Type I and II interferons inhibit Merkel cell carcinoma via modulation of the Merkel cell polyomavirus T-antigens

Christoph Willmes¹, Christian Adam¹, Miriam Alb¹, Lena Völkert¹, Roland Houben¹, Jürgen C. Becker², David Schrama¹²

¹ Department of Dermatology, University Hospital of Würzburg, Würzburg, Germany.
² Division of General Dermatology, Medical University of Graz, Graz, Austria.

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Precis: Merkel cell carcinoma, a rare but highly aggressive skin cancer driven by a polyomavirus tumor antigen, may be susceptible to interferon therapies found to modulate the T antigen’s expression.

Correspondence to:
Jürgen C. Becker
Department of Dermatology,
Medical University of Graz
A-8036 Graz, Austria
Phone: +43 316 385 12538
FAX: +43 316 385 13424
Juergen.becker@medunigraz.at

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Abstract (203 words)

Merkel cell carcinoma (MCC) is a rare and highly aggressive skin cancer associated with the Merkel cell polyomavirus (MCV). As MCC cell lines demonstrate oncogene addiction to the MCV T-antigens, pharmacological interference of the large T-antigen (LTA) may represent an effective therapeutic approach for this deadly cancer. In this study, we investigated the effects of interferons (IFNs) on MCC cell lines, especially on MCV positive (MCV+) lines. Type I IFNs (i.e. Multiferon, a mix of different IFN α subtypes, and IFN β) strongly inhibited the cellular viability. Cell cycle analysis demonstrated increased sub-G fractions for these cells upon IFN treatment indicating apoptotic cell death; these effects were less pronounced for IFN γ. Notably, this inhibitory effect of type I IFNs on MCV+ MCC cell lines was associated with a reduced expression of the MCV LTA as well as an increased expression of promyelocytic leukemia protein (PML), which is known to interfere with the function of the LTA. In addition, the intra-tumoural application of multiferon resulted in a regression of MCV+ but not MCV- MCCs in vivo. Together, our findings demonstrate that type I IFNs have a strong anti-tumour effect, which is at least in part explained by modulation of the virally encoded LTA.
Introduction

Merkel cell carcinoma (MCC) which is also known as neuroendocrine carcinoma of the skin is a rare, highly aggressive skin cancer, with a strong and continuous increase in incidence over the past years (1). UV exposure and immune suppression are known risk factors for MCC (2). Indeed, MCC is much more frequent in severely immunosuppressed populations caused by immune suppressive drugs in organ transplant patients, lymphoma or HIV infection (3). In accordance with the notion that many cancers with infectious etiologies are more prevalent in the context of immunosuppression, the Merkel cell polyomavirus (MCV) was identified clonally integrated in the genome of most MCC cells (4). Meanwhile, many studies confirmed the association of MCC with MCV (5, 6). MCV encodes the potential oncoproteins small and large T antigen (LTA) (7). Notably, it has been demonstrated that the maintenance of MCV positive (MCV+) MCC cell lines critically depends on the presence of MCV LTA sustaining the role of MCV in the pathogenesis of MCC (8, 9).

Interferon (IFN) has been first described as secreted macromolecule produced by cells after treatment with heat-inactivated influenza virus (10). Indeed, IFNs are a large family of multifunctional, secreted proteins which have antiviral, anti-tumoral and immune modulating effects mediated through IFN-stimulated gene (ISG) expression (11). Three types of IFNs have been described in mammals. Type I IFNs (α, β, ε, κ, ν, ω) as well as type III IFN (IFN γ) (12) are produced ubiquitously in response to viral infection, double stranded RNA or other stimuli. In contrast, type II IFN (γ) is only induced in activated T-lymphocytes and natural killer cells (11, 13).

The biological activities of IFNs are initiated by binding to their cognate receptors, i.e. predominantly the IFN-α/β receptor for type I, the IFN-γ receptor for type II and the IL10R2/IFNLR1 for type III IFN. Upon binding to the respective receptors different signal cascades are activated. The classical Jak/STAT pathway leads to the
transcription of a distinct set of genes which mediate the biological effects of these cytokines, i.e. anti-proliferative effects and antiviral activity for type I and III IFNs and immune modulatory effects for type II IFNs (11). It is important to note, however, that the cellular responses to engagement of the IFN-receptors are subject to several modulating factors, e.g., the activation status of the cells, binding of other cytokines/chemokines or environmental factors such as hypoxia.

The therapeutic use is currently largely restricted to type I IFNs. For example, recombinant IFN α2 has been FDA approved for the therapy of hairy cell leukemia or adjuvant therapy of melanoma (14, 15). In addition, IFN α is used for the treatment of hepatitis B and C and HIV-associated Kaposi sarcoma whereas IFN β is used for therapy of multiple sclerosis (16-18).

The antiviral activity of type I IFNs has also been investigated for polyomaviruses. In this regard, type I IFNs can both limit the replication of JC virus and interfere with expression of virally encoded genes (19). Similarly, IFN γ is able to suppress BK virus gene expression (20). Indeed, both publications demonstrate a down-regulation of polyomavirus LTA expression upon IFN treatment. Moreover, IFNs have been demonstrated to induce the expression of promyelocytic leukemia protein (PML); PML is known to modulate infection of cells by JC virus via interaction with LTA encoded by the polyomavirus (21). Given the recently demonstrated oncogenic addiction of MCV+ MCC cell lines towards LTA expression, IFNs appear as a promising therapeutic option for MCC. Indeed, Krasagakis and colleagues have already demonstrated sensitivity of a merkel cell carcinoma cell line towards IFN alpha (22). Furthermore, the clinical activity of IFN in MCC is demonstrated in anecdotal reports (23, 24). Here, we studied the impact of different types of Interferon, i.e. type I Multiferon ((MFN) a mix of 5 IFN α subtypes), IFN β-1a as well
as type II IFN γ, on MCV+ and MCV- MCC cell lines both in vitro and in vivo, revealing a striking effect of type I IFNs on the viability of MCV+ MCC cells.
Materials and Methods

Ethics statement. The presented work was conducted according to the principles expressed in the Declaration of Helsinki. The generation and characterization of MCC cell lines was approved by the Institutional Review Board of University Hospital Würzburg (sequential study number 124/05). All the animal experiments were approved by the local authorities (Regierung von Unterfranken; animal experiment request Az. 55.2-2531.01-59/06) according to the legal requirements.

Cell culture. The MCV+ cell lines WaGa, Broli, MKL-1 and MKL2 as well as the MCV- MCC cell lines UISO, MCC13 and MCC26 (25-27) have been described previously. For doxycyclin inducible T antigen knockdown, retroviral infected MKL-1 piH TA tet, MKL-2 piH TA tet and WaGa piH TA tet cell lines (9) were used. All cell lines were grown in RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma Aldrich, München, Germany).

Animal experiments. 5 week old female NOD.CB17/Prkdc<sup>scid</sup> mice were obtained from Harlan Winkelmann (Rossdorf, Germany), and housed under specific pathogen-free conditions. Tumours were induced by s.c. injection of 5*10<sup>6</sup> cells/100μl mixed 1:1 with MatriGel<sup>TM</sup> Matrix (Becton Dickinson, Heidelberg, Germany) into the lateral flank of the mice. MFN (Swedish Orphan, Stockholm, Sweden) treatment was started ten days after cellular injection. 10.000 Units of MFN in 50 μL Phosphat-buffered saline (PBS, PAN Biotech) or 50 μL PBS were injected i.t. every day (n=6 for each group) as previously described (28).
Immunoblotting.

Cell lysates of MCV+ MCC cell lines cultured for 4 days in 24 well plate at 1*10^6 cells per well with 50.000 Units/ml MFN; or IFN β (PeproTech, Hamburg, Germany) or 10.000 Units/ml IFN γ (PeproTech) were generated as previously described (9). After SDS-polyacrylamide gel electrophoresis, samples were transferred to nitrocellulose membranes (GE Healthcare, München, Germany), blocked 1 h with PBS (Sigma Aldrich) containing 0.05% Tween 20 (PBS-T) supplemented with 5% powdered skim milk and then incubated overnight with a primary antibody. Following three washing steps with PBS-T, membranes were incubated with a peroxidase-coupled secondary antibody (DAKO, Hamburg, Germany) followed by use of the Plus-ECL chemiluminescence detection kit (Thermo scientific, Rockford, Illinois, USA). Antibodies used were CM2B4 (1:1000) for MCV LTA protein, H-238 rabbit polyclonal antibody (1:200) for PML (both Santa Cruz Biotechnologies, Heidelberg, Germany) and TUB 2.1 (1:2500; Sigma Aldrich) for β-Tubulin.

MTS assay. Cell proliferation, metabolism and viability was measured with the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium] cell assay (Promega, Mannheim, Germany) according to the manufacturer’s instructions. MCC cell lines were cultured in triplicates at 1000 (MCC 13, MCC26), 3000 (UISO), 10.000 (WaGa) or 80.000 (BroLi, MKL-1, MKL-2) cells per well with 0, 781, 3125, 12500 or 50.000 Units/ml of type I or 0, 156, 625, 2500 and 10.000 Units/ml of type II IFN for 7 days. Cell proliferation of MCV+ cells upon T antigen knockdown was determined by culture of the respective cells for 5 days in the presence of 1μg/ml doxycyclin (Sigma Aldrich).
**Cell cycle analysis.** For cell cycle analysis $5 \times 10^5$ (MCC13, MCC 26, UISO) or $1 \times 10^6$ (MKL-1, MKL-2 WaGa, BroLi) cells per well were cultured for 7 days with 50000 Units/ml MFN or IFN $\beta$ or 10000 Units/ml IFN $\gamma$. Single cell suspensions were fixed with 5 ml of ice-cold ethanol (100%) overnight at 4°C; cell pellets were resuspended in 1 ml PBS supplemented with 1% FCS, 0.05 mg/ml propidium iodide (PI; Sigma Aldrich), and 0.1 mg/ml RNase A (Fermentas, St. Leon-Rot, Germany) and incubated for 1 h at 37°C. Flow cytometry was performed on a FACSCanto flow cytometer and analysis made with FlowJo analysis software (Tree Star, Inc., Ashland, USA).

**Immunohistochemistry (IHC) and immunofluorescence (IF).** IHC on formalin fixed and paraffin-embedded tissue was performed as previously described (9). For antigen recovery, de-paraffinized sections were incubated with DAKO Target Retrieval Solution (DAKO), pH 9.0 for 40 min at 90°C and rinsed twice with bidistilled water and once with PBS, incubated with blocking solution (DAKO) at room temperature and after two additional washes stained overnight with a rabbit monoclonal antibody specific for cleaved caspase 3 (D178;1:2000; Cell Signaling, Boston, USA). Detection of the antibody was performed with Dako Envision-HRP (DAKO) and Nova Red Substrate Kit (Vector Laboratories, Orton Southgate, UK) following the manufacturer’s protocol. To demonstrate possible interaction of PML and LTA the Duolink system was used according to the manufacture’s protocol (Olink Bioscience, Uppsala, Sweden). For microscopy a Leica DM750 microscope with ICC50 digital microscope camera (Leica, Heerbrugg, Switzerland) was used. For IF, cytospins of $6 \times 10^4$ WaGa cells cultured with 3500 Units MFN/ml for 3 days or of untreated cells, respectively were fixed in acetone for 10 min, rinsed with PBS and incubated with blocking solution (DAKO) for another 10 min. After another washing step, cells were stained first with PML H-238 rabbit polyclonal antibody (1:100; Santa
Cruz Biotechnologies) and with Cy3 labelled goat anti-rabbit IgG (H+L) secondary antibody (1:200; Dianova, Hamburg, Germany) each for 45 min. Slides were mounted with Vectashield with Dapi (Vector Laboratories) and analyzed with TCS SP2 confocal fluorescence microscope (Leica).

**Statistical Analysis.** Statistical analysis was performed with Prism 5.03 (GraphPad Software, Inc., San Diego, USA). The Wilcoxon test was applied to test sensitivity of MCV+ and MCV- cell lines to treatment with IFNs, the antiproliferative effect of Type I and Type II IFNs between MCV+ and MCV- cell lines and differences in the viability of MCV+ cells with T antigen knockdown compared with untreated controls. All analyses of IFN effect were performed for the highest IFN dosis used. Furthermore, t tests were performed to compare tumour volumes of WaGa xenografts in the *in vivo* experiments after the Kolmogorov-Smirnov test confirmed Gaussian distribution of tumor volumes.
Results

IFNs inhibit proliferation and viability of MCC cells

In a first series of experiments we tested the effect of MFN, IFN β and IFN γ on the proliferation, metabolism and viability of 4 MCV+ and 3 MCV- MCC cell lines using the MTS cell proliferation assay. These assays revealed on one hand that the MCV+ MCC cell lines are much more sensitive to IFN treatment (Wilcoxon Test: p=0.0039) and on the other hand that type I IFNs have a more pronounced effect (Wilcoxon Test: p=0.0313) than IFN γ (p=0.25) (Fig. 1). The strongest inhibition was observed for MFN and IFN β on MCV+ cell lines; notably, two of the MCV- MCC cell lines appeared insensitive to all IFNs. The effect of IFN γ was at best very weak and independent of the viral status of the cell line (Wilcoxon Test: p=0.25) (Fig. 1).

Type I and II IFNs variably induce apoptosis in MCC cells

To define the mechanisms of the inhibitory effects of the IFNs on MCC cell lines and to explore possible differences in MCV+ and MCV- cell lines, cell cycle analyses for all 7 MCC cell lines treated with the different IFNs in comparison to untreated controls were performed (Fig. 2). These analyses revealed that most of the cells lines, which displayed impaired proliferation and viability upon type I IFNs in the previous series of experiments, are characterized by an increase of the fraction of cells in the subG0 phase suggesting apoptotic cells death. However, this notion did not hold true for the MCV+ MCC cell line MKL-1. It should be further noticed that already in the absence of IFNs BroLi is characterized by a high frequency of cells in subG0 phase. More important, however, IFN γ did not affect the cell cycle distribution of any of the MCC cell lines tested.
Downregulation of T-antigens by IFNs

Expression of MCV LTA is necessary for the maintenance of MCV+ MCC cell lines (8, 9). We confirmed this notion by use of a doxycyclin inducible expression of shRNAs against MCV T antigens in MKL-1, MKL-2 and WaGa cell lines. Silencing of T antigen expression results in a clear reduction of cellular viability after 5 days compared to untreated control group (p=0.031; Fig. 3). Consequently, we next tested if the observed effects of type I IFNs on MCV+ MCC cell lines were mediated by a down regulation of LTA expression. Determination of protein expression of MCV LTA in MCC cell lines after seven days of incubation with type I and type II IFNs revealed a decrease in LTA expression in response to treatment with type I IFNs for all cell lines analysed; this downregulation is particularly pronounced for MFN (Fig. 4a). In contrast, incubation with IFN γ leads only in WaGa cells to a reduced expression level of MCV LTA.

Induction of the promyelocytic leukemia protein by IFNs

In addition to the negative regulation of the LTA expression, a number of proteins that interfere with the oncogenic proteins of viruses are regulated particularly by type I IFNs. The most prominent of these with respect to an impaired LTA function is the promyelocytic leukemia protein (PML). Indeed, we could demonstrate by Western blot analysis that PML is highly up-regulated in MCC cells upon treatment with IFNs (Fig. 4a). With the exception of WaGa cells, Type I IFNs cause a stronger PML induction than IFN γ. This induction of PML by type I IFN was further demonstrated by immunofluorescence of untreated or IFN treated WaGa cells. Here, a marked increase in the number of PML nuclear bodies was obvious (Fig 4b).
Anti-tumor activity of MFN against MCC in vivo

To translate these observations into the in vivo situation, we took advantage of recently established xenotransplantation mouse models for the MCV+ WaGa and the MCV- UISO cell lines. Therapy by intratumoral MFN injection was initiated ten days after inoculation of tumor cells. Subsequently, MFN was injected i.t. every day during the period of therapy. Upon MFN treatment the MCV+ WaGa derived xenografts not only stalled growth, but actually regressed whereas the controls injected with PBS alone progressed (Fig. 5a). Indeed, tumor volumes of MFN and PBS treated mice were significantly different at day 20 and at day 23, respectively (p=0.0004 and p<0.0001; unpaired T-test). In contrast, MCV-UISO xenografts did not alter their growth pattern as compared to those tumors injected with PBS alone. To elucidate the mechanism of impaired tumor growth in the MFN treated animals, we stained sections of the respective tumors for cleaved caspase 3 as a marker of apoptosis. Notably, MFN treatment results in a higher expression of cleaved caspase 3 in treated WaGa tumors (Fig. 5b); thus, the observed in vivo effects of MFN are not only due to an impaired proliferation, but also to an increased rate of apoptosis. This notion is in line with both with the in vitro findings as well as the active regression of the established tumors subsequent to MFN treatment. Our in vitro data suggests that PML might contribute to the regression of the tumors. In order to further expand this observation we performed duolink analysis to determine whether PML would interact with LTA; this technique generates positive signals only if the proteins are in close proximity (29). Analysis of MCV+ WaGa xenografts did indeed reveal positive signals primarily in the nucleus demonstrating a co-localisation of PML and LTA in WaGa cells (Fig. 5c). As a control, we could not observe such an interaction in MCV- UISO xenografts.
Discussion

Since their discovery in 1957 by Isaacs and Linderman (10), IFNs are regarded as drugs with a potential to treat cancer. Because of their ability to directly or indirectly interfere with the expression or function of oncogenic viral proteins, IFNs also seem to be particularly suitable to treat virally induced cancers. In the present report, we scrutinized the effects of type I and II IFNs on MCC, a highly aggressive skin cancer for which the viral oncogenesis has recently been indicated.

The impact of IFNs on MCC cells is characterized by an impaired proliferation, metabolism and viability particularly after type I IFN treatment; these effects were much more pronounced in MCV+ cell lines and largely associated with the induction of apoptosis. Notably, however, in the case of the MCV+ cell line MKL-1 the strong inhibiting effect of type I IFNs was not associated with the induction of apoptosis. Interestingly, a similar observation has been reported for human lung carcinoma cells (30). It should be further noted that the BroLi cell line harbors a significant proportion of apoptotic cells already in the untreated control group. This observation suggests that MCV infection itself may render cell prone to apoptosis. Indeed, MCC since its initial description by Toker (31) has been known for its high rate of apoptosis (32).

For several DNA viruses, including polyomaviruses, induction of apoptosis of the infected cell is part of the viral replication cycle ensuring the release of the virions. This host cell apoptosis is initiated by expression of the very late viral protein (33), which, however, has not been elucidated for MCV yet. It should be further noted, that specific miRNAs have been suggested to be involved in MCV virion release (34).

As mentioned above, recent reports described that IFN γ treatment interferes with LTA expression in BK virus infected cells (20) and that type I IFNs interfere with LTA expression in JC virus infected cells (19). These facts together with the profound effects of type I IFNs on the survival of MCV+ MCC cell lines and the recent
demonstration that MCV+ MCC lines critically depend on the expression of LTA (8, 9) prompted us to scrutinize the modulation of LTA expression of MCV+ MCC cells upon IFN treatment. This analysis revealed that type I IFNs, i.e. MFN and IFN β, strongly reduced the expression of MCV LTA. Interestingly, the strength of the effect of the different IFNs is not uniform for all MCV+ cell lines but rather individual, demonstrating the complexity of IFN signaling in general as well as in MCC.

Beside the direct inhibition of LTA expression, IFNs may also interfere indirectly with the function of the LTA. For example, the impact of a JC virus infection on human glial cells is reduced by an IFN-dependent induction of PML expression (21). This effect is based on a functional inhibition of LTA due to trapping of this protein within PML nuclear bodies via interaction the conserved LXCXE amino acid motif common to all viral oncoproteins that bind pRB (9, 35). In general, the antiviral activity of PML and PML nuclear bodies are well established (21, 36-38). As demonstrated here, IFN treatment strongly induces PML expression in MCV+ MCC cells; moreover, duolink immunohistochemistry of MCV+ WaGa xenotransplants revealed a co-localisation of PML and LTA. Thus, based on similar recent reports for other viruses (21, 35-38), upregulation of PML expression upon IFN treatment results in an increased interaction of both proteins and a thus a reduction of free LTA (21). These facts indicating that PML mediates the antiviral effect by sequestering viral and host proteins, that are indispensable for transcription of viral proteins. The effect of IFNs both on the expression as well as the functional activity of LTA is likely to explain both the robustness and the speed of the anti-MCC effect of IFNs that is actually faster and more pronounced than the genetic knock down of LTA (8). Importantly, the anti-tumor effect of the type I IFN MFN observed in vitro was also translated into the in vivo setting taking advantage of a newly established xenotransplantation model for the MCV+ WaGa cell line, but not the MCV- UISO cell line. Although we could detect
an antiproliferative effect of MFN on UISO cells in vitro, albeit to a lesser extent, there was no reduction in tumor growth of the in vivo xenotransplants. This observation might be due to the fact, that the UISO xenografts grew very rapidly and therefore the MFN dosage used might not be sufficient for a successful treatment. It should be noted, however, as this xenotransplantation model is based on severely immune deficient mice, immune modulating effects of IFNs could not be addressed.

The complexity of IFN signaling is reflected by the observation that the direct in vitro antiproliferative effect of IFNs - albeit less pronounced - is not restricted to MCV+ MCC cells. The fact that the MCV- UISO cell line is also affected by IFN suggests the involvement of additional mechanisms. For example, IFN α induces apoptosis in many kinds of cells by upregulation of tumor necrosis factor related-apoptosis inducing ligand (TRAIL). Interaction of TRAIL with its receptors results in a signal cascade which activates effector caspases such as cleaved caspase-3 (39). To this end, we observed an increased presence of activated caspase-3 in tumors of MFN treated mice harboring WaGa xenotransplants. Moreover, it has been demonstrated for myeloma cell lines that IFN α-induced apoptosis is at least in part mediated via PML by TRAIL induction (40). Thus, further experiments are warranted to elucidate the precise role of PML in the reduced viability of MCC cells after treatment with type I and II IFNs. Moreover, beside PML and TRAIL, there are a multitude of IFN stimulated genes, which are supposed to be involved in apoptotic cellular pathways that may explain the sensitivity of MCV- MCC cell lines to IFN treatment (reviewed in (41)) e.g. ISG54 (42) and USP18 (43) have recently been described as a mediator or regulator of IFN-induced apoptotic cell death. Still, MCC 13 and MCC 26 stayed nearly unaffected upon IFN treatment; a notion readily explained by the ability of cancer cells to become resistant to IFNs i.e. by overexpression of STAT5 as it has been previously shown for melanoma (44) or epigenetic silencing of genes involved
in IFN signaling (45, 46). Another report demonstrated the suppression of insulin-like growth factor-binding protein 7 (IGFBP7) in IFN α resistant hepatocellular carcinoma (HCC) cells (47). However, the crucial mechanism remains unknown. Further possible mechanisms for IFN resistance could be mediated by micro RNAs (miRNAs). Actually, it was recently described that sensitivity of HCC cells towards IFN α is regulated by miRNA-146a targeting the SMAD4 protein (48). All these reports illustrate the diversity of mechanisms underlying IFN resistance. Which of these multiple mechanisms mediates insensitivity in the two MCC cell lines has yet to be determined.

In summary the present work provides several lines of evidence that IFNs, particular type I IFNs, exert direct inhibitory effects on MCC cell lines *in vitro* and *in vivo*. Mechanistically, this effect seems largely due to an induction of apoptotic cell death. Treatment of MCC cell lines with the different IFNs inhibited the expression of virally encoded LTA and induced the expression of PML, which has been previously demonstrated to interfere with the functional activity of the LTA. Consequently, based on the here presented data as well as the well established immune modulating effects of IFNs such as reinduction of MHC class I molecules or the activation of immune competent cells, treatment of MCC with type I IFNs appears as a promising therapeutic option for MCC patients; a notion substantiated by several case reports on successful therapy of metastatic MCC lesions by localized type I IFN therapy; thus, these observations are advocating the investigation of especially type I IFNs for therapy of MCC in clinical trials.
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Figure legends

**Figure 1:** Type I and type II IFNs variably affect cellular proliferation of MCC cell lines. MTS based proliferation assay was used to determine the effect of IFN on different MCC cell lines. Depicted is the ratio of metabolic activity, i.e. measured extinction at 490 nm of the ATP dependent conversion of formazan, of MCV+ (A, C, E) and MCV- (B, D, F) cell lines subjected to the indicated concentrations of MFN (A,B), IFN β (C, D) or IFN γ (E, F) to medium. MFN and IFN β exert the strongest anti-proliferative effect on the MCV+ MCC cell lines MKL-1 (black line), MKL-2 (grey line), WaGa (black dashed) and BroLi (grey dashed). For IFN γ the effect on cellular proliferation is much less pronounced. For the MCV- MCC cell lines an anti-proliferative effect is only detectable for UISO (black dashed) treated with MFN and IFN β, whereas, MCC13 (grey line) and MCC26 (grey dashed) are resistant to IFN treatment.

**Figure 2:** Type I, but not type II IFNs, induce apoptosis in MCC cell lines. A-C, MKL-2 cell cycle analysis by propidium iodide staining after 7 day incubation with Multiferon (A), IFN β (B) or IFN γ (C). The histograms of the untreated control (grey) and of the respective IFN (black) are depicted. D-F, Percentage of cells in subG0 phase after treatment with Multiferon (D), IFN β (E) or IFN γ (F) (black bars) in comparison with the untreated control cells (white bars).

**Figure 3:** Silencing of the T antigens results in reduced viability of MCV+ MCC cells. 5 days after the doxycylin induced expression of shRNA against MCV T antigens in MCV+ MKL-1, MKL-2 and WaGa cell lines there is an explicit reduction in viability of the cells compared to the respective control groups without T antigen knockdown.
Figure 4: Treatment of MCV+ MCC cell lines with IFNs modulates the expression of MCV LTA and PML. A, After MCV+ MCC cell lines were cultured for seven days with IFNs, total cell lysates were analyzed for LTA expression by immunoblotting with antibody CM2B4. For all four cell lines there is a reduction in MCV LTA expression observable with the strongest effect for type I IFNs. Furthermore, an induction of PML could be detected in three of the four MCV+ MCC cell lines after treatment with IFNs. β-Tubulin served as internal loading control. B, Increased immunofluorescent detection of PML in MFN stimulated (3500 U) WaGa cells compared to control cells.

Figure 5: Application of MFN in xenografts of MCC cell lines results in reduced tumour growth for MCV+ WaGa but not for MCV- UISO cell lines. A, Treatment of MCV+ WaGa xenografts with MFN (n=6) reduces tumour growth in comparison with the PBS treated control group (n=6) with statistical significance after day 20 and 23 (unpaired t-test p=0.0004 after day 20 and p<0.0001 after day 23). In contrast, in MCV- UISO xenografts (for both groups n=6) no anti-tumour effect is detectable (unpaired t-test; p=0.9638). B, Cleaved caspase 3 immunohistochemistry of WaGa xenografts after i.t. injection of MFN and PBS as negative control demonstrate higher levels of activated caspase 3 in the MFN treated tumors in comparison with the PBS control. C, Duolink technology with antibodies reactive with PML and LTA reveal a co-localisation of these two proteins in MCV+ WaGa xenografts. This interaction is not present in UISO xenografts due to the lack of MCV LTA expression.
A

Tumor growth of in vivo tumors

relative change in tumor volume

days after cell injection

Waga / UISO

PBS control

Multiferon

*** ***

B

magnification 20x

PBS control

Multiferon

cleaved caspase 3

C

magnification 40x

UISO

WaGa

LTA & PML
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