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„*Anti-melanoma activity of IFN-α and Imiquimod: The role of TRAIL-induced apoptosis* “

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CONTENTS

1 INTRODUCTION

Malignant melanoma is a very aggressive form of skin cancer that often is refractory to standard treatments such as chemo- and radiotherapy. To this day, surgical resection of the primary tumor at an early stage is the only curative treatment for this disease. Therapeutic strategies aiming to activate the patient´s immune system to eliminate malignant cells may provide the basis for a more effective melanoma treatment.[1]

Immunoadjuvant therapy with Interferon-α (IFN-α) and topical treatment with the synthetic TLR 7/8 ligand Imiquimod (IMQ) have been shown to be clinically effective in melanoma patients.[2-5] Direct anti-tumor activity as well as stimulation of the immune system are thought to contribute to the efficacy of both agents.[6-11] The induction of the cytotoxic molecule tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), particularly on plasmacytoid dendritic cells (pDCs), might play an important role in IFN-α and IMQ-mediated melanoma containment.[12-14] Both in man and mice, the regression of cutaneous tumors upon topical IMQ-treatment was associated with an infiltrate of pDCs expressing TRAIL.[12, 13] Since IMQ-induced TRAIL expression was shown to be dependent on IFN-α *in vitro*, $TRAIL^+$ pDCs could also play a role in melanoma patients treated with IFN-α.[14]

In this diploma thesis, the role of TRAIL in immuno-adjuvant therapy of melanoma with IFN-α or IMQ was studied *in vitro* with a focus on TRAIL-mediated cytotoxic effector functions of pDCs.

We were particularly interested in:

1) Direct apoptotic and antiproliferative effects of IFN-α on melanoma cells.
2) Susceptibility of different melanoma cell lines to TRAIL and the correlation of TRAIL-sensitivity with TRAIL receptor (TRAIL R) expression.
3) Whether IMQ- and IFNA-activated pDCs acquire TRAIL-dependent cytotoxic effector functions *in vitro*.

In chapter 2 of this diploma thesis the literature relevant to this study is reviewed. It contains information on i) malignant melanoma and current therapeutic options, ii) the function of IFN-α and IMQ and their role in the treatment of malignant melanoma, iii) the cytotoxic molecule TRAIL and iv) dendritic cell biology with a focus on pDCs. Chapter 3 provides a detailed description of the materials and methods used in this study. We first characterized established human melanoma cell lines and such generated from stage IV melanoma patients with respect to their TRAIL and TRAIL R expression using flow cytometry and real-time PCR. The antiproliferative properties of IFN-α on melanoma cells were assessed using 3H-Thymidine incorporation and the apoptotic effects of TRAIL and IFN-α were studied using Annexin V/PI staining. The effect of IMQ or IFN-α-pretreatment on TRAIL-induced apoptosis in melanoma cells was also investigated. Finally, the cytotoxic potential of IFN-α and IMQ-activated, TRAIL+ pDCs against melanoma targets were assessed. The results from our research are presented in chapter 4 and conclusions are drawn in chapter 5.

Our findings implicate that, in addition to other immunostimulatory effects, the induction of TRAIL on immune cells could play a role in IFN-α and IMQ-mediated melanoma containment. Particularly pDCs may acquire important TRAIL-dependent cytotoxic functions in both therapeutic settings.

2 BACKGROUND

2.1 Malignant melanoma

Malignant melanoma is a neoplasm of melanocytes, the cells responsible for skin pigmentation. Although melanoma accounts for only 4% of skin cancer incidences, it is responsible for over 70% of deaths from skin cancer.[15] The incidence of melanoma in developed countries is approximately 9 per 100,000 in men and women. Since the 1980s this number has increased dramatically but mortality has not (Figure 1).[16] This is most probably due to earlier detection and surgical resection of the primary tumor rather than improved therapeutic options.

This chapter will provide an overview of melanoma development from benign melanocytes to metastatic melanoma and clinical melanoma staging. Further, therapeutic strategies against this disease will be discussed.

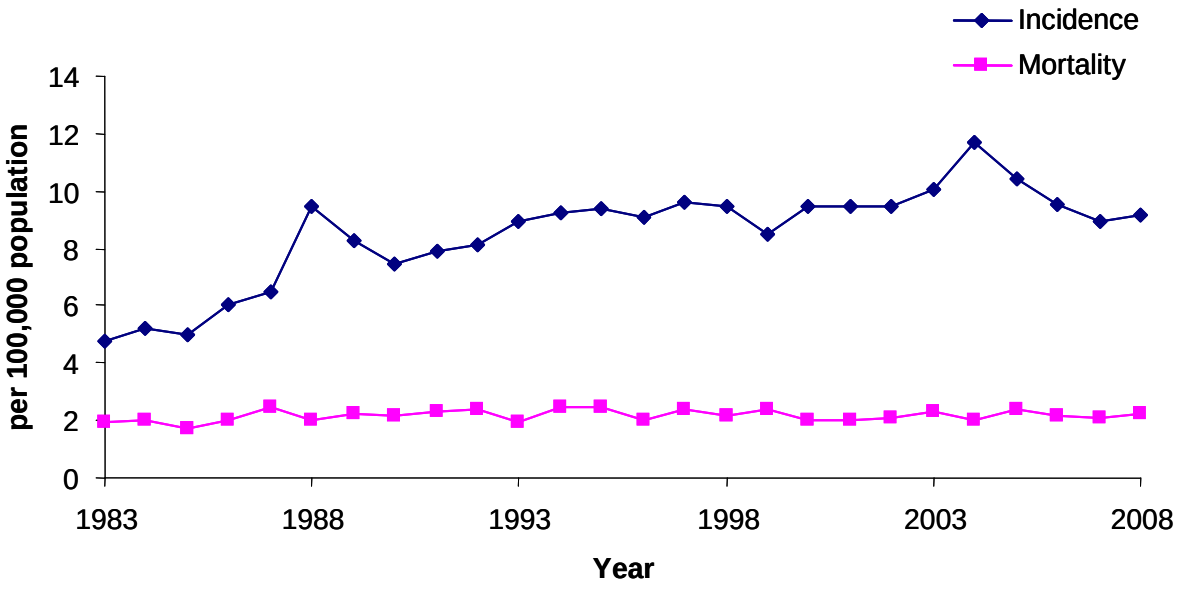

Figure 1: Melanoma incidence and mortality in Austria 1983-2008.[16]

2.1.1 Melanocytes

Melanocytes reside on the basement membrane of the epidermis but are also found in other organs like the eye, ear, leptomeninges, bones and heart.[17] Their main function is the production of the pigment melanin and its delivery to nearby keratinocytes where it absorbs UV radiation to shield the cellular DNA from UV-induced damage.[18] Skin tanning is a consequence of this mechanism, as melanin synthesis is particularly enhanced in response to UV exposure.

In contrast to keratinocytes, the predominant cell type in the epidermis, melanocytes are low in numbers (one melanocyte per 30-40 keratinocytes),[19] long-lived, and have a low proliferation potential.[18] To ensure melanocyte survival, they are endowed with antiapoptotic mechanisms such as the constitutive expression of the antiapoptotic protein Bcl-2.[20] Due to their longevity and their relative resistance to apoptosis melanocytes are prone to accumulate mutations over the years, potentially culminating in their malignant transformation to melanoma.[18]

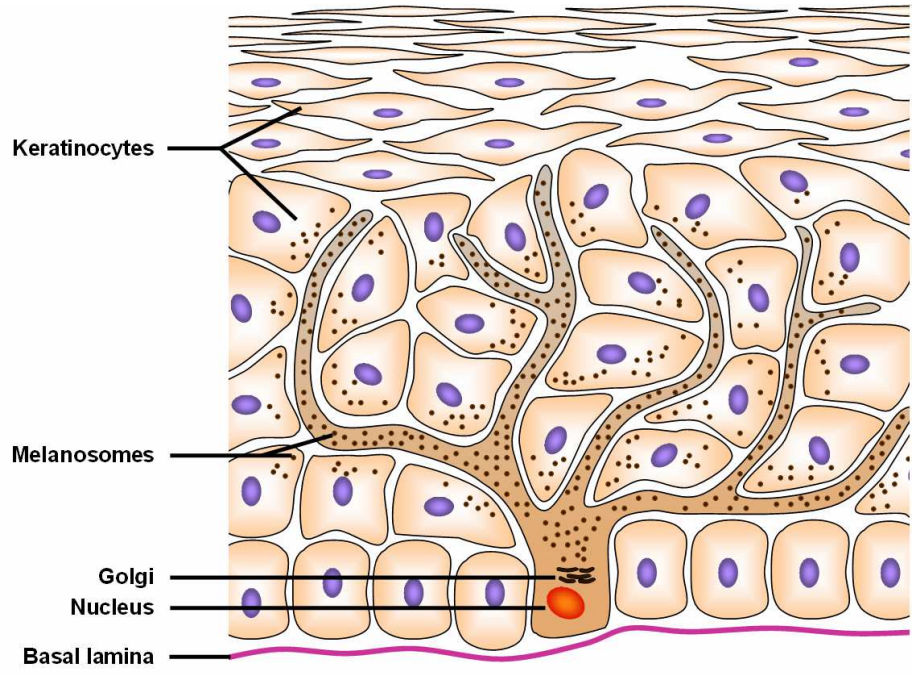

Figure 2: A melanocyte in its epidermal environment. The melanocyte is localized in the basal cell layer of the epidermis. Melanin-containing vesicles (melanosomes) are formed in the golgi apparatus and transported to keratinocytes via dendritic protrusions.

2.1.2 Incidence and risk factors

The greatest external risk factor for melanoma development is UV exposure. Melanoma incidence is higher in countries with high UV exposure. It is also associated with UV-sensitive skin types and the occurrence of severe sunburns, particularly during infancy and puberty. Whereas the risk of developing melanoma at UV-unexposed regions of the body (palms, soles, mucosa) is rare and similar in all ethnicities, caucasians bear a much higher predisposition toward the development of melanoma at other sites of the body. [21]

For the development of melanoma, a series of genetic lesions are required which usually take years to accumulate. Therefore, the incidence in children and young adults is rather low and it increases with age. In some families, there is an increased occurrence of melanoma. Members of these families often carry mutations in genes that regulate the cell cycle. A defect in CDKN2A, a gene that is involved in cell cycle control, is found in 25-40% of melanoma-prone families.[22]

Another risk factor for melanoma are multiple and atypic nevi. For patients with a history of melanoma the chance of developing a second primary tumor is greatly elevated (about 3% of patients develop a second melanoma within 10 years of resection of the first tumor). [1]

Regular dermatologic screenings are the most effective measure against melanoma. Depending on the number of nevi and other risk factors, examinations every 3-12 months are recommended.

2.1.3 Oncogenic transformation: The Clark model of melanoma progression

Melanoma is a polygenic disease that requires the progressive acquisition of multiple mutations in genes involved in cell cycle control, DNA repair and apoptosis (Figure 3). These genetic lesions can be related to histologic and phenotypic changes that are described in the Clark model of melanoma progression.[23] According to this model, the first event is the formation of a benign nevus. Neval melanocytes display abberant growth, often due to deregulation of the mitogen-activated protein kinase (MAPK) pathway (e.g. constant activation of BRAF in up to 82% of benign nevi).[24] Cell growth, although increased, is still limited in benign nevi, probably due to oncogene-induced senescence.[25] Such lesions are found on the skin of every person and rarely progress to cancer.

The development of a dysplastic nevus, either arising from an existing benign nevus or at a new site on the skin is the next step toward oncogenic transformation. It is marked not only by aberrant cell growth but also by defects in DNA repair and apoptotic pathways. In dysplastic nevi, the cell cycle control gene CDKN2A and PTEN, a gene involved in growth control and regulation of apoptosis, are often disrupted.[22]

During the next step, the radial growth phase, the cells proliferate mainly intraepidermally but a few cells can invade the papillary dermis.

The transition from radial to vertical growth phase is characterized by alterations in the expression of cell adhesion molecules (integrins and cadherins) causing increased motility and reduced adhesion to epidermal fibroblasts. This gives the cells the ability to further colonize the dermis and form expansive tumor nodules.

Integrins are a family of cell surface receptors that mediate cell attachment to the extracellular matrix (ECM) and transduce signals affecting proliferation and migration.[26] Alterations in integrin expression can increase melanoma cell motility through reorganization of the cytoskeleton and stimulation of the expression of matrix metalloproteinase 2 (MMP2), which degrades collagen in the basement membrane.[22]

Cadherins are a class of membrane proteins with an important role in cell adhesion. E-cadherin (epithelial) is expressed by epithelial cells like melanocytes and keratinocytes and mediates adhesion between these cell types. Downregulation of E-cadherin and switch to N-cadherin (neural) expression endows melanoma cells with the ability to form contacts with dermal fibroblast and other mesenchymal cells, facilitating the establishment of metastases.[27]

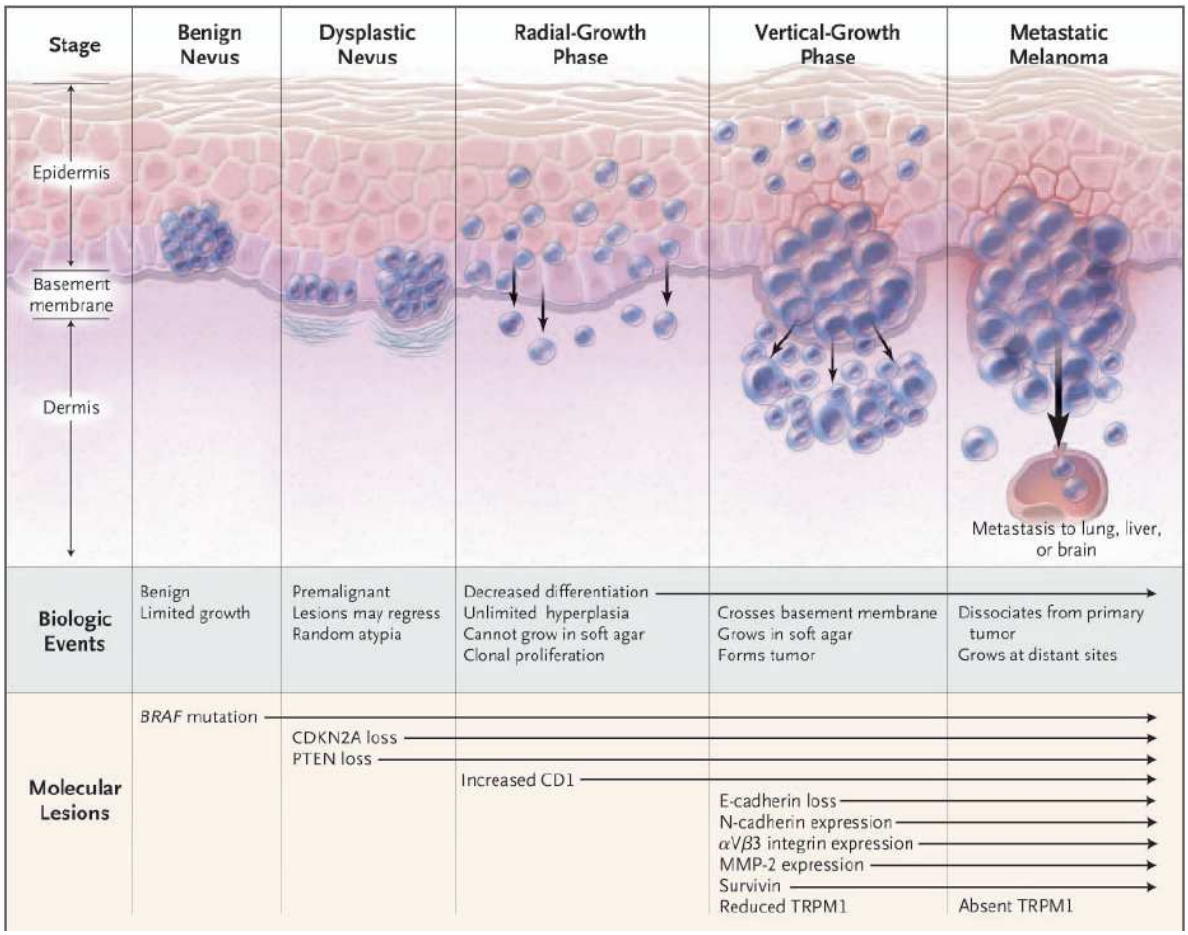

Figure 3: Model of melanoma progression and associated genetic lesions. Figure taken from Miller et al., 2006[22]

The final step, metastasis formation, is marked by spread to and successful colonization of other sites of the skin, draining lymph nodes (LNs) and finally distant organs by melanoma cells. Early metastases usually spread through the lymphatic system and occur either close to the primary tumor (satellite metastases, ≤2 cm from primary tumor), in the skin between 2 cm from the primary tumor and the sentinel LN (in transit metastases) or in the sentinel LN (LN metastases). The formation of distant metastases is mainly caused by the spread of tumor cells through blood vessels. The three most common sites for distant melanoma metastases are, in descending order, lungs, liver and brain.[28]

2.1.4 Clinical aspects of melanoma

In an effort to provide universal guidelines for the estimation of disease severity in cancer patients, the American Joint Committee on Cancer (AJCC) developed a Cancer Staging Manual, which is continually refined and updated since 1977.[29] The manual is based on the tumor-node-metastasis (TNM) classification system using prognostic markers to categorize melanoma patients to four different stages of disease (Table 1, Table 2).[30]

The T categories T1-4 are defined by the thickness of the tumor. The presence or absence of ulceration further divides the categories in subgroups (a: no ulceration, b: with ulceration). Melanoma ulceration is strongly connected with decreased patient survival. In fact, ulceration of the primary tumor is associated with survival rates similar to those of a patient with a non-ulcerated tumor of the next highest T category (Table 2).[30] The number of metastases in the regional LNs and in transit (in lymphatic vessel) or satellite metastases is the main criterion for the N classification. The three main categories N1-3 are further divided into subgroups a-c by the size and type of the metastases. The M classification groups distant metastases according to their location and the associated survival rates.

A staging system has been deduced from the TNM classification, grouping patients with localized melanoma and no evidence of metastasis to stage I and II, those with regional metastases to stage III and those with distant metastases to stage IV. According to the corresponding survival rates the stages are further divided into subgroups A-C (Table 2). Generally, there is a negative correlation between tumor stage and patient survival. Ulcerated primary tumors thicker than 4mm exhibit a particularly high risk for metastasis and, due to their aggressiveness, are associated with survival rates even poorer than those of stage III melanoma (Table 2).

Table 1: Melanoma TNM classification (adapted from Balch et al., 2001 and the AJCC cancer staging manual 7th edition)[29-31]

T classification	Thickness	Ulceration status
T0	No evidence of primary tumor	
Tx	Primary tumor cannot be assessed (e.g.: curettaged, regressed)	
T1	≤ 1.0 mm	a: w/o ulceration b: with ulceration
T2	1.01-2.0 mm	a: w/o ulceration b: with ulceration
T3	2.01-4.0 mm	a: w/o ulceration b: with ulceration
T4	> 4.0 mm	a: w/o ulceration b: with ulceration
N classification	**No. of metastatic nodes**	**Nodal metastatic mass**
N0	No evidence of local metastasis	
Nx	Regional nodes cannot be assessed	
N1	1 node	a: micrometastasis b: macrometastasis
N2	2-3 nodes	a: micrometastasis b: macrometastasis c: in transit/satellite metastases w/o metastatic nodes
N3	≥ 4 nodes or matted nodes or in transit/satellite metastases with metastatic nodes	
M classification	**Site**	**Serum lactate dehydrogenase**
M0	No evidence of distant metastasis	Normal
M1a	Distant skin, subcutaneous, nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	Other visceral metastases Distant metastases	Normal Elevated

Table 2: Melanoma TNM staging and 10-year survival rates (adapted from Balch et al., 2001)[30]

stage		T	N	M	10-year survival (+/- SD)
I	A	T1a	N0	M0	87.9 +/- 1.0
	B	T1b	N0	M0	83.1 +/- 1.5
		T2a	N0	M0	79.2 +/- 1.1
II	A	T2b	N0	M0	64.4 +/- 2.2
		T3a	N0	M0	63.8 +/- 1.7
	B	T3b	N0	M0	50.8 +/- 1.7
		T4a	N0	M0	53.9 +/- 3.3
	C	T4b	N0	M0	32.3 +/- 2.1
III	A	T1-4a	N1a	M0	63.0 +/- 4.4
		T1-4a	N2a	M0	56.9 +/- 6.8
	B	T1-4b	N1a	M0	37.8 +/- 4.8
		T1-4b	N2a	M0	35.9 +/- 7.2
		T1-4a	N1b	M0	47.7 +/- 5.8
		T1-4a	N2b	M0	39.2 +/- 5.8
		T1-4a/b	N2c	M0	-
	C	Any	N1b	M0	24.4 +/- 5.3
			N2b	M0	15.0 +/- 3.9
			N3	M0	18.4 +/- 2.5
IV		Any	Any	M1a	15.7 +/- 2.9
				M1b	2.5 +/- 1.5
				M1c	6.0 +/- 0.9

2.1.5 Melanoma therapy

The primary treatment for melanoma is surgical excision of all tumor material (primary tumor and metastases). For patients with resected stage I melanoma this treatment is often curative (80%-90% of patients with stage I melanoma survive for 10 years). To ensure complete removal of the primary tumor, it has to be removed with adequate surgical margins (Table 3).

Table 3: Recommended excision margins for melanoma

Tumor thickness	Excision margin
In situ (tumor has not penetrated basement membrane)	0.5 cm
< 2 mm	1 cm
> 2 mm	2 cm

Sentinel LN dissection for the detection of micrometastases may be performed in patients with primary tumors that display poor prognostic factors (tumor thickness > 1mm, ulceration, vertical growth). When micrometastases are found in the sentinel LN specimen, radical LN dissection is recommended since 5-12% of affected patients have involvement of non-sentinel nodes. Radical LN dissection is also standard therapy if LN macrometastases are identified clinically.[1]

Although surgery is the preferential treatment for melanoma, complete removal is not always possible due to extension and localization of the tumor/metastases or the patient´s condition. In this case primary tumors of the lentigo maligna type, regional LN metastases and skin metastases may also be controlled by radiation therapy. Further, radiation therapy has an important role in the palliation of bone and brain metastases.[1]

Chemotherapy with cytotoxic agents is used primarily in a palliative mode, aiming at prolongation of patient survival and the reduction of tumor burden to ease symptoms in stage IV melanoma patients with little chance for cure. With dacarbazine (DITC), the best established monotherapy agent, objective remission (>50% reduction in tumor mass) can be achieved in 5.3-28.6% of patients. Comparable remission rates can be achieved with both IFN-α and IL-2.[1] The combination of different cytotoxic agents (polychemotherapy) and the combination of cytotoxic agents and cytokines (chemoimmunotherapy) could delay the development of drug resistance. These combination therapies have been shown to increase the response rate but at the cost of higher toxicity. It also appears that they do not significantly affect overall survival.[1]

Spontaneous tumor regression has been observed more frequently in melanoma than in most other cancers.[32-35] This indicates that melanocytic malignancies may be particularly susceptible to effector mechanisms of the immune system. Therapies aiming at enhancement of anti-melanoma immunity could therefore be effective against this type of cancer.

Patients with no evidence of metastases but at high risk for tumor spread (stage II and stage III) may benefit from adjuvant therapy. IFN-α (Roferon®-A, IntronA®) can be given as an adjuvant to stage IIB and stage III melanoma patients after resection of the primary tumor (Table 4). A meta-analysis of 14 randomized controlled trials assessing the efficacy of adjuvant IFN-α in high risk stage II and III melanoma patients revealed a statistically significant benefit of IFN-α on disease-free and overall survival.[5] As some studies indicate, this effect may be more pronounced in patients receiving high-dose IFN-α.[36, 37]

Another cytokine, Interleukin-2 (IL-2), has been shown to be beneficial for patients with metastatic melanoma. An analysis of eight clinical trials between 1985 and 1993, involving 270 patients with metastatic melanoma receiving high-dose IL-2, found that 16% of the patients responded to this treatment.[38] IL-2 was approved by the FDA for the therapy of metastatic melanoma in 1998. Studies using a variety of other cytokines and immunostimulatory agents (Bacille Calmette Guerin – BCG, IFN-γ) in an adjuvant setting have shown no significant efficacy.[1]

Table 4: **Dosage schedules for adjuvant therapy of melanoma with interferon-α.** (s.c.: subcutaneous; i.v: intravenous, adapted from Garbe et al., 2010)[1]

Schedule	Dose	Frequency	Duration	Indication
Low dose	3 MIU (s.c.)	Days 1, 3, and 5, every week	18 months	Stages II-III
High dose				
Initiation	20 million IU/m^2 intravenous (i.v.) rapid infusion	Days 1–5 every week	4 weeks	Stage III
Maintenance	10 million IU/m^2 subcutaneous (s.c.)	Days 1, 3 and 5 every week	11 months	Stage III

In certain patients, cutaneous melanoma metastases and lentigo maligna melanoma have also been shown to respond to topical treatment with IMQ, a synthetic TLR 7/8 agonist.[3, 4]

Antibodies that block inhibitory immunological pathways may also enhance anti-melanoma immunity. Ipilimumab, an antibody targetet at Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) has been shown to improve overall survival of patients with metastatic melanoma and has recently been approved by the FDA for the treatment of late-stage melanoma.[39, 40]

There is also strong evidence that the transfer of *ex vivo* expanded tumor-infiltrating T cells can induce regression of melanoma, probably via $CD8^+$ T cell-mediated cytotoxicity.[41-43]

A new class of drugs, specifically targeting products from genes that are frequently mutated in melanoma such as BRAF and NRAS, has been shown in clinical trials to significantly improve progression-free survival when compared to DITC (for a review, see Eggermont et al., 2011).[44] These substances may be of great value in the future of melanoma therapy but additional strategies will be needed to successfully treat advanced melanoma.

2.2 Interferon-α

Type I IFNs were first described in 1957 by Isaacs and Lindenmann for their ability to interfere with viral replication in cell culture.[45] Later, it was discovered that they have a very important role in both antiviral and anticancer immunity, due to their ability to inhibit cell growth and induce apoptosis in malignant and virally infected cells and their anti-angiogenic and immune-stimulatory properties.[7, 9, 46]
Interferon-α (IFN-α) is used as an adjuvant treatment in high-risk stage IIB and stage III melanoma patients after surgical resection of the primary tumor (see 2.1.5). It is a member of the type I IFN family, a group of cytokines with great stuctural homology. All type I IFNs are encoded by a single gene cluster on chromosome 9 in humans and chromosome 4 in mice.[47, 48] In humans they include 13 subtypes of IFN-α (IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21), IFN-β, IFN-ε, IFN-κ and IFN-ω.[47] They all are induced by similar mechanisms and trigger similar but not identical responses, depending on their differential affinities to the subunits of their common receptor.[49]

This chapter will provide a review of type I IFN signaling. Since the present study focuses on the role of IFN-α in melanoma therapy, the anti-cancer activity of this cytokine and its role as an adjuvant in melanoma therapy will be discussed in depth.

2.2.1 The type I IFN signaling network

Most cell types can produce type I IFN when properly stimulated. In immune cells, induction of type I IFN expression requires the engagement of immunological pattern recognition receptors (PRR), such as toll like receptors (TLRs) 3, 4, 7, 8 and 9.[50, 51] Somatic cells mainly produce type I IFN in response to viral infection. They sense viral nucleic acids via cytoplasmatic sensors such as DAI and RIG-1.[52, 53] Sensing of viral nucleic acids is followed by the phosphorylation of interferon regulatory factors (IRFs).[50-55] These transcription factors regulate the expression of the IFN genes.
Most cell types constitutively express IRF-3 which, upon activation, translocates into the nucleus and induces the transcription of IFN-β (Figure 4). Via a positive feedback loop active type I IFN signaling then induces the production of IRF-7, the transcription factor required for efficient IFN-α expression and for boosting the expression of IFN-β.[56] PDCs, however, constitutively express IRF-7 which allows for the rapid

production of large amounts of IFN-α upon recognition of viral nucleic acids via TLR 7 and 9 (see 2.5.2.1).

The most thoroughly studied pathway induced by type I IFNs is the JAK-STAT pathway. It involves the activation of gene expression by signal transducers and activators of transcription (STATs) upon Janus family tyrosine kinase (JAK)-mediated phosphorylation.[57, 58] All type I IFNs signal via the IFN-α receptor (IFNAR) on the cell surface, a heterodimer formed by two peptide chains, IFNAR1 and IFNAR2.[47] The intracellular domains of the receptor subunits are associated with the Janus kinases (JAKs) Tyk2 (IFNAR1) and Jak1 (IFNAR2). IFN-α binding leads to heterodimerization of IFNAR1 and IFNAR2 and cross-phosphorylation of Jak1 and Tyk2.[59] This activates the kinase activity of Jak1 and Tyk2 which then phosphorylate the receptor subunits to provide a docking platform for SH2-domain-containing STAT proteins.[59, 60] These proteins are also phosphorylated by Jak1 and Tyk2, which allows them to form homo- or heterodimers and translocate into the nucleus where they act as transcriptional activators on genes containing an IFN-γ-activated site (GAS) in their promoter region.[61] Another important transcription factor formed by activated STATs is the IFN-stimulated gene factor 3 (ISGF3). This complex is formed by a STAT1/2 heterodimer and IRF9. It activates the transcription of genes controlled by an interferon-stimulated response element (ISRE).[62]

Other pathways required for full transcriptional activation of interferon-stimulated genes (ISGs) which are induced by IFN-α include the phosphatidyl-inositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) pathways (Figure 4, right side).[61, 63]

Most somatic cells express IFNAR,[64] but they respond to IFN signaling in very different ways. Depending on the cell type and additional factors, such as signals from other immunological pathways, ISGs are differentially activated. Thus, IFN-α can elicit various effects, including inhibition of proliferation and induction of apoptosis in malignant and virally infected cells as well as stimulation of innate and adaptive immunity (Table 5, Figure 4).[9, 45, 46, 65-73]

Table 5: Effects of type I IFNs

Target	Type	Source
Anti-Tumor	Inhibition of proliferation	Sangfelt et al., 1997[7] Grimley et al., 1998[74]
	Induction of apoptosis	Thyrell et al., 2002, 2004[8, 75] Sangfelt et al., 1997[7]
	Sensitization to apoptosis-inducing drugs	Sabaawy et al., 1999[76]
	Antiangiogenic	Sidky et al., 1987[46] Indraccolo et al., 2010[77]
	Increased MHC class I expression	Makridis et al., 1994[78] Yang et al., 2004[79]
Antiviral	Induction of apoptosis in infected cells	Tanaka et al., 1998[71]
	Increased MHC class I expression	Makridis et al., 1994[78] Yang et al., 2004[79]
	Inhibition of viral life cycle	Isaacs et al., 1957[45] Der et al., 1995[73]
Stimulation of immunity	NK cell activation; DC differentiation, maturation and migration; enhanced phagocytosis by macrophages; B cell isotype switching; promotion of T_H1-response; Promotion of $CD8^+$ T cell effector function and survival	Hervas-Stubbs et al., 2011[9] (review article)

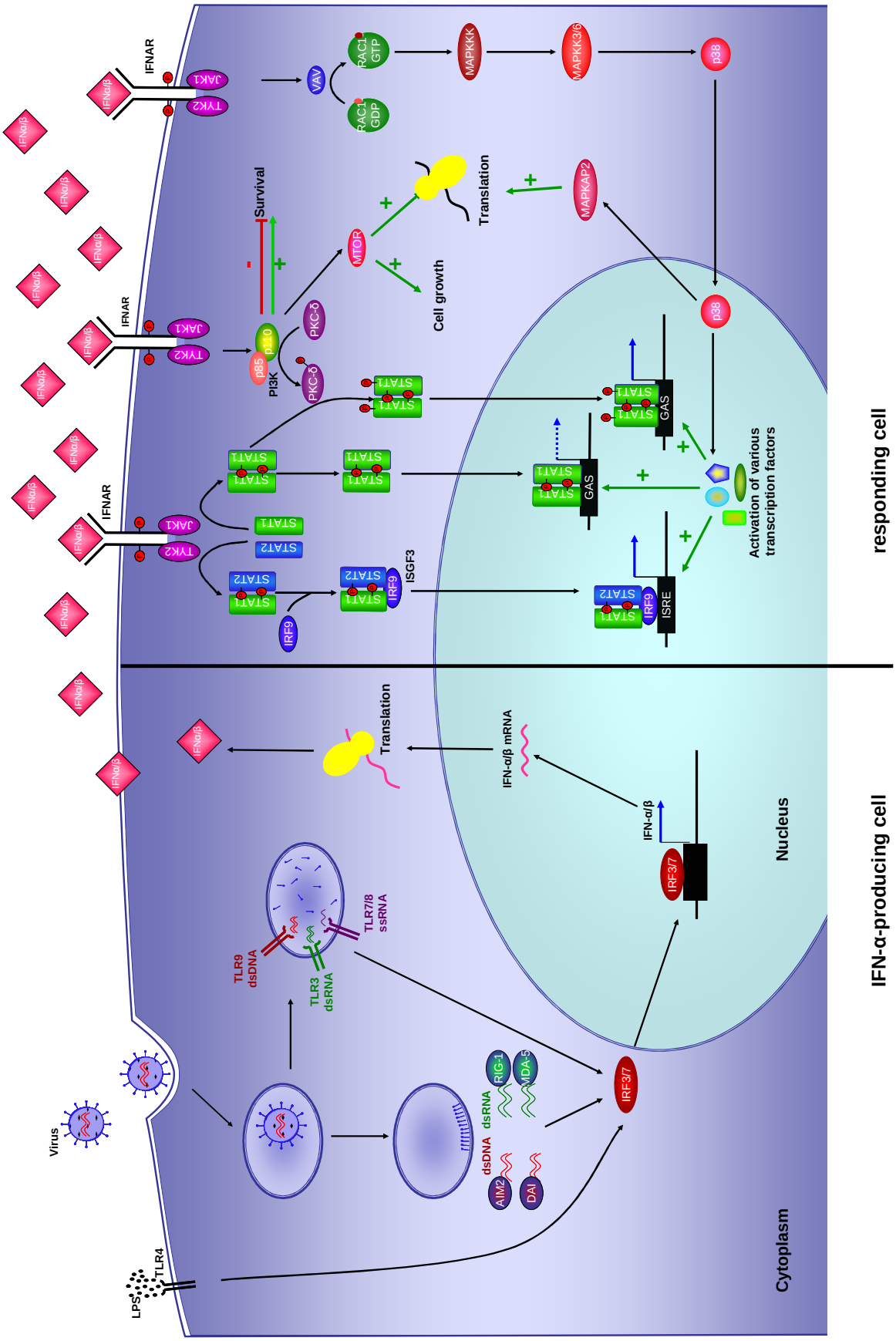
IFN-α-producing cell
responding cell
Cytoplasm
Nucleus
Virus
LPS
TLR4
TLR9
dsDNA
TLR7/8
ssRNA
TLR3
dsRNA
RIG-I
MDA-5
dsRNA
dsDNA
AIM2
DAI
IRF3/7
IFN-α/β
IFN-α/β mRNA
Translation
IFNAR
JAK1
TYK2
STAT1
STAT2
IRF9
ISGF3
ISRE
GAS
Activation of various transcription factors
PI3K
p85
p110
PKC-δ
Survival
MTOR
Cell growth
Translation
MAPKAP2
VAV
RAC1 GTP
RAC1 GDP
MAPKK
MAPKK3/6
p38

Figure 4 (previous page): The type I IFN signaling network. Overview of signals leading to type I IFNs induction (left side) and signaling pathways triggered by type I IFN (right side). Immune cells produce type I IFN upon sensing viral nucleic acids via endosomal TLR3/7/8/9 or extracellular bacterial lipopolysaccharides via TLR4 on the cell surface. Type I IFN production by somatic cells is dependent on cytoplasmatic sensors of infection such as AIM2 and DAI (dsDNA) or RIG-1 and Mda-5 (dsRNA). These signals lead to the activation of NF_KB and IRFs, which translocate into the nucleus and activate the transcription and secretion of type I IFNs.

All type I IFNs bind to IFNAR, the intracellular domains of which are constitutively associated with the Janus family tyrosine kinases JAK-1 and TYK-2. JAK-1 and TYK-2 cross-activate each other upon type I IFN binding to IFNAR and dimerization of the receptor subunits. Subsequently, they phosphorylate the intracellular domains of IFNAR whereupon STATs associate with the phosphorylated receptor subunits. This brings them in close proximity to the receptor-associated Janus kinases, leading to their activation. Upon phosphorylation, STATs can form homo- or heterodimers which translocate into the nucleus where they bind to GAS sites in the promoter region of various ISGs and activate the transcription of these genes (only the STAT1 homodimer is depicted in this figure). The STAT1/STAT2 heterodimer can associate with IRF9, forming the transcription factor ISGF3 which can also pass the nuclear membrane and activates the transcription of genes containing an ISRE in their promoter. Another target of type I IFN signaling is phosphatidyl-inositol 3-kinase (PI3K). After receptor engagement, the catalytic subunit of PI3K is activated via an adaptor protein which leads to an additional phosphorlyation of STAT1. This is required for full transcriptional activation of GAS-controlled ISGs by STAT1 homodimers. Further, PI3K signaling can also promote apoptosis or survival in a cell type-dependent manner and enhance mRNA translation via the activation of mTOR.[61, 80] The p38 MAPK pathway also has a role in IFN-α-mediated signaling. After IFN-α-dependent activation, Jak1/Tyk2 can mediate the phosphorylation of guanine-nucleotide exchange factors (GEFs), particularly VAV, resulting in the exchange of GDP for GTP in RAC1 and possibly other small GTPases. This drives the activation of a MAPK cascade ultimately leading to the phosphorylation of p38.[61, 81] Downstream of p38 effector kinases like MAPKAP2 and a variety of transcription factors are activated and regulate ISG expression on transcriptional as well as post-transcriptional levels.[61, 82-87]

2.2.2 Anticancer effects of IFN-α

IFN-α has been shown to exert both direct and indirect antitumor effects. It is currently used in the therapy of various solid and haematologic tumors, including hairy cell leukemia, follicular non-Hodgkin's lymphoma, renal cell carcinoma, AIDS-related Kaposi's sarcoma and melanoma.[88]

Direct effects on cancer cells:

In cell culture, IFN-α is able to directly inhibit cancer cell proliferation and induce apoptosis in malignant cells or sensitize them to other apoptosis-inducing chemotherapeutic drugs (e.g. 5-fluorouracil and doxorubicin).[7, 8, 74, 76, 89-91] IFN-α also enhances MHC class I expression on most cells, facilitating MHC class I-restricted antigen presentation to $CD8^+$ T-cells.[78, 79]

Indirect anticancer effects

IFN-α also exerts indirect anticancer effects. It can cause hypoxia and necrosis in tumors due to its antiangiogenic properties.[77] Further, it stimulates innate and adaptive immune responses.

Activating effects of IFN-α on the innate immune system include the promotion of DC differentiation and activation[92-94] and NK cell effector function.[95] The adaptive immune system is also affected: IFN-α pushes $CD4^+$ T cells toward the T_H1 phenotype[96] and promotes the effector function of $CD8^+$ T cells[97, 98] as well as the survival, proliferation and isotype switching in B cells (Table 5).[99-102]

Many cells, including B cells,[103] T cells,[104] NK cells[105] and pDCs,[14] express TRAIL in response to IFN-α stimulation.[14, 103-105] The relevance of this protein in tumor immunity is discussed in more detail in chapter 2.4.3.

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2.2.3 Anti-melanoma activity of adjuvant IFN-α

In 2010, Mocellin et al. published a meta-analysis of 14 randomized controlled trials assessing the efficacy of adjuvant IFN-α in high risk stage II and III melanoma patients.[5] A total of 8122 patients were enrolled in these trials, 4362 of which were allocated to the IFN-α arm. Whilst previous studies, covering smaller numbers of patients, had only found a significant increase in disease-free survival and trend toward an increase in overall survival, this study revealed a statistically significant benefit of IFN-α on both disease-free and overall survival.

Many of the potential anti-tumor mechanisms mentioned in 2.2.2 might contribute to the observed clinical benefit from adjuvant IFN-α therapy. This benefit appears to be attributable to direct anti-tumor effects as well as immunostimulatory effects. A better understanding of the mechanisms underlying the efficacy of IFN-α will reveal new strategies to maximize its positive effects whilst minimizing its systemic side effects.

In vitro, IFN-α has been shown to reduce proliferation and increase MHC class I expression in human melanoma cells.[106] Some human melanoma cell lines underwent apoptosis when treated with type I IFN, particularly IFN-β.[107] IFN-β was also shown to sensitize melanoma cells to TRAIL.[108] Further, the production of vascular endothelial growth factor (VEGF) was repressed in melanoma cells in the presence of IFN-α, indicating a decreased angiogenic capacity.[109]

IFN-α was also shown to promote NK cell activity and decrease metastasis in murine melanoma models.[110, 111]

Several immunologic changes were observed in human melanoma patients receiving IFN-α. Already in 1985, Hersey and colleagues reported an increase in the $CD4^+/CD8^+$ T lymphocyte ratio in the blood and an enhanced cytotoxic activity of NK cells against a melanoma target cell line during IFN-α treatment in patients with metastatic melanoma.[112] One study performed by Ascierto et al. in 22 melanoma patients with different disease stages, showed that before IFN-α therapy the levels of blood regulatory T cells (T_{regs}) were significantly elevated in melanoma patients compared with healthy controls. This might be associated with a repression of an anti-melanoma immune response. Upon high dose IFN-α treatment, a trend toward a decrease in T_{reg} levels was observed.[113]

Another report shows that in melanoma patients receiving adjuvant IFN-α therapy serum levels of angiogenic and growth factors (VEGF, epidermal growth factor, and

hepatocyte growth factor) are decreasing during treatment whereas the levels of IP-10, a IFN-γ-inducible protein with antiangiogenic and chemoattractant properties (recruitment of monocytes/ macrophages, NK cells, T cells and DCs) are significantly raising.[114]

The lymphocytic infiltrate in the tumor also seems to be affected by IFN-α. An increase in endotumoral $CD4^+$ T cells, $CD11c^+$ mononuclear cells and $CD86^+$ cells and a slight decrease in $CD83^+$ cells (both markers of DC activation) were observed in a study comparing biopsies of LN melanoma metastases before and after 4 weeks of IFN-α therapy. These effects were more pronounced in patients with clinical response.[115] The biopsies were also examined immunohistochemically for changes in tumor cell proliferation, angiogenesis and apoptosis but no significant differences were found.

2.3 Imiquimod

IMQ is a small molecular weight TLR7/8 ligand of the imidazoquinoline family. It has been approved under the name Aldara® by the FDA for topical treatment of anogenital warts, actinic keratoses and superficial BCC.[116] Interestingly, results from studies conducted so far show that this immune response modifier also has great therapeutic potential against melanoma.[1, 4, 117, 118]

The following chapter will present an overview of the mode of action of IMQ. Since the role of IMQ in melanoma therapy was investigated in this study, special interest is paid to the anti-melanoma activity of this drug.

2.3.1 Mode of action

The presence of nucleic acids in endosomal compartments presents a danger signal for the innate immune system. DNA and RNA do not normally occur in this localization, except during infection or after uptake of cellular debris from the extracellular space. The endosomal TLRs 3, 7, 8 and 9 can recognize these molecules (TLR3 – double stranded RNA, TLR7/8 – single stranded RNA, TLR9 – unmethylated CpG DNA) and initiate the transcription of cytokines that modulate the immune response.[119, 120]

The structure of IMQ resembles that of the purine bases of nucleic acids, particularly adenine (Figure 5).[121] This allows for its recognition via TLR 7 and 8. Upon ligand binding, TLR7/8 activate the transcription factor NFκB and IRFs via the adaptor protein MyD88, leading to the production of proinflammatory cytokines.[119]

IMQ has been shown to induce the production of IFN-α, TNF-α, IL-1α, IL-1β, GM-CSF, G-CSF, MIP-1α and IL-1 receptor in human PBMCs *in vitro.*[122] In DCs, IMQ treatment leads to maturation.[14, 123-125] However, particular DC subsets are differentially activated in terms of cytokine production and the expression of cytotoxic molecules. $CD11c^+$ myeloid dendritic cells (mDCs) produce IL-12 and granzyme B and perforin whereas pDCs excrete large amounts of IFN-α and express TRAIL when stimulated with IMQ *in vitro* (Figure 6).[12, 126]

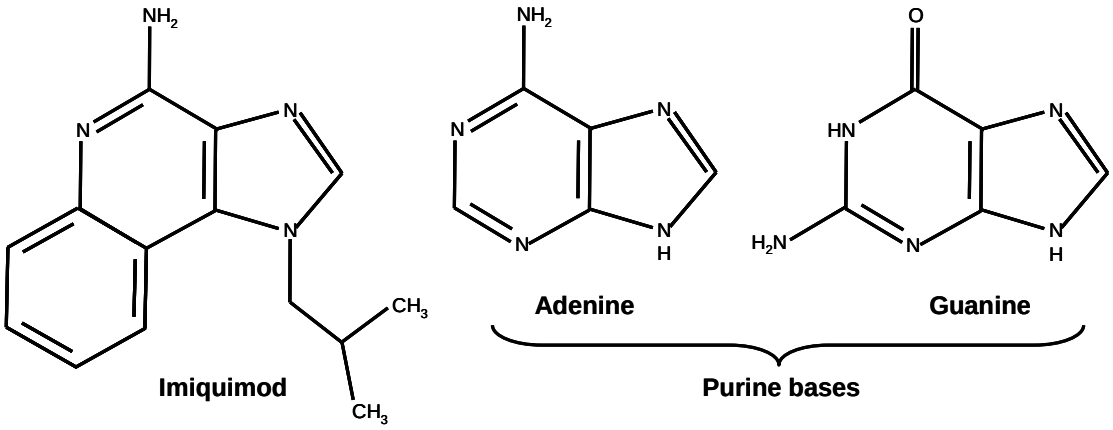

Figure 5: IMQ and the purine bases adenine and guanine. IMQ and purine bases share a very similar chemical structure.

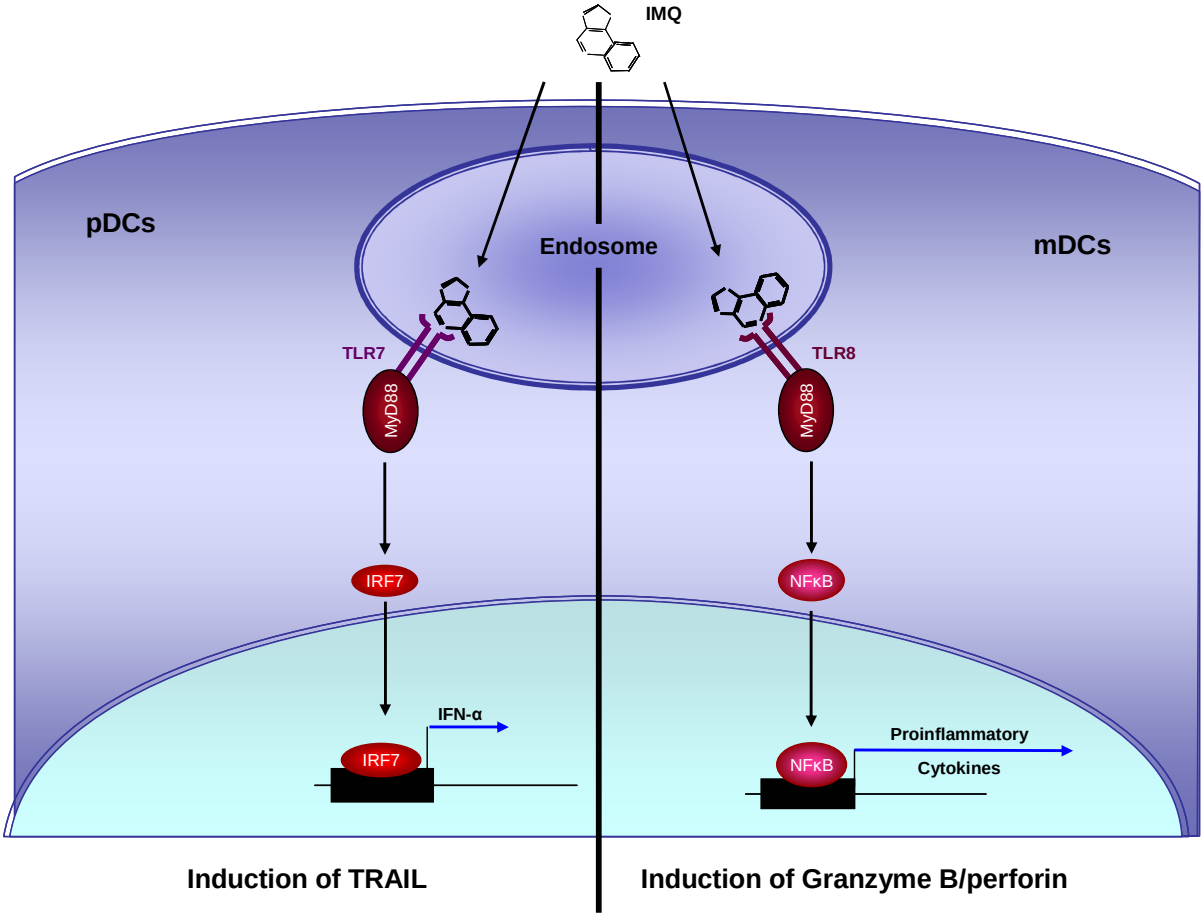

Figure 6: Different response of pDCs and mDCs to stimulation with IMQ. In pDCs, IMQ activates TLR 7. Subsequently, IRF7 is activated via the adaptor protein MyD88. This transcription factor is expressed constitutively only in pDCs, leading to the production of large amounts of IFN-α. Stimulation of mDCs results in activation of NF_KB and the expression of proinflammatory cytokines such as IL-12. Both cell types express cytotoxic molecules in response to IMQ stimulation *in vitro.* PDCs produce TRAIL whereas mDCs express granzyme B and perforin.

One study indicates that in TLR7/8 negative cell lines (Jurkat, K562, MeWo), IMQ may also act in a TLR-independent fashion via interaction with adenosine receptor signaling and induce the expression of proinflammatory cytokines such as TNFa, IL-1β, IL-6, and IL-8.[127]

IMQ has antiviral properties *in vivo*, as first demonstrated in a guinea pig model of herpes simplex virus (HSV) infection. Intravaginal administration of the drug was 100% protective of primary disease and also significantly reduced disease recurrence.[128, 129]

Several studies also demonstrate the antitumoral properties of IMQ against superficial cutaneous malignancies. Regression of various cutaneous tumors such as BCC, squamous cell carcinoma (SCC), Bowen´s disease and cutaneous melanoma have been observed upon topical IMQ treatment. [12, 130-132]

The anti-tumor effect of IMQ appears to be associated with its immune-activating properties. In 2007, Wolf et al. reported that the inflammatory infiltrate in cutaneous tumors (BCC, SCC, melanoma in situ and melanoma metastases) treated with IMQ contains increased numbers of T lymphocytes and pDCs.[11] This phenomenon was associated with tumor regression. A study by Stary et al. also showed that in BCC and melanoma, tumor regression upon IMQ treatment was associated with an influx of pDCs.[12]

2.3.2 IMQ in melanoma therapy

Although its use in melanoma therapy is experimental, IMQ has very efficiently been used to treat cutaneous melanoma lesions in some patients. Complete remission of lentigo maligna melanoma could be achieved in 75-88% of patients treated with Aldara® cream.[1] Complete or partial responses of cutaneous melanoma metastases have also been observed with this therapy.[4, 117, 118]

These positive effects of IMQ have mainly been attributed to indirect anti-cancer activity via stimulation of anti-melanoma immunity but direct effects on the tumor have also been proposed.

Indirect anticancer effects of IMQ:

In mouse skin, topical IMQ treatment leads to an influx of pDC-like cells and a local increase of IFN-α and TNF-α, two cytokines with known antitumoral properties. [10, 133] Topical treatment with IMQ after cryosurgery enhanced the systemic immunity against new melanoma challenges in a B16 murine melanoma model.[134]

In humans, the treatment of melanoma lesions with IMQ leads to local inflammation. An increase in T cells (both $CD4^+$ and $CD8^+$), TIA-1^+ (T-cell-restricted intracellular antigen-1) and granzyme B^+ cytotoxic cells and pDCs in the tumor infiltrate.[11] Enhanced numbers of $CD4^+$ T cells in the sentinel LNs were also reported.[11, 135] In mice too, melanoma regression after IMQ treatment was associated with an influx of pDC-like cells.[11, 133] Expression of cytotoxic molecules was not assessed in these studies but analysis of a similar infiltrate in human BCC upon IMQ treatment revealed that intralesional pDCs expressed TRAIL.[12] These findings indicate that pDCs might have a direct cytotoxic effector function in IMQ-treated cutaneous malignancies (see 2.5.2.1).

Direct anticancer effects of IMQ:

IMQ might also affect melanoma cells themselves. High doses of IMQ have been shown to induce apoptosis in melanoma cell lines *in vitro* in a TLR-independent fashion.[6] In murine macrophages and human BCC cells, IMQ can also induce autophagy.[136, 137] Whether the latter applies to melanoma too, remains to be elucidated.

Many patients with benign or semi-malignant skin cancers clearly benefit from topical IMQ treatment.[130, 138, 139] Future studies will reveal whether IMQ could also serve as a non-invasive treatment for superficial melanoma lesions.

2.4 TRAIL

In 1995, Wiley et al. first described a new member of the tumor necrosis factor (TNF) family, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[140] Other members of this family include TNF-α, TNF-β and Fas ligand. They are capable of inducing apoptosis in a broad range of cells via interaction with specific death receptors (DRs) but they can also mediate inflammation and the transcription of survival factors.[119] Among these proteins, TRAIL has the remarkable ability to induce apoptosis in tumor cells but not in normal cells.[141] This makes it an interesting subject for cancer research and a promising candidate for new therapeutic approaches against cancer.[140]

Since the ability of TRAIL to induce apoptosis in melanoma cells was the main focus of our work, this chapter will give an overview of TRAIL-induced apoptotic signaling and discuss its role in tumor immunity.

2.4.1 TRAIL and TRAIL receptors

TRAIL can exist in a trimeric membrane-bound form (memTRAIL) or it can be cleaved from the cell surface, probably via cysteine proteases, to form soluble TRAIL (sTRAIL).[142] In humans, four cellular receptors, TRAIL R1-4 can bind to TRAIL. TRAIL R1 and TRAIL R2, also termed death receptors (DR), possess an intracellular death domain (DD) that can activate the apoptotic cascade. TRAIL R1 can be activated by both memTRAIL and sTRAIL whereas TRAIL R2 signaling is triggered by memTRAIL or secondarily crosslinked sTRAIL only.[143]

TRAIL R4 has a truncated intracellular domain lacking the DD and TRAIL R3 completely lacks the intracellular domain. This renders them both incapable of transducing an apoptotic signal. They are thought to act as decoy receptors via competition for TRAIL binding. Indeed, overexpression of these receptors has been shown to be protective against TRAIL-induced apoptosis.[144, 145] However, other studies show that TRAIL susceptibility is largely independent of TRAIL R3/4 levels.[146, 147]

2.4.2 TRAIL signaling pathways

Binding of TRAIL to one of its DRs induces the extrinsic (receptor-mediated) apoptotic pathway. This pathway requires the binding of a cytotoxic ligand to a DR on the cell surface. Receptor trimerization upon ligand binding leads to a conformational change in the intracellular DD of the receptor. Thereby activated, the receptor DD can interact with the DD of the adaptor protein FAS-associated death domain (FADD) which in turn recruits procaspase-8, forming the death-inducing signaling complex (DISC). Within this complex, procaspase-8 is cleaved autoproteolytically into caspase-8. This enables it to activate the downstream effector caspase-3 which then initiates the apoptotic programme (Figure 7).[148, 149]

Some cells require an amplification of the TRAIL apoptotic signal via the intrinsic (mitochondrial) apoptotic pathway. The two pathways are linked via caspase-8, which can also cleave the pro-apoptotic protein Bid. Truncated Bid induces the formation of pores in the outer mitochondrial membrane, causing the release of mitochondrial cytochrome-c into the cytosol. This initiates the assembly of the apoptosome, where procaspase-9 is cleaved to active caspase-9. Caspase-9 also cleaves procaspase-3, providing an amplification loop for the extrinsic apoptotic pathway (Figure 7).[149]

Based on the apoptotic pathways involved, type I (extrinsic only) and type II (additional activation of intrinsic pathway required) TRAIL-sensitive cells can be distinguished.[149] It has also been proposed, that the requirement of activation of the intrinsic pathway of apoptosis might partially be a dose-dependent phenomenon, since the protective effect of blocking the mitochondrial pathway in Jurkat cells was limited to low doses of TRAIL.[150]

Under certain conditions, such as the inhibition of protein synthesis or a low pH of the cell culture medium, TRAIL can also induce necrosis in tumor cells via activation of receptor-interacting protein (RIP) kinase.[151, 152] It has been suggested that this pathway might play a major role in TRAIL-mediated cytotoxicity against solid tumors with an acidic microenvironment.[151]

An alternative pathway leading to the activation of NFκB and promoting cell survival can be activated via TRAIL R1, 2 and 4.[153, 154] The balance between apoptotic signaling and NFκB activation is thought to be one factor determining TRAIL susceptibility. Additionally, a variety of antiapoptotic proteins (cFLIP, XIAP, Bcl-2)[155-158] can interfere with the apoptotic cascade at different levels to inhibit the activation of caspase-3. Such proteins are often overexpressed in tumor cells (eg. FLIP, Bcl-2

and Mcl-1 in melanoma), thus limiting the efficacy of apoptosis-inducing drugs. [159, 160, 161]

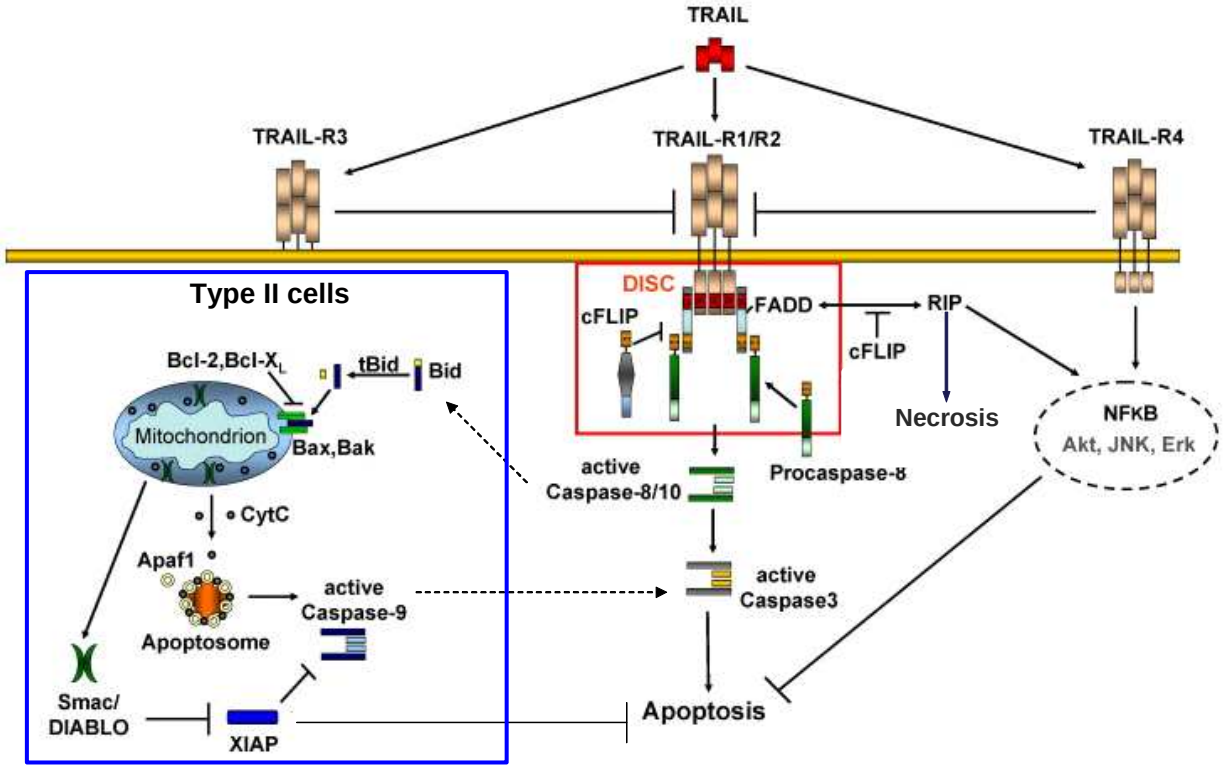

Figure 7: Scheme of the TRAIL signaling cascade. Binding of TRAIL to TRAIL R1/2 induces the assembly of the death-inducing signaling complex (DISC), the activation platform of caspase-8. Active caspase-8 then cleaves the effector caspase-3, initiating the apoptotic programme. In most cells, additional activation of the intrinsic pathway is required for the induction of apoptosis. In these cells, termed type II cells, active caspase-8 truncates the proapoptotic protein Bid, inducing the release of cytochrome c from the mitochondria. This leads to the assembly of the apoptosome, where caspase-9 is activated. Caspase-9 further promotes apoptosis via the activation of caspase-3. In contrast to apoptosis, TRAIL can also induce necrotic cell death via activation of the kinase RIP. Conversely, TRAIL R1/2 and TRAIL R4 engagement can also promote cell survival via NF_KB activation. Figure adapted from Falschlehner et al., 2007.[162]

2.4.3 The role of TRAIL in anti-tumor immunity

Not only the selective induction of apoptosis in tumor cells indicates that TRAIL has an essential role in tumor immunity. A broad range of human immune cells including B cells,[103] T cells,[104] NK cells,[105, 163] and pDCs[14, 164] can be induced to express TRAIL on their surface when treated with certain immunostimulatory agents, particularly IFN-α. TRAIL-mediated cytotoxicity of such stimulated lymphocytes against tumor cells has been demonstrated in several *in vitro* studies. [14, 164-166]

In vivo evidence for the importance of TRAIL in cancer immunity has been generated in TRAIL and TRAIL R knockout mice which display increased susceptibility to tumor formation and metastasis.[167-169] Further, murine liver NK cells that constitutively express TRAIL were demonstrated to have a role in the suppression of liver metastasis.[170, 171]

TRAIL itself is currently being assessed as a potential anti-cancer drug. Results obtained in phase I and II clinical trials using recombinant human TRAIL (rhTRAIL) or antagonistic antibodies against TRAIL DRs show that these drugs are generally well tolerated but the clinical effect is limited (for a review, see Bellail et al., 2009).[172] It has been shown that tumor cells acquire TRAIL resistance rather quickly when cultured in the presence of TRAIL. This might be the reason for the limited efficacy of monotherapy with TRAIL-based drugs.[173]

Various studies demonstrate that the combination of recombinant human TRAIL (rhTRAIL) with other proapoptotic agents or even radiotherapy can have synergistic effects or sensitize cancer cells to TRAIL-induced apoptosis.[174-178] This indicates that TRAIL-based drugs may be more efficient when used in combination with other therapies.

In summary, the current state of knowledge points toward an important function for TRAIL in natural and therapy-induced tumor immunity. In the future it may play a significant role in the combat against cancer either via the induction of immune cells to express TRAIL or as an anti-cancer drug itself.

2.5 Dendritic cells

The following chapter will provide an introduction into DC biology. Since the present study aims to assess the cytotoxic potential of pDCs, this particular DC subset is discussed in detail.

2.5.1 Antigen presentation

DCs are professional antigen presenting cells with the unique ability to induce primary immune responses. Immature DCs in the periphery have a high capacity to take up antigens from the environment. Within endosomes the antigen is proteolytically degraded and loaded onto MHC class II molecules, which are recognized only by $CD4^+$ T cells (Figure 8). Activation of $CD8^+$ T cells requires antigen presentation via MHC class I molecules, which usually display peptides derived from proteins that were generated in the cytoplasm. DCs can also transport endosomal antigens into their cytosol, allowing for their presentation to $CD8^+$ T cells in the context with MHC class I (cross-presentation).[119]

Recognition of pathogens, leading to DC maturation occurs via general pattern recognition receptors (e.g. TLRs, C-type lectin receptors). This induces the upregulation of costimulatory molecules such as CD83 and CD86 and migration to the draining LNs. There, mature DCs present processed antigens to naive T cells, leading to their activation. The ability of DCs to activate naive T cells depends on their maturation status and the expression of costimulatory molecules. Antigen recognition without costimulation causes tolerance rather than immunity. [119, 179]

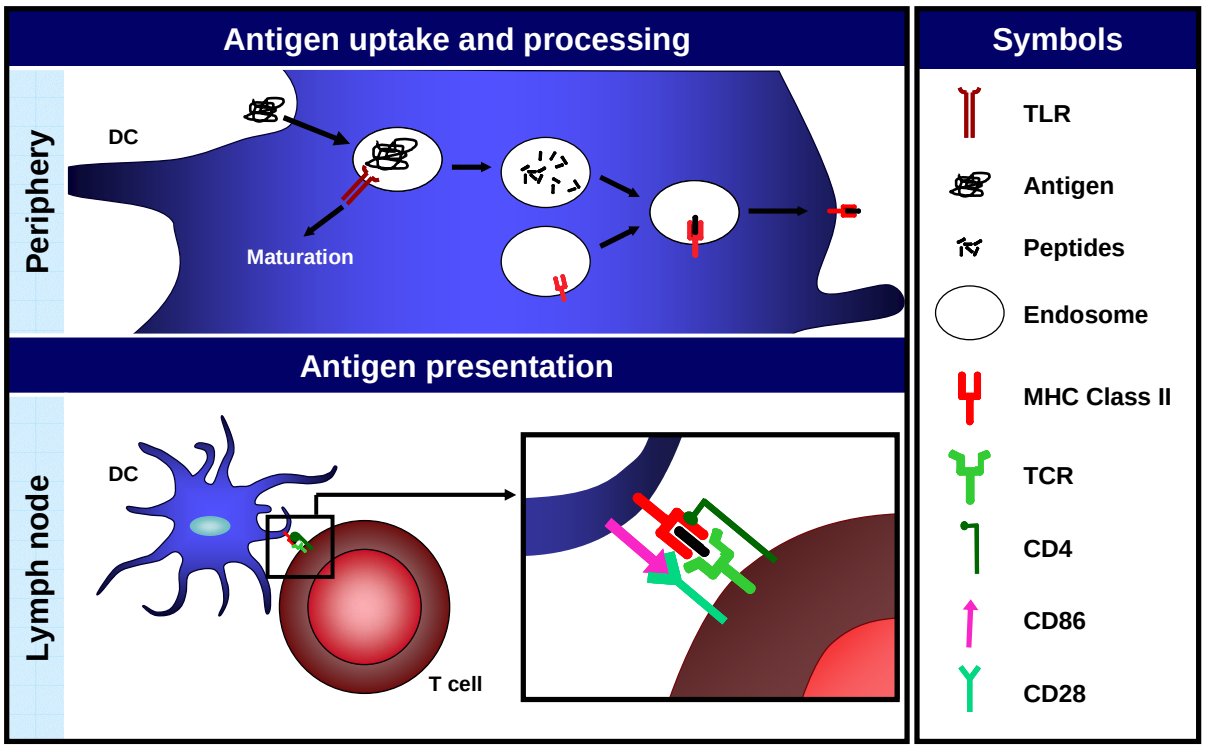

Figure 8: MHC class II-restricted antigen presentation by DCs. In contrast to somatic cells, DCs are capable of presenting antigen to naive T cells in the context of MHC class II molecules. In the periphery, DCs take up extracellular antigens via endocytosis. In endosomes, the antigen is then cleaved into small peptides (processed antigen). The processed antigen is then loaded onto MHC class II molecules and presented on the cell surface. Via cross-presentation, DCs can also present peptides to $CD8^+$ T cells in the context with MHC class I (not shown). Upon activation via pattern recognition receptors (e.g. TLRs) DC maturation is induced. Activated DCs migrate to the draining LNs where the antigen is presented to naive $CD4^+$ T cells. Interaction of the T cell receptor with a specific MHC class II bound antigen in context with stimulation via costimulatory molecules (CD86) leads to T cell activation.

2.5.2 Blood dendritic cells subsets

DCs are characterized by the expression of MHC class II (HLA-DR) and the lack of hematopoietic lineage (Lin) markers (e.g. CD3, CD19, CD14, CD56) on their surface. Human blood contains three major $Lin^-HLA\text{-}DR^+$ subsets: $CD34^+$ precursors, $CD303^+$ (blood dendritic cell antigen-2, BDCA-2) plasmacytoid DCs (pDCs) and $CD11c^+$ myeloid DCs (mDCs), which can be further divided into subgroups expressing CD1c (BDCA-1), CD141 (BDCA-3) and CD16. [180]

DC subsets do not only differ in their expression of surface markers, they also express distinct sets of immunological pattern recognition receptors, the best

characterized of which are TLRs. The expression of TLRs in pDCs and mDCs is summarized in Table 6.

Table 6: TLR expression in human pDCs and mDCs.[126, 181-184] (+: present; -: absent; mRNA: reported on mRNA level only; +/-: present in some studies)

TLR	Ligand	localization	Expression in	
			pDCs	mDCs
1	bacterial products	Cell membrane	mRNA	+
2	bacterial products	Cell membrane	-	+
3	dsRNA	Endosomes	-	+
4	bacterial products	Cell membrane	-	+/-
5	bacterial products	Cell membrane	-	+
6	bacterial products	Cell membrane	mRNA	+
7	ssRNA	Endosomes	+	+/-
8	ssRNA	Endosomes	-	+
9	CpG DNA	Endosomes	+	-

The differential expression of TLRs indicates distinct functions for different DC subsets. MDCs predominantly sense bacterial products via extracellular TLRs and TLR signaling results in the activation of NFκB, leading to the secretion of proinflammatory cytokines such as IL-6, IL-12 and TNF-α. In contrast, pDCs specialize in detecting viral nucleic acids via endosomal TLRs and produce large amounts of IFN-α.[179]

2.5.2.1 Plasmacytoid dendritic cells

Although they only account for 0.01-0.05% of PBMCs, pDCs are the primary source of type I IFNs.[185, 186] This is made possible by the constitutive expression of IRF7, rendering the cells capable of producing copious amounts of type I IFN, independent of IFNAR-mediated feedback signaling (see 2.2.1).[187]

Classical functions of pDCs:

PDCs sense viral nucleic acids via endosomal TLR7 (ssRNA) and TLR9 (unmethylated CpG DNA) and can initiate a multitude of IFN-dependent and IFN-independent immunological functions.

Upon activation pDCs produce large amounts of type I IFN along with other cytokines and chemokines including TNF-α, IL-6, IL-8 and CXCL10 (reviewed by Fitzgerald-Bocarsly et al., 2009).[188] Activated human pDCs promote the migration of NK cells and activated T cells,[189] the maturation of mDCs[190] and plasma cells[191] *in vitro* and

can also function as antigen presenting cells themselves, albeit less efficiently than mDCs.[192] Furthermore, pDC-derived type I IFNs can directly interfere with viral replication and tumor growth as discussed in 2.2.2.

Cytotoxic pDCs:

Interestingly, pDCs have also been found to have cytotoxic functions. Freshly isolated pDCs have been shown to express granzyme B. Upon stimulation with endogenous or exogenous type I IFN, viruses or synthetical TLR7 and TLR9 agonists pDCs also express TRAIL. This enables them to directly exert cytotoxic effector functions against tumor cells and virally infected cells (see 2.4.3).[12-14, 164, 193]

The role of pDCs in tumor immunity:

The role of pDCs in tumor immunity is not yet clearly understood, but tumor-infiltrating pDCs have been found in human melanoma,[194, 195] breast cancer,[196] head and neck cancer[197] and BCC. [11]

In early stage breast cancer, infiltration with pDCs was associated with decreased patient survival,[196] which might be due to tolerogenic properties of immature pDCs.[192]

Several studies indicate that pDCs might have an important role in the efficacy of topical IMQ against cutaneous malignancies. In a mouse melanoma model, the number of tumor infiltrating pDC-like cells correlated well with the clinical response to IMQ.[13, 133] A recent study by Drobits et al. further demonstrated that pDCs actively participate in melanoma clearence in a mouse melanoma model.[13] In humans, pDCs were found in the lymphocytic infiltrate of melanoma, squamous cell carcinoma and BCC during IMQ treatment.[11] Our group has shown that in human BCC tumor regression upon topical IMQ treatment was associated with an influx of pDCs that expressed TRAIL and that IMQ-stimulated pDCs acquire TRAIL-dependent cytotoxic effector functions *in vitro*.[12] The current data suggests that the contribution of pDCs to tumor immunity might not only be confined to the production of IFN-α and the stimulation of the immune system. PDCs themselves might acquire cytotoxic effector functions against tumor cells. To gain a better understanding of this phenomenon was an aim of this study.

3 MATERIALS AND METHODS

All plastic material (multiwell plates, cell culture flasks, pipet tips) was purchased from Eppendorf, Becton Dickinson or Corning Incorporated, if not stated differently.

3.1 Cell lines and cell culture

All working steps with cell lines were carried out under sterile conditions in a laminar air flow hood.

Standard cell culture medium:

RPMI 1640 (Invitrogen, Cat. No.: 52400-041)

10% v/v FCS (Invitrogen, Cat. No.: 10500-064)

1% v/v Penicillin-Streptomycin (Invitrogen, Cat. No.: 15070-063), containing 5,000 IU of penicillin (base) and 5,000 µg of streptomycin (base) per ml.

Melanoma cell lines

Four established malignant melanoma cell lines from American Type Culture Collection (ATCC) or Wistar Institute (SkMel2, SkMel28, MeWo, WM793) and four melanoma cell lines generated from metastases from stage III and IV melanoma patients by Dr. F. Koszik (Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna), Pat1-4, were used in this study. The melanoma identity of the cell lines was confirmed prior to this study by assessing the intracellular expression of the melanoma markers HMB45, MelanA and S100 using flow cytometry.

Table 7: List of melanoma cell lines, patient information and split ratio. N.a.: information not available.

Cell line	Patient age	Patient gender	Metastatic site	Split ratio	Catalog number
SkMel2	60	Male	Skin	1:8	ATCC HTB-68
SkMel28	51	Male	N.a.	1:6	ATCC HTB-72
MeWo	78	Male	Lymph node	1:6	ATCC HTB-65
WM793	N.a	N.a	N.a	1:6	Wistar Institute
Pat1	52	Female	Dermal	1:8	-
Pat2	30	Male	Illeum	1:6	-
Pat3	57	Male	Ascites	1:4	-
Pat4	53	Female	Axilla	1:6	-

Jurkat cells

Jurkat cells are a line of immortalized T-lymphocytes established from the peripheral blood of a 14-year old boy with T cell leukemia. Because of their susceptibility to TRAIL [198], they served as a model cell line for TRAIL-mediated killing in this study.

3.1.1 Harvesting/passaging of melanoma cells

Trypsin/EDTA

Trypsin/EDTA (Invitrogen, Cat.No: 14190-169)

PBS/EDTA 0.02%

Dulbecco´s Phosphate buffered saline (Invitrogen, Cat. No.: 14190-169)

0.02% w/v EDTA

Cells were passaged by trypsinization when about 90% confluent. Medium was removed completely and 5-10ml Trypsin/EDTA (Invitrogen, Cat.No: 14190-169) were added. After detachment from the culture flask surface, the cells were transferred into a 50ml falcon tube and washed once with standard medium. After resuspension in 10ml standard medium and depending on the split ratio (Table 7), 2-5ml of the cell suspension were seeded into a new 75cm^2 cell culture flask containing 15-18ml of culture medium.

In order to avoid proteolytic cleavage of surface antigens, the cells were harvested using PBS/EDTA for extracellular FACS staining. This procedure was carried out like trypsinization but with a longer incubation time before cell detachment.

3.1.2 Passaging of Jurkat cells

Jurkat cells were passaged every 2-3 days by removing 15ml of cell suspension and adding 15ml of fresh culture medium.

3.1.3 Freezing and thawing

Freezing medium:

FCS (Invitrogen, Cat. No.: 10500-064)

10% v/v DMSO (Merck, Cat. No.: 1.09678.0100)

To ensure consistent quality of the cells, frozen stocks of all cell lines were prepared and cells were discarded after a maximum of two months in culture.

Cells grown to confluency in four 225cm^2 culture flasks were trypsinized, pooled and washed once in sterile PBS before they were resuspended in freezing medium to a final concentration of 1x10^6-10x10^6 cells/ml. Aliquots of 1ml were filled in Cryo.S cryotubes (Greiner Bio One, Cat. No.: 122263) and cooled down to -80°C over night in a freezing container (Qualifreeze, Qualilab). The next day, cells were transferred to liquid nitrogen.

Cells were thawed in a 37°C waterbath, washed once in culture medium and seeded to 75cm^2 polypropylene plastic flasks in a total of 20ml culture medium. Before they were used in experiments, cells were passaged at least once.

3.1.4 Determination of cell number

After harvesting, cells were resuspended thoroughly in culture medium, and 10µl of cell suspension were mixed with 10µl of trypan blue (Sigma, Cat.No.: T8154). 10µl of this solution were then loaded on a disposable C-Chip hemacytometer (Digital Bio, Cat.No.: DHC-N01). Cells in all four squares were counted and the average value calculated. The result was multiplied with 2x10^4 to obtain the number of cells per ml of the suspension.

3.2 DAPI staining for mycoplasm contamination

Mycoplasma are a genus of small intra- or extracellular bacteria often found as contaminants in cell culture. Due to their small size, they can pass through standard filters. Also, many strains are resistant to antibiotics commonly used in cell culture. Infection with mycoplasma can inhibit cell proliferation by nutrient withdrawal and, more importantly, it can also affect cell physiology and thus influence the outcome of an experiment. Cell cultures therefore have to be tested for contamination with these bacteria.[199] To this aim the cells were stained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) which intercalates in the nuclear DNA of eukaryotic cells as well as mycoplasma DNA. In contaminated samples, mycoplasma appear as small spots of extranuclear fluorescence in and around the cells.

3.2.1 Experimental procedure

Methanol:

Methanol (Fisher Scientific, Cat. No.: M/4000/15)

DAPI stock solution:

10mg DAPI (Roche, Cat. No.: 10 236 276 001)

10ml ddH_2O (B. Braun Melsungen AG, Cat. No.: 0082479E)

DAPI Stock solution was diluted 1:1000 in methanol before use (DAPI/methanol, final concentration: 1µg/ml).

Cells were grown for 2-3 passages in antibiotic-free medium in 25cm^2 culture flasks and then seeded on Chamber slides (Lab-Tek, Cat. No.: 70379-42). When confluency reached 50%-70% the medium was removed and chambers were rinsed once with DAPI/methanol. Cells were then covered with DAPI/methanol and incubated for 15min at 37°C in the dark. After washing once with pure methanol the chambers were removed and a coverslip was placed on the sample using PermaFluor (Thermo Scientific, Cat. No.: TA-006-FM) as mounting medium. Within the next days, slides were examined for mycoplasm contamination under a Zeiss Axiophot fluorescence microscope.

3.3 Preparation of blood cells

3.3.1 Preparation of PBMC

PBMCs were prepaired from buffy coats (Austrian Red Cross, Cat. No.: E2776V00). Buffy coats were diluted in PBS to a final volume of 280ml. 35ml of this solution were carefully layered over 15ml of Ficoll Paque™ Plus (GE Healthcare, Cat. No.: 17-1440-03) per 50ml Falcon and centrifuged for 20min at 601xg without break.

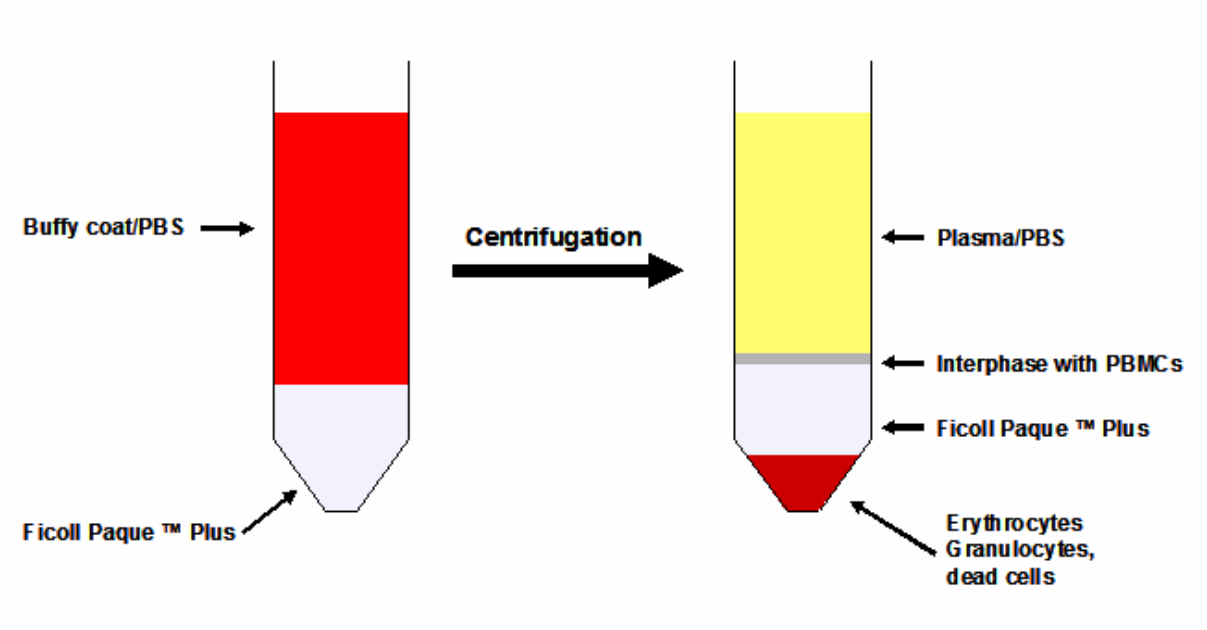

Figure 9: Schematic depiction of PBMC isolation using Ficoll Paque™ Plus. After centrifugation the components are separated according to their specific densities. PBMCs are located in the interphase between Ficoll Paque™ Plus and plasma/PBS.

PBMCs in the interphase were pooled into a new 50ml Falcon tube and washed three times (601xg-338xg-601xg). After the last washing step PBMCs were either resuspended in MACS buffer for isolation of pDCs or in RPMI 1640 with 10%v/v FCS for over night cultivation.

3.3.2 Isolation of pDCs

MACS buffer stock solution 20x:

PBS

40mM EDTA

10% w/v BSA (Sigma, Cat. No.: A9647-500G)

MACS buffer stock solution was diluted 1:20 in PBS and sterile filtered prior to use.

PBMCs generated from buffy coats were used for the isolation of pDCs. After the last wash, the cells were resuspended in MACS buffer to a concentration of $3x10^8$ cells/ml. First the cell suspension was depleted of hematopoietic progenitor cells and lineage positive (lin+) cells, such as T cells, B cells, monocytes, macrophages, NK cells or granulocytes. To this end the cell suspension was incubated for 15min with primary mouse IgG1 antibodies against CD3, CD11b, CD16, CD19, CD34, CD41, CD56 and CD235a (Beckman Coulter, Cat. No.: IM1304, IM0190, IM0813, IM1313, IM1869, IM0145, IM1844, IM2210). Cells were washed once in MACS buffer (4°C, 601xg, 10min) and resuspended in MACS buffer at a concentration of $1.25x10^8$ cells/ml. Subsequently 250µl of Rat Anti-mouse IgG1 MicroBeads (Miltenyi biotec, Cat. No.: 130-047-102) were added per ml cell suspension. Following another incubation for 15min in the fridge, lin+ cells were immunomagnetically depleted using the appropriate amount of CS Columns (Miltenyi Biotec, Cat. No.: 130-041-305).
Plasmacytoid DCs were isolated from lineage-depleted PBMCs using the CD304 (BDCA-4/Neuropilin-1) MicroBead kit (Miltenyi Biotec, Cat. No.: 130-090-532) according to the manufacturer´s protocol. Purity of isolated pDCs, as assessed by flow cytometry, was greater than 95% and contaminations with CD3+, CD14+, CD19+ and CD56+ cells were smaller than 1%.

3.3.3 Stimulation of blood cells

IFN-α2a

Roferon®-A 6x10^6 IU/ml (Hoffmann – La Roche, Cat. No.: B2070), stored at +4°C

Imiquimod (IMQ)

Imiquimod 5mg (Invivogen, Cat. No.: tlrl-Imq)

To induce expression of TRAIL, PBMCs or pDCs were stimulated over night with different concentrations of Roferon®-A or IMQ or a combination of both reagents.

3.4 Flow cytometry

FACS buffer

PBS

1% w/v BSA

0.1% w/v Azide

The expression of TRAIL, TRAIL receptors and MHC molecules on melanoma cells as well as the different subpopulations of PBMCs were assessed using flow cytometry.

The cells were harvested using PBS/EDTA, washed twice in ice-cold PBS and resuspended in ice-cold FACS buffer. 2% mouse serum (Dako, Cat. No.: X0910) was added to the cell suspension to block unspecific binding of antibodies. Labeled antibodies against surface antigens (Table 8) were pipetted into 1.4ml ScreenMates tubes (Thermo Scientific, Cat. No.: 4140), and 50µl cell suspension was added to each tube. After 15min incubation at 4°C in the dark, cells were washed twice in ice-cold FACS buffer and antigen expression was analyzed using a 4-colour BD FACSCalibur™ flow cytometer. Expression was considered positive, when the mean fluorescence intensity MFI (geo $mean_{sample}$-geo $mean_{isotype}$) was positive in three independent experiments and the mean MFI was greater than one. A mean MFI greater than 10 was defined as strong expression.

Table 8: List of antibodies used for flow cytometry

Antibody	Isotype	Label	Company	Cat. No.
TRAIL	mIgG1	PE	eBioscience	12-9927-71
TRAIL R1	mIgG1	PE	R&D	FAB 347P
TRAIL R2	mIgG1	PE	R&D	FAB6311P
TRAIL R3	mIgG2b	PE	R&D	FAB6302P
TRAIL R4	mIgG1	PE	R&D	FAB633P
HLA-DR	mIgG2a	FITC	BD	347400
HLA-DR	mIgG2a	Per-CP	BD	347402
HLA-ABC	mIgG2a	FITC	seroTec	MCA81P
BDCA-2	mIgG1	APC	Miltenyi Biotec	130-090-905
CD83	mIgG1	FITC	BD	556910
CD19	mIgG1	PerCP	BD	345790
CD14	IgG2b	FITC	BD	345784
CD3	mIgG1	APC	BD	345767
mIgG1	mIgG1	PE	BD	345816
mIgG2b	mIgG2b	PE	southern biotech	0104-09
mIgG2a	mIgG2a	PE	BD	349053

3.5 Real-time PCR

All reagents and plastic material used were of PCR quality.

DEPC-treated water:

0.1ml DEPC (Carl Roth GmbH, Cat. No.: K028.1)

100ml Aqua bidestillata (Mayrhofer Pharmazeutika GmbH, Cat. No.: 15.533)

Reagents were stirred over night at 37°C and autoclaved two times. 50ml aliquots were prepared and stored at -20°C.

75% Ethanol:

37.5ml Absolute Ethanol (Merck, Cat. No.: 1.00983.1000)

12.5ml DEPC-treated water

3.5.1 RNA isolation

$1x10^6$-$5x10^6$ cells were harvested, washed twice in 1x PBS, resuspended in 1ml TRI reagent (Sigma-Aldrich Co., Cat. No.: T9424) and stored at -20°C. For RNA isolation samples were thawed on ice and incubated at room temperature for 5min. Then 200µl chlorophorm (Merck, Cat. No.: 1.02445.0250) were added and samples were vortexed for 15sec. After a 2-10min incubation, samples were centrifuged (4°C, 12000xg, 5min). The aqueous (upper) phase was transferred into a new Eppendorf tube and mixed with 500µl isopropanol (Sigma-Aldrich, Cat. No.: 19516). After a 10min incubation period, samples were centrifuged again (4°C, 12000xg, 10min) and the supernatants were discarded. The pellets were washed once in 75% ethanol and then air-dried for 5-10min. After dissolving the pellets in 40µl DEPC-treated water, the RNA obtained was incubated for 10min at 55°C on an Eppendorf Thermomixer 5436 heat shaker. RNA concentration was determined using a NanoDrop ND-1000 Spectrometer (Peqlab). If not used immediately, RNA samples were stored at -80°C.

3.5.2 Reverse transcription

For reverse transcription of isolated RNA, the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Cat. No.: 4374966) was used.

Reaction mix:

4ng RNA (or maximal 27.4µl)
1.6µl dNTP mix (25x)
4µl RT buffer (10x)
4µl random primers (10x)
2µl MultiScribe reverse transcriptase
1µl RNAse inhibitor
DEPC-treated water was added to a reaction volume of 40µl.

The reaction mix was incubated for 10min at 25°C, followed by 120min at 37°C and 5min at 85°C in a Perkin Elmer 480 Thermocycler. cDNA samples were stored at 4°C (short-term) or -20°C (long-term).

3.5.3 Real time PCR

The reaction was carried out on ice. TaqMan Gene Expression Mastermix (Applied Biosystems, Cat. No.: 4369510) and Taqman Gene Expression Assays (Applied Biosystems, Cat. No.: 4331182, 4351372) were used for real time PCR.

2µl of cDNA per well were mixed with 10µl TaqMan Gene Expression Mastermix (2x), 1µl TaqMan Gene Expression Assay (20x), and 7µl DEPC-treated water in a 96-well PCR plate. After sealing the plate with an optical adhesive film, the plate was centrifuged briefly (1min, 234xg) and quantitative PCR was performed using a StepOne Plus PCR machine with the following parameters:

Table 9: PCR parameters

Real-time PCR conditions	**1**	**2**	**3 (40 cycles)**	
Temperature	50°C	95°C	95°C	60°C
Time	2min	10min	15sec	1min

All reactions were run in duplicates. To compare the mRNA levels of TRAIL and its receptors within the cell lines, the fold difference (d) between the target gene and the reference gene (GAPDH) was calculated using the formula $d=2^{-\Delta CT}$ (ΔCT=difference in threshold cycles for target gene and reference gene).

3.6 Annexin V/PI staining for apoptosis

Annexin V is a cellular protein with a binding specificity for phosphatidyl serine residues. It can be used to detect inversion of the cell membrane in apoptotic cell death. During this process, phosphatidyl serine residues, which are located on the inner cell membrane in living cells, are transported to the cell surface. This makes them accessible to binding of Annexin V, which can be fluorescently labeled and subsequently detected via flow cytometry.

SuperKillerTRAIL™ 0.1mg/ml:

20µl SuperKillerTRAIL™ (Alexis biochemicals, Cat. No.: ALX-201-115-C010)

80µl Killer TRAIL Storage and Dilution Buffer (Alexis biochemicals, Cat. No.: ALX-505-005-R500)

10µl aliquots were stored at -80°C.

1x Annexin binding buffer

4ml Annexin V Binding Buffer, 10X Concentrate (BD, Cat. No.: 556454)

36ml ddH_2O (B. Braun Melsungen AG, Cat. No.: 0082479E)

For Annexin staining, FITC-labelled Annexin V and Propidium Iodide (PI) from the Bender MedSystems Apoptosis Detection Kit (Cat. No.: BMS500FI/100CE) were used.

Melanoma cells were seeded to 24-well plates with an expected density of 60-90% confluency for the next day and allowed to adhere to the surface over night before treatment. The next day, the medium was removed and wells were rinsed to remove unattached and dead cells. Then 0.5ml standard medium containing the effector agent was added.

For the evaluation of susceptibility of melanoma cells to TRAIL, standard medium containing different concentrations of SuperKillerTRAIL™ (skTRAIL) or the TRAIL storage and dilution buffer as mock control were added to the wells and incubated for 16h. To investigate IFN-α-induced apoptosis, cells were incubated with standard medium containing 100-10000U/ml Roferon®-A for 24h. Culture medium with the

same volume of PBS served as negative control. The effect of IFN-α-pretreatment on TRAIL-induced apoptosis was analyzed using cells that had been pretreated with 1000IU/ml Roferon®-A for 24h.

After incubation cells were trypsinized and transferred into a FACS tube. Cells were centrifuged and supernatants were removed completely before cell pellets were carefully resuspended in 100µl 1x Annexin binding buffer (10x Annexin binding buffer diluted 1:10 in ddH_2O (B. Braun Melsungen AG, Cat. No.: 0082479E). Subsequently, 1.5µl Annexin V-FITC was added to each tube. After an incubation period of 10min at room temperature in the dark, cells were washed once with 1x binding buffer and pellets were cerefully resuspended in 150µl 1x binding buffer. 7µl of PI was added immediately before flow cytometric analysis. The percentage of Annexin V single positive and Annexin V/PI double positive cells was then determined (total of early and late apoptotic cells). Sensitivity was defined as follows: <10% apoptosis compared to untreated cells: resistant; 10%-40%: moderately sensitive; >40%: highly sensitive.

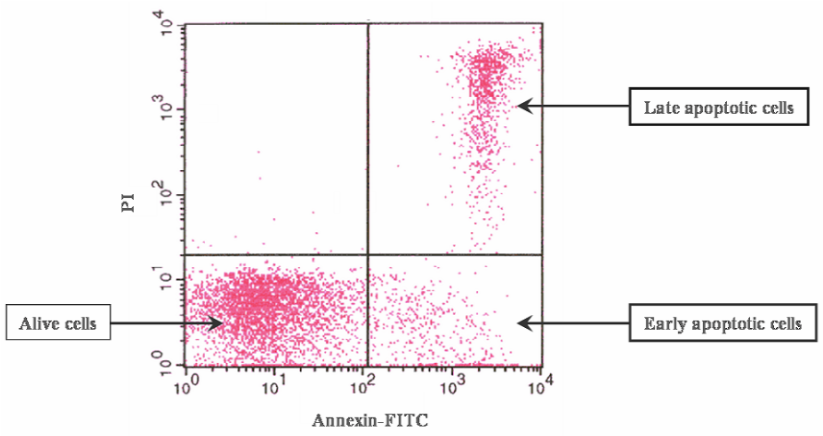

Figure 10: Measurement of apoptosis using Annexin V/PI staining. Early apoptotic cells are Annexin V positive but PI negative (lower right panel), Late apoptotic cells additionally stain positive for PI (upper right panel). Living cells are not stained by either reagent (lower left panel). Figure taken from Bordón et al., 2009[200]

3.7 Europium-TDA release assay

The Europium-TDA release assay can be used to measure cell-mediated cytotoxicity. [12, 201] Target cells are labeled with a fluorescence enhancing ligand (BATDA) before incubation with the effector cells. Inside the cells, BATDA is hydrolysed, forming a compound (TDA) which can no longer penetrate the cellular membrane. During cell lysis, cytoplasmic TDA is released into the cell culture supernatant by labeled cells. When the supernatant is then introduced into the europium solution, TDA forms a highly fluorescent chelate with europium (EuTDA, Figure 11). This fluorescence can be detected by time-resolved fluorescence measurement and correlates directly with target cell lysis.

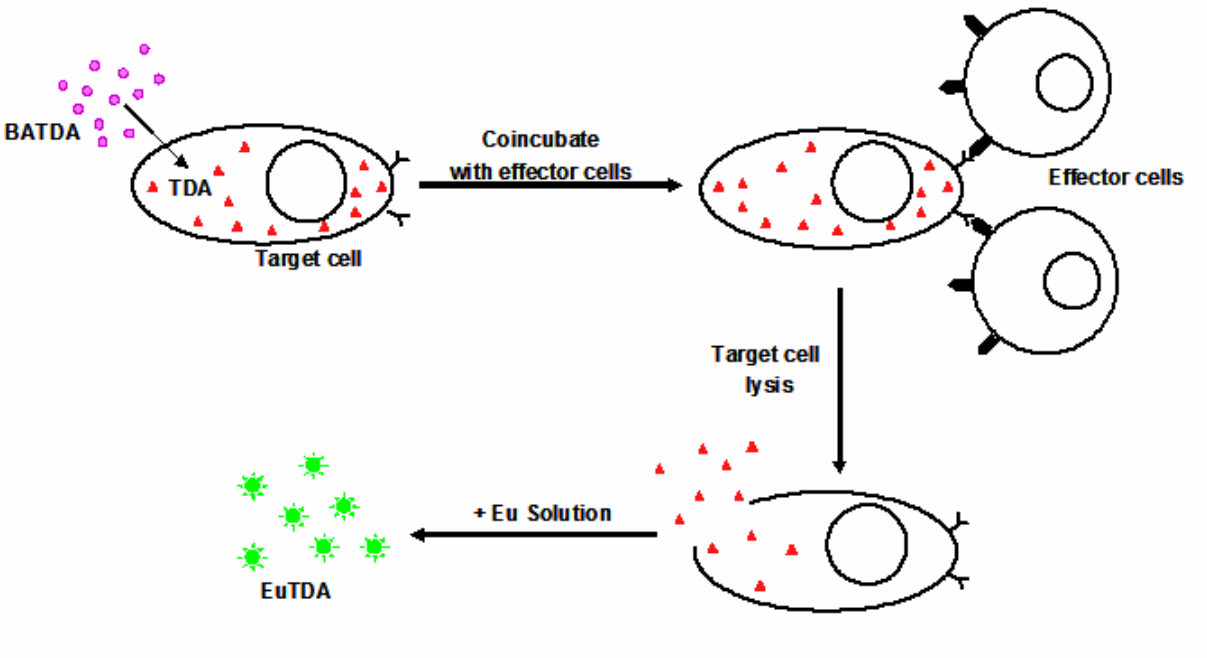

Figure 11: Principle of the Europium-TDA release assay

3.7.1 Experimental procedure

Europium solution:

Europium solution (Perkin Elmer, Cat. No.: C135-100)

BATDA reagent:

BATDA reagent (Perkin Elmer, Cat. No.: C136-100)

Lysis buffer:

Culture medium

2% v/v Triton X (Neuber, Cat. No.: 12298.0100)

Effector cells (pDCs) were isolated the day before the assay and were either stimulated with Roferon®-A or IMQ over night, or left unstimulated.
On the day of the assay, target cells were harvested and resuspended in standard medium to a final concentration of 10^6 cells/ml. Cells were then labeled for 30min in a 37°C waterbath with 4µl/ml (Jurkat) or 2µl/ml (melanoma) BATDA reagent. After labeling cells were washed five times in antibiotic-free medium to remove excess labeling reagent.
2-3x10^3 target cells were coincubated with pDCs in duplicates in a round-bottom 96-well plate at an effector to target ratio (E:T) of 20:1. 100 ng/ml SuperKillerTRAIL™ served as a positive control for TRAIL-mediated killing. TRAIL-dependence of pDC-mediated killing was assessed by pretreating the stimulated pDCs with a neutralizing antibody against TRAIL (R&D, Cat. No.: MAB375) for 30 min before the assay. A mouse IgG1 isotype served as negative control. For spontaneous and maximum release cells were treated with 100µl medium alone and lysis buffer, respectively. The inherent cytotoxicity of the labeling agent BATDA limited maximum incubation time in a cell type-dependent manner. After 2h (Jurkat), 4h (SkMel2) or 6h (WM793) incubation plates were centrifuged for 5min at 601xg and 20µl cell culture supernatant were transferred into a 96-well FluoroNunc™ plate (Nunc, Cat. No.: 437869). 200µl Europium solution was added to each well containing supernatant and the plate was incubated for 15min on a shaker at room temperature in the dark. Fluorescence was determined using a Wallac 1234 Delfia® Fluorometer. Specific Lysis (L) was calculated in percent using the formula L=(E-S)/(M-S)x100 (E=counts in wells treated with effector cells or SuperKillerTRAIL™; S=spontaneuos Lysis in wells with medium only; M=maximum Lysis in wells treated with lysis buffer).

3.8 ^{3}H-Thymidine Proliferation assay

^{3}H-Thymidine solution

0.5ml ^{3}H-Thymidine (Perkin Elmer, Cat. No.: Net027005MC)

9.5ml RPMI 1640

All steps involving radioactivity were performed with the help of Dr. F. Koszik, radiation safety officer.

Measurement of cell proliferation was performed in triplicates. Proliferation rates were assessed 24h, 48h and 96h after IFN-α-stimulation. For each of the three timepoints one flat-bottom 96-well plate was prepared. Melanoma cells were seeded at a density of 3000-10000 cells/well. The next day, 0-10^{5} U/ml Roferon®-A were added. 3.7x10^{4}Bq ^{3}H-Thymidine were added to each well 0h, 24h, or 72h after IFN-α stimulation. After 24h of ^{3}H-Thymidine incorporation plates were frozen at -20°C.

3.8.1 Detection of radioactivity

For the detection of radioactivity plates were thawed at 37°C and radioactively labeled DNA was transferred to a glass filtermat (Wallac, Cat. No.: 1205-401) using a Tomtec Mach II M Harvester. Together with a MeltiLex™ Melt-on Scintillation Sheet (Perkin Elmer, Cat. No.: 1205-441) the filtermat was then heat-sealed in a plastic bag and radioactivity was determined using a Wallac 1205 Betaplate Liquid Scintillation counter.

4 RESULTS

4.1 Characterization of melanoma cell lines

Before their use in this study, the established melanoma cell lines SkMel2, SkMel28 and MeWo as well as the melanoma cell lines Pat1-4, generated from metastases of stage IV melanoma patients, were tested for contamination with mycoplasma. Subsequently the cells were examined for their expression of MHC class I and II, which is often aberrant in tumor cells as well as for TRAIL and TRAIL R expression.

4.1.1 Melanoma cell lines are free of mycoplasma contamination

As shown by the lack of small spots of extranuclear fluorescence, the cell lines used were free of mycoplasma contamination (Figure 12).

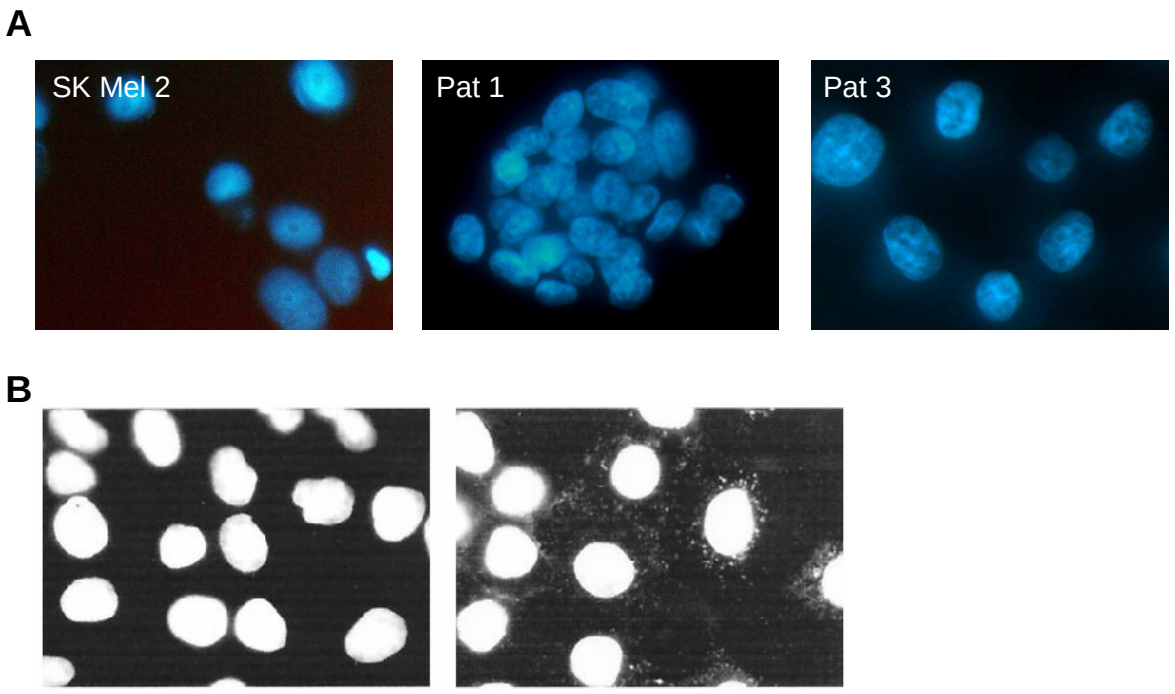

Figure 12: Melanoma cell lines are free of mycoplasma. A) DAPI-stained nuclei of three selected cell lines. No extracellular fluorescence indicating contamination was detected after a minimum of two passages in antibiotic free medium. B) A mycoplasma-free sample (left) compared with a contaminated culture (right). Photos in B were taken from Nir-Paz et al., 2002.[202]

4.1.2 Surface expression of MHC class I and II

In this study, the expression of MHC class I (HLA-ABC) and MHC class II (HLA-DR) on the cell surface was assessed using flow cytometry. The results obtained show that all cell lines except for Pat2 cells expressed high levels of MHC class I on their surface. MHC II was found only on Pat1 cells (Figure 13, Table 10).

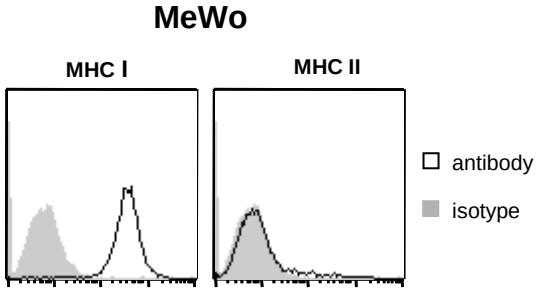

Figure 13: Surface expression of MHC class I and II on MeWo cells. Figure shows a representative example of FACS analysis using MeWo cellls.

Table 10: Surface expression of MHC class I and II on melanoma cell lines. Data obtained by flow cytometry in a minimum of three independent experiments. (++: strong expression (MFI>10); +: weak expression; -: no expression)

Cell line	MHC I	MHC II
SkMel2	++	-
SkMel28	++	-
MeWo	++	-
Pat1	++	++
Pat2	-	-
Pat3	++	-
Pat4	++	-

4.1.3 Expression of TRAIL and TRAIL receptors

The expression of TRAIL and its receptors was assessed at the protein and mRNA level.

TRAIL and TRAIL R surface expression was assessed using flow cytometry. None of the melanoma cell lines tested expressed TRAIL on their surface. Of the DRs, TRAIL R2 was found on all cell lines, whereas TRAIL R1 was not detected. Minute levels of TRAIL R3 were found on SkMel28, Pat1, Pat2 and Pat3 cells. Only Pat3 cells showed TRAIL R4 expression, albeit at a very low level (Figure 14, Table 11).

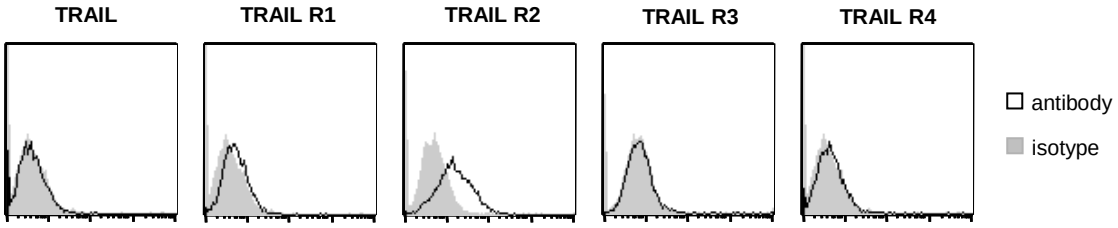

Figure 14: Surface expression of TRAIL and TRAIL R on MeWo cells. Figure shows a representative FACS analysis of TRAIL and TRAIL R expression on MeWo cells.

Table 11: Surface expression of TRAIL and TRAIL R on established and patient-derived melanoma cell lines. Data obtained by flow cytometry in a minimum of three independent experiments. (++: strong expression (MFI>10); +: weak expression; -: no expression)

Cell line	TRAIL	TRAIL R1	TRAIL R2	TRAIL R3	TRAIL R4
SkMel2	-	-	+	-	-
SkMel28	-	-	+	+	-
MeWo	-	-	++	-	-
Pat1	-	-	++	+	-
Pat2	-	-	++	+	-
Pat3	-	-	+	+	+
Pat4	-	-	++	-	-

Next, mRNA levels of TRAIL and TRAIL R were determined using real time PCR and the results were compared to the flow cytometry data. Figure 15 shows the relative mRNA levels of TRAIL and TRAIL R. The mRNA expression in Pat3 cells could not be compared to those of the other cell lines, due to a great difference in the levels of the reference gene GAPDH. This was also the case when another housekeeping gene (β-Actin) was used as reference (data not shown).

The results of flow cytometry were reflected in the mRNA data by the fact that TRAIL R2 mRNA levels were generally 2log higher than those of the other receptors or TRAIL itself.

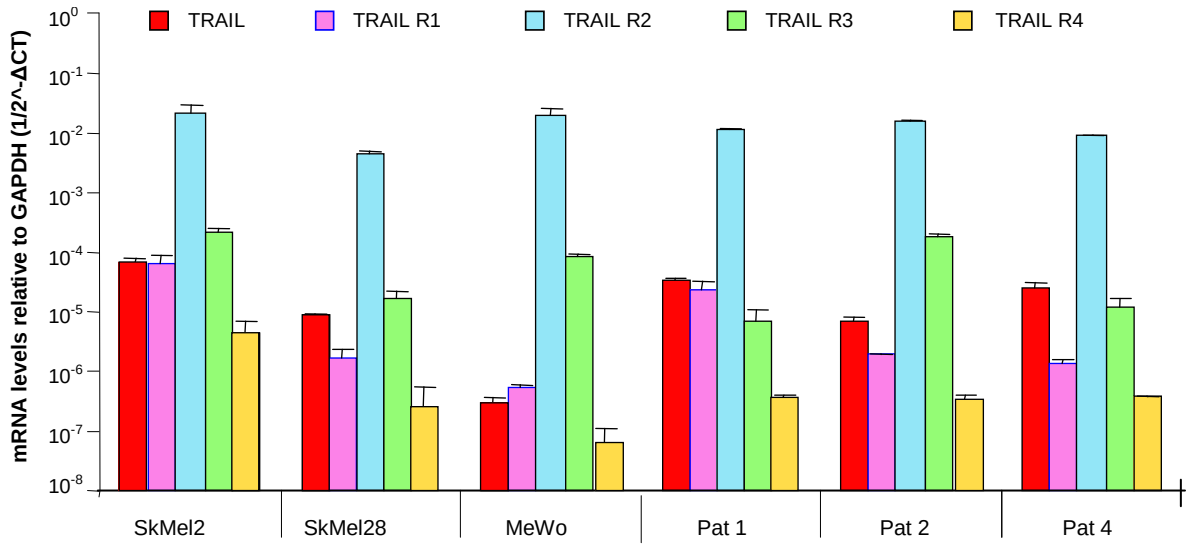

Figure 15: TRAIL R2 mRNA levels were generally 2-log higher compared to those of other TRAIL receptors. Levels of mRNA were determined by real time PCR and normalized to GAPDH expression. Data show mean RNA levels ± SD of two independent experiments performed in duplicates.

Taken together, the results from flow cytometry and real time PCR show that due to their expression of TRAIL R2, all melanoma cell lines tested are potential targets for TRAIL-mediated cytotoxicity.

4.2 Apoptotic and antiproliferative effects of IFN-α on melanoma cells

It has previously been been shown that IFN-α may influence tumor progression not only due to its immune-activating effects but also via direct effects on tumor cells (see 2.2.2).[7, 8] We therefore assessed the effect of IFN-α on melanoma cell proliferation and apoptosis.

4.2.1 Selection of IFN-α

Two recombinant IFN-α compounds, Roferon®-A (IFN-α2a) and Intron®A (IFN-α2b), are available for adjuvant melanoma therapy in Austria. IFN-α2a and IFN-α2b are two allelic variants of the IFN-α2 gene. Although they only differ in two amino acid positions[203], this distinction might affect the response of IFN-α receptor-bearing cells. Further, the composition of the drugs (e.g. salt, pH) might influence their activity. To ensure that the effects observed in this study are not attributable to unique properties of one IFN-α2 compound, Intron®A and Roferon®-A were compared in their ability to induce TRAIL on different PBMC subsets and their effect on melanoma cell proliferation and viability using SkMel2 cells.

The expression of TRAIL on PBMCs was assessed after over night incubation of isolated PBMCs with Roferon®-A or Intron®A (50,000 IU/ml) and, in addition IMQ (5µg/ml). The expression of TRAIL on CD3+ (T cells), CD14+ (monocytes), and CD19+ (B cells) subsets was assessed the next day using flow cytometry.

Both IFNs induced comparable levels of TRAIL on all subsets analyzed (Figure 16). Stimulation of TRAIL expression was strongest in CD14+ monocytes and weakest in CD3+ T cells. IMQ did not induce TRAIL in any of the subsets tested. As we have previously shown, IMQ-induced expression of TRAIL on pDCs depends on the induction of type I IFNs.[14] The lack of TRAIL expression on B cells, T cells and monocytes upon IMQ-stimulation is probably due to insufficient induction of type I IFNs on these cell types. [204, 205]

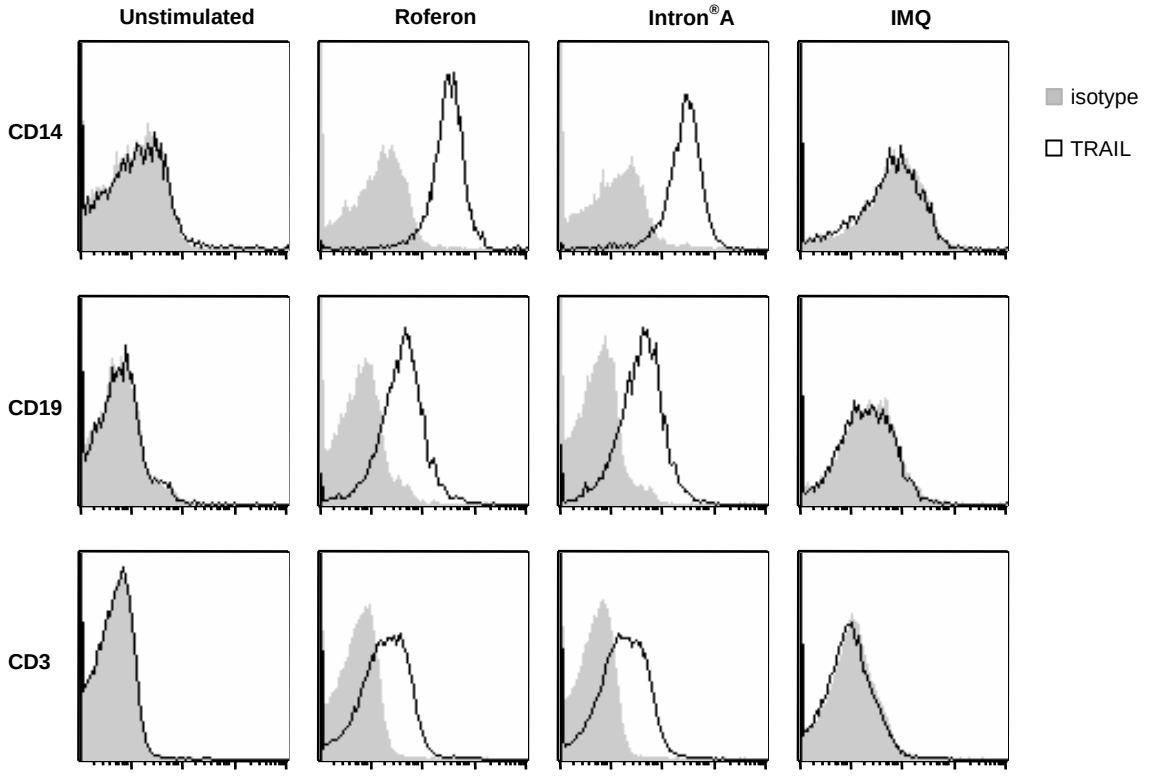

Figure 16: Roferon®-A and Intron®A induce comparable levels of TRAIL on monocytes, B cells and T cells whilst IMQ does not induce TRAIL. TRAIL expression was determined by flow cytometry after 24h stimulation with *Roferon®-A* Intron®A (50,000 IU/ml) or IMQ (5 µg/ml). Cells were gated on CD14 (monocytes), CD19 (B-cells) and CD3 (T-cells). Histograms show results of one experiment performed.

To assess the effect of Roferon®-A and Intron®A on melanoma cell viability, SkMel2 cells were treated with Roferon®-A or Intron®A for 24-96h. The percentage of viable cell was determined via Annexin V/PI staining.

Both IFNs lead to a slight decrease in viablility of SkMel2 cells after 96h treatment with 1,000 IU/ml. The results obtained were nearly identical with both compounds (Figure 17 A).

Proliferation of SkMel2 cells was assessed after 24-96h treatment with Roferon®-A or Intron®A via 24h ^{3}H-Thymidine incorporation. Both IFN-α compounds caused a comparable, concentration-dependent decrease in proliferation (Figure 17 B).

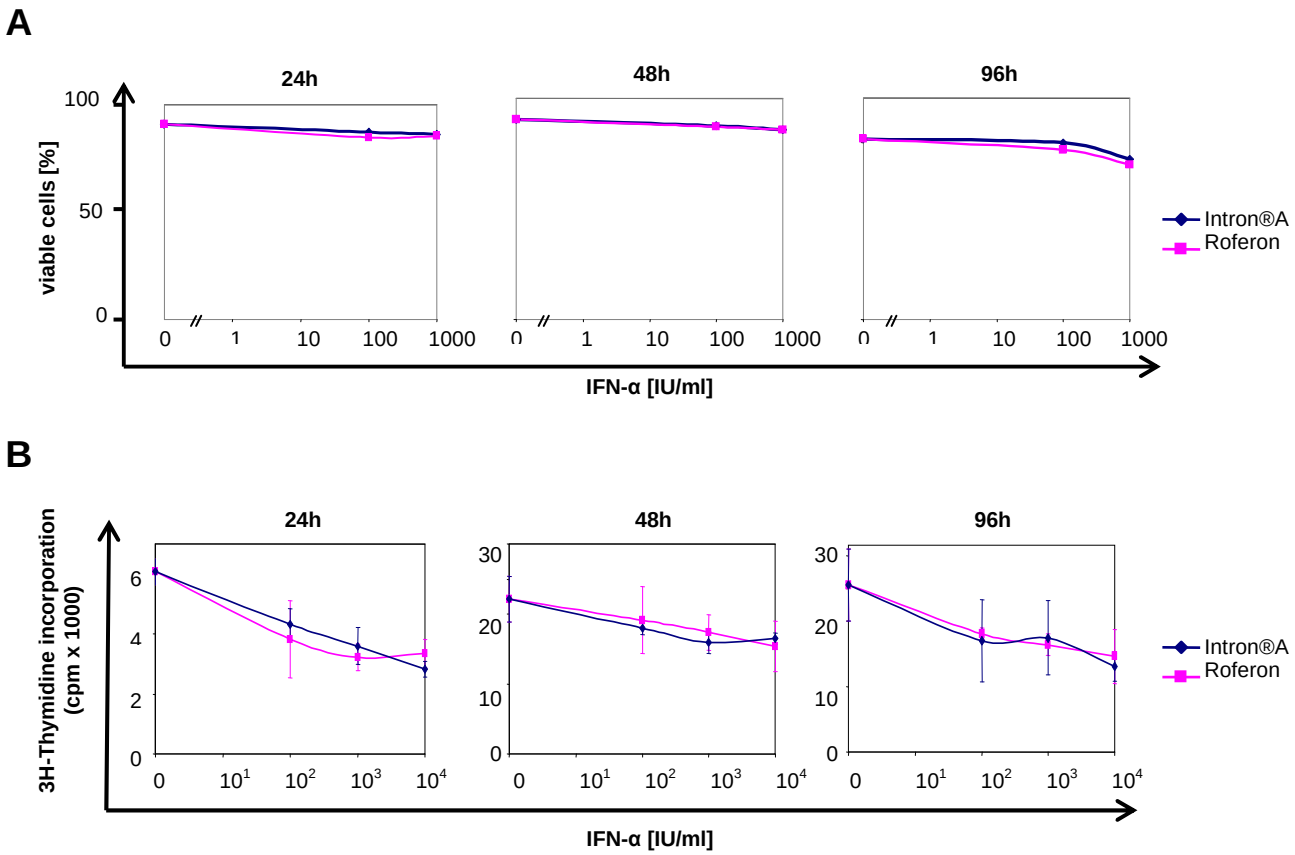

Figure 17: Roferon®-A and Intron®A have similar effects on SkMel2 viability and proliferation. A) Viability of SkMel2 cells after 24, 48 and 96h treatment with Roferon®-A or Intron®A determined by Annexin V/PI staining (n=1). B) Proliferation of SkMel2 cells determined by ^{3}H-Thymidine incorporation. Data shown represent mean ^{3}H-Thymidine incorporation +/- SD of one experiment performed in triplicates.

Since no differences were found between the effects of Intron®A and Roferon®-A in any of the above experiments, Roferon®-A was arbitrarily chosen to be used in this study.

4.2.2 IFN-α inhibits proliferation but does not induce apoptosis in melanoma cells

We next assessed the apoptotic and antiproliferative effects of IFN-α on melanoma cells.

Apoptosis induced by IFN-α was measured after 24-72h treatment with different doses of IFN-α using Annexin V/PI staining. In general the melanoma cells were resistant to IFN-α-induced apoptosis. Even when stimulated with 10,000 IU/ml and after 72h of treatment, apoptosis was not induced in most of the cell lines. Only Pat4 cells showed low sensitivity (12%) to IFN-α after treatment with the maximum dose for 48h and above (Figure 18 A).

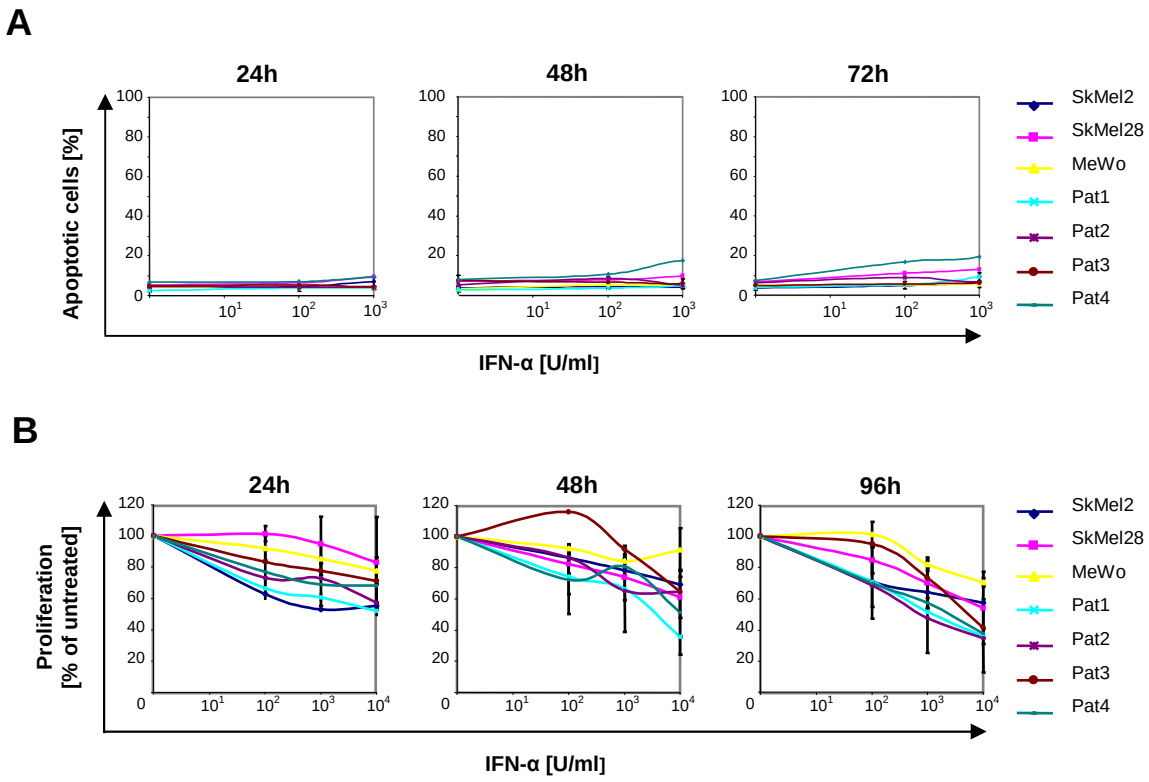

Figure 18: Effect of IFN-α on melanoma cell apoptosis and proliferation. A) Apoptosis of melanoma cells upon treatment IFN-α for 24, 48 and 72h as determined by Annexin V/PI staining. Data shown represent mean apoptosis ± SD (n=2). B) Melanoma cell proliferation upon treatment with different concentrations of IFN-α for 24, 48 and 96h. Data show mean proliferation ± SD of two independent 24h ^{3}H-Thymidine incorporation assays performed in triplicates.

Melanoma cell proliferation was measured via 24h 3H-Thymidine incorporation after 24-96h of incubation with IFN-α (0-10,000 IU/ml). Consistent with previous findings,[7, 74] IFN-α inhibited melanoma cell proliferation in a concentration-dependent fashion (Figure 18 B). This effect was most pronounced in Pat1 and Pat2 cells (max. Reduction of proliferation: about 60% after 96h treatment with 10,000 IU/ml Roferon®-A compared to untreated). The smallest reduction of proliferation compared to untreated cells was seen in MeWo cells (about 30%).

Taken together these results show that IFN-α affects melanoma cell proliferation but does not induce apoptosis in the melanoma cell lines tested.

4.3 TRAIL-induced apoptosis in melanoma cells

Next, we assessed the sensitivity of the melanoma cell lines to skTRAIL using Annexin V/PI staining. In three independent experiments, the sensitivity of the melanoma cells to TRAIL was determined after 16h incubation with skTRAIL (1-100 ng/ml). Results obtained show that SkMel2 and SkMel28 cells were moderately sensitive to a 1 ng/ml dose of skTRAIL and highly sensitive when incubated with 10 ng/ml or 100 ng/ml. MeWo, Pat1, Pat2 and Pat4 cells showed low sensitivity to skTRAIL only at the highest concentration. Pat3 cells were resistant to all concentrations of skTRAIL tested (Figure 19 A).

Interestingly, the sensitivity of a given cell line to skTRAIL did not correlate with the level of surface expression of TRAIL R2, the DR detected on our cells, or its mRNA levels (Figure 19 B, C). Indeed, three of the cell lines with low susceptibility to TRAIL (MeWo, Pat2, Pat4) showed very high surface expression of TRAIL R2, whilst TRAIL R2 expression on the highly susceptible cell lines SkMel2 and SkMel28 was rather low.

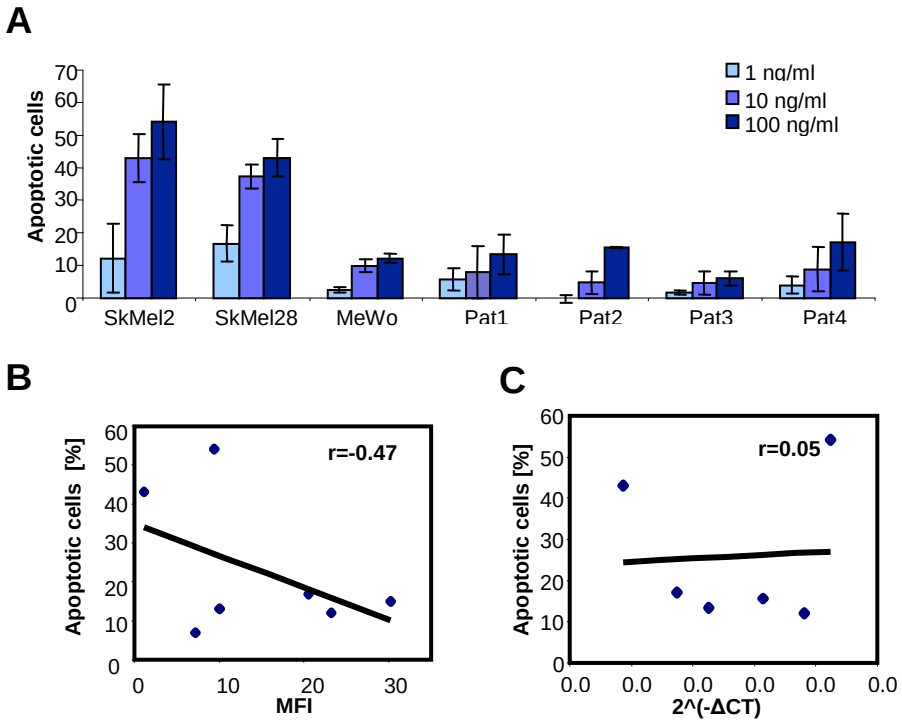

Figure 19: TRAIL induces varying degrees of apoptosis in melanoma cells, irrespective of their TRAIL R expression. Data in A show mean levels of apoptosis (± SD) induced by over night treatment with different concentrations of skTRAIL and normalized to untreated cells (n=3). B and C) Correllation of sTRAIL-induced apoptosis with mean fluorescence intensities of TRAIL R2 expression, determined by flow cytometry (B) and TRAIL R2 mRNA levels relative to GAPDH expression (C). (r indicates Pearson correlation coefficient).

4.3.1 IFN-α but not IMQ enhances TRAIL-induced apoptosis in melanoma cells

Effects of IFN-α on TRAIL-susceptibility:

In a patient treated with IFN-α, melanoma cells may be simultaneously exposed to TRAIL induced by IFN-α and IFN-α itself. To examine whether IFN-α influences the extent of TRAIL-induced apoptosis, the susceptibility of melanoma cells to skTRAIL (100 ng/ml) was re-assessed after pretreatment with IFN-α. Whilst IFN-α alone did not induce apoptosis in melanoma cells, as previously shown (see 4.2), it significantly enhanced TRAIL-induced apoptosis in five out of seven cell lines tested (Figure 20). The effect was most pronounced in Pat4 cells which were only moderately sensitive to TRAIL alone (17% apoptosis), but highly sensitive when pretreated with IFN-α (48% apoptosis). These data demonstrate that IFN-α has a synergistic effect on TRAIL-induced apoptosis in melanoma.

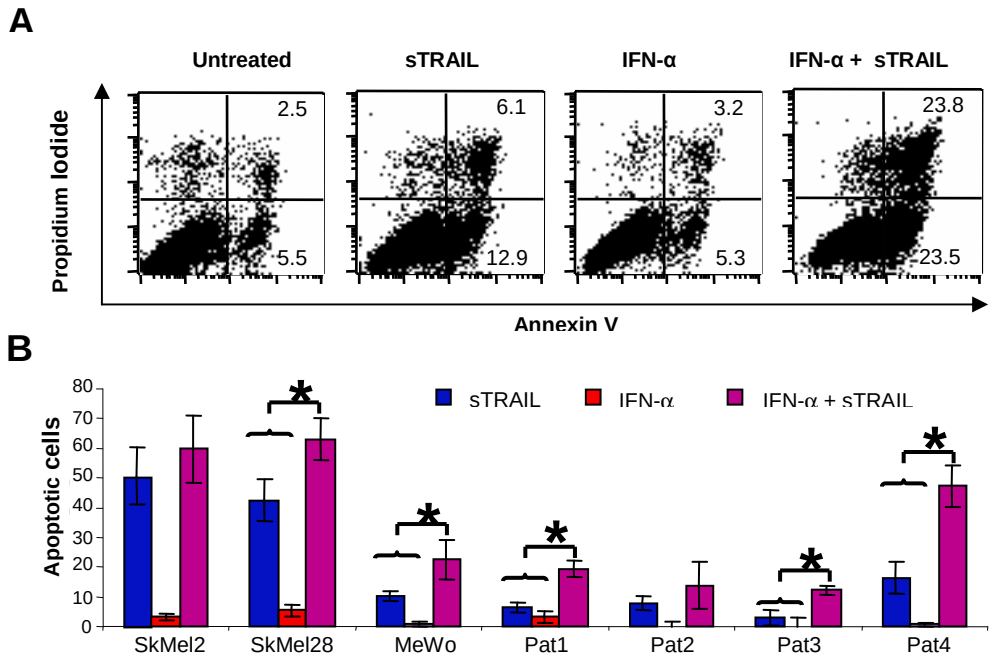

Figure 20: IFN-α-pretreatment of melanoma cells enhances TRAIL-induced apoptosis. Representative dot plots (A) and quantitative analysis (B) of apoptosis induced by skTRAIL (100 ng/ml) with or without 24h IFN-α pretreatment (1,000 IU/ml). Data in B show mean levels of apoptosis ± SD normalized to untreated cells (n=3-5). (∗Significant difference between TRAIL-induced apoptosis after IFN-α-pretreatment compared to the sum of apoptosis induced by TRAIL and IFN-α alone (paired, two-tailed t-test; $p<0.05$).

IFNs have been shown to modulate the expression of TRAIL Rs *in vitro*.[206, 207] Table 12, however, shows that in our study TRAIL-R and also TRAIL-expression on melanoma cells generally remained unchanged upon IFN-α treatment. The upregulation of MHC class I upon IFN-α treatment served as a positive control.[78, 79] Our data indicate that the enhancing effect of IFN-α on TRAIL-mediated apoptosis involves intracellular pro- or antiapoptotic molecules downstream of the TRAIL-TRAIL R interaction.

Table 12: Surface levels of TRAIL and TRAIL R are generally not altered by IFN-α. ++: strong expression; +: weak expression; -: no expression; (↓): downregulation; (↑): upregulation upon 24h treatment with IFN-α (1,000 IU/ml) as determined by flow cytometry (n=3).

Cell line	TRAIL	TRAIL R1	TRAIL R2	TRAIL R3	TRAIL R4	MHC I
SkMel2	-	-	+	-	-	++ (↑)
SkMel28	-	-	+	+	-	++ (↑)
MeWo	-	-	++	-	-	++ (↑)
Pat1	-	-	++	+	-	++ (↑)
Pat2	-	-	++	+	-	-
Pat3	-	-	+	+	+ (↓)	++ (↑)
Pat4	-	-	++	-	-	++ (↑)

Effect of IMQ on TRAIL susceptibility

It has been shown that viral infections can increase cell susceptibility to TRAIL.[208-210] Since RNA viruses are natural ligands for TLR7/8, we asked whether the artificial TLR7/8 ligand IMQ might have a similar effect. We therefore assessed the effect of melanoma cell pretreatment with IMQ and/or IFN-α on TRAIL-induced apoptosis. However, our data indicate that IMQ does not affect melanoma cell sensitivity to TRAIL (Figure 21).

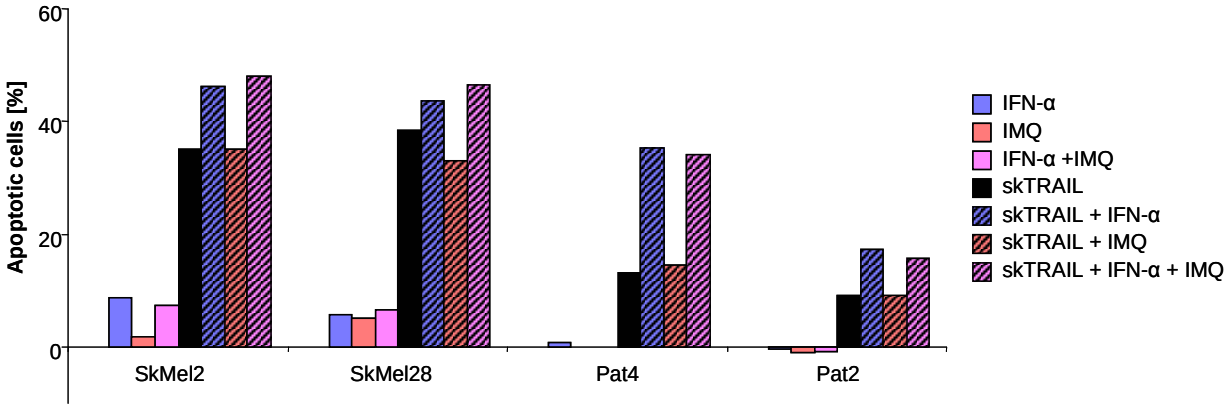

Figure 21: IMQ does not enhance TRAIL-induced apoptosis in melanoma cells. Data show apoptosis induced by skTRAIL (100 ng/ml), IFN-α (1,000 IU/ml) and/or IMQ (5 µg/ml) as determined by Annexin V/PI staining in one experiment per cell line.

4.4 Induction of TRAIL on pDCs by IMQ and IFN-α

Type I IFNs have previously been shown to induce expression of TRAIL on pDCs.[14, 164] The synthetic TLR7/8 ligand IMQ is another strong inducer of TRAIL on this cell type.[12, 14, 164] To compare the effectivity of TRAIL-induction by both stimuli, human pDCs isolated from buffy coats were treated over night with different concentrations of IFN-α or IMQ and TRAIL expression was assessed the next day using flow cytometry. Our results show that both compounds induce TRAIL expression on pDCs. Interestingly, the maturation marker CD83 was upregulated upon IMQ stimulation, but not when pDCs had been stimulated with IFN-α (Figure 22).

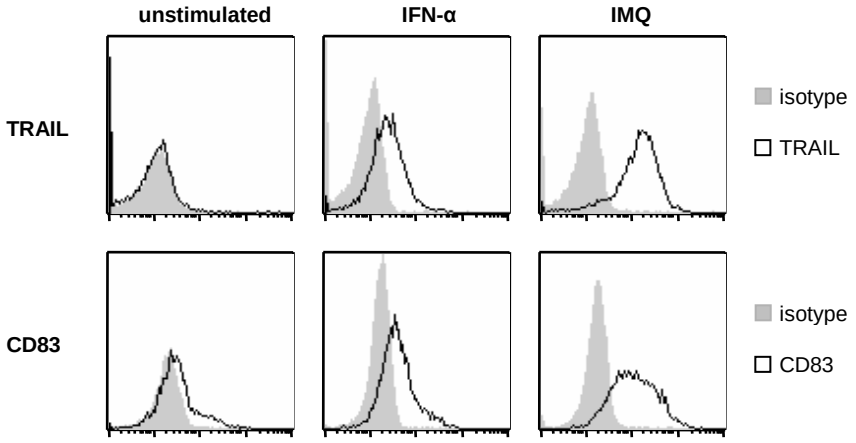

Figure 22: Induction of TRAIL and CD83 on pDCs by IFN-α and IMQ. Expression of TRAIL and CD83 on pDCs freshly isolated from buffy coats, stimulated with IFN-α (10,000 IU/ml) or IMQ (5 µg/ml) over night was assessed by FACS analysis. Cells were gated on HLA-DR and BDCA-2 and purity was greater than 95%. Histograms shown are representative of five independent experiments.

We and others have previously shown that IMQ-induced expression of TRAIL on pDCs is dependent on IMQ-induced expression of IFN-α.[14, 164, 211] However, our results demonstrate that IMQ is a stronger inducer of TRAIL expression than IFN-α (Figure 22). This indicates that TLR7/8 activation could provide a costimulatory signal for TRAIL expression.

To test this hypothesis, isolated pDCs were incubated over night with increasing concentrations of IMQ in presence or absence of IFN-α (10,000 IU/ml). At a concentration of 0.05 µg/ml, IMQ did not induce TRAIL when used alone. In combination with IFN-α, however, low-dose IMQ enhanced TRAIL as well as CD83

expression, resulting in levels comparable to those seen when using high-dose IMQ (Figure 23).

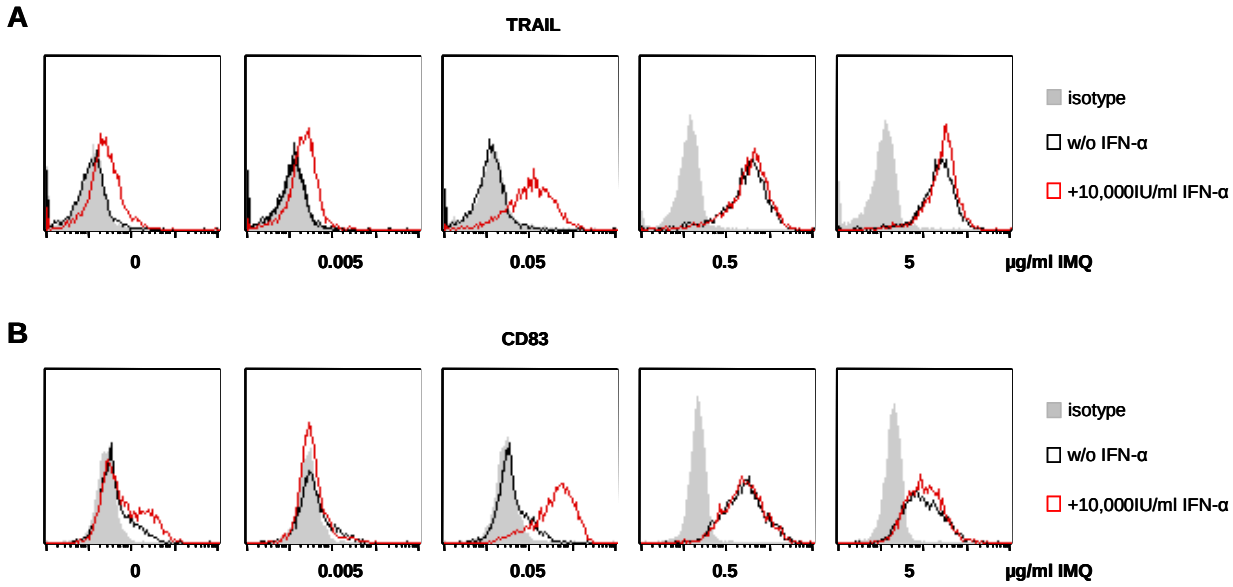

Figure 23: IFN-α-induced expression of (A) TRAIL and (B) CD83 on pDCs is enhanced by IMQ. FACS staining after over night incubation of pDCs isolated from buffy coats with different concentrations of IMQ (as indicated under the histograms) with (red line) or without (black line) 10,000 IU/ml IFN-α. (n=1)

Next, the effect of low-dose IMQ (0.05 µg/ml) on TRAIL-induction with different concentrations of IFN-α was assessed. Low-dose IMQ without IFN-α did not stimulate TRAIL expression (Figure 24A). IFN-α alone induced expression of low levels of TRAIL already at concentrations as low as 50 IU/ml. In presence of low-dose IMQ, TRAIL levels were markedly increased. The expression of the activation marker CD83 was also enhanced by low-dose IMQ (Figure 24B).

Our findings demonstrate that TLR7/8 engagement has a costimulatory effect on induction of TRAIL on pDCs by IFN-α.

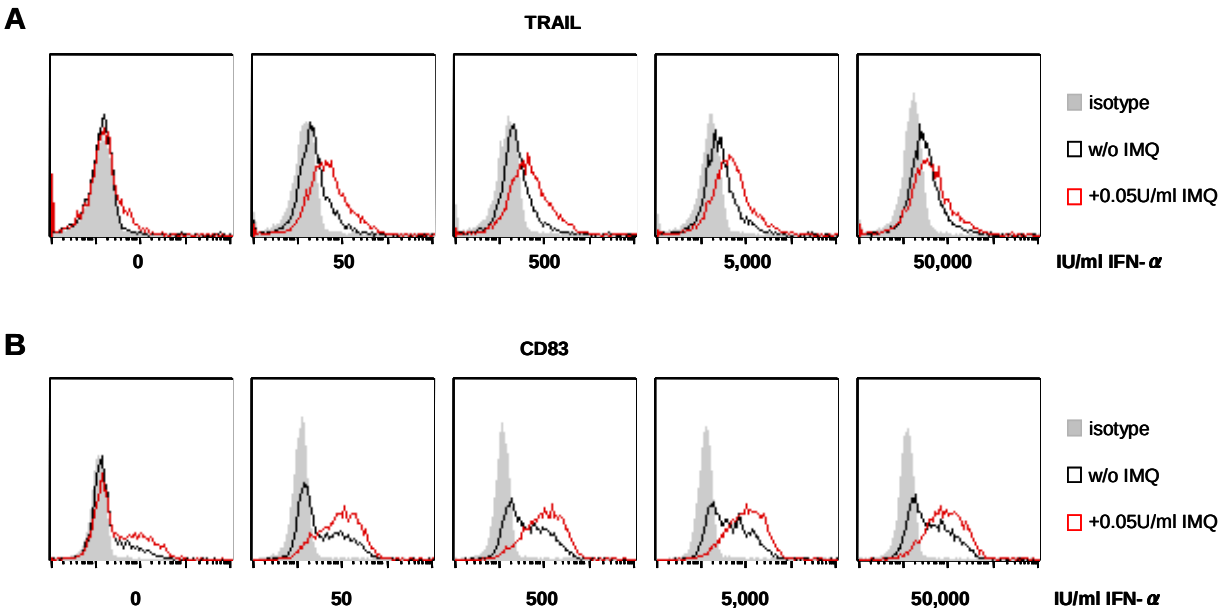

Figure 24: Low-dose IMQ enhances IFN-α-induced expression of TRAIL (A) and CD83 (B) on pDCs. Human pDCs isolated from buffy coats were assessed using flow cytometry after over night incubation with different concentrations of IFN-α (as indicated under the histograms) with (red line) or without (black line) 0.05 µg/ml IMQ. Histograms shown are representative of three independent experiments. This data is also shown in Kalb et al., 2012.[14]

4.5 Analysis of tumoricidal properties of pDCs

4.5.1 TRAIL$^+$ pDCs are capable of lysing TRAIL-sensitive cells

To verify that TRAIL$^+$ pDCs have cytotoxic potential, a conventional 2h EuTDA release cytotoxicity assay was performed using the highly TRAIL-susceptible Jurkat cell line as targets. Expression of TRAIL was confirmed on the day of assay using flow cytometry (Figure 25A).

As expexted, unstimulated pDCs that do not express TRAIL did not exert cytotoxic effects. Both, IMQ- and IFN-α-stimulated pDCs effectively lysed Jurkat cells. Consistent with the higher induction of TRAIL, lysis was greatest when pDCs had been stimulated with IMQ rather than IFN-α (Figure 25B). These results show that IMQ- as well as IFN-α-stimulated pDCs are capable of lysing TRAIL-sensitive cells.

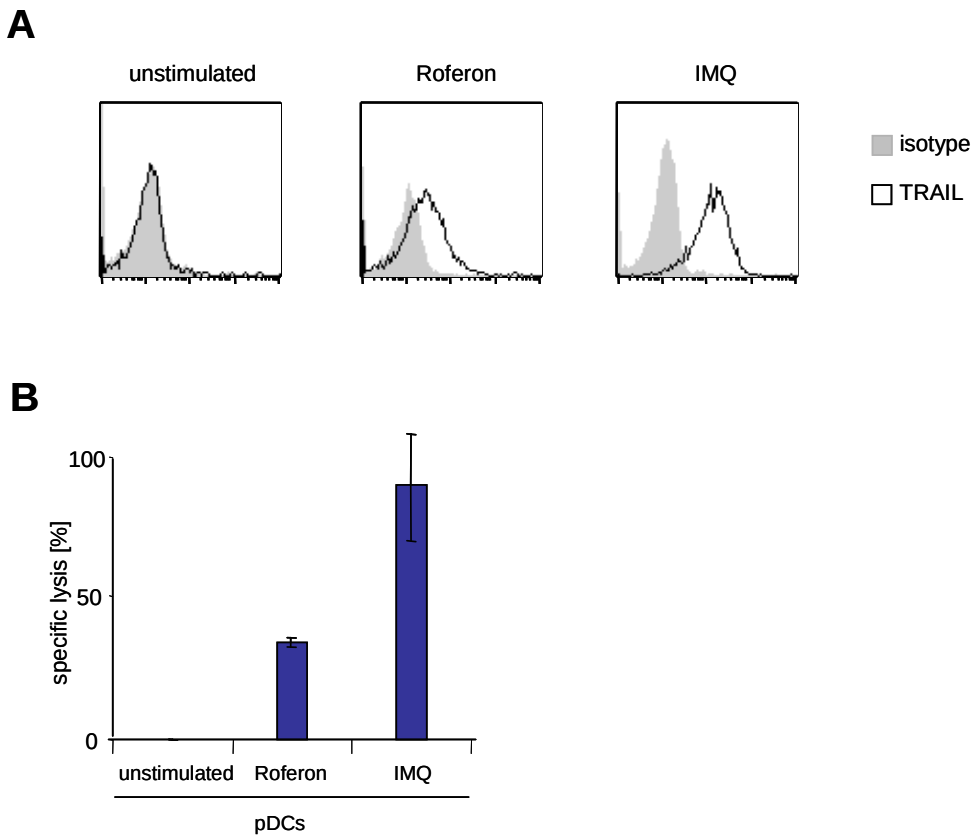

Figure 25: PDCs acquire TRAIL expression and are able to lyse Jurkat cells upon over night stimulation with IFN-α or IMQ. A) Induction of TRAIL on pDCs by IFN-α (50,000 IU/ml) and IMQ (5 µg/ml) as determined by flow cytometry. Data in B represent mean lysis of one 2h EuTDA release assay performed in duplicates (E:T=20:1).

4.5.2 IFN-α- and IMQ- stimulated pDCs effectively lyse melanoma cells

Topical IMQ and systemic IFN-α have been shown to be clinically effective in melanoma patients.[2-5] In man and mice, IMQ-induced regression of skin tumors was associated with an influx of pDCs that expressed TRAIL.[12, 13, 133] To test whether TRAIL$^+$ pDCs could have a role in IFN-α- and IMQ-mediated anti-melanoma activity, we studied the ability of human pDCs to lyse melanoma cells. SkMel2 and WM793 cells, both highly TRAIL susceptible melanoma cell lines due to their expression of TRAIL R2 (Figure 26), were used as target cells. SkTRAIL served as a positive control.

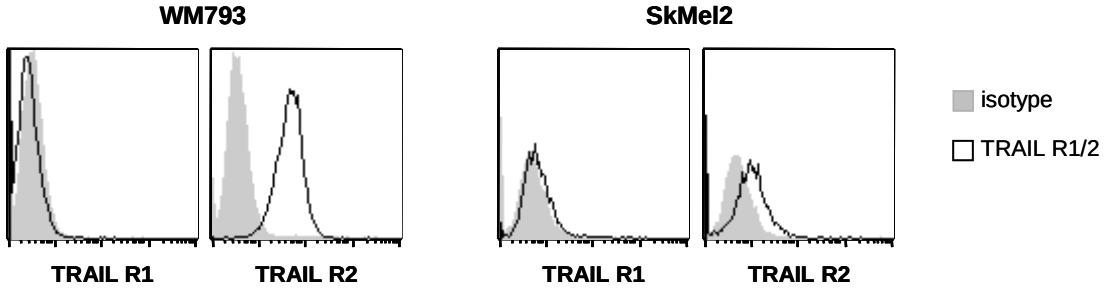

Figure 26: WM793 and SkMel2 cells express the proapoptotic receptor TRAIL R2 but no TRAIL R1. Histograms show flow cytometry results representative of a minimum of three independent experiments.

Figure 27 clearly demonstrates that both IFN-α and IMQ- activated pDCs effectively lyse melanoma targets. There was no significant difference in melanoma cell lysis induced by IFN-α or IMQ-activated pDCs (T-test: p=0.43 and p=0.23 for SkMel2 and WM793, repectively). In all experiments lysis was reduced to baseline level when stimulated pDCs were pretreated with a neutralizing anti-TRAIL antibody, demonstrating the TRAIL-dependence of the cytotoxic effect.

Taken together, these results show that IMQ- as well as IFN-α-stimulated pDCs are capable of killing susceptible melanoma cells in a TRAIL-dependent fashion.

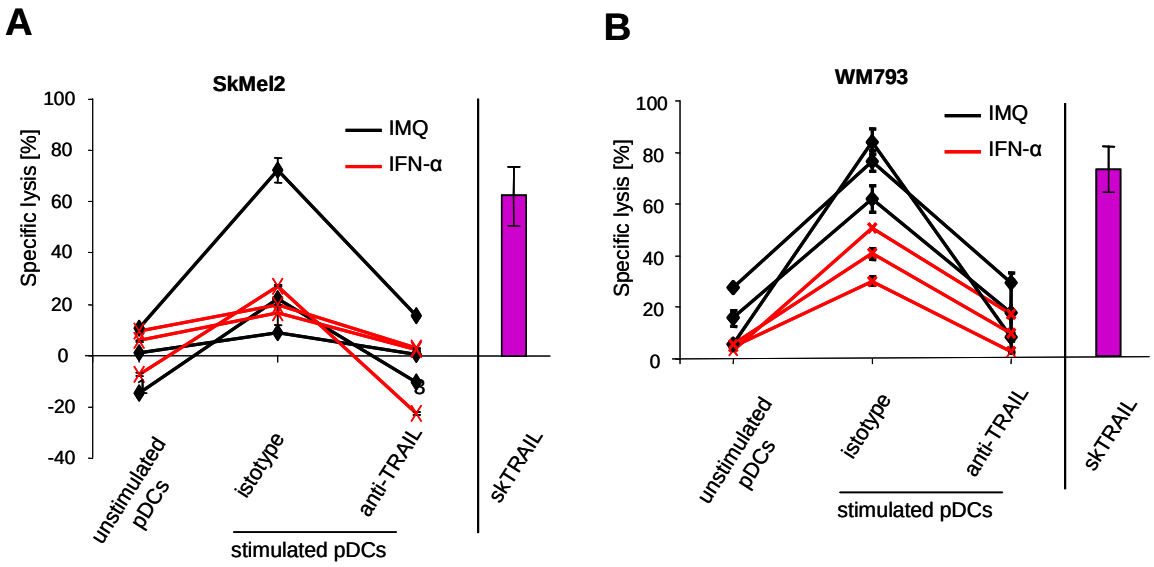

Figure 27: IMQ- and IFN-α-stimulated pDCs lyse TRAIL-susceptible melanoma cells in a TRAIL-dependent fashion. Figure shows results of EuTDA release assays with SkMel2 cells (A) and WM793 cells (B) as targets. PDCs stimulated with IMQ (5 µg/ml) or IFN-α (25,000 IU/ml) were used as effector cells. Baseline levels of cytotoxicity were determined using unstimulated pDCs. TRAIL-dependence of lysis was demonstrated using a neutralizing antibody against TRAIL (anti-TRAIL). SkTRAIL served as positive control for TRAIL-mediated lysis of melanoma cells. (n=3)

5 DISCUSSION

Patients with high-risk primary melanoma may benefit from treatment with IFN-α in an adjuvant setting.[2, 5] At the same time, there exist data that the topical treatment of cutaneous melanoma metastases with the TLR7/8 agonist Imiquimod, an IFN-α-inducer on pDCs, may lead to the disappearance of the tumor.[4] Many effects, including stimulation of the immune system and direct induction of apoptosis have been suggested to contribute to the positive effects observerd with these therapies.[6-10, 122] In this diploma thesis, we sought to gain better insight in the mechanisms behind the anti-melanoma activity of IFN-α and IMQ with particular interest on the role of the cytotoxic molecule TRAIL.

The initial characterization of the melanoma cell lines used in this study by flow cytometry showed that, among the different TRAIL R, only TRAIL R2 was expressed abundantly on all melanoma cell lines. Some cell lines also stained positive for TRAIL R3 (SkMel2, Pat1, Pat2, Pat3). TRAIL R4 was only detected on Pat3 cells, whereas all cell lines lacked TRAIL R1 and TRAIL (Figure 14). These results were reflected in our real-time PCR data as the mRNA levels of TRAIL R2 were generally 2log higher than those of TRAIL or the other receptors (Figure 15). The presence of the DR TRAIL R2 on the cell surface indicates that all melanoma cell lines used in this study are potential targets for TRAIL-mediated cytotoxicity. Since only minute amounts of TRAIL R3 and TRAIL R4 were found on the melanoma cells, they are unlikely to interfere with TRAIL sensitivity by acting as decoy for TRAIL binding.[145] In accordance with our results, Ren et al. showed that the majority of uveal melanoma cell lines express high levels of TRAIL R2, whereas expression of the other receptors for TRAIL generally was rather low.[212] Another study, however, demonstrates that many melanoma cell lines express all four TRAIL receptors whereas some express none at all.[146]

Previous studies have shown that about 66% of human melanoma cell lines are susceptible to TRAIL.[213] In the present study, the melanoma cell lines used displayed different levels of TRAIL-sensitivity (Figure 19). Those derived from melanoma patients were generally less susceptible than the established cell lines from ATCC (6%-17% vs. 12%-54% apoptosis). This finding was not entirely unexpected, as the patient cell lines were derived from metastases of late stage

melanoma patients that had undergone previous treatments. The fact that these patients developed metastases indicates that these melanomas have developed resistance against therapy-induced anti-melanoma mechanisms. As TRAIL is suggested to be involved in tumor immunity, the patient cells may have encountered TRAIL and may have been selected for TRAIL resistance within the body prior to their isolation. Consistent with this hypothesis, Nguyen et al. have shown that TRAIL-sensitive melanoma cell lines rapidly acquire resistance by downregulating TRAIL R expression when cultured in the presence of TRAIL.[214]

It has been proposed that, in melanoma, TRAIL-sensitivity is largely dependent on the expression of TRAIL R2.[146, 215, 216] This finding is partially reflected in our results by the fact that all melanoma cells expressed TRAIL R2 but not TRAIL R1. It is therefore plausible that the apoptotic signal is transduced via this receptor. However, in our system, TRAIL sensitivity did not correlate with TRAIL R2 protein or mRNA expression (Pearson correlation coefficient r=-0.47 and r=0.05, respectively; Figure 19). This demonstrates that other factors also decide upon the faith of a cell after TRAIL-TRAIL R2 interaction. A multitude of pro- and antiapoptotic factors such as Bcl-2 have been shown to affect TRAIL signaling.[155-157] Such proteins are likely to be key regulators of TRAIL-sensitivity and their expression in melanoma should be the subject of further investigation.

Next, we assessed the antiproliferative and proapoptotic effects of IFN-α on melanoma cells. We found that IFN-α inhibits melanoma cell proliferation but does not induce apoptosis in melanoma cells (Figure 18). In 1997, Sangfelt et al. showed that growth arrest and induction of apoptosis are independent reactions to stimulation with IFN-α.[7] Inhibition of cell growth by IFN-α appears to be associated with prolonged activation of STAT1 whereas an intact PI3K/mTOR pathway seems to be necessary for the induction of apoptosis.[74, 89, 217] The activation of this pathway upon IFN-α-stimulation in melanoma cells may be of interest in future studies.

Since IFN-α has previously been shown to enhance the sensitivity of malignant cells to apoptosis-inducing agents,[76, 218] we also assessed the effect of melanoma cell pretreatment with IFN-α on TRAIL-induced apoptosis. Although no direct proapoptotic effect of IFN-α on melanoma cells was detected, IFN-α-pretreatment was found to enhance melanoma cell sensitivity to TRAIL (Figure 20). The increase in apoptotic cell death was significant in five out of seven cell lines and most pronounced in Pat4 cells (from 17% to 48%). Even the TRAIL-resistant melanoma

cell line Pat3 became moderately sensitive upon IFN-α pretreatment. This interesting finding suggests effects of IFN-α on the expression of TRAIL R or other molecules of the apoptotic cascade. It has been previously proposed that IFN-α can affect the TRAIL-susceptibility of Daudi B lymphoma cells and human hepatoma cells through alteration of TRAIL R surface expression.[206, 219] However, we detected no effect of IFN-α on TRAIL or TRAIL R expression on melanoma cells (Table 12). This indicates that IFN-α acts on apoptotic factors downstream of the TRAIL-TRAIL R interaction. Microarray studies have shown that type I IFN affect the expression of a variety of genes involved in apoptosis, including X-linked inhibitor of apoptosis-associated factor-1 (XAF1).[70] This protein appears to be one regulator of TRAIL sensitivity in melanoma as overexpression of this protein potently sensitized A375 melanoma cells to TRAIL.[220] The proteins responsible for enhanced TRAIL-sensitivity upon IFN-α treatment might have potential as therapeutic targets themselves and should therefore be subjected to further examination.

Another factor that is known to increase cellular sensitivity to TRAIL is viral infection.[210, 221, 222] We therefore asked whether, by mimicking a viral infection in melanoma cells, IMQ could also enhance TRAIL-mediated cytotoxicity. Direct effects of IMQ on melanoma cells have already been proposed. Schön et al. have shown that high doses of IMQ can induce apoptosis in melanoma cells.[127] However, they demonstrate that this phenomenon is independent of the activation of TLR7/8 and rather functions via interaction with the adenosine receptor pathway. Cellular systems for the detection of viral infection are highly sensitive and capable of detecting minute amounts of virus components. We therefore chose a lower concentration of IMQ that would not induce apoptosis by itself to study the effects of IMQ on TRAIL-induced apoptosis. In our system, IMQ did not sensitize melanoma cells to TRAIL. Consistent with this finding, Goto et al. found that melanoma cell lines express little or no mRNA for TLR7 and TLR8.[223] Our findings indicate that, although high concentrations may directly induce apoptotic cell death in melanoma cells, IMQ has no enhancing effect on TRAIL-induced apoptosis. However, topical treatment with IMQ is known to cause an infiltration of pDCs.[11, 12] These cells produce large amounts of IFN-α when stimulated with IMQ.[224] PDC-derived IFN-α could therefore sensitize melanoma cells to TRAIL in IMQ-treated patients.

Taken together, our data on TRAIL-induced apoptosis in melanoma cells indicate that TRAIL could have a role in anti-melanoma immunity but melanoma cells often acquire resistance to TRAIL in affected patients. During topical IMQ treatment and IFN-α therapy, however, TRAIL sensitivity of melanoma cells may be restored.

To conclude the study, we looked into one potential aspect of IFN-α- and IMQ-induced anti-melanoma immunity, i. e. the generation of cytotoxic pDCs.[12, 13] The finding that DCs can acquire cytotoxic molecules upon proper stimulation challenges the classical view of DCs being antigen-presenting cells only.[119] Interestingly, the expression of these molecules in DCs is subtype-dependent. Stimulation with IMQ leads to the expression of granzyme B and perforin in mDCs whereas pDCs are induced to express TRAIL on their surface.[14]

PDCs appear to be particularly important in the treatment of cutaneous malignancies with topical IMQ.[11, 12, 133] Recently, it has been shown in a murine melanoma model that topical treatment with IMQ leads to tumor clearence via the conversion of pDCs into $TRAIL^+$ and Granzyme B^+ cytotoxic effector cells.[13] Our group has further demonstrated that the induction of TRAIL on pDCs by IMQ is dependent on IFN-α and that IFN-α itself can stimulate pDCs to express TRAIL *in vitro*.[14] We therefore asked whether cytotoxic $TRAIL^+$ pDCs could play a role in IMQ- and IFN-α-mediated tumor containment in melanoma patients. To this end, we first assessed the expression of TRAIL on human peripheral blood pDCs after overnight stimulation with IMQ/IFN-α using flow cytometry. Both compounds induced the expression of TRAIL on pDCs (Figure 22). IMQ strongly induced the expression of the maturation marker CD83 on pDCs whereas little or no upregulation of CD83 was detected upon IFN-α stimulation (Figure 22), indicating that pDC maturation was not required for the induction of TRAIL.

Interestingly, TRAIL expression on pDCs was considerably higher upon IMQ stimulation than after incubation with IFN-α (Figure 22). Since we have previously shown that IMQ-mediated induction of TRAIL is dependent on IFN-α,[14] this result sugests that TLR7 engagement can provide an enhancing stimulus for IFN-α-induced TRAIL expression. Indeed, when pDCs were incubated with IFN-α and a dose of IMQ that was too low to induce TRAIL on pDCs by itself, TRAIL expression was increased significantly and could also be induced using lower concentrations of IFN-α (Figure 23 and Figure 24). This finding indicates that combination of adjuvant

IFN-α therapy with subclinical doses of IMQ might potentiate TRAIL-mediated cytotoxicity of pDCs in melanoma patients. Combination therapy with IFN-α and IMQ also might reduce the dosage of IFN-α required for the induction of TRAIL on pDCs thus lowering systemic side effects.

Our results confirm that both, IFN-α and IMQ, are potent inducers of TRAIL on human peripheral blood pDCs. Together with the finding that i) a portion of melanoma cell lines is sensible to TRAIL and ii) pDCs are found in the inflammatory infiltrate of IMQ-treated skin tumors,[11] this supports the notion that $TRAIL^+$ pDCs may acquire cytotoxic effector functions and promote tumor clearence in IFN-α- and IMQ-treated melanoma patients. It has previously been shown that the human pDC model cell line GEN2.2 lyses melanoma cells in a TRAIL-dependent fashion.[164] In the present study, the cytotoxic potential of freshly isolated pDCs from human blood was assessed using the EuTDA release assay. Our data clearly demonstrate that freshly isolated pDCs from human blood stimulated with IFN-α or IMQ lyse TRAIL-sensitive melanoma cells *in vitro* (Figure 27). The cytotoxicity of pDCs was dependent on TRAIL, as lysis was reduced to baseline levels in presence of a neutralizing anti-TRAIL antibody. This finding suggests that cytotoxic pDCs play a role in the anti-melanoma effects of adjuvant IFN-α and topical treatment with IMQ. Further studies on murine melanoma models and on tumor samples from patients treated with IMQ or IFN-α will be needed to evaluate the *in vivo* relevance of this mechanism.

Taken together, our data indicate that the induction of TRAIL on immune cells could play a role in IFN-α- and IMQ-mediated melanoma containment. Particularly pDCs may acquire important TRAIL-dependent cytotoxic effector functions in both therapeutic settings.

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7 APPENDIX

7.1 List of abbreviations

AJCC	American Joint Committee on Cancer
ATCC	American Type Culture Collection
BCC	basal cell carcinoma
BDCA	blood dendritic cell antigen
BSA	bovine serum ablumin
DC	dendritic cell
mDC	myeloid dendritic cell
pDC	plasmacytoid dendritic cell
DD	death domain
FADD	Fas-associated DD
DEPC	diethylpyrocarbonate
DISC	death-inducing signaling complex
DITC	dacabazine
DMSO	dimethyl sulfoxide
DR	death receptor
dsRNA	double-stranded RNA
E:T	effector to target ratio
Eu	Europium
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GAS	interferon-γ-activated sites
IFN	interferon
IFN-α	interferon-α
IFNAR	IFN-α receptor
IL-2	interleukin-2
IMQ	imiquimod
IRFs	interferon regulatory factors
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
JAKs	janus kinase
lin^-	lineage negative
LN	lymph node
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MFI	mean-fluorescence intensity
MHC	major histocompatibility complex

NK cells	natural killer cells
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
SCC	squamous cell carcinoma
SD	standard deviation
ssRNA	single-stranded RNA
STATS	signal transducers and activators od transcription
TLRs	toll-like receptors
TNF	tumor necrosis factos
TNM classification	tumor-node-metastasis classification
TRAIL	TNF-related apoptosis-inducing ligand
memTRAIL	membrane-bound TRAIL
rhTRAIL	recombinant human TRAIL
skTRAIL	SuperKillerTRAIL™
sTRAIL	soluble TRAIL
TRAIL R1-4	TRAIL receptor 1-4
T_{regs}	regulatory T cells

7.2 List of figures

7.3 List of tables

7.4 Abstract

Patients with high-risk primary melanoma may benefit from treatment with Interferon-α (IFN-α) in an adjuvant setting. At the same time, there exist data that the topical treatment of cutaneous melanoma metastases with the TLR7/8 agonist Imiquimod (IMQ), an IFN-α-inducer on plasmacytoid dendritic cells (pDCs), may lead to the disappearance of the tumor. Direct anti-tumor activity as well as stimulation of the immune system are thought to contribute to the efficacy of both agents. The induction of the cytotoxic molecule tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), particularly on pDCs, might play an important role in IFN-α- and IMQ-mediated melanoma containment. Both in man and mice, the regression of cutaneous tumors upon topical IMQ-treatment was associated with an infiltrate of pDCs expressing TRAIL. Since IMQ-induced TRAIL expression was shown to be dependent on IFN-α *in vitro*, $TRAIL^+$ pDCs could also play a role in melanoma patients treated with IFN-α.

In this diploma thesis, we focus on the role of TRAIL in the treatment of melanoma with IFN-α or IMQ. We were particularly interested in: i) direct apoptotic and antiproliferative effects of IFN-α on melanoma cells ii) susceptibility of different melanoma cell lines to TRAIL, iii) the correlation of TRAIL-sensitivity with TRAIL receptor (TRAIL R) expression and iv) whether IMQ- and IFN-α-activated pDCs acquire TRAIL-dependent cytotoxic effector functions against melanoma cells *in vitro.*

To this end, we first characterized established melanoma cell lines and such generated from stage IV melanoma patient metastases with respect to their TRAIL and TRAIL receptor expression using flow cytometry and real-time PCR. We found that the death receptor TRAIL R2 was expressed abundantly on all melanoma cell lines, making them potential targets for TRAIL-mediated cytotoxic effects. We next assessed the susceptibility of melanoma cell lines to TRAIL by flow cytometric Annexin V/PI staining. Our results show that overnight treatment with soluble TRAIL induced different levels of apoptosis in the melanoma cell lines. Apoptosis was further increased by pretreatment of the melanoma cells with IFN-α. Finally, we assessed the cytotoxic potential of IFN-α or IMQ-activated pDCs against melanoma targets. Using Europium-TDA release cytotoxicity assays, we show that both, IMQ-

and IFN-α-activated pDCs effectively lyse melanoma cells in a TRAIL-dependent fashion.

Our data indicate that the induction of TRAIL on immune cells could play a role in IFN-α- and IMQ-mediated melanoma containment. Particularly pDCs might acquire important TRAIL-dependent cytotoxic effector functions in both therapeutic settings.

7.5 Zusammenfassung

Adjuvantes Interferon-α (IFN-α) kann in Hochrisiko-Melanompatienten das rezidivfreie Überleben und das Gesamtüberleben in geringem Maße verlängern. Weiters gibt es Hinweise darauf, dass topische Applikation des TLR7/8 Agonisten Imiquimod (IMQ) - einem Aktivator von IFN-α-Expression in plasmazytoiden dendritischen Zellen (pDZ) - zur Regression von kutanen Melanommetastasen führen kann.
Sowohl direkte Effekte gegen Tumorzellen, als auch eine Aktivierung des Immunsystems dürften zur Wirkung beider Substanzen beitragen. Die Induktion des zytotoxischen Moleküls Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), insbesondere auf pDZ, könnte hierbei eine wichtige Rolle spielen. Sowohl bei Menschen als auch bei Mäusen war die Regression von Hauttumoren nach topischer IMQ-Behandlung mit einem Infiltrat TRAIL-exprimierender pDZ assoziiert. Da nachgewiesen wurde, dass die Induktion der TRAIL-Expression durch IMQ *in vitro* von IFN-α abhängig ist, könnten $TRAIL^+$ pDZ auch in der Therapie von Melanompatienten mit IFN-α eine Rolle spielen.

Diese Diplomarbeit beschäftigt sich mit der Bedeutung von TRAIL in der Behandlung von Melanomen mit IFN-α oder IMQ. Besonderes Interesse gilt dabei i) direkten apoptotischen und antiproliferativen Effekten von IFN-α auf Melanomzellen, ii) der Empfindlichkeit verschiedener Melanomzelllinien gegen TRAIL, iii) der Korrelation der TRAIL-Sensitivität mit der TRAIL-Rezeptorexpression und iv) der Frage, ob *in vitro* mit IMQ und IFN-α aktivierte pDZ TRAIL-abhängige zytotoxische Effektorfunktionen erwerben.

Hierfür wurden zunächst etablierte und aus Metastasen von Melanompatienten im vierten Tumorstadium generierte Melanomzelllinien bezüglich TRAIL- und TRAIL-Rezeptorexpression mittels Durchflusszytometrie und real-time PCR charakterisiert. Es zeigte sich, dass der Death Receptor TRAIL R2 auf allen Melanomzelllinien exprimiert ist, was diese Zellen als potentielle Ziele für TRAIL-vermittelte zytotoxische Effekte ausweist. Anschließend wurde die Empfindlichkeit der Melanomzelllinien gegenüber TRAIL mit Hilfe von durchflusszytometrischer Annexin V/PI Färbung untersucht. Die erworbenen Ergebnisse zeigen, dass die Behandlung

von Melanomzellen mit löslichem TRAIL über Nacht Apoptose in unterschiedlichem Ausmaß induziert, welches insbesondere durch Vorbehandlung der Zellen mit IFN-α erhöht wird. Letztendlich wurde das zytotoxische Potential von mit IFN-α oder IMQ stimulierten pDZ gegen Melanomzellen evaluiert. Mit Europium-TDA release cytotoxicity assays wurde gezeigt, dass sowohl IMQ-, als auch IFN-α-aktivierte pDZ Melanomzellen effektiv und TRAIL-abhängig lysieren können.

Die Daten dieser Arbeit zeigen, dass die Induktion von TRAIL auf Immunzellen eine wesentliche Bedeutung bei der Kontrolle von Melanomen durch IFN-α und IMQ haben könnte. Insbesondere pDZ könnten hierbei wichtige TRAIL-abhängige zytotoxische Effektorfunktionen erwerben.

7.6 Curriculum vitae

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Sep. 7 – 10. 2010 Barcelona	41st Annual Meeting of the European Society for Dermatological research
Poster Presentation:	**Astrid Glaser**, Madeleine L. Kalb, Frieder Koszik, Georg Stingl. *IMQ- or IFN-α-stimulated pDCs kill melanoma cells in a TRAIL-dependent manner*