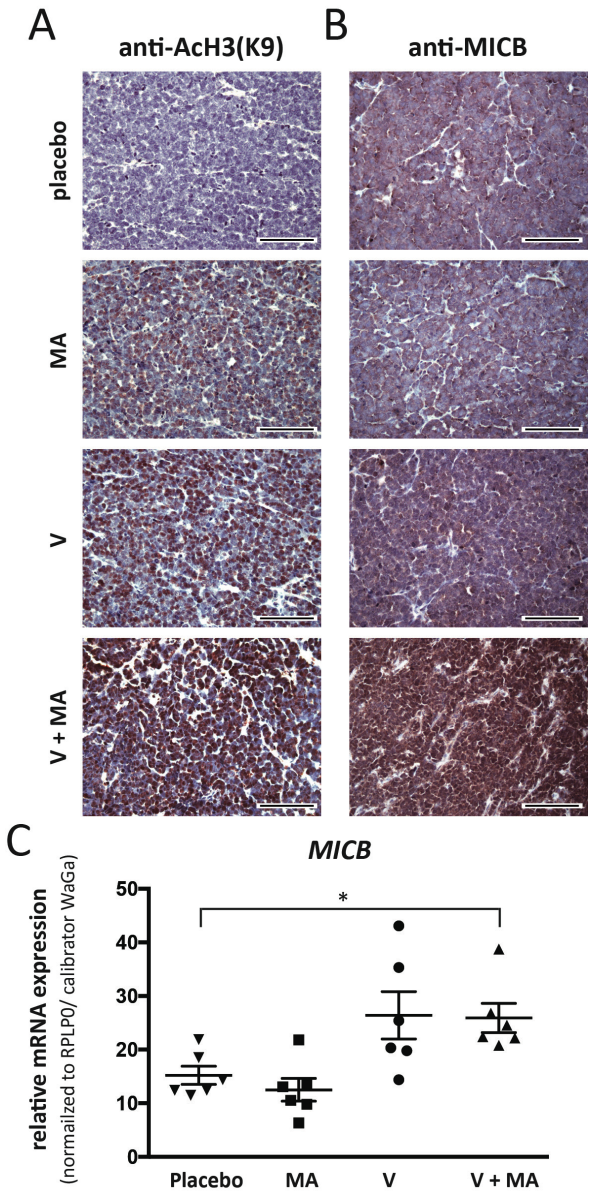

Fig. 6. Vorinostat and mithramycin A treatment induces histone H3K9 acetylation and MICB expression in MCC cells in vivo.
NOD.CB17/Prkdc^{scid} mice (n=6 for each treatment group) bearing subcutaneous xenotransplants of WaGa cells were treated with placebo, vorinostat (V) mithramycin A (MA), or the combination thereof (V+MA) as described in materials and methods. Immunohistochemistry on FFPE fixed tumor samples obtained after two weeks of treatment was performed using antibodies specific against AcH3K9 (**A**) or MICB (**B**). Representative examples are depicted at 40x magnification, scale bar is 100µm. (**C**) mRNA was isolated from cryopreserved tumors and qRT-PCR was performed using primers specific for *MICB*. C_T values were normalized to RPLP0 and calibrated to in vitro cultured WaGa cells. Statistical analysis was performed using the Kruskal-Wallis test.

Supplementary

Supplementary material and methods:

Tissue microarrays

Tumor samples of 134 MCC lesions comprising primary lesions, recurrences, skin and nodal metastasis of 99 patients were included in the tissue microarray (TMA). All patients had MCC, as assessed by two or more pathologists. Diagnoses occurred between the years 1985 and 2011. All materials were obtained from the MCC Data and Tissue Repository at the University of Washington/Fred Hutchinson Cancer Research Center [Seattle, WA; Institutional Review Board (IRB) approval #6585]. Tissue cores of 0.6 mm in diameter were taken in triplicates from each FFPE tumor sample. Specimens were categorized into MICA/B positive or negative in a blinded fashion.

Immunohistochemistry of tissue microarrays

Sections were deparaffinized in xylene, rehydrated with 100%, 96%, 70%, and 50% ethanol and rinsed with demineralized water. After citrate antigen retrieval, samples were blocked with peroxidase blocking solution (Dako). Samples then were incubated for 60 minutes at room temperature with an antibody recognizing both MICA and MICB (clone 6D4) diluted 1:20 in antibody diluent (Dako). After incubation with a biotinylated secondary antibody and streptavidin peroxidase, detection was carried out with Vector NovaRED Peroxidase Substrate (Vector Laboratories) according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin (Dako). Slides were dehydrated and mounted in Shandon Hypermount (Thermo Scientific) glass mounting medium.

Chromatin immunoprecipitation of Sp1 at the MICA and MICB promoter

Chromatin immunoprecipitation was performed as described in material and methods before but here with an SP1 specific antibody (cat. no. 5931, Cell Signaling) for untreated and vorinostat plus mithramycin A treated cells. Enrichment of Sp1 at the MICA and MICB promoter was calculated after normalization to background and relative to input using the following equation:

enrichment to input= 100*2^(adjusted input-C_T IP)

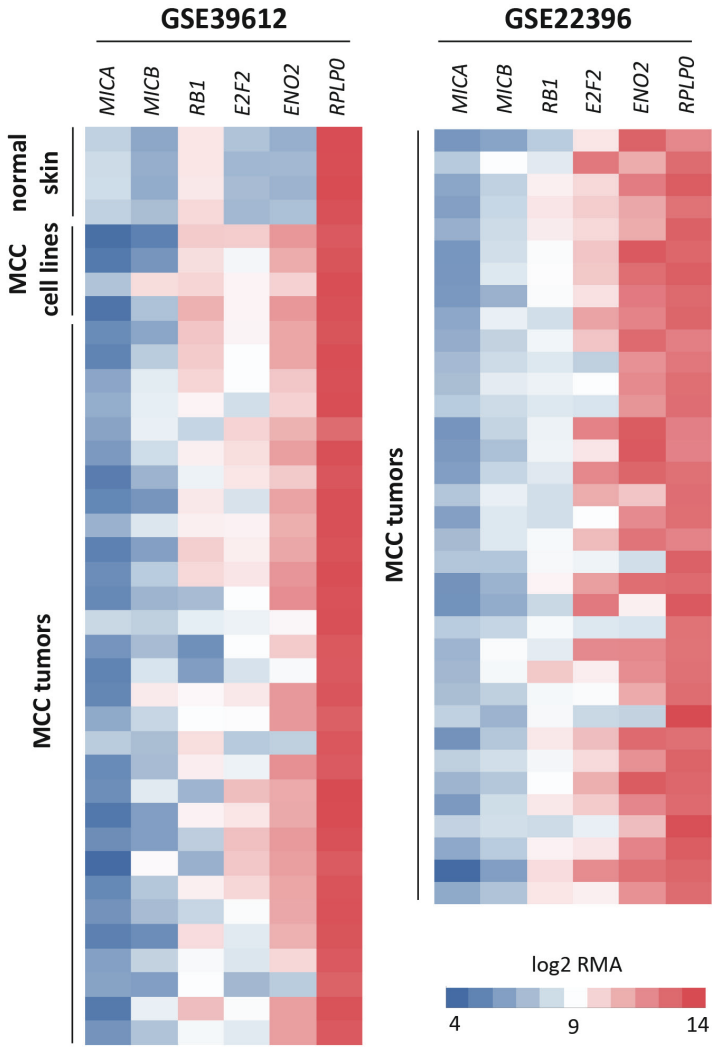

Fig. S1. MICA and MICB mRNA expression in MCC lesions in situ.
Robust multi array average (RMA) normalized expression values of two gene expression arrays (GSE22396 and GSE39612) were obtained from the Gene Expression Omnibus (GEO) database. In GSE 22396 the gene expression profiles of 35 MCC tumors of 34 patients were analyzed. In GSE39612 data of 30 MCC tumors of 27 patients and 4 MCC cell lines renormalized with normal skin samples were provided. RMA values were log2 transformed and are depicted as heat map with expression values ranging from 4 (blue = low expression) to 14 (red = high expression). MICA and MICB mRNA expression is shown in comparison to RB1, E2F2, ENO2 and RPLP0.

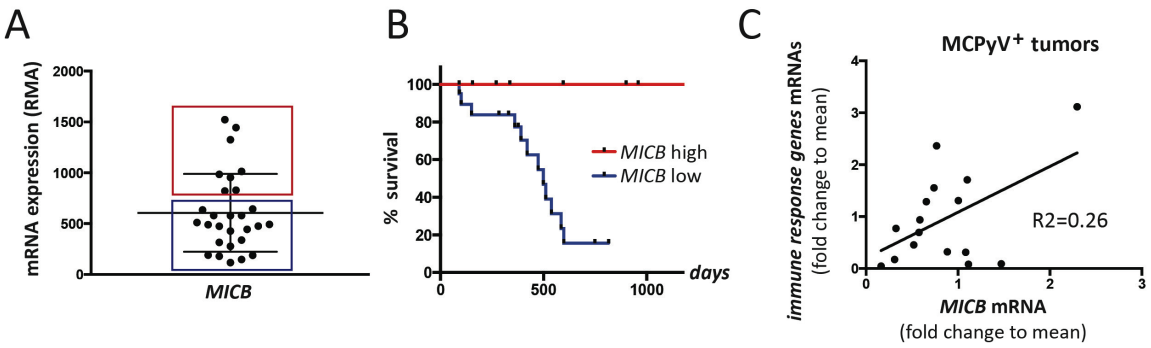

Fig. S2. High *MICB* expression in MCC tumors is associated with an improved survival and immune infiltration.

Robust multi array average (RMA) normalized expression values of MICB mRNA were obtained from the Gene Expression Omnibus (GEO) database (GSE22396). (**A**) Tumors were categorized according their MICB mRNA expression level: The low group persists of tumors with a MICB mRNA expression below or equal to average (within blue square), the high group includes tumors with a MICB mRNA expression markedly above average (within red square). (**B**) MCC-specific survival was analyzed using the Kaplan-Meier method for the MICB low (blue line) or high (red line) mRNA expressing tumors; p=0.009 (**C**) MICB mRNA expression was correlated with the expression of immune gene signature associated with a good prognosis (i.e. ALDH1A, AMICA1, BHLHE41, CCL 19, CCR2, CD8a, CGA, CHI3L1, CHIT1, CHRNA9, FAM46C, FBP1, GZMA, GZMB, GZMH, GZMK, HLA-DPA1, HLA-DRB5, IGJ, IGKC, ITGBL1, KLRK1, LYZ, MMP7, POUF2AF1, PROM1, SLAMF1, TRBC1) for MCPyV positive tumors by linear regression analysis (p=0.04 , R2=0.26).Tumors were classified into MCPyV positive and negative by their MCPyV T-Antigen mRNA expression state.

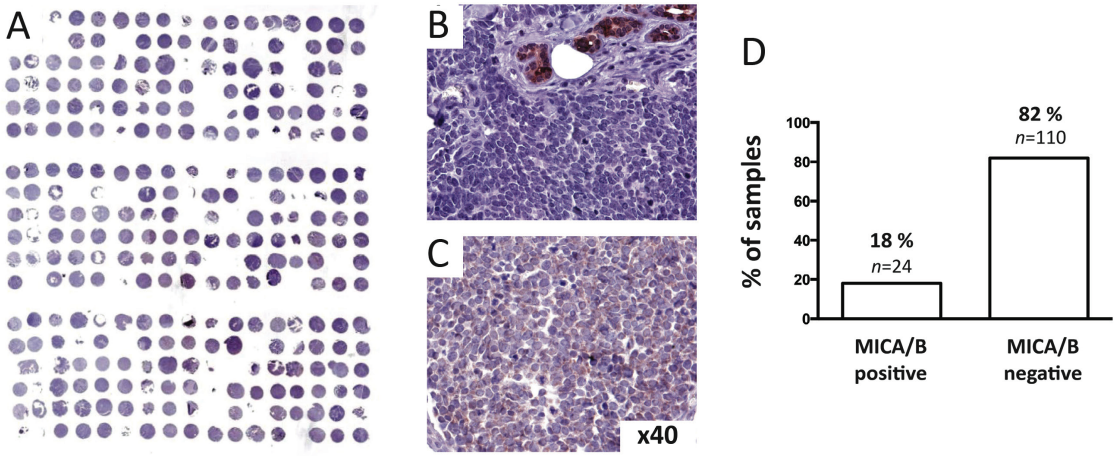

Fig. S3. in situ expression of MICA/B in MCC lesions in tissue microarrays.
Triplicates of 134 MCC tumors of 99 patients were arranged on a tissue microarray as shown (**A**). MICA/B expression was determined by immunohistochemistry using an antibody recognizing both MICA and MICB (clone 6D4) as described in supplementary material and methods. Samples were classified as positive or negative and representative sections for negative (**B**) and positive (**C**) lesions are depicted at 40x magnification. Percent of MICA/B positivity is given on a lesion based calculation (**D**).

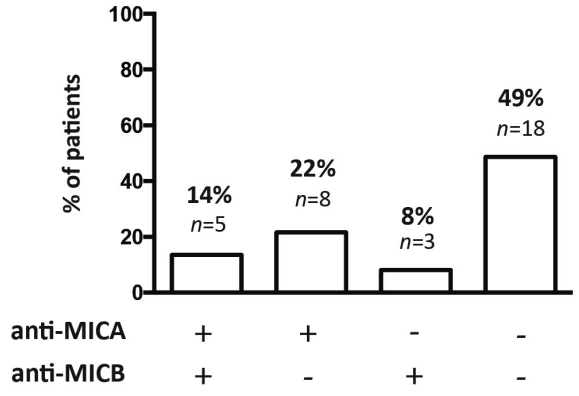

Fig. S4. Frequency of in situ expression of MICA and MICB in sections of MCC lesions calculated on a per patient basis.

50 MCC tumor samples of 34 patients were analyzed by immunohistochemistry for expression of MICA and MICB. The intra-individual heterogeneity of the analyzed tumor lesions is negligible. Patients were classified into 4 groups: Double positive (+/+, 14%, n=5) or double negative (-/-, 49%, n=18) for the expression of MICA and MICB, or only positive for MICA (+/-, 22%, n=8) or MICB (-/+, 8%, n=3). Groups are depicted as percentage of all patients (n=34).

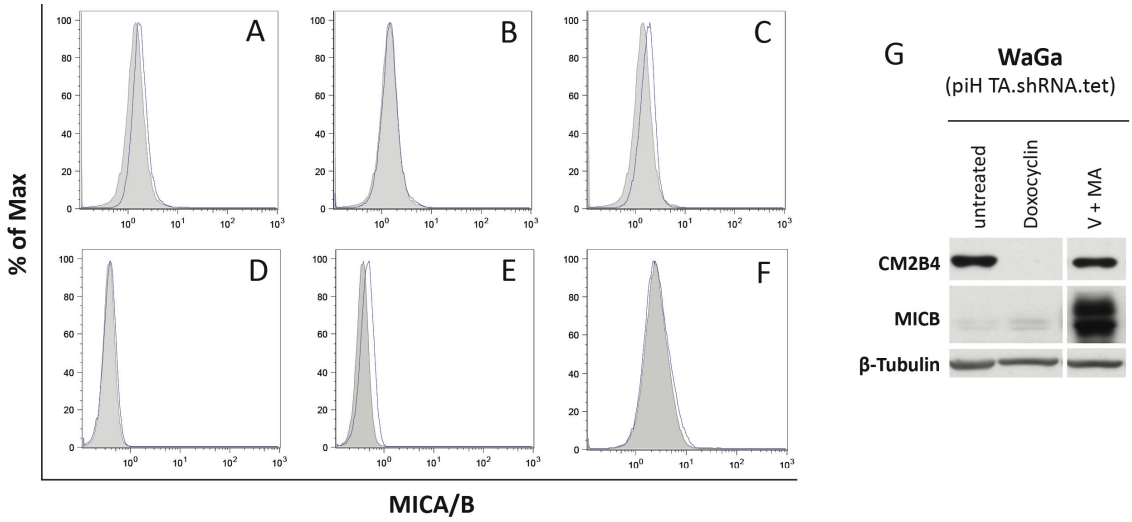

Fig. S5. Cell stress does not induce MICA/B protein expression in a MCC cell line.

MICA/B cell surface expression of WaGa cells under standard culture conditions (grey filled area) or after (**A**) serum starvation, (**B**) 5% DMSO, (**C**) heat shock at 41,5°C for 24hrs, the presence of 1000U/ml (**D**) interferon α or (**E**) interferon γ for 48h, or (**F**) after silencing of the MCPyV-encoded T antigens as illustrated under (**G**) was determined by flow cytometry using an antibody recognizing both MICA and MICB (clone 6D4). A previously described inducible MCPyV T-antigen knockdown was used to silence both MCPyV large and small T-antigens (*54*). WaGa (piH TA.shRNA.tet) cells were cultured for 5 days in the absence or presence of 1 µg/ml doxycycline; silencing of LTA was confirmed by immunoblot using the LTA specific antibody CM2B4. MICB protein expression of whole MCC cell lysates was determined by immunoblot; β-tubulin served as a loading control; WaGa (piH TA.shRNA.tet) cells treated with the combination of vorinostat and mithramycin A served as positive control.

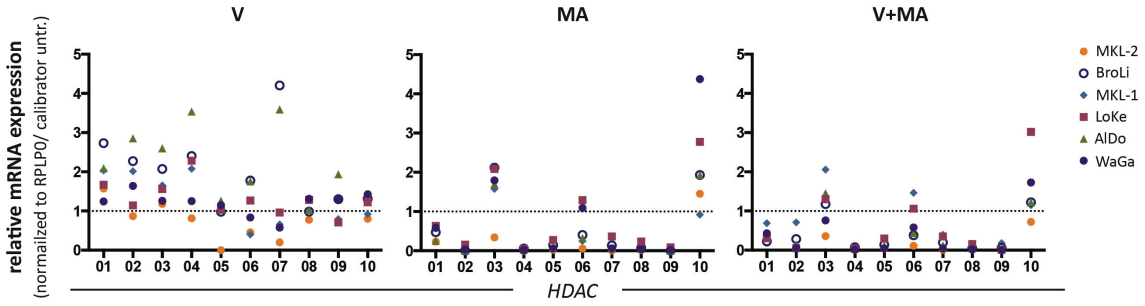

Fig. S6. Mithramycin A inhibits vorinostat induced *HDAC* mRNA transcription. RNA was isolated from the indicated MCC cell lines (MKL-2 [yellow dot], BroLi [blue circle], MKL-1 [blue diamond], LoKe [pink square], AlDo [green triangle], and WaGa [purple dot]) either untreated or treated with vorinostat (V), mithramycin A (MA), or the combination thereof (V+MA) treated as described in Material and Methods. qRT-PCR was performed using primers specific for class I and II *HDACs 1-10* listed in supplementary Table 1. C_T values were normalized to RPLP0. The relative mRNA expression compared to the respective untreated cell lines is depicted.

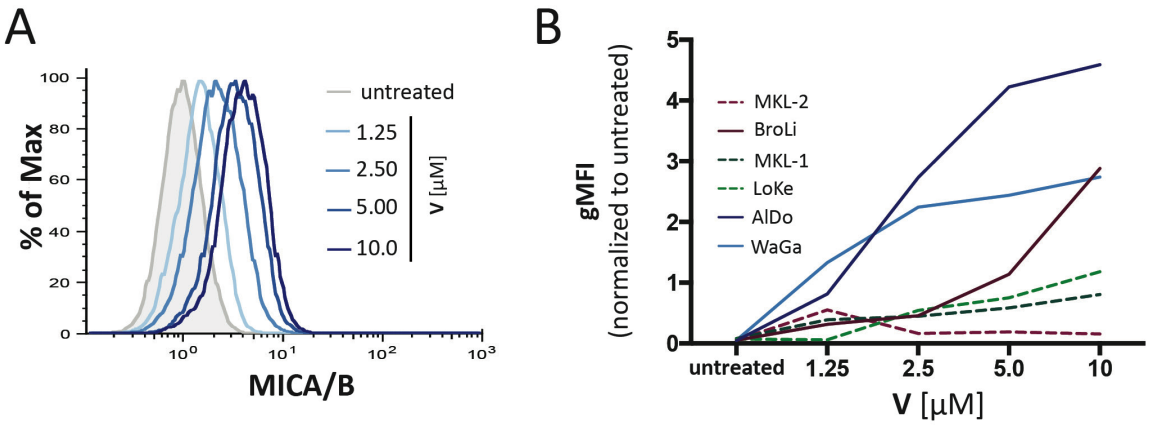

Fig. S7. Induction of MICA/B surface expression by vorinostat is dose dependent.

MICA/B cell surface expression MCC cell lines (MKL-2 [purple dashed line], BroLi [purple line], MKL-1 [dark green dashed line], LoKe [light green dashed line], AlDo [dark blue line], and WaGa [light blue line]) cells subjected to increasing concentrations of vorinostat (V)(none, 1.25, 2.50, 5.00 and 10.00 µM) for 24h was determined by flow cytometry using an antibody recognizing both MICA and MICB (clone 6D4), which is exemplified for AlDo (**A**). The summary for all cell lines is depicted as the geometric mean fluorescence intensity (gMFI) normalized to the respective untreated cell lines (**B**).

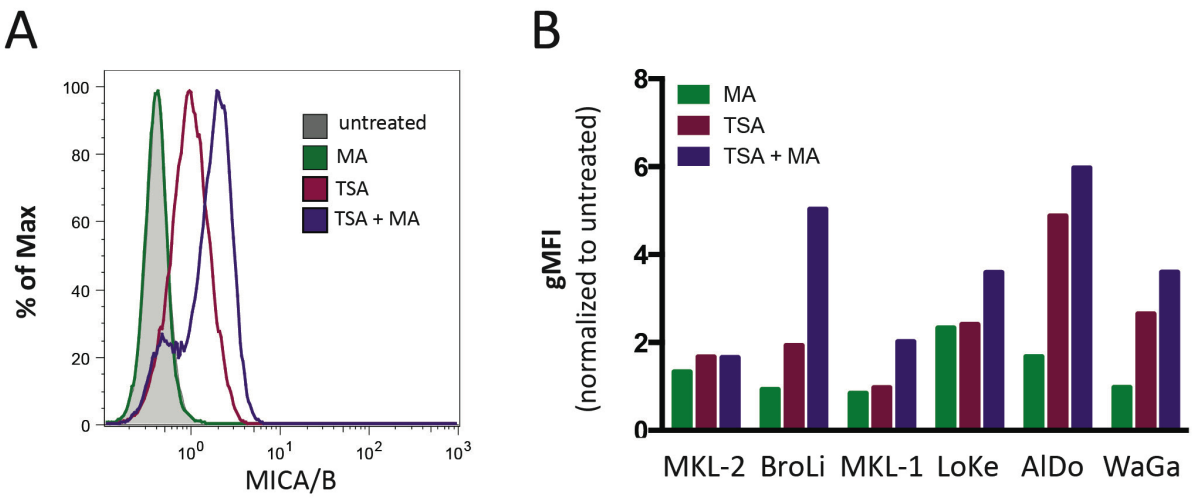

Fig. S8. Induction of MICA/B expression by trichostatin A alone or in combination with mithramycin A.

Cell surface MICA/B expression on untreated MCC cell lines (MKL-2, MKL-1, BroLi, and WaGa) was compared to the expression after treatment with mithramycin A (MA, green), trichostatin A (TSA, red), or the combination thereof (TSA+MA, purple) was determined by flow cytometry using an antibody recognizing both MICA and MICB (clone 6D4), which is exemplified for WaGa (**A**). The data for all cell lines is depicted as the geometric mean fluorescence intensity (gMFI) of MICA/B staining, normalized to the respective untreated cell line (**B**).

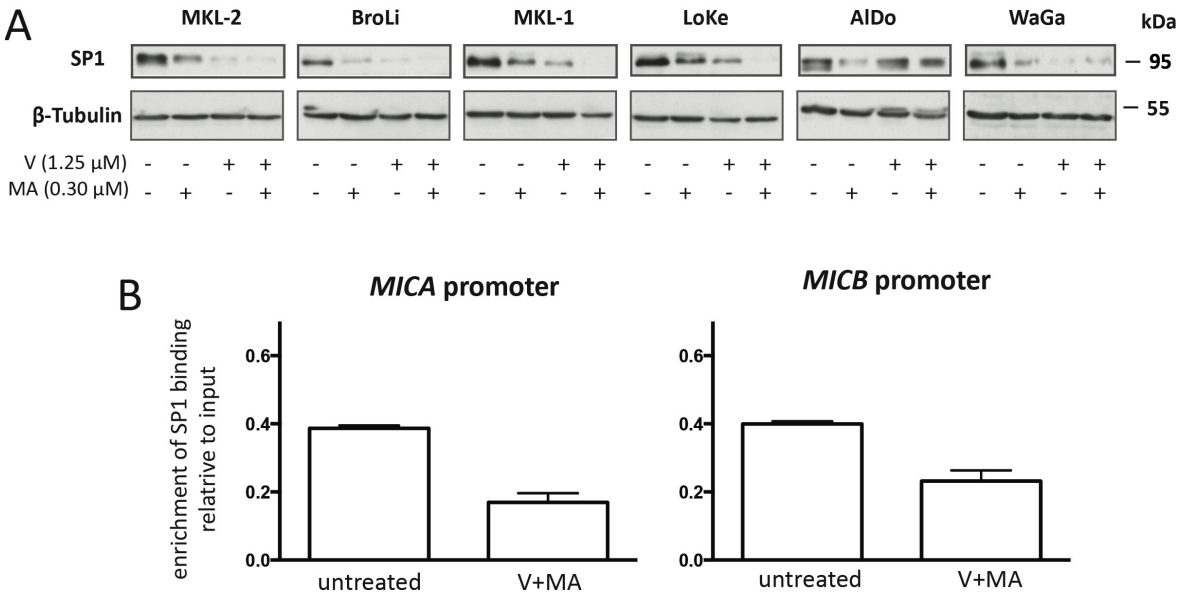

Fig. S9. Reduced SP1 expression and *MICA* and *MICB* promoter binding after vorinostat and mithramycin A treatment.

(**A**) Sp1 expression in whole cell lysates of either untreated or treated with vorinostat (V), mithramycin A (MA), or the combination thereof (V+MA) MCC cell lines was detected by immunoblot using a Sp1 specific antibody; β-tubulin served as loading control. (**B**) Chromatin immunoprecipitation (ChIP) assay was performed with untreated and vorinostat plus mithramycin A (V+MA) treated WaGa cells followed by a qRT-PCR using MICA or MICB promoter specific primers. Sp1 enrichment at the MICA and MICB promoter relative to input was calculated as described in supplementary material and methods. qRT-PCR was performed in duplicates and results are expressed as mean ± SEM.

Table S1. HDAC 1-10 qRT-PCR primer

	forward	reverse
HDAC1	CACGGACCGGGTCATGACTGT	CTTGCCTTTGCCAGCCCCGA
HDAC2	TCAAGGAGGCGGCAAAAA	TGCGGATTCTATGAGGCTTCA
HDAC3	CTGTGTAACGCGAGCAGAAC	GCAAGGCTTCACCAAGAGTC
HDAC4	CTGGTCTCGGCCAGAAAGT	CGTGGAAATTTTGAGCCATT
HDAC5	CCATTGGAGACGTGGAGTACCT	GCGGAGACTAGGACCACATCA
HDAC6	GGAATGGCATGGCCATCATTAG	CGTGGTTGAACATGCAATAGC
HDAC7	CTCAGTGGCCATCGCCTGCC	TTGCTGGGTGCCGTTGCCAT
HDAC8	GACCGTGTCCCTGCACAAA	CAACATCAGACACGTCACCTGTT
HDAC9	CTTCTCACGGACAACAGGGT	GCTCAGCAAAGAATGCACAG
HDAC10	ATGTTGCAGTGCCATCCT	GTGTAAATGCTCCCACCTTG

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HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery

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Merkel cell carcinoma (MCC), HLA class I, antigen processing, Histone deacetylase inhibitor (HDACi), epigenetic silencing, immune evasion, immunotherapy.

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Abstract

Merkel cell carcinoma (MCC) is an aggressive skin tumor associated with the Merkel cell polyomavirus (MCPyV). Despite the constant expression of immunogenic viral antigens, advanced MCCs tumors are able to escape host's immune system. HLA class I down-regulation is held responsible for this escape. Here, we not only confirm a reduced expression of HLA class I in 75% of MCC tumors *in situ* in an independent set of 56 MCC lesions of 40 patients and on four out of six MCC cell lines. More importantly, we demonstrate that reduced HLA class I surface expression is primarily due to epigenetic silencing of key components of the antigen processing machinery (APM), including *LMP2*, *LMP7*, *TAP1* and *TAP2,* via histone hypoacetylation. Consequently, the re-expression of APM components by HDAC inhibitors restored HLA class I surface expression on MCC cells both *in vitro* and in a pre-clinical mouse xenotransplantation model *in vivo*. HLA class I antigen re-induction on MCC cell surface increased the susceptibility of MCC cells to HLA class I dependent cytotoxicity. In summary, our data suggest that restoration of HLA class I expression on MCC cells by epigenetic priming is likely to enhance the therapeutic efficacy of T cell-based immunotherapy.

Introduction

Merkel cell carcinoma (MCC) is still a rare neuroendocrine cancer of the skin, but its incidence has tripled over the last 20 years (*1*). Based on the disease specific death rate, it is more lethal than melanoma, with no approved treatment for advanced stages (*2*). At least 80% of MCCs are associated with a polyomavirus, which was first identified in this tumor and was hence named Merkel cell polyomavirus (MCPyV) (*3*). The polyomavirus is integrated in the MCC cell genome, and tumor cell proliferation is dependent on expression of the oncogenic viral proteins, i.e. large T (LT) and small T antigens (sT) (*4*, *5*). This expression of viral proteins translates into an exceptional immunogenicity of MCC tumors. Accordingly, $CD8^+$ T cells specific for MCPyV derived epitopes are present in the peripheral blood of most MCC patients (*6*, *7*) and an intratumoral infiltration of $CD8^+$ T cells is associated with improved prognosis (*8*, *9*). Although spontaneous remissions of primary tumors as well as metastatic lesions can be observed (*10*), strong intratumoral $CD8^+$ T cell infiltration is a rare event in MCC tumors, being detectable in only approximately 5% of patients (*9*). This circumstance is most likely owing to the sophisticated immune escape strategies exploited by MCCs such as the inhibition of cellular immune responses via PD1/PD-L1 signaling (*11*, *12*). An additional immune escape strategy utilized by

MCCs is the stable, yet reversible reduction of human leukocyte antigens (HLA) class I surface expression, as adaptive T-cell responses against tumor cells critically depend on tumor antigen derived peptides presented by HLA class I antigens (*13*). HLA class I molecules are heterodimers of the HLA heavy chain and the β_2-microglobulin (β_2m) light chain (*14*). This complex is more frequently expressed on the cell surface if stabilized by binding of a suitable peptide. Recognition of specific peptide-HLA class I complexes via the cognate T cell receptor (TCR) on $CD8^+$ T cells triggers their cytotoxic activity (*15*). A complex network of proteases, peptidases and transporters, *aka* the antigen processing machinery (APM), is necessary to achieve correct peptide processing, transportation and loading on HLA class I molecules in the endoplasmatic reticulum (ER) (*15*). Key components of the APM are proteasome, composed of different catalytic subunits, including the interferon-inducible subunits LMP2 and LMP7, as well as the transporter subunits TAP1 and TAP2, mediating peptide transfer from the cytosol into the ER for loading onto HLA class I molecules (*15*). Aberrations in antigen processing and presentation have been linked to disease aggressiveness and outcome in a variety of malignancies including colorectal, cervical, ovarian, and prostate cancer as well as melanoma (*15*). In cancer, but also in virally infected cells, APM expression may be altered by genetic and/or epigenetic mechanisms. The latter includes promoter methylation and/or histone hypoacetylation of genes involved in antigen processing and presentation (*16*, *17*). Notably, since most genes encoding the APM components as well as for the *HLA* genes (*HLA-A, -B* and *-C*) are located in the major histocompatibility complex (*MHC)* gene cluster on chromosome 6p, epigenetic silencing of this chromatin region via histone hypoacetylation appears to be an effective way to silence HLA class I mediated antigen presentation (*17*). No information is available about the mechanism(s) underlying HLA class I antigen down-regulation in MCC cells, although this information may suggest the rationale design of strategies to restore HLA class I antigen expression on MCC cells and to counteract their escape from recognition and destruction by cognate T cells. Therefore in the present study after having confirmed our original finding of HLA class I antigen down regulation in MCC cells in an independent cohort of 56 MCC tumors, we have shown that HLA class I antigen down regulation in MCC cells is caused by epigenetic silencing of APM genes via histone hypoacetylation. Furthermore we have shown that pharmacological inhibition of HDACs could restore histone acetylation and thereby re-induce not only HLA class I antigen expression but also immune recognition of MCC cells.

Results:

Reduced HLA class I antigen expression is common in MCC both *in situ* and *in vitro*

In the first series of experiments we confirmed in an independent set of MCC lesions the recent report by Paulson *et al.* that HLA class I expression is down-regulated in the majority of MCC tumors (*13*). HLA-A expression *in situ* was detected by immunohistochemistry (IHC) in a cohort of 56 MCC lesions from 40 patients using an HLA-A specific antibody (clone EP1395Y; Fig. 1A-D). The HLA-A staining score was compiled by multiplying the scores for staining intensity and frequency of positive cells (Fig. 1E). In line with Paulson *et al.'s* observations 37% (n=20) MCC lesions entirely lacked HLA-A expression (HLA score 0,) 42% (n=24) were characterized by a low expression (HLA score 1-4), whereas only 21% (*n=12*) of the analyzed tumors expressed high amounts of HLA-A molecules on most tumor cells (HLA score 6-12). To establish whether the available MCC cell lines reflect the *in situ* situation and thus can be used as models to study HLA class I regulation in MCC *in vitro,* we analyzed six MCC cell lines for their HLA class I surface expression with an HLA-A, -B and –C detecting antibody (clone W6/32) via flow cytometry. Four out of six tested MCC cell lines were negative (LoKe) or expressed only low to intermediate levels of HLA class I molecules (MKL-1, BroLi and WaGa) while only two cell lines expressed high levels of total HLA class I molecules on their cell surface (MKL-2 and AlDo; Figure 1F-G).

Reduced HLA class I cell surface expression on MCC cells is associated with down-regulation APM components

To elucidate the mechanism(s) of reduced HLA class I expression in MCC, we analyzed gene expression data from 35 cryopreserved MCC tumors, accessible at the gene expression omnibus (GEO) database (GSE22396), for *HLA-A* and *HLA-C* as well as the light chain β_2 *microglobulin* ($\beta_2 m$). Similar to the expression pattern of HLA-A protein (Fig. 1E) ~25% of tumors (*n=8*) expressed low or intermediate levels of HLA-A mRNA. However the majority (~75%, *n=27*) of tumors contained high amounts of HLA-A specific mRNA (Fig. 2A). *HLA-C* and $\beta_2 m$ mRNAs were also expressed at intermediate to high levels in the majorities of tumors. In Addition we further analyzed the mRNA expression of the APM components *TAP1*, *TAP2*, *LMP2* and *LMP7* in the same data set (GSE22396). To this end, *TAP1* and *TAP2* mRNAs were expressed at very low levels in all analyzed tumors and *LMP2* and *LMP7* mRNAs at low to intermediate levels in ~75% of tumors (*n=27*) (Fig. 2A). To determine whether these results indeed reflect transcript levels in MCC cells and not

cells of the tumor microenvironment, we next analyzed four MCC cell lines for their *HLA-ABC* and β_2*m* as well as the APM component mRNA expression. In line with the expression values obtained *in situ*, *HLA-ABC* and β*2m* mRNAs were expressed at relatively high levels in all MCC cell lines (Fig. 2B; supplementary Fig. 1), irrespective of the MHC class I surface expression (Fig. 1G). But the three HLA class I-low/HLA class I-negative MCC cell lines (BroLi, MKL-1 and WaGa) contained diminished TAP1, TAP2, LMP2 and LMP7 mRNA levels (Fig. 2B). Notably, only MKL-2 i.e. the MCC cell line with a high HLA class I surface expression, contained high levels of APM specific mRNA (Fig. 2B, Supplementary Fig. 1). To determine whether the association of low APM expression and HLA class I surface expression also persists on the protein level we performed immunoblots of total cell lysates with antibodies specific for HLA-A, HLA-B/C, β_2m and the APM components in four MCC cell lines (MKL-2, BroLi, MKL-1 and WaGa) (Fig. 2C). In line with the mRNA expression patterns, HLA-A was expressed in all analyzed MCC cell lines, while β_2m and HLA-BC was expressed in concordance with HLA class I surface expression only in MKL-2 and WaGa. Most important, protein expression of the APM components TAP1 and LMP7 were largely restricted to MKL-2 cells and absent or only weakly expressed in the HLA class I-low/ -negative MCC cell lines (Fig. 2C).

HLA class I surface expression of MCC cells is stabilized by addition of MCPyV derived peptides

Most MCCs express ample amounts of both *HLA* heavy and β_2*m* light chain mRNA whereas the respective expression of APM constituents is scarce. Therefore, the lack of HLA class I surface expression might be due to deficient antigen processing. HLA class I molecules not loaded with a suitable processed peptide are less stable, and such empty HLA complexes are rapidly removed from the cell surface and subsequently degraded (*18*, *19*). Notably, in cells with deficient antigen processing such as the TAP deficient T2 cell line, an excess of externally provided peptide binding to the respective HLA molecules stabilizes HLA class I complexes in an APM independent manner (*20*). Indeed, when incubated in the presence of a mixture of high affinity HLA-A binding peptide epitopes derived from MCPyV large T (LT), small T antigen (sT) and VP1, the MCC cell lines MKL-1, BroLi and WaGa increased their HLA class I surface expression (Fig. 2D,E). All peptides were described before (*7*), and peptides for the cocktail were selected according to the HLA-A type of the respective MCC cell line (Supplementary Tab. S1). A representative example is given for BroLi in figure 2D. Thus, reduced HLA class I cell surface expression in MCC is at least in part due to an impaired transcription of APM components.

Transcription of APM genes is epigenetically silenced in MCC and is re-induced by histone deacteylase (HDAC) inhibition

Expression of APM genes in cancer can be silenced by DNA methylation and/or histone hypoacetylation at the *MHC* gene cluster (*17*). Since treatment of MCC cell lines with high doses (5 μM) of two different methyltransferase inhibitors (5 azacytidine and RG108) had no effect on HLA class I surface expression (Supplementary Fig. 2), we focused on histone hypoacetylation as a potential mechanism of APM silencing. Histone hypoacetylation at the promoter regions of genes is attained by a family of enzymes, so called histone deacetylases (HDACs), and renders the chromatin in a condensed, transcriptionally inactive state (*21*). First, we analyzed data extracted from GSE22396 to establish whether an increased *HDAC* expression was associated with a reduced transcription in the *MHC* locus. Indeed, a high abundance of *HDAC* mRNAs (sum of *HDAC 1-10*) was negatively correlated with the mRNAs specific for *TAP1*, *TAP2*, *LMP2* and *LMP7* (R squared = 0.32, p = 0.0006; Supplementary Fig. S3). Thus, in MCC APM genes appear to be negatively regulated by HDACs. To test this notion, we determined the level of histone acetylation at the *MHC* locus by chromatin immune precipitation (ChIP) with an anti-acetyl-histone H3 lysine 9 (acH3K9) antibody. Acetylation at lysine 9 of histone H3 in promoter regions is a marker for transcriptional activity (*23*). Studying histone acetylation of the HLA-A promoter region, we detected that around 50% of histones were acetylated in untreated WaGa cells (Fig. 3A). In line with the intermediate histone acetylation state, WaGa cells are characterized by an intermediate HLA class I surface expression (Fig. 1 F,G). Similarly, global Histone H3K9 acetylation was very low, i.e. at or below the detection level, in the three MCC cell lines (BroLi, MKL-1, WaGa) with a reduced HLA class I surface expression (Fig. 3B).

Next, we tested if the promoter specific and/or global histone hypoacetylation can be reversed by HDAC inhibition. For this purpose, WaGa cells were subjected to 1.25 μM of the HDAC inhibitor vorinostat (mean plasma concentration observed under current treatment regimens) (*23*), that within 24 hours resulted in an induction of histone acetylation in the *HLA-A* promoter (Fig. 3A). However, with only ~60% of histones in the *HLA-A* promoter being acetylated after treatment, this induction was rather modest. Thus, we combined vorinostat with mithramycin A, a clinically available drug that can synergize with HDAC inhibitors by (i) transcriptionally inhibiting the compensatory inductions of certain HDACs (*24*) and (ii) by preventing the formation of SP1/HDAC inhibitory complexes at the promoters' GC box (*25*).

While mithramycin A alone had no effect on histone acetylation in the *HLA-A* promoter region, the combination of vorinostat and mithramycin A induced a strong H3K9 acetylation in the *HLA-A* promoter region, resulting in acetylation of 100% of histones (Fig. 3A). In line with these results, vorinostat alone increased global H3K9 acetylation levels of MCC cell lines, which could be further enhanced by addition of mithramycin A; however, compared to HLA-A promoter specific histone acetylation the synergistic effect on global H3K9 acetylation was less pronounced (Fig. 3B).

The following experiments addressed if the pharmacological induction of histone acetylation actually leads to an increased transcription of genes in the *MHC* locus. mRNA expression of *HLA-A*, β_2*m* and APM components (*TAP1, TAP2, LMP2, LMP7*) was measured by qRT-PCR before and after HDAC inhibition (Fig. 3C and Supplementary Fig. 4). *HLA-A* and β_2*m* mRNA, already highly expressed in all untreated MCC cell lines (Fig. 2B and Supplementary Fig. 1), were only moderately induced by HDAC inhibition (Fig. 3C and Supplementary Fig. 4). In contrast, expression of APM genes was strongly induced by the treatment with vorinostat in combination with mithramycin A in all HLA class I-low/-negative MCC cell lines (BroLi, MKL-1, WaGa) with initially low APM mRNA and HLA class I surface expression. Notably, the induction of the APM genes was up to 100 fold, relative to the expression of the respective untreated cell line and is therefor depicted on a logarithmic (log10) scale. For MKL-2 it is important to note that this cell line is characterized by already strong expression of the APM genes in untreated cells (Fig. 3C and Supplementary Fig. 4). However, the effect of HDAC inhibition on the protein expression of APM constituents was not ascertainable as the amounts of TAP1 and LMP7 remained below detection level (Fig. 3D). Interestingly, though, HDAC inhibition by vorinostat plus mithramycin A had a marked enhancing effect on HLA-ABC and β_2m protein expression in general as well as with respect to cell surface expression (Fig. 3D,E,F). These findings suggest that HDAC inhibition post-transcriptionally contributes to the stability of HLA molecules and surface expression of HLA class I complexes in MCC cells.

HDAC inhibition induced susceptibility of MCC cells to LAK cell mediated cytotoxicity is blocked by anti-HLA-ABC antibodies

To assess the functional significance of the restoration of HLA class I antigen expression on MCC cells treated with HDAC inhibitors, we tested the recognition and susceptibility of treated cells to lysis by cytotoxic cells. For these experiments we had to rely on lymphokine activated killer cells (LAK cells). LAK cell mediated MCC cell lysis was measured via a flow cytometry based cytotoxicity assay. The gating

strategy to differentiate dead from living target cells is exemplarily depicted in Fig. 4A for untreated BroLi cells at an effector to target ratio of 40:1. The majority of LAK cells (on average over 80 %) utilized for these experiments were characterized as $CD3^+/CD56^-$ (T cell type) while only around ~8% were $CD3^-/CD56^+$ (NK cell type) or $CD3^+/CD56^+$ (NKT cell type; Supplementary Fig. 5) suggesting that the main effector cells in this heterogeneous population are T cell derived LAK cells (*26*).

Upon HDAC inhibition by vorinostat in combination with mithramycin A, LAK cell mediated cytotoxicity against MKL-2 and BroLi cells was increased as compared to untreated cells (Fig. 4B). Blocking experiments using saturating amounts of an anti-HLA class I antibody confirmed that increased lysis of MCC cells was indeed dependent on the cell surface expression of HLA class I molecules. Hence, HDAC inhibition mediated induction of HLA class I molecules is responsible for the augmented sensitivity of treated MCC cells towards LAK cell mediated lysis.

HDAC inhibition induces histone acetylation and HLA class I stabilization *in vivo*

A recently established MCC xenotransplantation model (*27*) was utilized to translate our *in vitro* observations into an *in vivo* preclinical setting. MCC tumors were induced by s.c. injection of WaGa cells in NOD/SCID mice. After the tumors reached a volume of approximately 100 mm^3, treatment with i.p. injections of the drug carrier polyethylene glycol or vorinostat in combination with mithramycin A was started. Mice were treated for two weeks at concentrations equivalent to those applied in humans. 48h after the last dosage animals were sacrificed, and the xenotransplants subjected to detailed characterization. Immunohistochemistry of these xenotransplants demonstrated that tumors treated with placebo exhibited hypoacetylated histones while histone H3K9 acetylation was induced in tumors of mice *in vivo* after treatment with vorinostat in combination with mithramycin A (Fig. 5A). Real time PCR revealed no increase in *HLA-ABC* and β_2m mRNA expression in xenotransplants after treatment with vorinostat plus mithramycin A, supporting our assumption that HDAC inhibitor mediated HLA class I induction is not dependent on the induction of *HLA-ABC* and β_2m transcription. (Supplementary Fig. 6). This treatment, however; significantly increased the transcription of *LMP2* and *LMP7* in xenotransplanted MCC tumors *in vivo* in comparison to the placebo group ($p < 0.05$; Fig. 5B); the induction of *TAP2* mRNA was less pronounced and the expression of *TAP1* was not further increased. The latter is explained by the fact that *TAP1* expression was already strongly induced after xenotransplantation as compared to *in vitro* cultured WaGa cells (Supplementary Fig. 7), and thus could not be further increased by treatment

with vorinostat and mithramycin A. Finally, we determined the HLA-A protein expression in these xenotransplants. In accordance with our *in vitro* observations, treatment with vorinostat in combination with mithramycin A markedly increased HLA-A protein expression in tumors (Fig. 5C).

Discussion

Virally associated malignancies like Merkel cell carcinoma are highly immunogenic and are consequently subjected to a particularly harsh immunoselective pressure. Thus, these cancers can only progress if efficient immune escape strategies are operative. Indeed, although most MCC patients harbor $CD8^+$ T cells specific to virus derived epitopes (*6*, *7*), these T cells only rarely infiltrate into MCC tumors (*9*). MCC has a poor prognosis in general and among those presenting with disease in the lymph nodes, over 50% of patients will succumb to this disease (*1*). Notably, absence of a T-cell infiltrate further diminishes the prognosis (*8*, *9*). Still, due to the exceptional immunogenicity of MCC tumors, immunotherapy is regarded as a promising therapy option for MCC patients and is therefore currently tested in several clinical trials including: (i) autologous MCPyV specific $CD8^+$ T cell transfer (NCT01758458), (ii) CTLA-4 blocking antibody ipilimumab (NCT02196961), (iii) PD-L1 blocking antibody MSB0010718C (NCT02155647), or cytokine based therapies using (iv) tumor-stroma targeting antibody-IL2 fusion proteins (NCT02054884). All of these approaches aim at activating MCC-specific cytotoxic T cells, i.e. all crucially depend on recognition of tumor cells by cytotoxic T cells. T-cell mediated lysis of tumor cells is initiated by cognate interaction of the T-cell receptor with suitable peptide/HLA class I complexes presented on the surface of MCC tumor cells. Indeed, $CD8^+$ T cell infiltration in various tumor types critically depends on an ongoing, i.e. an intact, antigen processing and HLA class I surface expression (*15*, *28*). The molecular mechanisms resulting in HLA class I loss or down-regulation vary widely depending both on the type of tumor as well as on the individual patient (*29*). This is not surprising since impaired HLA class I expression in cancer is mainly due to selective pressure caused by ongoing immune responses (*30*, *31*). To this end, in line with a recent report by Paulson *et al.* we demonstrate, that HLA class I expression is frequently lost or down-regulated in MCC tumors *in situ* and in MCC cell lines *in vitro* (*13*). More importantly, we scrutinized the underlying molecular mechanisms of HLA class I deregulation in MCC, revealing that this immune escape mechanism is mediated by epigenetic silencing of the antigen presentation machinery (APM) via histone de-acetylation. In detail, the transporter subunits TAP1

and TAP2 as well as immunoproteasome subunits LMP2 and LMP7 are simultaneously down-regulated in the majority of MCC tumors *in situ* and MCC cell lines *in vitro*. While defects in a single component of the APM can result in a decreased HLA class I expression in several malignancies (*15*), the synchronized down-regulation of APM components is an even more efficient way to inhibit HLA class I surface expression (*31*). Viral infection of cells is frequently associated with a strong inflammatory response; thus, infected cells are exposed to cytokines such as interferon gamma, which results in the replacement of certain catalytic subunits of the proteasome by LMP2 and LMP7 and thereby the formation of the immunoproteasome (*32*). The immunoproteasome prevents random cleavage of proteins and favors cleavage with specific patterns, generating a broader spectrum of peptides that can be presented on HLA class I complexes (*33*). Notably, viral proteins that are only poorly processed by the regular proteasome are more efficiently processed by the immunoproteasome (*34*). Thus, epigenetic silencing of immunoproteasome subunits LMP2 and LMP7 appears to be particularly suitable means of immune evasion for cancers with a viral carcinogenesis such as MCC.

Despite the fact that epigenetic silencing of gene expression via histone hypoacetylation is stable and efficient, it can be effectively targeted and reversed pharmacologically (*35*). Indeed, treatment of MCC cells with the HDAC inhibitor vorinostat in combination with the Sp1 inhibitor mithramycin A (MA) efficiently re-induced the transcription of the APM components and subsequently restored HLA class I surface expression in MCC *in vitro*; notably, we observed this effect already at clinically relevant concentrations (*23*). It has been reported before that genes of the HLA class I locus are re-inducible by pharmacological inhibition of HDACs (*36*-*38*). We could further extend these findings by translating them into a pre-clinical *in vivo* setting. Moreover, it has been demonstrated that HDACi act as immune sensitizers for melanoma cell lines resistant to cytotoxic T cells (*39*, *40*); this effect was at least in part due to an increased HLA class I expression (*40*). In line with these reports, we observed that HDAC inhibition renders MCC cells more sensitive to HLA class I dependent immune recognition. For these experiments, we had to rely on LAK cells as effector cells because of the lack of MCC-reactive T cell lines. Nevertheless, LAK cells appear to be a valid choice as they constitute a clinically applicable heterogeneous population of activated cytotoxic immune cells (*41*). Moreover, we are currently conducting a clinical trial based on the antibody targeted delivery of Interleukin 2 (IL2) to the MCC tumor microenvironment (www.immomec.com). However, it appears that increased expression of HLA class I is not the sole effect of HDAC inhibition induced susceptibility of MCC cells towards cellular cytotoxicity:

While LAK cell mediated lysis of untreated MKL-2 cells was very low, after HDAC inhibition MKL-2 cells were effectively lysed. This was a bit unexpected as MKL-2 are characterized by high HLA class I expression, which was not further increased after HDAC inhibition. Increased susceptibility to cellular cytotoxicity in MKL-2 by HDAC inhibition may be due to the induction of other immune stimulatory ligands (42) and/or a change the composition of the peptidome on the cell surface by altering the expression of APM components (*43*). Notably, for MCC there is no strong correlation between the level of HLA class I expression and immune cell infiltration(data not shown and (*13*) ; this notion further supports the hypothesis that not only the number of HLA class I complexes, but also the presented peptidome as well as other factors, such as expression of immune regulatory molecules or characteristics of the microenvironment might be of importance for immune recognition of MCC cells. We and others have previously demonstrated that interferons are potent inducers of HLA class I surface expression in MCC *in vitro* and *in vivo* (*13,7*), but there are substantial advantages of the use of HDAC inhibitors for this purpose. While HDAC inhibitors have been reported to induce the expression of activating and co-activating molecules, like NKG2D ligands (*42*), interferons induce inhibitory ligands like PD-L1 on tumor cells (*44*). Similarly, interferons can paradoxically promote immune evasion of virally induced cancers by inhibiting the expression of tumor antigens (*45*). Indeed, we have recently reported that interferons reduce the expression of MCPyV large T antigen (*27*), i.e. the source from which most of the immune dominant T cell epitopes are derived (*7*).

Taken together, we believe that immunotherapeutic protocols (especially those employing the action of tumor-specific cytotoxic T-cells), will need to be individualized based on the HLA class I expression status of the respective tumors. In this regard “epigenetic priming” by HDAC inhibition appears to be an attractive intervention since it is an efficient and well tolerated means to re-induce APM components, in particular the immunoproteasome and thereby peptide epitope processing, HLA class I expression, and antigen presentation on MCC tumors.

Material and Methods:

Patients

A total of 56 archived formalin-fixed and paraffin-embedded MCC tumor samples from 40 MCC patients were utilized from the Department of Dermatology, Medical University of Graz. All tumors were histologically confirmed Merkel cell carcinoma and had been excised for therapeutic reasons from 1992 to 2013. Specimens included primary lesions, nodal metastases, recurrences and skin metastases.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed and paraffin-embedded (FFPE) tissue using the Autostainer Link 48 (Dako, Glostrup, Denmark). After deparaffinization in xylene, sections were rehydrated with 100%, 96%, 70%, and 50% ethanol for 5 minutes each, and finally rinsed with demineralized water. Antigens were retrieved with a citrate buffer (Dako retrival solution, cat. no. S1699, pH 6) in a steamer at 100°C for 30 minutes. After cooling for 20 minutes and two additional washing steps, sections were blocked with peroxidase blocking solution (Dako) followed by incubation for 1 hour with an HLA-A specific antibody (clone EP1395Y, Abcam) diluted 1:1000 in antibody diluent (Dako). After washing steps, incubation with a biotinylated secondary antibody, further washing steps and addition of streptavidin peroxidase, the detection was obtained using ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. After counterstaining of nuclei with haematoxylin (Dako), sections were dehydrated and mounted in Tissue Tek glass mounting medium (Sakura Finetek, Torrance, CA, USA). Three independent investigators (CR, DS, JCB) classified the staining intensity on a scale from zero to three and the percentage of HLA-A positive cells as follows: 0%=0, 1%-25%=1, 26%-50%=2, 51%-75%=3, >75%=4. The HLA-A staining score was calculated by multiplying the score for staining intensity and percentage of positive cells (min=0, max=12).

Cell culture

The MCC cell lines LoKe, BroLi, MKL-1, WaGa, MKL-2, AlDo have been described previously (*46*). All cell lines were maintained in RPMI-1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Biochrome, Berlin, Germany). For the cell line AlDo the medium was additionally supplemented with 30% fibroblast conditioned medium. For treatment with specific inhibitors, cells were cultured at a

concentration of $1x10^6$ cells/ml in 6-well plates. Inhibitors were dissolved according to the manufacturers' guidelines and used at 1.25 µM for vorinostat (Selleckchem, Munich, Germany), 0.3 µM for mithramycin A (Sigma), 5 µM for RG108 and 5-Azacytidine (Selleckchem) for 24 hours if not otherwise stated.

Quantitative real time-PCR

RNA of *in vitro* propagated cells or cryopreserved xenotransplants was isolated using PeqGOLD total RNA Kit (Peqlab, Erlangen, Germany) and transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Quantitative real time polymerase chain reactions (qRT-PCR) were performed using SYBR green or TaqMan PCR master mix (Sigma) on the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). *RPLP0* served as endogenous control, and was detected with the sense-primer: 5'-CCA TCA GCA CCA CAG CCT TA-3', the antisense-primer: 5'-GGC GAC CTG GAA GTC CAA CT, and the probe ATC TGC TGC ATC TGC TTG GAG CCC A-3'. The mRNA expression of *HLA-ABC*, *B2M*, *TAP1*, *TAP2*, *LMP2*, *LMP7* was detected using SYBR green assays with specific primers listed in supplementary table S1. Relative quantification was calculated by the ΔΔCt method using the respective untreated control or as indicated.

Immunoblot

Cell lysates were generated by lysing $3x10^6$ cells per sample in protein extraction buffer supplemented with a proteinase inhibitor cocktail as described before (*47*). Lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page), protein samples were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), blocked for 1 hour in blocking buffer, and then incubated overnight at 4°C with primary antibodies diluted in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBST) according to data sheet: anti-HLA-A (clone EP1395Y) 1:4000, anti-HLA-B/C (clone HC10) 1:1000, anti-acetyl-histone H3Lys9 (clone C5B11), anti-β-Tubulin (Sigma) 1:200 in PBST, anti-B2M, anti-TAP1 and anti-LMP7 each 1:500 in PBST. Antibodies have been described before (*48*) . After membranes were washed trice for 10 minutes in tris buffered saline and tween (TBST), they were incubated for 1 hour with the appropriate peroxidase-coupled secondary antibodies (Dako), followed by visualization using the ECL Western Blotting Substrate (Pierce).

Flow Cytometry

Cell surface expression of HLA class I was determined by flow cytometry. $1x10^6$ cells were washed with ice cold PBS and incubated with the FITC linked anti-HLA-ABC antibody (clone W6/32, Biolegend, San Diego, CA, USA) in PBS with 0.1% bovine serum albumin (BSA) for 90 minutes at 4°C in the dark. After a washing step, cells were stained with 10 µg/ml 7-aminoactinomycin (7AAD, Sigma) to allow viable/non-viable discrimination, and measured on a FC500 Flow Cytometer (Beckman Coulter, Brea, CA, USA). Flow cytometry data were analyzed with FlowJo Version 8.7 software (TreeStar, Sunnyvale, CA, USA).

HLA class I stabilization assay

MCC cell lines were incubated with 10 µM of a MCPyV LT, sT and VP1 derived peptide mixture (supplementary table S2; *9*) specific for their respective HLA-A subtype or an irrelevant peptide in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin until the maximal HLA class I stabilization was observed i.e. 24 hours for WaGa and MKL-1 and 48 hours for BroLi. Subsequently, cells were stained with an anti-HLA-ABC antibody (clone W6/32, Biolegend) and analyzed for their HLA-ABC surface expression via flow cytometry.

Chromatin immune precipitation (ChIP)

ChIP assays were performed using the SimpleChiP® Enzymatic Chromatin IP Kit with Agarose beads (Cell Signaling). In brief, proteins were cross-linked to DNA with 1.5 % formaldehyde for 10 minutes. Nuclear membranes were broken up using the UP50H Sonicator (Hielscher, Teltow, Germany) set to 100%, 0.9 output for 20 seconds, 6 times in a row with incubation on ice for 30 seconds between sonication pulses. Subsequently, antibodies against Histone H3 (cat. no. 6420), Acetyl-Histone H3Lys9 (AcH3K9, cat. no. 9671) and rabbit IgG control (Cell Signaling) were used for immunoprecipitation. The immunoprecipitated DNA was then analyzed by real time PCR using primers specific to the *HLA-A* promoter region in a SYBR green assay. Primers: *HLA-A* promoter-sense: 5'-CAC AGR AGC AGA GGG GTCA G-3', anti-sense 5'-AAA CTG CGG AGT TGG GGA AT-3'. The percentage of acetylated histones (AcH3K9) was normalized to total Histones H3 and calculated using the following equation:

% AcH3K9 over total H3 = $100*2\hat{}(C_T$ H3- C_T AcH3K9)

Flow cytometry based cytotoxicity assay

Peripheral blood mononuclear cells were isolated via gradient centrifugation with Lymphoprep™ (Stemcell Technologies, Vancouver, BC, Canada), and cultured for 3 days in CellGro®SCGM (CellGenix, Freiburg, Germany) supplemented with 10% FBS (Biochrom) and 500 IU interleukin 2 (IL2) per ml (Miltenyi Biotec, Bergisch Gladbach, Germany) to generate lymphokine-activated killer (LAK) cells. MCC cell lines served as target cells either without or with inhibitor (vorinostat, mithramycin A, or combination thereof) treatment for 12 hours at the concentrations described above. The shortened incubation time was chosen to assure that cells are indeed vital and target cell membranes are fully intact for the cytotoxicity assay. After target cells were washed 3 times in RPMI with 10% FBS, they were labeled by incubation in 3 µM CFSE (Sigma) in pre-warmed RPMI with 10% FBS for 10 minutes at 37°C, followed by another round of 3 washing steps to remove any excessive CFSE. $2x10^4$ CFSE labeled target cells were incubated alone to establish spontaneous cell death, or co-incubated at varying effector:target ratios, i.e. 40:1, 20:1, 10:1, for 4 hours at 37°C in a total volume of 100 µl. Prior to flow cytometry, cells were incubated in 10 µg/ml 7AAD (Sigma). Dead target cells were defined as $CFSE^+/7AAD^+$, and the percentage of cytotoxicity was calculated as following:

% cytotoxicity = (% dead target cells experimental - % dead target cells spontaneous)/(100-% dead target cells spontaneous)*100

For blocking experiments target cells were incubated with saturating concentrations of blocking antibodies against HLA-ABC (clone W6/32; Biolegend) or isotype control for 2 hours at 37°C prior to incubation with LAK cells. Pre incubation with F(ab')2 fragments for 30 minutes (Life Technologies) was performed to avoid Fc-receptor mediated antibody-dependent cell-mediated cytotoxicity.

Xenotransplantation experiments

Six-week-old female NOD.CB17/Prkdc^{scid} mice were obtained from Charles River Laboratories (Erkrath, Germany) and housed under specific pathogen-free conditions. Tumors were induced by s.c. injection as described before (*27*). Twenty-four days after tumor cell inoculation, when the tumors reached a volume of approximately 100 mm^3, treatment was started. Mice were divided into four groups of six mice each ensuring an equal overall tumor burden. Immediately before injection a 1 M (264.3 mg/ml) vorinostat stock solution was diluted in 45% Polyethylene glycol

(PEG-400, Sigma) to 10 mg/ml and 60 mg/kg bodyweight were administered intraperitoneally (i.p.) per mouse. For mithramycin A, a 5 mg/ml stock solution was diluted in water to 0.33 mg/ml and 0.2 mg/kg bodyweight were injected i.p. per mouse. The placebo group received the same volume of the respective solvents. Mice were treated five consecutive days per week for a total of two weeks. Thereafter, the tumor tissue was excised, formalin fixed and paraffin embedded for IHC or cryo-preserved for RNA isolation.

Statistics

Statistical analyses were performed using Graphpad Prism 6.0 Software (Graphpad Software Inc., San Diego, CA, USA). Cell culture experiments were analyzed using Friedman test, a paired non-parametric ANOVA. A p-value smaller than 0.05 was considered significant and indicated by * in the figures.

Study approval

All studies on human material were approved by the institutional review board of the Medical University of Graz (24-295 ex 11/12). All animal studies were approved by the Austrian ministry of education and science according to the regulations for animal experimentation (BMWF-66.010/0151-II/3b/2012).

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Author contributions

CR, AP, DS and JCB designed the study, wrote the manuscript and evaluated the immunohistochemistry stainings; CR and KF performed the experiments and analyzed the data. SRH provided the MCPyV derived peptides. SF provided AMP component specific antibodies. All authors reviewed the final manuscript.

The authors have declared that no conflict of interest exists.

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Figures

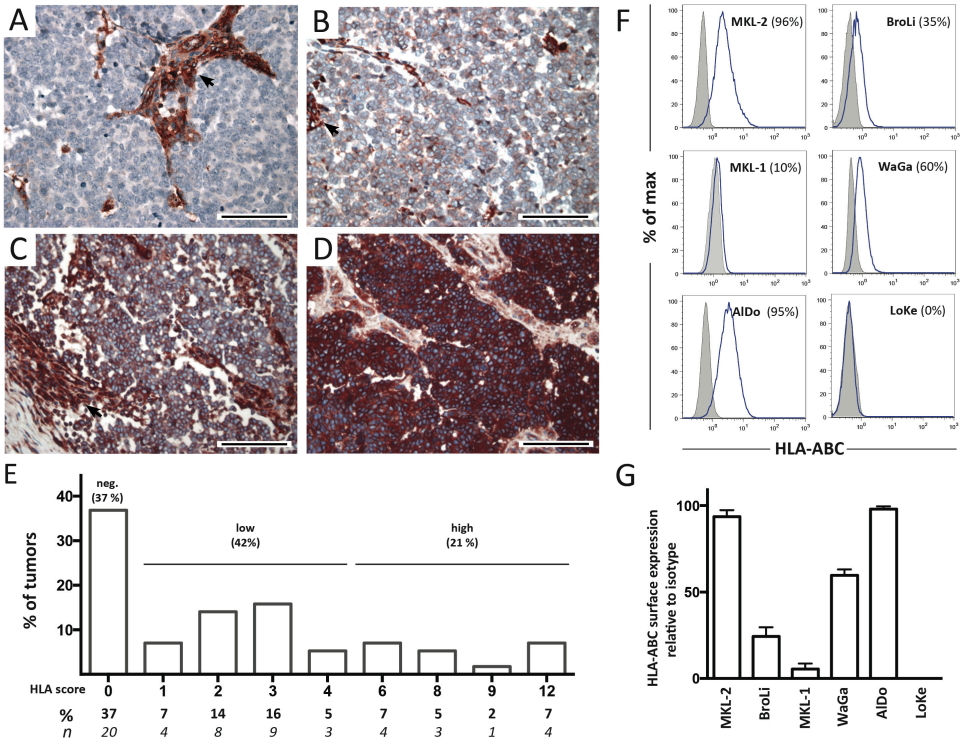

Fig. 1: HLA class I expression by MCC tumors *in situ* and MCC cell lines *in vitro.*

(A-D) 56 MCC tumor samples of 40 patients were analyzed by immunohistochemistry for the expression of HLA-A. Tumors were classified by their staining intensity and one representative tumor for each staining intensity (0-3) is depicted; (A=0, B=1, C=2, D=3). Arrows indicate stromal cells as an internal positive control; scale bar represents 100 µm. **(E)** Samples were scored from 0 to 3 for staining intensity and from 0 to 4 for the frequency of positive tumor cells; a combined HLA-A expression score was calculated by multiplying both scores. **(F)** Six MCPyV^{+} MCC cell lines were analyzed for their HLA class I expression *in vitro* by flow cytometry using an HLA-ABC detecting antibody (clone W6/32, blue line); matched isotype controls are depicted as grey filled histogram. One representative histograms is shown for each cell line. **(G)** Percent of HLA class I expression normalized to isotype control was calculated in three independent experiments, and the results are depicted as mean + SEM for all analyzed cell lines.

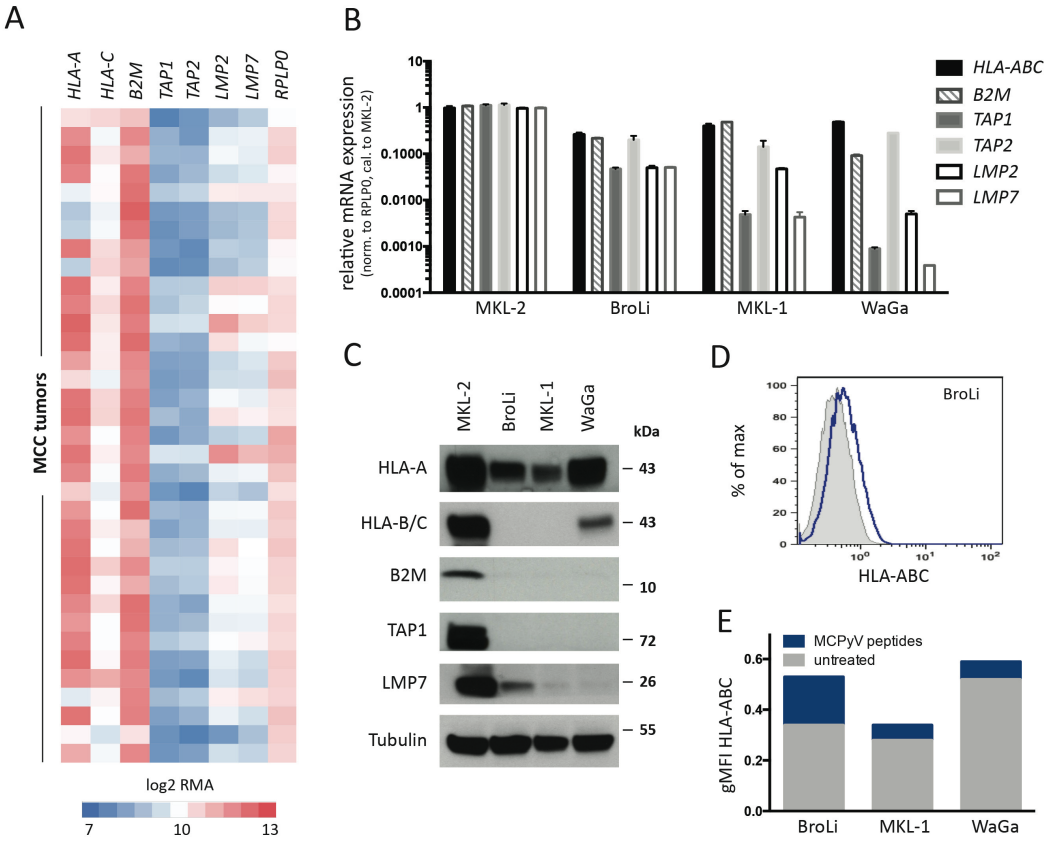

Fig. 2: Reduced HLA class I expression in MCC is associated with an impaired antigen processing machinery (APM).

(A) RMA normalized expression values of gene expression array GSE22396, were obtained from the GEO database. RMA values were log2 transformed and are depicted as heat map with expression values ranging from 4 (blue = low expression) to 14 (red = high expression). *HLA-A, HLA-C, B2M, TAP1*, *TAP2*, *LMP2* and *LMP7* mRNA expression is shown in comparison to RPLP0. **(B)** mRNA expression of *HLA-ABC, B2M, TAP1*, *TAP2*, *LMP2* and *LMP7* in 4 MCC cell lines was determined by qRT-PCR in triplicates using specific primers; C_T values were normalized to *RPLP0* and calibrated to a set of ΔC_Ts of MKL-2; relative mRNA expression is depicted as mean + SEM. **(C)** Protein expression in 4 MCC cell lines was determined by immunoblot of whole cell lysates using antibodies specific for HLA-ABC, B2M, TAP1, and LMP7; β-tubulin served as loading control. **(D,E)** MCC cell lines with low (BroLi, MKL-1) and intermediate (WaGa) HLA class I surface expression were incubated with saturating amounts (10 µM) of MCPyV encoded large and small T antigen and VP1 derived epitopes binding with high affinity to the respective HLA-A molecules or an irrelevant peptide cocktail for at least 24h (WaGa and MKL-1) or 48h (BroLi). HLA class I surface expression was determined by flow cytometry using an HLA-ABC detecting antibody. Induction of HLA class I surface expression after stabilization with the specific (blue line) or an irrelevant peptide cocktail (grey filled) is depicted exemplarily for BroLi cells **(D)**. Comparison of HLA class I surface expression is depicted as geometric mean fluorescence intensity (gMFI) after incubation with MCPyV derived high affinity (blue line) or an irrelevant peptide control (grey filled) for all analyzed MCC cell lines **(E)**.

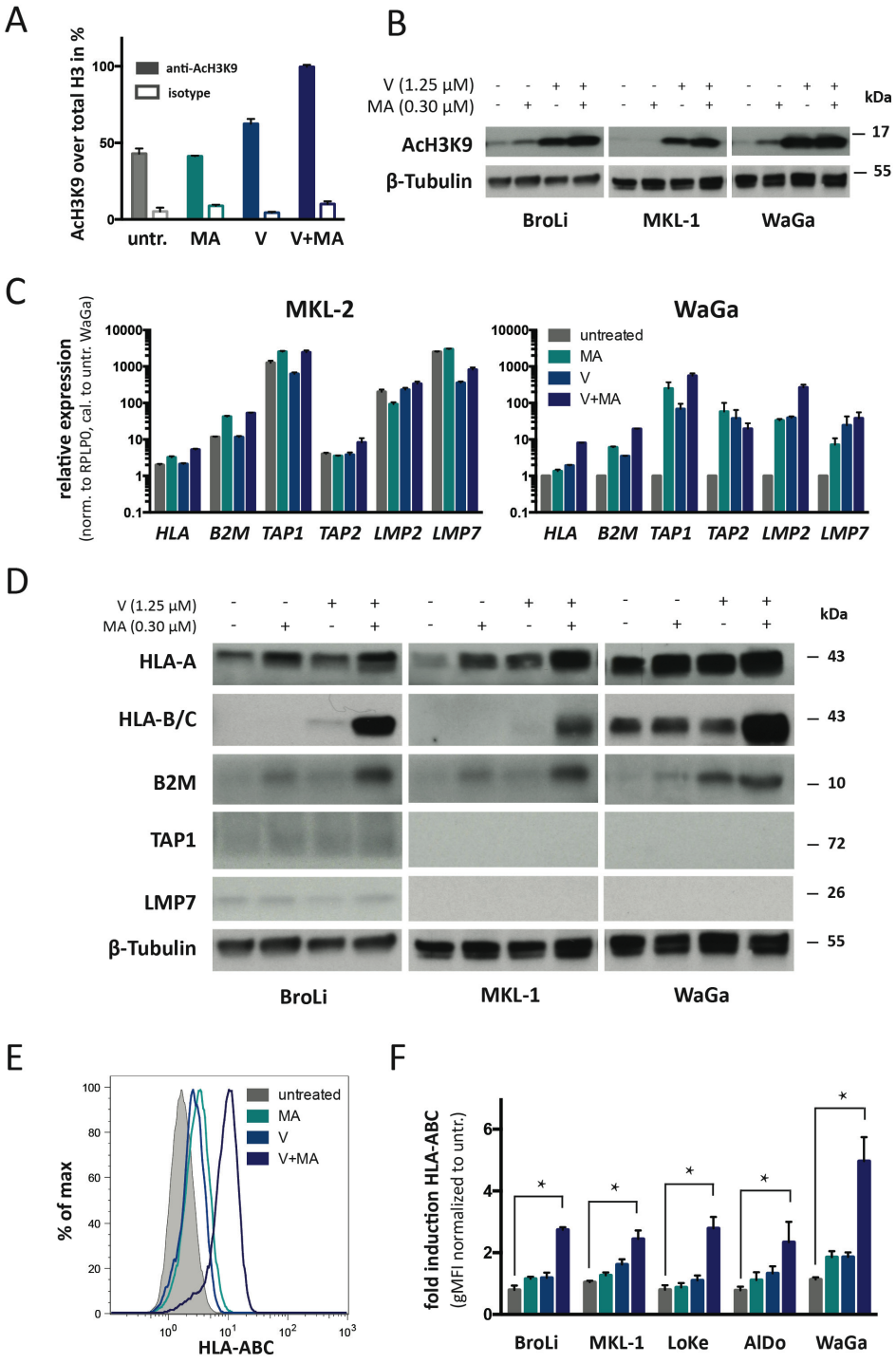

Fig. 3: Reduced expression of antigen processing machinery genes is mediated by histone hypoacetylation and is increased by pharmacologic histone deactylase inhibition.

For all experiments the indicated MCC cell lines were analyzed without treatment (grey) and after treatment with mithramycin A (MA, turquoise), vorinostat (V, light blue), or the combination thereof (V+MA, dark blue). **(A)** Chromatin immunoprecipitation (ChIP) of differentially treated WaGa cells was followed by qRT-

PCR using *HLA-A* promoter specific primers. C_T values of anti-acetyl-H3K9 (AcH3K9) antibody or rabbit IgG isotype control precipitated DNA were normalized to total histone H3 antibody as described in materials and methods. Open bars represent the percentage of acetylated H3K9, filled bars the respective control. Experiments were performed in duplicates and results are expressed as mean + SEM. (B) Global H3K9 acetylation of variably treated BroLi, MKL-1 and WaGa cells was determined by immunoblot with the same AcH3K9 antibody used in the ChIP assay; β-tubulin served as loading control. **(C)** mRNA expression of *HLA-ABC*, *TAP1*, *TAP2*, *LMP2*, *LMP7* and *B2M* was determined by qRT-PCR in triplicates; C_T values were normalized to *RPLP0* and calibrated to the ΔC_T value of untreated WaGa cells; relative mRNA expression is depicted on a logarithmic scale (log 10) + SEM for WaGa and MKL-2. **(D)** Protein expression of whole cell lysates was determined by immunoblot using antibodies specific for HLA-ABC, B2M, TAP1, and LMP7; β-tubulin served as loading control. **(E, F)** HLA class I cell surface expression was determined by flow cytometry using a HLA-ABC specific antibody (clone W6/32) as exemplified for WaGa **(E);** the results for all cell lines analyzed are depicted as the geometric mean fluorescence intensity (gMFI) of HLA class I (HLA-ABC) staining, normalized to the respective untreated cell lines + SEM in three independent experiments **(F)**. Statistical analysis was performed using the Friedman test as indicated.

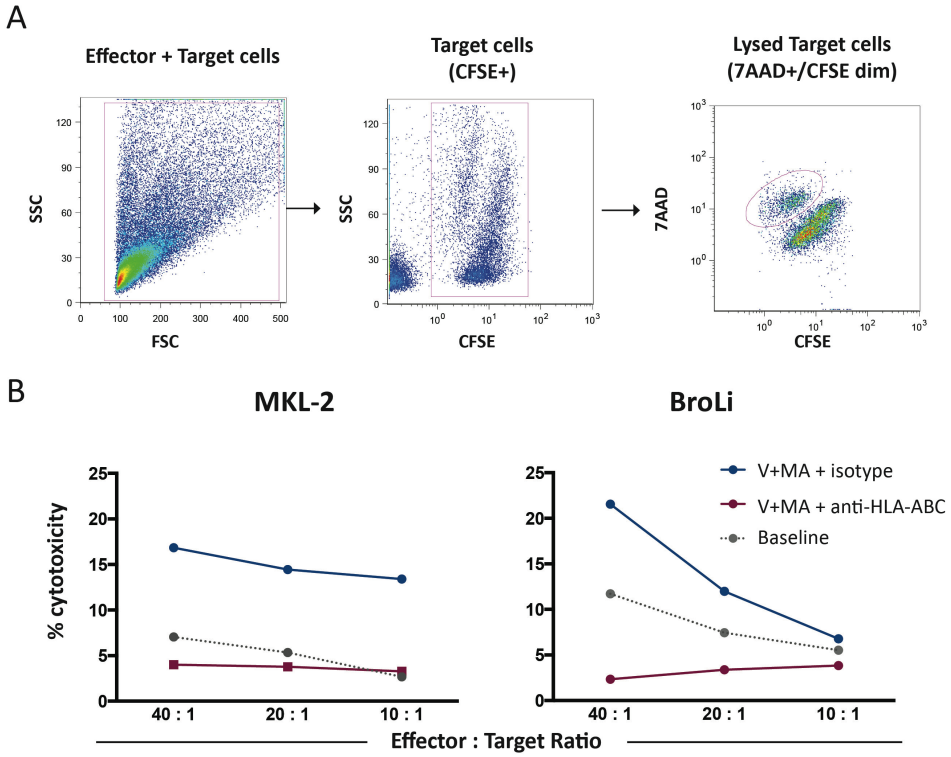

Fig. 4: Histone deactylase inhibition increases LAK cell mediated lysis of MCC cell lines, which is inhibited by a HLA class I blocking antibody.
A flow cytometry based cytotoxicity assay was performed as described in material and methods. **(A)** The gating strategy is illustrated with BroLi cells as targets at an effector to target ratio of 40:1. Target cells were gated as CFSE positive cells in an FSC/CFSE plot, lysed target cells were defined as 7AAD/CFSE double positive cells and are quantified as percentage of all target cells. **(B)** MKL-2 and BroLi cells were treated for 12 h with vorinostat and mithramycin A, and after two washes cells were co-incubated with LAK cells at the indicated effector to target ratios for 4h in the presence of saturating amounts of an isotype control antibody (blue) or a HLA-ABC blocking antibody (red); Fc-receptors were blocked in either case with F(ab)2 fragments. The baseline lysis of untreated MCC cells by LAK cells is depicted as grey dotted line.

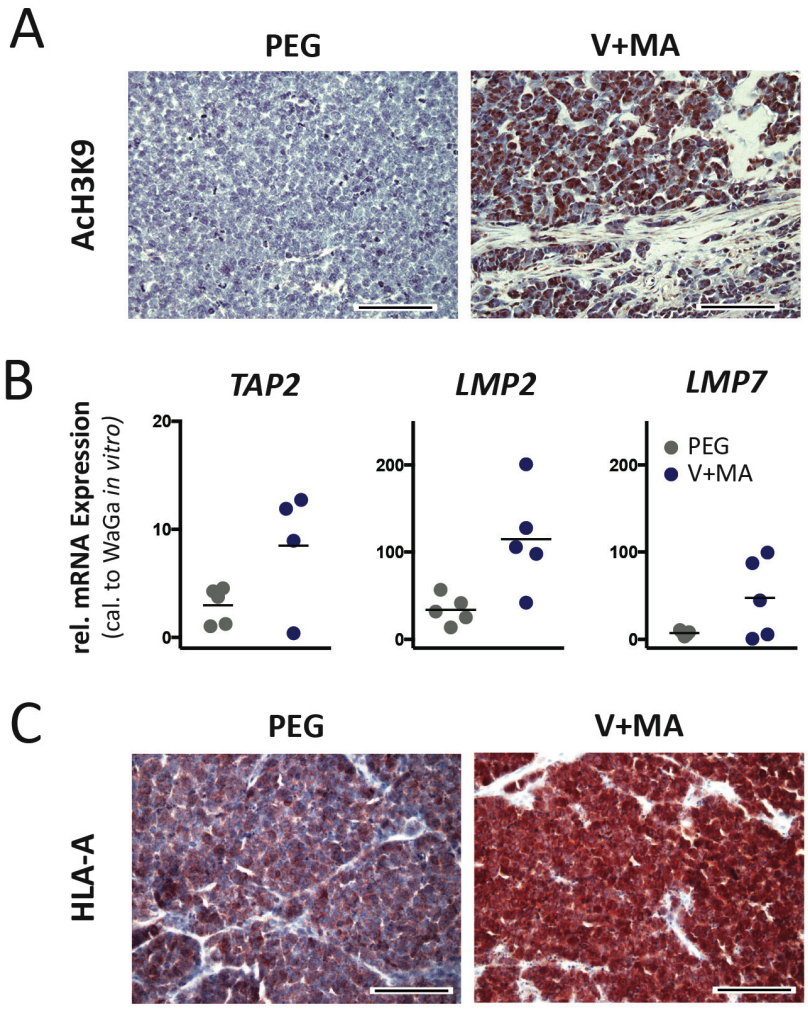

Fig. 5: Histone deactylase inhibition induces histone H3K9 acetylation and HLA class I expression *in vivo*.
NOD.CB17/*Prkdc*^{scid} mice (*n=6* for each treatment group) bearing xenotransplanted WaGa tumors were treated with the drug carrier polyethylene glycol (PEG) (grey), or the combination of vorinostat and mithrmaycin A (V+MA, dark blue) as described in materials and methods. **(A)** Immunohistochemistry was performed on sections of FFPE fixed tumors using antibodies specific against AcH3K9. A representative example for each group is depicted. **(B)** mRNA was isolated from cryopreserved tumors and qRT-PCR was performed using primers specific for the APM components *TAP2*, *LMP2* and *LMP7*. C_T values were normalized to *RPLP0* and calibrated to *in vitro* cultured WaGa cells. **(C)** Immunohistochemistry was performed on sections of FFPE fixed tumors using an antibody specific against HLA-ABC. A representative example for each group is depicted. Scale bars represent 100 µm.

Supplementary data

Table S1:

	forward	reverse
HLA-ABC	GCGGCTACTACAACCAGAGC	GATGTAATCCTTGCCGTCGT
B2M	TCTCTGCTGGATGACGTGAG	TAGCTGTGCTCGCGCTACT
TAP1	TCAGGGCTTTCGTACAGGAG	TCCGGAAACCGTGTGTACTT
TAP2	ACTGCATCCTGGATCTCCC	TCGACTCACCCTCCTTTCTC
LMP2 (PSMB9)	TCAAACACTCGGTTCACCAC	GGAGAAGTCCACACCGGG
LMP7 (PSMB8)	CATGGGCCATCTCAATCTG	TCTCCAGAGCTCGCTTTACC

Table S2:

Cell line	HLA-A type	LT peptide	sT peptide	VP1 peptide
BroLi	A11/A2*	ASFTSTPPK	IMMELNTLWSK	ASVPKLLVK
MKL-1	A3	ASFTSTPPK	IMMELNTLWSK	KMALHGLPR
WaGa	A1/A2	P**V**IMME<u>L</u>N**T**L	KTLEETDYCLL	ALHGLPRYFNV

Supplementary figures

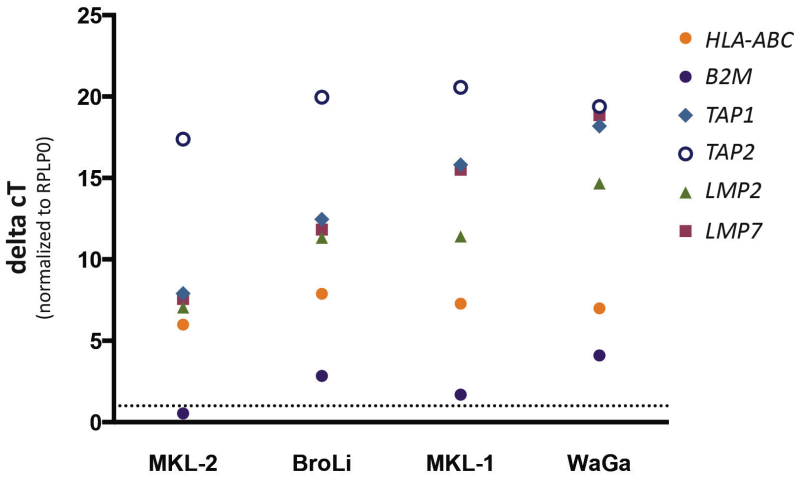

Fig. S1: *HLA-ABC* and *B2M* mRNA is expressed in high abundance, whereas antigen processing machinery (APM) genes are variably expressed in MCC cell lines.

HLA-ABC (yellow dot), *B2M* (purple dot), *TAP1* (blue diamond), *TAP2* (blue circle), *LMP2* (green triangle) and *LMP7* (red square) mRNA expression in the MCC cell lines MKL-2, BroLi, MKL-1 and WaGa was determined by qRT-PCR using specific primer sets. Depicted are ΔC_T values ($C_{T;\ target\ gene}$ - $C_{T;\ RPLP0}$), which correlate inversely with expression.

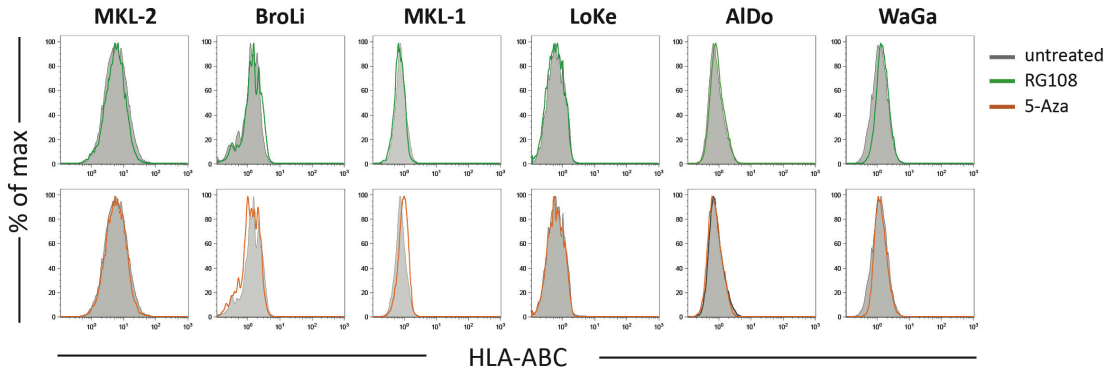

Fig. S2: Methyltransferase inhibitors have no effect on HLA class I expression in MCC.

MCC cell lines were left untreated (grey filled) or treated with 5 µM of the methyltransferase inhibitors RG108 (green line) or 5-azacytidine (orange line) for 24h. HLA class I surface expression was determined by flow cytometry using an HLA-ABC specific antibody (clone W6/32).

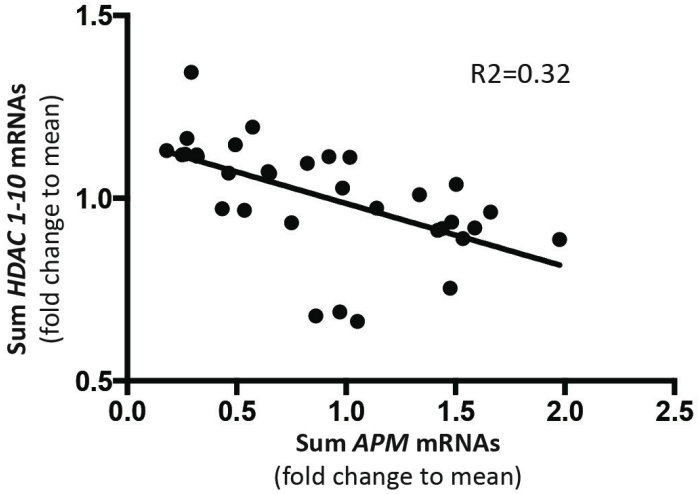

Fig. S3: Histone deactylase (HDAC) mRNA expression is inversely correlated with expression of antigen processing machinery (APM) genes.
RMA normalized mRNA expression values of class I and class II HDACs (*HDAC1-10*) and genes of the antigen processing machinery (APM), i.e. *TAP1*, *TAP2*, *LMP2*, *LMP7,* were extracted from a gene expression array in the GEO database (GSE22396). Fold change expression to mean was calculated for each individual sample and gene. The sum of relative expression of *HDAC* genes was correlated with the sum of genes of APM; R square = 0.32, p = 0.0006.

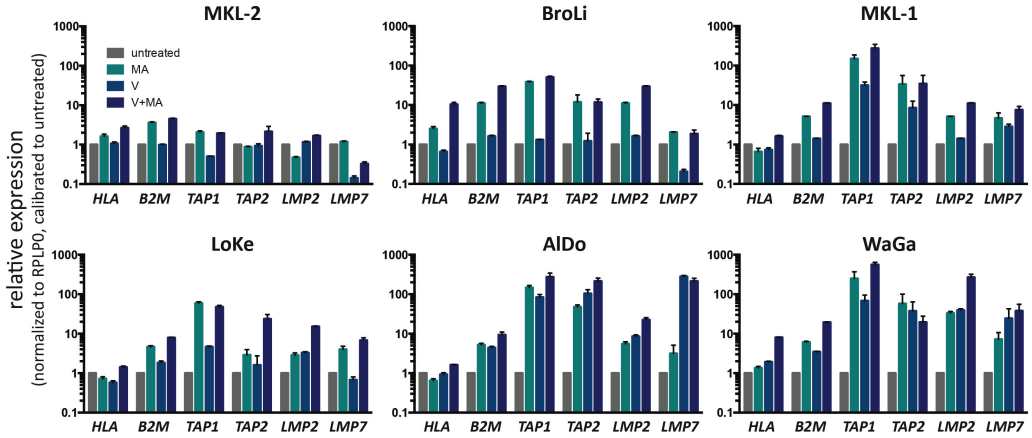

Fig. S4: Induction of antigen processing machinery (APM) gene expression by vorinostat in combination with mithramycin A in MCC cell lines.
The mRNA expression of the indicated MCC cell lines without treatment (grey) was compared to the respective expression after treatment with mithramycin A (MA, turquoise), vorinostat (V, light blue), or the combination thereof (V+MA, dark blue). mRNA expression of *HLA-ABC (HLA), TAP1*, *TAP2*, *LMP2*, *LMP7* and *B2M* was determined by qRT-PCR in triplicates; C_T values were normalized to *RPLP0* and calibrated to the ΔC_T value of the respective untreated cells; relative mRNA expression is depicted on a logarithmic scale (log 10) + SEM for all analyzed MCC cell lines.

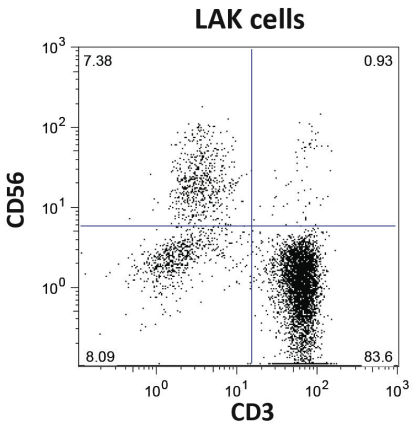

Fig. S5: Characterization of lymphokine activated killer (LAK) cells
LAK cells were generated by stimulating freshly isolated peripheral blood mononuclear cells with Interleukin 2 (IL2) as described in material and methods. The phenotype of LAK cells was determined by flow cytometry using antibodies specific for the NK cell marker CD56 and the T cell marker CD3. One representative staining example is depicted.

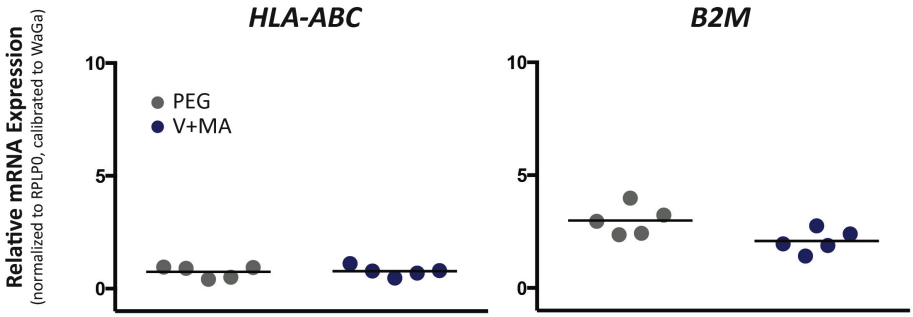

Fig. S6: *HLA-ABC* and *B2M* mRNA expression in xenotransplanted WaGa tumors is not altered by histone deacetylase inhibition *in vivo.*
NOD.CB17/*Prkdc*^{scid} mice (*n=6* for each treatment group) bearing xenotransplanted WaGa tumors were treated with drug carrier polyethylene glycol (PEG, grey), or the combination thereof (V+MA, dark blue) as described in materials and methods. mRNA was isolated from cryopreserved tumors and qRT-PCR was performed using primers specific for *HLA-ABC* and *B2M*. Relative expression was calculated with RPLP0 as endogeneous and calibrated to the ΔC_T of untreated WaGa cells *in vitro*.

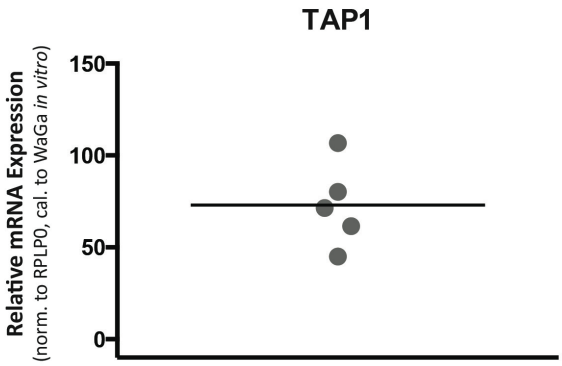

Fig. S7: *TAP1* mRNA expression is increased after xenotransplantation.
WaGa cells were xenotransplanted into NOD.CB17/*Prkdc*^{scid} mice (*n*=5) and treated with the drug carrier polyethylene glycol (PEG) as described in materials and methods. mRNA was isolated from cryopreserved tumors and qRT-PCR was performed using primers specific for *TAP1*. Relative expression was calculated by DDC_T method with *RPLP0* as endogeneous control and untreated WaGa cells *in vitro* as calibraton.

Discussion

A potential role of the PI3K-AKT signaling in MCC immune escape

We and others have demonstrated that the PI3K-AKT pathway is activated in the majority of MCCs [122, 123]. The oncogenic PI3K-AKT pathway is well known to be of vital importance at multiple levels of cancer development, proliferation and metastasis [124]. Furthermore, recent studies provide evidence, that it is also involved in viral and tumor immune escape [125]. In HIV infected cells for example, the down-regulation of MHC class I is dependent on the activation of the PI3K-AKT pathway [126]. In human glioblastoma cells, the overexpression of IGF-1 stimulation results in an increased AKT phosphorylation which is in turn associated with a reduced antigen processing and MHC class I surface expression [127]. In line with this we and others demonstrated that, although the PI3K-AKT pathway is activated in 80% of MCCs, MHC class I expression is reduced in the majority of MCC tumors [107]. Studies in melanoma, breast and prostate carcinoma revealed that the expression of the immune suppressive ligand PD-L1 is dependent on the activation of the PI3K-AKT pathway [128-130]. In line with this, loss of the PI3K-AKT pathway negative regulator PTEN, induced PD-L1 expression on colorectal carcinoma cell lines [131]. In glioma cells PTEN expression is negatively correlated with PD-L1 expression and loss of PTEN leads to increased PD-L1 expression and increased immune resistance of the affected cells [132]. Notably most MCC tumors exhibit a reduced PTEN expression [133], and PD-L1 is expressed on around 50% of $MCPyV^+$ MCC tumors [104]. However, it has to be determined whether PTEN loss and the subsequent activation of the PI3K-AKT pathway are involved in PD-L1 expression in those tumors. Inhibition of the PI3K-AKT signaling has already been demonstrated to increase antitumor immune responses in other malignancies. Hähnel *et al.* reported that PTEN silencing and subsequent AKT activation mediates immunoresistance of colorectal cancer cells against cytotoxic T cells *in vitro* and *in vivo*, and that the inhibition of the PI3K-AKT downstream effector mTOR leads to an immune sensitization of those cells [134]. Strikingly, Noh *et al.* described AKT activation as a major mechanism for cancer immune escape in HPV associated tumors [135]. They generated a human papillomavirus type 16 (HPV-16) E7-expressing tumor cell line that acquired resistance to E7 specific $CD8^+$ T cell mediated lysis after E7-specific vaccination *in vivo*. They further demonstrated that this acquired immune-resistance was due to AKT hyper-activation [135]. In a first pre-clinical trial Marshall and

colleagues reported that PI3K inhibitors in combination with TLR agonists as a direct therapy or as part of a DC vaccine promote interferon γ- and IL-17- secreting T cells, leading to eradication of a variety of murine tumors *in vivo* [136]. In light of these findings, the PI3K-AKT signaling pathway might also be involved in MCC tumor immune escape via the induction of several immune escape strategies (Figure 6).

Potential benefits and limitations of PI3K inhibitors in MCC immune recognition

The main question at hand is, how to pharmacologically target a universal pathway like the PI3K-AKT signaling pathway specifically in MCC cells in order to increase their immune recognition. Several PI3K inhibitors are already tested in clinical trials, unfortunately exhibiting severe side effects [137]. This is not surprising given the fact that the PI3K-AKT pathway is required for normal cell functions and proliferation [137]. Another severe drawback of using PI3K inhibitors in combination with immunotherapy is that the main effector cells of the innate and adaptive immune system also critically depend on an intact PI3K-AKT signaling for adequate activation and functionality. For example, the PI3K pathway plays an essential role in the activation of innate immune cells like neutrophils, mast cells and macrophages [138]. Moreover, the delta isoform of PI3K is crucial for NK cell maturation and cytokine production [139]. Also, it has been demonstrated that the IL-15-PI3K-mTor pathway is critical for NK cell effector function against virus infected and malignant cells [140], as well as for $CD8^+$ memory T cell development [141]. In this case NKG2D controls formation and commitment of $CD8^+$ memory T cells via PI3K signaling [142]. In murine epidermal γδ T cells, NKG2D induced cytotoxicity is mediated via an PI3K dependent pathway [143]. Generally, the activation and co-activation of immune cells via the NKG2D receptor is mediated via the PI3K-AKT pathway [144, 145]. Therefor the challenge in implementing PI3K inhibitors into the immunotherapy of MCC patients is to selectively target only PI3K-AKT dependent MCC cells, while leaving immune effector cells, which themselves also critically depend on PI3K signaling, unharmed. One approach to solve this dilemma could be the employment of isoform specific PI3K inhibitors [146]. Another promising way to target PI3K-AKT signaling indirectly in MCC cells might be via epigenetic drugs, like HDAC inhibitors. Notably in the previously mentioned AKT dependent immune resistant HPV associated tumor model [135] an HDAC inhibitor (AR-42) was able to enhances E7-specific $CD8^+$ T cell-mediated response after HPV DNA vaccination against those immune resistant

tumor cells. This was mainly mediated via the AR-42 induced MHC class I expression on tumor cells [147], demonstrating that AKT mediated immune resistance can indeed be reversed via pharmacological inhibition of HDACs.

PI3K pathway and epigenetics in MCC

The underlying molecular processes leading to the activation of the PI3K-AKT pathway in MCC are not fully understood yet. Notably, we did not detect any oncogenic mutations in the *PIK3CA* and *AKT1* genes in the majority of MCCs [122]. Moreover, one genomic allele of PTEN is frequently lost in MCC tumors [148]. Since the majority of MCC tumors exhibit a completely diminished PTEN protein expression [133], an additional silencing mechanism likely affects the second PTEN allele. In summary, this hints towards an epigenetic silencing mechanism of the PI3K-AKT pathway in MCC, probably via epigenetic silencing of PTEN, a frequently observed event in other human cancers [149]. PTEN expression can be silenced epigenetically via DNA methylation in the PTEN promoter region [150, 151], via PTEN targeting microRNAs [149], or via binding of the Sp1-HDAC1 complex in the PTEN promoter region and subsequent histone hypoacetylation [152]. In line with this, the down-regulation of another putative negative regulator of AKT signaling, PIB5PA, was recently demonstrated to be modulated epigenetically via the recruitment of HDAC2, HDAC3 and Sp1 to the PIB5PA gene promoter in the majority of melanomas [153]. Intriguingly, the PI3K-AKT pathway not only seems to be regulated epigenetically, but its activation in turn can also induce major epigenetic changes in human cancer cells. In breast carcinoma cell lines for example, the activation of the PI3K-AKT pathway mediates global epigenetic alterations via histone tri-methylation (repressive mark) and DNA methylation resulting in a reduced transcription of several tumor suppressor genes [154]. It has also been demonstrated that pancreatic cancer cells undergo massive re-organization of histone acetylation in response to metabolic changes in an AKT activation dependent manner [155]. These findings suggest that the activation of the PI3K-AKT pathway might be a key mediator in an “epigenetic feedback loop” in MCC cells, that ultimately results in a variety of immune escape mechanisms like the induction of PD-L1 as well as the epigenetic silencing of MICs and other components of the antigen presenting machinery (Figure 6), which will be discussed in the next chapters.

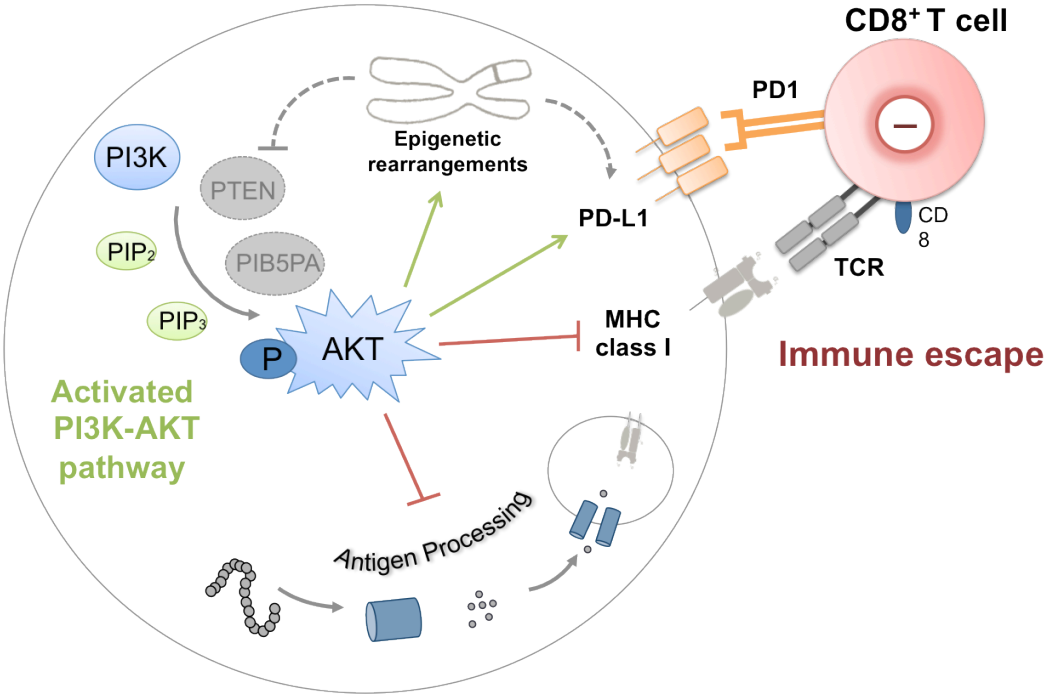

Figure 6: Possible PI3K-AKT pathway mediated immune escape mechanisms of MCC cells.
The activation of the PI3K-AKT pathway after PTEN in MCC tumors might play an essential role in MCC immune escape via the inhibition of antigen processing and MHC class I expression and induction of PD-L1 expression on MCC cell surface. An activated PI3K-AKT pathway can also mediate epigenetic rearrangements resulting in an "epigenetic feedback loop" and inhibition of AKT signaling repressors like PTEN and PIB5PA and further activation of PI3K-AKT signaling and additional immune escape mechanisms.

Epigenetic immune escape via histone hypoacetylation

During the last decade a variety of epigenetic strategies of immune escape exploited by viruses and tumors have been discovered. The most important mechanisms described so far include microRNAs, DNA methylation and histone de-acetylation [156-158]. Considering that several genes coding for immune activating ligands, as well as most genes necessary for antigen processing and presentation are all located in the HLA complex, a chromosomal region on the short arm of chromosome 6 (Figure 7). Epigenetic silencing of this entire region via chromatin rearrangement appears to be a very efficient immune escape strategy. The manuscripts presented in this thesis demonstrate, that in MCC several genes encoded in the HLA complex on chromosome 6, like stress induced NKG2D ligands MICA and MICB and key mediators in the antigen processing (TAPs and LMPs) are silenced or down-

regulated via histone de-acetylation in their promoter region. In line with our findings, epigenetic silencing via histone hypoacetylation of genes in the HLA complex has been demonstrated in a variety of other human cancers. For example Kato *et al.* described, that MICA and MICB expression was silenced via histone acetylation mediated chromatin rearrangements in leukemic cells [159]. It also has been demonstrated that APM components like TAPs and LMPs are silenced due to HDAC mediated histone hypoacetylation in several malignancies [157, 160].

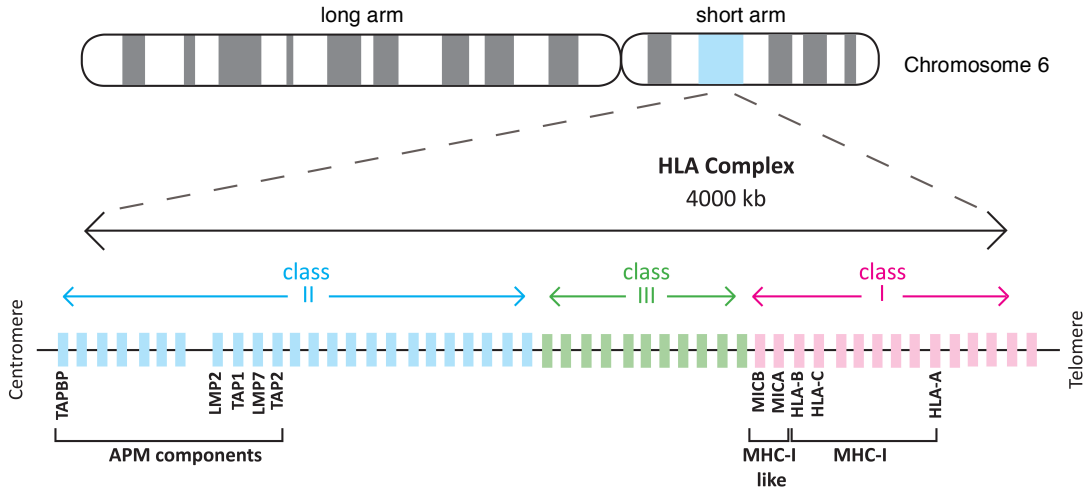

Figure 7: Genes encoded in the HLA complex on chromosome 6
The human MHC gene complex is located on the short arm of chromosome 6 (6p21.3) and encodes 224 genes of which 128 are thought to be expressed. A majority of the genes located in the MHC complex are involved in humoral and adaptive immune responses. Among those genes are components of the antigen processing machinery like Tapasin (*TAPBP*), immunoproteasome subunits *LMP2* and *LMP7*, transporter components *TAP1* and *TAP2*. The cluster also encodes for NKG2D activating ligands *MICA* and *MICB* and MHC class I heavy chain alleles *HLA-A*, *B* and *C*.

Epigenetic silencing of the expression of APM components like TAPs and LMPs, via histone hypoacetylation also seems to be a major mechanism of immune escape in viral associated cancers [161]. For example in adenovirus transformed cells, the viral protein E1A mediates histone de-acetylation in an de-acetylating repressor complex, which results in decreased MHC class I expression in those cells [162]. In HPV16 associated tumors MHC class I deficiency is also mediated epigenetically via silencing of APM components [163]. An extraordinary example for epigenetic camouflage of cancer cells against the immune system is the devil facial tumor disease (DFTD), a tumor of a marsupial called Tasmanian devil. Due to its superior immune escape strategies DFTD is a contagious malignancy, in which tumor cells spread from one individual to another [164]. One of the main escape strategies of the DFTD is the lack of MHC class I molecules on the cell surface of tumor cells. Siddle

et al. demonstrated that the reduced MHC class I surface expression is at least partially due to histone de-acetylation in several genes of the MHC class I antigen processing machinery. Strikingly MHC class I surface expression could be restored after treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A [164]. On the one hand, these findings impressively demonstrate how efficient and stable epigenetic immune escape of cancer cells via histone hypoacetylation can be, but on the other hand also demonstrates that epigenetic immune escape can be reversed pharmacologically.

Pharmacological reversal of epigenetic immune escape in cancer

In the here presented thesis it was demonstrated, that pharmacological HDAC inhibition in MCC cells lead to an increased transcription of components of the antigen processing machinery and MICs as well as a significant induction of MHC class I and MIC surface expression *in vitro* and *in vivo*. This induction of MICs and APM components after HDAC inhibition has been demonstrated before in other malignancies. Skov and colleagues showed that different HDAC inhibitors induced MIC surface expression on a variety of cancer cell lines [165]. Kato *et al.* demonstrated that the HDAC inhibitor Trichostatin A induced MIC surface expression on leukemic cell lines [159] and Armeanu and colleagues reported that MIC expression was induced after treatment with the HDAC inhibitor sodium valproate on hepatoma cells [166]. The exact underlying mechanisms by which HADC inhibitors mediate MIC induction are not fully understood, but seem to include activation of DNA damage response [167], activation of the GSK3 kinase [165], inhibition of MIC targeting microRNAs [168] and direct acetylation of histones in the MIC's promoter regions [169], as we also demonstrated it to be the case in MCC.

HDAC inhibitor mediated induction of antigen processing and presentation has been described before in other malignancies. The HDAC inhibitors Trichostatin A and Sodium butyrate for example, were demonstrated to induce MHC class I surface expression on several human cancer cell lines [170]. Trichostatin A and Valporic acid also induced APM component expression and MHC class I surface expression in murine melanoma and colon carcinoma cell lines [160]. Moreover, Kortenhorst *et al.* identified the up-regulation of *HLA* and *B2M* genes in human prostate cancer cells after treatment with Vorinostat or Valporic acid, in a high throughput bioinformatic approach [171].

Strikingly in our experiments HDAC inhibition sensitized MCC cells towards lymphokine activated killer (LAK) cell mediated lysis in an MHC class I and MIC dependent manner (Figure 8).

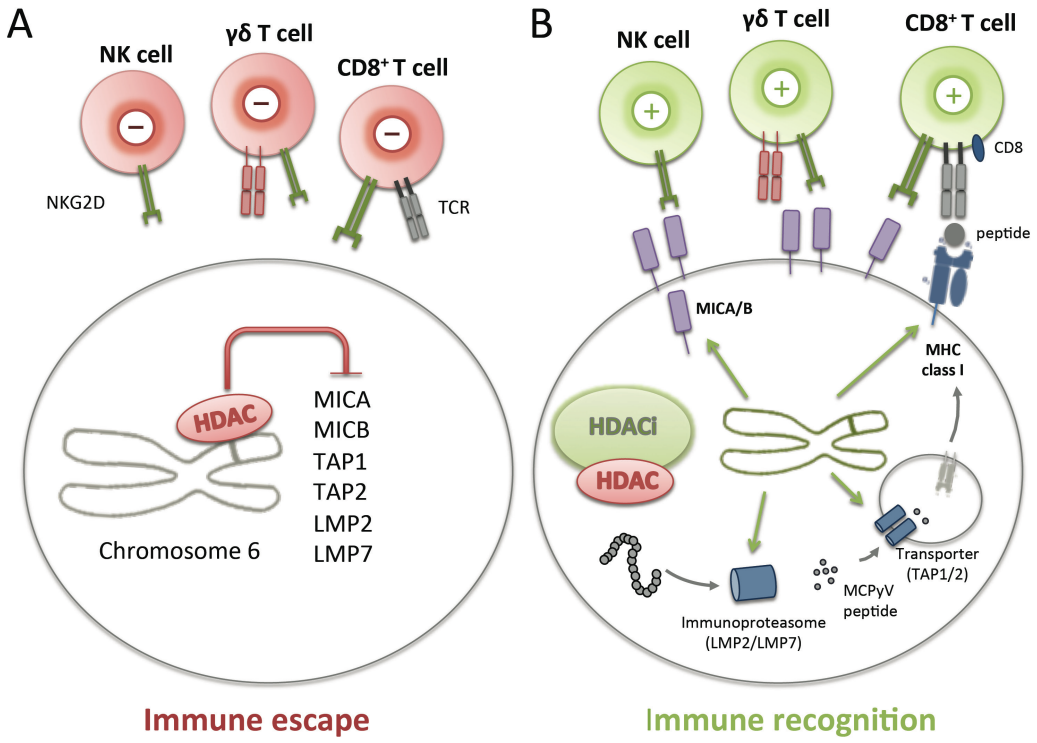

Figure 8: HDAC inhibitor induced immune responses towards MCC.
(A) In untreated MCC cells immune regulatory genes like NKG2D ligands MICA and MICB as well as components of the antigen processing machinery like transporter subunits TAP1 and TAP2 and immunoproteasom subunits LMP2 and LMP7 are silenced epigenetically via HDAC mediated histone deacetylation. As a result MCC cells are able to escape immune responses. **(B)** Treatment of MCC cells with HDAC inhibitors results in an increased antigen processing and surface expression of MHC class I, MICA and MICB. As a result MCC cells can be detected and eliminated by NKG2D and/or TCR expressing cytotoxic lymphocytes, like NK cells, γδ T cells and $CD8^+$ T cells.

Our findings are in line with the results reported by several other groups, which described an increased NK and T cell mediated lysis of breast cancer, hepatoma, leukemic and B cell lymphoma cell lines after pharmacological HDAC inhibition [159, 165, 166, 172]. We thus conclude that HDAC inhibitor mediated immune sensitization of MCC cells might be a valuable addition to immunotherapeutic regimens against MCC.

Advantages and limitations of HDAC inhibitors in MCC immune recognition

The rational for the use of HDAC inhibitors in the treatment of cancer traditionally has been based on their anti-proliferative capacities [173]. However HDAC inhibitors have been clinically applied for the treatment of other diseases owing to their ability to manipulate immune responses (Table 2)[174]. Indeed more recently increasing evidence has been accumulated that these immune modulatory properties of HDAC inhibitors could largely enhance the effectiveness of immunotherapeutic approaches [175]. Complementing this finding it has been reported that the beneficial effects of HDAC inhibitors observed in past clinical trials were largely dependent on a functional immune system [176]. West and colleagues reported that HDAC inhibitors Vorinostat and Panobinostat are significantly less efficient against B-cell lymphomas and colon carcinomas in mice lacking a functional immune system (*Rag2*$^{-/-}\gamma c^{-/-}$ mice) than in their wild type counterparts [176].

In general HDAC inhibitors are well tolerated alone or in combination, with manageable side effects [177]. This is owed to the fact that tumor cells are significantly more sensitive towards HDAC inhibitor mediated effects than healthy cells [165, 166]. Reasons for this increased “epigenetic vulnerability” of tumor cells might be their altered HDAC expression and activity profile, their oncogene addiction and their dependence on immune escape strategies [174].

In addition to the afore mentioned ability of HDAC inhibitors to induce antigen processing and surface expression of MHC class I and NKG2D ligands on MCC and other cancer cells [165, 166, 172, 178, 179], they exhibit several additional immune stimulatory features. The induction of MICs in HDAC inhibitor treated tumor cells, seem to not only stimulate local immune responses, but possibly also systemic immune responses. In this regard, MICB protein was found to be increased in exosomes isolated from Entinostat treated liver cancer cells. Co-culture of those exosomes with NK cells significantly increased their cytotoxicity and proliferation [180]. Another advantage of HDAC inhibitors in the setting of immunotherapy is, that they are able to induce an *immunogenic death* of cancer cells. During this form of cell death, dying tumor cells stimulate the uptake, activation and cross-presentation of antigen presenting cells (APC) and thereby provoke immune responses against remaining tumor cells [181]. In this regard Vorinostat has been demonstrated to stimulate the expression and release of important immunogenic cell death mediators, like HMGB1 and calreticulin by dying tumor cells [182, 183]. Further it has been demonstrated that fragments of dying colon carcinoma cell were taken up efficiently

by antigen presenting cells after treatment with Vorinostat [184]. In line with this, vaccination of mice with Vorinostat treated mesothelioma cells resulted in an increased infiltration of $CD8^+$ T cells into tumors *in vivo* [185]. HDAC inhibitors have also been demonstrated to increase or induce *de novo* expression of tumor-associated antigens (TAA). Treatment with the HDAC inhibitor Romidepsin for example, could increase the expression of the TAA gp-100 in a murine melanoma model [194]. Consequently a combination therapy with Romidepsin and gp-100 specific autologous $CD8^+$ T cells increased immune recognition and killing of tumor cells [194].

Table 2: HDAC inhibitors with immunomodulatory capacities.

HDACi	Specificity	Clinical Application	Reference
Trichostatin A (TSA)	pan HDAC	pre-clinical	[186]
Vorinostat (SAHA)	pan HDAC	FDA approved	[187]
Sodium Butyrate (SB)	pan HDAC	pre-clinical	[188]
Panobinostat (LBH589)	pan HDAC	phase III clinical trial	[189]
Valporic Acid	pan HDAC	phase III clinical trial	[190]
Dacinostat (LAQ824)	pan HDAC	phase I clinical trial	[191]
Romidepsin	HDAC1/2	FDA approved	[192]
Entinostat	class I HDAC	phase III clinical trial	[193]

Trichostatin A could induce the *de novo* expression of the TAA MAGE-A in selected solid cancer cell lines [195]. Due to their potential to modulate the composition of the proteasome, HDAC inhibitors might also be able to alter the peptidome presented on HLA complexes on cancer cells, which in turn can lead to the attraction and activation of $CD8^+$ T cells [196]. In line with this several studies reported, that HDAC inhibitors are able to enhance the activation and infiltration of $CD8^+$ T cells due to their ability to stimulate the expression of certain cytokines. The HDAC inhibitor Panobinostat for example, increased craft versus host disease after organ transplants in mice, by increasing IFN-γ and TNF-α levels, resulting in an increased infiltration of $CD8^+$ T cells into transplanted organs. Moreover, it has also been described that HDAC inhibitors can revert the exhausted phenotype of $CD8^+$ T cells in chronic viral infections [197]. This could emerge as a very useful feature in the treatment MCC patients, since MCPyV reactive $CD8^+$ T cells in MCC patients indeed present an exhausted phenotype [104, 105], which might be reversible with HDAC inhibitors. Treatment of murine $CD8^+$ T cells with Trichostatin A, for example, induces their activation and memory in a similar way than pro-inflammatory cytokines, like IL-

12 and TNF-α [198]. Additionally, Panobinostat enhances $CD8^+$ T cell proliferation and activation in mice [199, 200]. In general HDAC inhibitors seem to have mainly positive effects on $CD8^+$ T cells, but under certain conditions, some HDAC inhibitors also seem to negatively influence cytotoxic lymphocytes. It was demonstrated that in cutaneous T cell lymphoma patients the frequency of $CD8^+$ and $CD4^+$ T cells was decreased by 50% after treatment with the HDAC inhibitor Romidepsin [201]. And panHDAC inhibitors Valporic acid (VPA) and Vorinostat reduced the cytolytic capacity and proliferation of NK cells when administered before their activation with IL-2 [202]. However, HDAC inhibitor sensitivity seems to be dependent on the activation state of the NK cells [203].

Combination of HDAC inhibitors and immunotherapy in the treatment of solid tumors

HDAC inhibitors have been successfully implemented in the treatment of hematologic malignancies, like cutaneous and peripheral T cell lymphomas [204]. Motivated by these success stories, several clinical trials were conducted employing HDAC inhibitors for the treatment of solid tumors [205]. Unfortunately it became apparent that HDAC inhibitors were not efficient as monotherapy for the more complex and harder to reach solid tumors [205]. To overcome the limitations of HDAC inhibitors for the treatment of solid malignancies, they have been frequently combined with chemotherapeutic drugs and small molecule inhibitors revealing some remarkable anti-cancer effects [206-208]. Given the afore mentioned synergism and interdependence of HDAC inhibitors and the immune system [176] there is a strong indication, that HDAC inhibitors might reveal their true potential in combination with immunotherapy. Indeed several pre-clinical studies accumulated evidence that HDAC inhibitors are able to potentiate the effects of various immunotherapeutic approaches towards solid tumors *in vitro* and *in vivo* [175]. The HDAC inhibitor Romidepsine (FK228) for example was able to induce immune sensitization in a murine melanoma model *in vivo* [194]. Moreover, Schmudde *et al.* demonstrated that four different HDAC inhibitors synergized with IL-2 stimulated PBMCs in the eradication of several solid human cancer cell lines *in vitro* [178]. In line with this, the class I HDAC inhibitor Entinostat was demonstrated to synergize with IL-2 in the treatment of murine model of renal cell carcinoma [209]. Notably the survival benefit of HDACi and IL-2 treated mice was diminished after depletion of $CD8^+$ T cells [209]. The panHDAC inhibitors Dacinostat and Panobinostat potentiated the effectiveness of adoptively transferred tumor specific $CD8^+$ T cells in a B16 murine melanoma

model [200, 210]. Vo *et al.* also demonstrated that tumors of HDAC inhibitor treated mice exhibited an increased expression of the tumor antigen gp100 and MHC class I complexes and a higher infiltration of adoptively transferred T cells [210]. Lisero *et al.* additionally showed that the number of regulatory T cells was decreased in the tumors of mice treated with Panobinostat [200]. PanHDAC inhibitors Vorinostat and Panobinostat synergized with immune stimulating antibodies against CD137 and CD40, resulting in long lasting memory responses in pre-clinical mouse models of several solid malignancies [184]. Lee *et al.* demonstrated that the efficiency of HPV DNA vaccination could be increased by co-administering the HDAC inhibitor AR-42 in a murine model *in vivo* [147].

Based on these encouraging pre-clinical results, several clinical trials are currently recruiting patients to explore the potential of HDAC inhibitors in combination with immunotherapy for the treatment of solid tumors. For example stage III/IV melanoma patients are recruited for the treatment with Panobinostat in combination with the CTLA-4 specific antibody Ipilimumab (NCT02032810). Entinostat is tested in combination with Aldesleukin (IL-2) for the treatment of patients with metastatic kidney cancer (NCT01038778). A Phase II clinical trial is recruiting non small cell lung cancer patients for “epigenetic priming” with Entinostat and the methyltransferase inhibitor Azacitidine prior to PD-1 blockade with Nivolumab (NCT01928576). Moreover, a phase I clinical trial will soon start recruiting breast cancer patients to test the best dose and side effects of Entinostat with Nivolumab and Ipilimumab (NCT02453620).

Outlook on MCC immunotherapy

Clinical trials only recently started recruiting patients with solid tumors for the treatment with HDAC inhibitors in combination with immunotherapy. Therefor no information about effectiveness, response rates and long-term survival of the different combinatory approaches are available. Nevertheless, several issues already need to be addressed in order to successfully implement HDAC inhibitors in the immunotherapeutic treatment of MCC. Increasing evidence is accumulating, that the right timing of HDAC inhibitor administration will be crucial for their successful application together with immunotherapy. As previously mentioned, Ogbomo *et al.* demonstrated that Valporic acid inhibited the proliferation and activation of NK cells [202]. Schmudde *et al.* however demonstrated that this inhibitory effects did not occur, if NK cells were activated with IL-2 prior to the addition of HDAC inhibitors

[211]. Furthermore Bridle *et al.* demonstrated that the HDAC inhibitor Entinostat only enhanced tumor specific $CD8^+$ T cell function when administered at the time of the booster vaccination, but not when given at the prime vaccination [212]. Tumor heterogeneity will also be an issue that needs to be address in the immunotherapy of MCC. While we could demonstrate that MICs were inducible via HDAC inhibition in the majority of MCC cells, a small population of around 10% of cells remained resistant to HDACi induced MIC induction. Indeed it is becoming apparent that tumors are composed of heterogeneous cell populations [213]. Adding a further level of complexity, the different populations seem to be plastic and able to interconvert into each other as a response to therapy [214]. In line with this it has been demonstrated, that cancer cell plasticity and the maintenance of the different subpopulation is mainly mediated via epigenetic mechanisms [215]. Particularly intriguing here are the frequent mutations in genes of major players of the epigenetic machinery in cancer cells, which are increasingly recognized [215]. While this epigenetic mediated plasticity is a challenge in the treatment of cancer, it also demonstrates the immense opportunities epigenetic drugs hold to overcome therapy resistance and immune escape of cancer cells [215]. Another major challenge in the implementation of HDAC inhibitors in MCC immunotherapy will be to determine compounds, that specifically increase MCC cell immunogenicity, leave healthy cells unharmed, cause minimal side effects and at the same time stimulate cytotoxic immune cells. One auspicious drug in this regard is the narrow spectrum HDAC inhibitor Entinostat. Entinostat does not inhibit NK cell function, and on the contrary, it even increased NKG2D expression on NK cells [216]. It further boosts their cytolytic activity against tumor cells *in vitr*o and, when adoptively transferred, *in vivo* [216]. Furthermore, Entinostat has been shown to suppress regulatory T cells, thus releasing the breaks on the immune system [217]. In Addition, Entinostat was able to induce the expression of MICs *in vitro* and *in vivo* [216]. These results emphasize that class selective HDAC inhibitors can prime tumor cells epigenetically and activate immune cells at the same time. To select promising specific epigenetic compounds for the treatment of MCC, it will be crucial to determine which members of the large HDAC family are deregulated and involved in the epigenetic silencing of MICs and antigen processing in MCC. With this knowledge classically used pan and class specific HDAC inhibitors could be replaced with new and emerging narrow spectrum HDAC inhibitors like Entinostat. Indeed, some isoform specific HDAC inhibitors are already tested in clinical trials for their effectiveness against cancer and other disease. Examples are the HDAC3 specific inhibitor RG2883, which is tested in phase I clinical trials for neurodegenerative diseases, the HDAC6 specific inhibitor

Rocolinostat (ACY1215), that is currently tested alone or in combination in phase II clinical trials (NCT01323751, NCT01997840 and NCT01583283) for hematological malignancies. Additional there are several newly developed isoform specific HDAC inhibitors in preclinical testing stages [174, 218]. It will also be of vital importance to determine biomarkers for the response to HDAC inhibitors and immunotherapy in MCC patients. From the insights obtained in the manuscripts presented in this thesis such biomarkers could be aberrant mRNA and protein expression levels of specific HDAC isoforms, MICs and APM components. Moreover, the “epigenetic state” of MCC cells might serve as a potential biomarker for the response to HDAC inhibitor boosted immunotherapy. Additionally the activation of the PI3K-AKT pathway could be determined using specific biomarker panels including genomic aberrations, but also epigenetic markers (histone acetylation, miRNAs) and the activation state of PI3K downstream effectors to generate a “PI3Kness” score [219, 220]. At the same time not only tumor cells have to be characterized, but also the presence and activation state of peri- and intratumoral as well as systemic tumor specific T cells and NK cells have to be determined in order to optimize treatment regimens. Based on the individual results of the different biomarker panels personalized immunotherapeutic regimens could be designed (Figure 9).

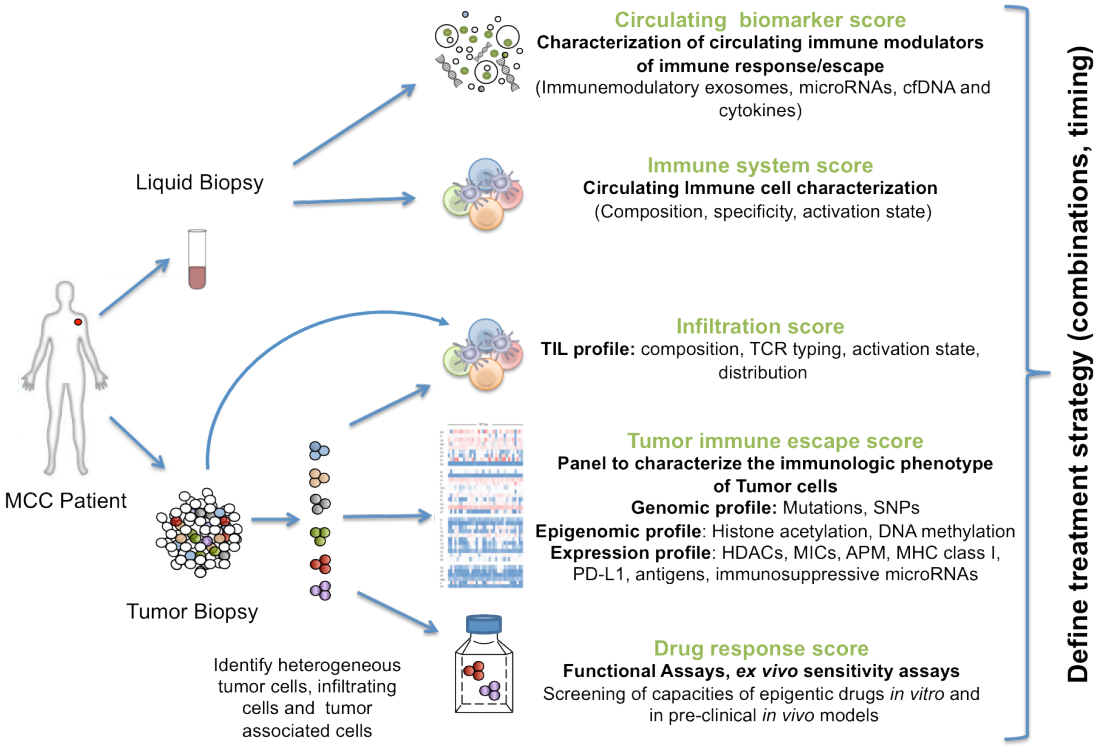

Figure 9. Personalized immunotherapy for MCC patients. To personalize MCC therapy different variables have to be taken into account and weighed against each other. Scores for the presence of circulating biomarkers, the activation state of cytotoxic immune cells and the infiltration of those immune cells into the tumor microenvironment, as well as for immune escape mechanisms and for drug responsiveness will be helpful tools in this reagard. Based on the individual scores the effectiveness of treatment strategies could be predicted.

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Abbreviations

4E-BP1	4E-binding protein 1
AIDS	Acquired immunodeficiency syndrome
APM	Antigen processing machinery
bcl-2	B-cell lymphoma 2
CD	Cluster of differentiation
$CD4^+$	CD4 positive
$CD8^+$	CD8 positive
cm	Centimeter
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CVID	Common variable immunodeficiency
DC	Dentritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia*
EBV	Epstein Barr Virus
Ep-cam	Epithelial cell adhesion molecul
et *al.*	et alii (and others)
Fab	Antigen-binding fragment
FDA	U.S. Food and drug administration
GLA-SE	Glucopyranosyl lipid adjuvant–stable emulsion
gp-100	Glycoprotein 100
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HHV	Human Herpes Virus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HPV	Human Papilloma Virus
IFN	Interferon
IGF-1	Insulin-like growth factor 1
KIR	Killer-cell immunoglobulin-like receptors
LAK	Lymphokine-activated Killer
LIR	Leucocyte inhibitory receptor
LMP2	Large multifunctional peptidase 2
LMP7	Large multifunctional peptidase 7
LTA	Large T antigen
MA	Mithrmaycin A
MC	Merkel cell
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MCpyV+	Merkel cell polyomavirus positive

mDC	Myeloid dendritic cells
MHC	Major histocompatibility complex
MHC class I	Major histocompatibility complex class I
MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein B
MM	Malignant melanoma
mTOR	Mechanistic target of rapamycin
MUC-1	Mucin-1
NCCN	National comprehensive cancer network
NK	Natural killer
NKG2D	NK group 2D
NKG2DL	NKG2D ligand
NKT	Natural killer T
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-1L	Programmed cell death ligand 1
PI3K	Phosphoinositide 3-kinase
PP2A	Protein phosphatase 2A
PP4C	Protein phosphatase 4C
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
Sp1	Specificity protein 1
sTA	Small T antigen
TAA	Tumor-associated antigen
TAP1	Transporter 1
TAP2	Transporter 2
TCR	T cell receptor
TIL	Tumor infiltrating lymphocytes
Tim-3	T-cell immunoglobulin domain 3
TLR	Toll like receptor
TNF	Tumor necrosis factor
T_{regs}	Regulatory T cells
TSA	Trichostatin A
ULBP	UL-16-binding proteins
VP	Viral protein
VPA	Valporic acid
β2m	β2-microglobulin

Journal Articles

Schrama D, Ugurel-Becker S, Sucker A, Ritter C, Zapatka M, Schadendorf D, Becker JC. **STAT3 single nucleotide polymorphism rs4796793 SNP does not correlate with response to adjuvant IFNα therapy in stage III melanoma patients**. *Front Med*. 2014 Nov 28;1:47. doi: 10.3389/fmed.2014.00047. eCollection 2014.

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Presentations and Posters

Ritter C, Fan K, Schrama D, Houben R, Becker JC
Enhanced MHC-class I expression on Merkel cell carcinoma by HDAC inhibitors. Presentation and poster at the 42nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung, 2015

Schrama D, Ritter C, Utikal J, Becker JC, Houben R
Titel: Large T antigen truncating mutations accur before Merkel cell polyomavirus integration in Merkel cell carcinoma cells. Poster at the 42nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung, 2015

Ritter C, Fan K, Paulson KG, Schrama D, Becker JC
Epigenetic silencing of MHC class I chain-related protein A and B in Merkel cell carcinoma: Improved immune recognition after reversal. Poster at the 7th German-Israeli Cancer Research School in Systems Medicine, 2014

Ritter C, Becker JC, Schrama D
Increasing immunogenicity of Merkel cell carcinoma by HDAC inhibitors. Poster for the European Society for Dermatological Research, 2014

Ritter C, Schrama D, Roland Houben, Becker JC
Enhanced MHC class I molecule expression on Merkel cell carcinoma by HDAC inhibitors. Presentation at the 24. German Skin Cancer Congress (ADO) and at the ADO Student Retreat, 2014

Fan K, Ritter C, Schrama D, Roland Houben, Becker JC
Ectopic expression and secretion of miR-375 in MCC – impact on the tumor microenvironment. Presentation at the 24. German Skin Cancer Congress (ADO) and at the ADO Student Retreat, 2014

Ritter C, Schrama D, Becker JC
Re-induction of epigenetically silenced NKG2D ligands MICA and MICB in Merkel cell carcinoma. Presentation at the 23. German Skin Cancer Congress (ADO) and Student Retreat, 2013

Schrama D, Ritter C, Becker JC
PDGFR alpha und das Merkelzellkarzinom - p.S478P Polymorphismus und PDGFR-Inhibitoren. Poster at the 23. German Skin Cancer Congress (ADO), 2013

Ritter C, Schrama D, Ugurel S, Odum N, Becker JC
microRNA regulated processes in Merkel cell carcinoma. Poster for funding of the Ph.D. Programm “Moleculare Fundamentals of Inflamation” (MOLIN) by the FWF
The Poster and the presentation of data was graded as excellent, 2011

Curriculum Vitae

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School career:

1991-1995	Hans Memling Grundschule, Mömlingen, Germany
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University career:

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10-12/2010	Center for Cancer Immuno Therapy (CCIT), Herlev Hospital, Herlev, Denmark
2011-2014	Department of Dermatology, Medical University of Graz, Austria
09/2014	Department of Translational Skin Cancer Research, German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ)

Eidesstattliche Erklärung

Ich Erkläre hiermit an Eides statt, dass ich die vorliegende kumulative Dissertation mit dem Titel „Scientific basics for new immunotherapeutic approaches towards Merkel cell carcinoma / Grundlagen neuer immuntherapeutischer Ansätze gegen das Merkelzellkarzinom" selbstständig angefertigt habe und dabei keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Ich erkläre außerdem, dass ich die vorliegende Dissertation weder in gleicher noch in ähnlicher form bereits in einem anderen Prüfungsverfahren vorgelegt habe.

Weiterhin habe ich keinen weiteren akademischen Grade, außer den mit dem Zulassungsantrag urkundlich vorgelegten, erworben oder zu erwerben versucht.

Würzburg, im Juni 2015

Cathrin Ritter

Author Contributions

Article

Activation of the PI3K/AKT pathway in Merkel cell carcinoma

Journal: PLoS One. 2012;7(2):e31255

Authors: Christian Hafner, Roland Houben, Anne Baeurle, Cathrin Ritter, David Schrama, Michael Landthaler, Jürgen C. Becker

I hereby certify that Cathrin Ritter contributed to the concept and experimental design of this article. She performed PI3K inhibitor experiments and immunoblots to analyze the phosphorylation state of AKT in MCC cell lines.

Regensburg 15.06.15

Place, Date **Christian Hafner**

Article

Activation of the PI3K/AKT pathway in Merkel cell carcinoma

Journal: PLoS One. 2012;7(2):e31255

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Würzburg, 11.6.15

Place, Date Roland Houben

Article

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ESSEN, 11.06.15

Place, Date

Cathrin Ritter

Article

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Frankfurt a. M., 12.06.2015

Place, Date

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Article

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Würzburg, 11-6-15
Place, Date

David Schrama

Article

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Article

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Essen 11.6.15

Place, Date

Jürgen C. Becker

Manuscript

Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

Journal: Submitted to Science Translational Medicine

Authors: Cathrin Ritter, Kaiji Fan, Kelly G. Paulson, Paul Nghiem, David Schrama, Jürgen C. Becker

I hereby certify that Cathrin Ritter was the main contributor to the concept and experimental design of this manuscript. She was the principal scientist in the planning and execution of the experiments and in the writing of the manuscript.

ESSEN, 11.06.15
Place, Date

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Manuscript

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Essen, 11/06/2015
Place, Date

Kaiji Fan

Author Contributions

Manuscript

Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

Journal: Submitted to Science Translational Medicine

Authors: Cathrin Ritter, Kaiji Fan, Kelly G. Paulson, Paul Nghiem, David Schrama, Jürgen C. Becker

I hereby certify that Cathrin Ritter was the main contributor to the concept and experimental design of this manuscript. She was the principal scientist in the planning and execution of the experiments and in the writing of the manuscript.

Seattle, WA, USA
11 June 2015

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Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

Journal: Submitted to Science Translational Medicine

Authors: Cathrin Ritter, Kaiji Fan, Kelly G. Paulson, Paul Nghiem, David Schrama, Jürgen C. Becker

I hereby certify that Cathrin Ritter was the main contributor to the concept and experimental design of this manuscript. She was the principal scientist in the planning and execution of the experiments and in the writing of the manuscript.

Seattle, WA, June 11, 2015

Place, Date

Paul Nghiem

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Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

Journal: Submitted to Science Translational Medicine

Authors: Cathrin Ritter, Kaiji Fan, Kelly G. Paulson, Paul Nghiem, David Schrama, Jürgen C. Becker

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Würzburg, 11-6-15

Place, Date

David Schrama

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Reversal of epigenetic silencing of MHC class I chain-related protein A and B improves immune recognition of Merkel cell carcinoma

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Essen 11.6.15

Place, Date

Jürgen C. Becker

Manuscript

HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

Journal: Submitted to Journal of Clinical Investigation

Authors: Cathrin Ritter, Kaiji Fan, Annette Paschen, Sine Reker Hardrup, Soldano Ferrone, Paul Nghiem, David Schrama, Jürgen C. Becker

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Place, Date

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Essen, 11/06/2015
Place, Date

Kaiji Fan

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Journal: Submitted to Journal of Clinical Investigation

Authors: Cathrin Ritter, Kaiji Fan, Annette Paschen, Sine Reker Hardrup, Soldano Ferrone, Paul Nghiem, David Schrama, Jürgen C. Becker

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HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

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Copenhagen, June 14th 2015

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Manuscript

HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

Journal: Submitted to Journal of Clinical Investigation

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I hereby certify that Cathrin Ritter was the main contributor to the concept and experimental design of this manuscript. She was the principal scientist in the planning and execution of the experiments and in the writing of the manuscript.

Boston, MA 06/11/2015 Soldano Ferrone

Manuscript

HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

Journal: Submitted to Journal of Clinical Investigation

Authors: Cathrin Ritter, Kaiji Fan, Annette Paschen, Sine Reker Hardrup, Soldano Ferrone, Paul Nghiem, David Schrama, Jürgen C. Becker

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Seattle, WA, June 11, 2015

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HDAC-inhibition re-induces HLA class I expression in Merkel cell carcinoma via activation of the antigen processing machinery.

Journal: Submitted to Journal of Clinical Investigation

Authors: Cathrin Ritter, Kaiji Fan, Annette Paschen, Sine Reker Hardrup, Soldano Ferrone, Paul Nghiem, David Schrama, Jürgen C. Becker

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Würzburg, 11-6-15

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Manuscript

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Jürgen C. Becker

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