Immunogenicity of B16 melanoma cells after radiochemoimmunotherapy and cell death modulation by zVAD-fmk

Immunogenität von B16 Melanomzellen nach Radiochemotherapie und Zelltodesmodulation durch zVAD-fmk

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Contents

1 Introduction ............................................................................. 6

1.1 Metastatic Melanoma .............................................................. 6

1.2 Anti-tumor immune response ................................................... 7

1.2.1 Immunosurveillance and immunoevasion ............................... 7

1.3 Cell death ............................................................................. 9

1.3.1 Apoptosis ......................................................................... 9

1.3.2 Necrosis .......................................................................... 10

1.3.3 Necroptosis ..................................................................... 11

1.3.4 zVAD-fmk ....................................................................... 12

1.3.5 Immunogenicity of dying and dead cells ................................. 13

1.4 Danger signals ...................................................................... 13

1.4.1 HMGB1 .......................................................................... 13

1.4.2 HSP70 ........................................................................... 14

1.4.3 ATP ................................................................................ 14

1.5 Radiotherapy ........................................................................ 15

1.6 Chemotherapy ...................................................................... 16
1.7 Hyperthermia ................................................................. 16
1.8 Scope of this thesis ............................................................. 18

2 Material and Methods ......................................................... 19
2.1 Reagents and laboratory materials ........................................... 19
2.2 Cell culture ................................................................. 24
2.3 Treatment of B16-F10 cells .................................................. 24
2.4 Cell death determination ..................................................... 25
2.5 Analysis of cell cycle ......................................................... 26
2.6 Detection of danger signals .................................................. 26
2.7 Isolation of peritoneal macrophages ........................................ 27
2.8 Generation of bone marrow-derived dendritic cells ...................... 27
2.9 Analysis of expression of activation markers on dendritic cells and macrophages 28
2.10 Detection of inflammatory cytokines secreted by DCs and macrophages .......... 28
2.11 Phagocytosis assay ......................................................... 29
2.12 Migration assay ............................................................ 29
2.13 Analysis of presentation of the model tumor antigen OVA .................... 30
2.14 Cultivation of murine NK cells ............................................ 30
2.15 Analysis of the maturation status of NK cells ............................. 30
2.16 Kill of B16-F10 cells by NK cells .......................................... 31
2.17 Detection of secreted INFγ by NK cells after activation with treated melanoma cells 31
2.18 Mixed lymphocyte reaction with CD4+ or CD8+ T cells ...................... 32
2.19 Analysis of proliferation of OVA-specific CD8+ T cells .................... 32
2.20 Detection of various cytokines secreted by T cells after activation .............. 33
2.21 Induction of B16-F10 melanomas in C57/BL6, MyD88 KO or RAG KO mice ...... 33
2.22 Treatment of B16-F10 melanomas with ionizing irradiation, hyperthermia, dacarbazine, zVAD-fmk, Apyrase and NK cell depleting antibody................................. 34
2.23 Analysis of immune cell infiltration into the tumor by flow cytometry............... 35
2.24 Analysis of immune cell infiltration into draining lymph nodes by flow cytometry 36
2.25 Analysis of OVA-specific T cell activation by using OT1 mice and B16-OVA melanoma cells........................................................................................................... 36
2.26 Analysis of in vivo proliferation of OVA-specific CD8+ T cells ....................... 37
2.27 Statistical analysis................................................................................................ 37

3 Results ...................................................................................................................... 38

3.1 In vitro immunogenic potential of melanoma cells after treatment with RT, DTIC and/or HT in absence or presence of zVAD-fmk ....................................................... 38
  3.1.1 Melanoma cell apoptosis after combination of DTIC with HT ......................... 38
  3.1.2 Melanoma cell necrosis especially after RT and HT ....................................... 39
  3.1.3 Necroptosis is inducible in B16 melanoma cells .............................................. 40
  3.1.4 zVAD-fmk induces a timely-restricted G2 cell cycle arrest ............................. 42

3.2 Analysis of the in vitro immunogenic potential of B16 cells ......................... 43
  3.2.1 The release of HMGB1 but not that of Hsp70 is enhanced by zVAD-fmk........ 43
  3.2.2 Increased surface expression of MHCII and CD86 on macrophages induced by supernatants of melanoma cells whose cell death was modulated with zVAD-fmk ...... 44
  3.2.3 zVAD-fmk increased the secretion of certain inflammatory cytokines by peritoneal macrophages .............................................................................................................. 48
  3.2.4 Increased surface expression of MHCII and CD86 on DCs induced by supernatants of RT, DTIC and HT treated melanoma cells whose cell death was modulated with zVAD-fmk 49
3.2.5 zVAD-fmk increased the secretion of certain inflammatory cytokines by bone marrow derived DCs ........................................................................................................... 53
3.2.6 Single and multimodal melanoma treatments in the absence or presence of zVAD-fmk do not impair phagocytosis of the tumor cells and migration of macrophages and DCs towards SN of the tumor cells ................................................................................. 55
3.2.7 Presentation of tumor antigen by DCs is not influenced by SN of treated melanoma cells .......................................................................................................................... 57
3.2.8 SN of treated melanoma cells do not impair activation status, maturation status, or kill activity of NK cells .............................................................................................................. 58
3.2.9 SN of melanoma cells do not impact on DC-induced T cell proliferation, but on cytokine secretion when gathered from melanoma cells treated with RT, DTIC and HT in combination with zVAD-fmk ........................................................................................................ 60

3.3 In vivo impact of zVAD-fmk on tumor growth retardation induced by RT or combination of RT, DTIC and HT .................................................................................................................. 62

3.3.1 Combination of fractionated RT DTIC and HT reduces tumor growth significantly and addition of zVAD-fmk further retards it .................................................................................. 62
3.3.2 Combination of fractionated RT, DTIC and HT with zVAD-fmk impacts on immune cell infiltration into the draining lymph nodes and the presentation of tumor antigen ... 66
3.3.3 Combination of fractionated RT, DTIC and HT with zVAD-fmk do not influence the proliferation, but affects the expression of immune stimulatory cytokines of T cells ...... 68
3.3.4 Combination of fractionated RT, DTIC and HT with zVAD-fmk retards tumor growth in a T cell dependent manner ................................................................. 70
3.3.5 Nucleotides and HMGB1 contribute to anti-tumor effects induced by zVAD-fmk in combination with fractionated RT, DTIC and HT ........................................................................ 72

4 Discussion ....................................................................................................................... 74

4.1 B16-F10 cells as mouse model for malignant melanoma ........................................ 74
4.2 Immune therapy as a treatment option against melanoma .............................................. 74

4.3 Immunogenic cell death ............................................................................................ 75

4.4 Necrosis and necroptosis in melanoma cells .............................................................. 76
   4.4.1 Impact of multimodal treatments on melanoma cell death forms ......................... 76
   4.4.2 Induction of melanoma necroptosis by zVAD-fmk .............................................. 77

4.5 Effects of zVAD-fmk on the immunogenic potential of B16 melanoma cells in vitro 77
   4.5.1 Effects of zVAD-fmk on the cell cycle ................................................................. 77
   4.5.2 Effects of zVAD-fmk on the secretion of DAMPs .............................................. 78
   4.5.3 Effects of zVAD-fmk on macrophages and DCs ................................................. 78
   4.5.4 Effects of zVAD-fmk on T cells ......................................................................... 80
   4.5.5 Effects of zVAD-fmk on NK cells ..................................................................... 81

4.6 zVAD-fmk induced B16-F10 tumor growth retardation in vivo ............................... 81
   4.6.1 Immune stimulatory effects of zVAD-fmk in vivo .............................................. 82
   4.6.2 Infiltration of immune cells into the tumor ...................................................... 82
   4.6.3 Tumor growth retardation in immune deficient mice ....................................... 83

4.7 Conclusion .................................................................................................................. 84

4.8 Outlook ...................................................................................................................... 86

References ....................................................................................................................... 87

Publications ..................................................................................................................... 96
Abbreviations

APCs: Antigen presenting cells
ASC: Apoptosis associated speck-like protein
ATP: Adenosin-5′-triphosphate
BRAF: v-raf murine sarcoma viral oncogene homolog B1
Caspase: Cysteine aspartyl-specific proteases
CD: cluster of Differentiation
CFSE: carboxyfluorescein succinimidyl ester
CT: Chemotherapy
CTL: cytotoxic T lymphocyte
CTLA4: cytotoxic T-lymphocyte-associated Protein 4
CYLD: Cylindromatosis
DAMP: Damage associated molecular pattern
DCs: Dendritic cells
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DTIC: Dacarbazine
FADD: Fas associated protein with death domain
FBS: fetal bovine serum
FDA: Food and Drug Administration
FITC: Fluorescein isothiocyanate
Gy: Gray
HLA: Human Leukocyte Antigen
HMGB1: High mobility group protein B1
HSPs: Heat shock proteins
HT: Hyperthermia
IDO: Indoleamine 1,2-dioxygenase
IL: Interleukin
i.p.: Intraperitoneal
i.v.: Intravenous
INF: Interferon
LPS: Lipopolysaccharid
MAPK: BRAF mitogen-activated protein kinase pathway
MDSCs: myeloid derived suppressor cells
MFI: mean fluorescence intensity values
MHC: Major Histocompatibility Complex
Nec-1: Necrostatin-1
NK cells: Natural killer cells
NKT cells: Natural killer T cells
NLRP3: NOD-like receptor family, pyrin domain containing-3 protein
P2RX7: Purinergic receptor P2x
PAMP: Pathogen associated molecular pattern
PCR: Polymerase Chain Reaction
PGE2: Prostaglandin E2
PRR: Pattern recognition receptor
PS: Phosphotidylserin
RAGE: receptor for advanced glycation end product
RHIM: RIP homotypic interaction motif
RIP: Receptor interacting protein
RT: Radiotherapy
SN: Supernatant
TAA: tumor associated antigens
TAM: Tumor associated macrophages
TGF: Transforming growth factor
T_H: T helper cells
TLR: Toll like receptor
TNF: Tumor necrosis factor
TNFR: Tumor necrosis factor receptor
TRADD: TNF receptor associated death domain
TRAF: TNF receptor associated factor
TRAIL: Tumor necrosis factor related apoptosis inducing ligand
Tregs: Regulatory T cells
X-ray : ionizing irradiation
zVAD-fmk: Z-Val-Ala-Asp-fluoromethylketone
List of figures

Figure 1: Radio(chemo)therapies are capable of inducing various cell death forms that differ in their immunogenicity.......................................................... 10
Figure 2: Necroptosis as an alternative form of programmed cell death. ........ 12
Figure 3: Dot plot of an Anx5-FITC/PI staining of B16 melanoma cells 24h after treatment with 2Gy.......................................................... 25
Figure 4: Histogram of the cell cycle phase of B16 melanoma cells 24h after treatment with 2Gy.......................................................... 26
Figure 5: Dot plot of a CFSE/ F4/80 staining of Macrophages and B16 melanoma cells after phagocytosis assay.......................................................... 29
Figure 6: Histogram of a CFSE staining of CD4+ T cells after incubation with LPS activated DCs for 5 days .......................................................... 32
Figure 7: Pictures of local irradiation with the linear accelerator PRIMART of C57/BL6 mice with B16-F10 tumors.......................................................... 34
Figure 8: A schematic diagram and pictures of the local hyperthermia of a local hyperthermia treatment of a C57/BL6 mouse with a B16-F10 tumor. ............ 35
Figure 9: Apoptosis of B16 melanoma cells after single and combined treatment with RT, DTIT and/or HT.......................................................... 38
Figure 10: Necrosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT.......................................................... 39
Figure 11: Necrosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT in the absence or presence of nec-1 or zVAD-fmk. . 40
Figure 12: Impact of necrostatin-1 on zVAD-fmk-induced B16 necrosis. ........ 41
Figure 13: G2 cell cycle arrest of B16 cells after single and combined treatment with RT, DTIC and/or HT in the absence or presence of zVAD-fmk.............. 42
Figure 14: Release of the dangers signals HMGB1 and HSP70 of melanoma cells after single or multimodal treatments with RT, DTIC and/or HT in the absence and presence of zVAD-fmk......................................................... 43

Figure 15: Representative histograms of the expression of MHCII or CD86 on macrophages after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk................................................................. 44

Figure 16: Surface expression of activation markers on macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.................................................................................. 45

Figure 17: Representative histograms of the expression of MHCII or CD86 on macrophages after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk................................................................. 46

Figure 18: Surface expression of activation markers of macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.................................................................................. 47

Figure 19: Secretion of inflammatory cytokines by macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk. ........................................................................................................... 48

Figure 20: Representative histograms of the expression of MHCII or CD86 on DCs after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk. ........................................................................................................... 49

Figure 21: Surface expression of activation markers of dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.................................................................................. 50
Figure 22: Representative histograms of the expression of MHCII or CD86 on DCs of MyD88KO mice after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk................................................................. 50

Figure 23: Representative histograms of the expression of MHCII or CD86 on DCs of C57/BL6 mice after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk................................................................. 51

Figure 24: Surface expression of activation markers of dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.......................................................................................... 52

Figure 25: Impact of anti-HMGB1 antibody on the surface expression of activation markers on DCs after contact with supernatants of treated melanoma cells in the presence of zVAD-fmk. ................................................................. 53

Figure 26: Secretion of inflammatory cytokines by dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk. .................................................................................................. 54

Figure 27: Impact of nucleotides and Toll-like receptor signaling on the secretion of the inflammatory cytokine TNFα by dendritic cells after contact with supernatants of melanoma cells........................................................................... 55

Figure 28: Phagocytosis of melanoma cells by macrophages and dendritic cells and the migration of those towards supernatants of the tumor cells. .............. 56

Figure 29: Presentation of the model tumor antigen OVA by dendritic cells after contact with B16-OVA melanoma cells. ................................................................. 57

Figure 30: Phenotype of NK cells after contact with SN of treated melanoma cells in absence or presence of zVAD-fmk................................................................. 58

Figure 31: Impact of treatment of melanoma cells on killing activity of NK cells. ................................................................................................................................. 59
Figure 32: Release of INFγ by NK cells after contact with SN of treated melanoma cells. ................................................................. 59

Figure 33: Proliferation of T cells after co-incubation with allogeneic or syngeneic DCs pre-incubated with SN of treated melanoma cells. ............... 61

Figure 34: Secretion of cytokines by T cells after contact with dendritic cells after activation by SN of treated melanoma cells. ........................................ 62

Figure 35: Timetable of treatment and in vivo growth of B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk. ................................................................. 63

Figure 36: Infiltration of cells of the innate immune system into B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence and presence of zVAD-fmk. ................................................................. 64

Figure 37: Infiltration of cells of the adaptive immune system into B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence and presence of zVAD-fmk. ................................................................. 65

Figure 38: Immune cell infiltration into draining lymph nodes of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk. ................................................................. 67

Figure 39: Immune cell infiltration into draining lymph nodes of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk. ................................................................. 68

Figure 40: In vivo proliferation of specific CD8+ T cells in B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk. ................................................................. 69
Figure 41: Intracellular expression of INF y and IL2 of CD8+ T cell of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk and ex vivo restimulation with OVA peptide. ............... 69

Figure 42: In vivo growth of B16-F10 tumors in RAG KO mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk. ..................... 71

Figure 43: In vivo growth of B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk and in presence of Apyrase........................................................... 72

Figure 44: In vivo growth of B16-F10 tumors in MyD88 KO mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk. .......... 73

Figure 45: Combination of RT, DTIC, HT with zVAD-fmk is capable of activating both innate and adaptive immune cells against the tumor cells. .................. 84
List of tables

Table 1: Cell lines.................................................................................................................. 19
Table 2: Laboratory materials................................................................................................. 19
Table 3: Instruments ............................................................................................................... 20
Table 4: Reagents .................................................................................................................... 21
Table 5: Antibodies ................................................................................................................ 22
Abstract

Standard tumor therapies like radiotherapy (RT) or chemotherapy (CT) do not only kill the tumor cells directly to destroy the primary tumor but can also induce anti-tumor immune responses against the primary tumor and metastasis. The latter is achievable by rendering the tumor cells immunogenic. Tumor cell death forms like necrosis or its programmed form necroptosis are immunogenic and one basis for the induction of anti-tumor immunity. These cell death forms can be induced by distinct combinations of stress stimuli or by inhibition of apoptosis with the pan-caspase inhibitor zVAD-fmk. Since melanomas are very resistant to ionizing radiation applied in radiotherapy (RT) or chemotherapeutics, the identification of multimodal therapies that induce immunogenic melanoma cell death is a big challenge. Since hints exist that additional immune stimulation by hyperthermia (HT) augments the efficacy of melanoma therapies and that tumors can be sensitized for RT with zVAD-fmk, we asked whether combinations of RT with the chemotherapeutic agent dacarbazine (DTIC) and/or HT induce immunogenic melanoma cell death and how this is influenced by zVAD-fmk. We revealed that necroptosis is inducible in poorly immunogenic B16-F10 melanoma cells with all standard therapies. The highest percentage of necrosis was detected after treatments including RT and HT. zVAD-fmk generally increased melanoma necrosis independent of the treatment, concomitantly with an increased release of danger signals like ATP and especially HMGB1. Supernatants of melanoma cells whose cell death was modulated with zVAD-fmk induced an up-regulation of the activation markers CD86 and MHCII on macrophages. The same was seen on dendritic cells (DCs), but here only when zVAD-fmk was added to multimodal tumor treatments including DTIC. zVAD-fmk also led to an enhanced release of inflammatory cytokines like TNFα or IL-6 by both, macrophages and DCs. DCs or macrophages of MyD88 KO mice and mice treated with apyrase did not increase CD86 and MHCII on their surface. This suggests that nucleotides like ATP and HMGB1 might induce these effects. The presence of zVAD-fmk in combination with RT, DTIC and HT further activated NK cells to produce and secrete INFγ and CD4+ T cells to produce IL-6 and INFγ.
In *in vivo* experiments we revealed that zVAD-fmk added to a combined treatment of the tumor with fractionated RT, DTIC and HT retards the tumor growth significantly and results in significantly reduced infiltration of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Further it increased the infiltration of DCs, NK cells and CD8+ T cells. Concomitantly, a significantly increased NK cell, DCs and CD8+ T cell infiltration into the draining lymph nodes was induced. Especially CD8+ DCs were found that are predestinated for cross presentation of tumor antigen. Furthermore an increased secretion of INF-γ by CD8+ T cells after *ex vivo* re-stimulation was observed when taking the cells of tumor bearing mice that had been treated with fractionated RT, DTIC and HT in combination with zVAD-fmk. Of note is that zVAD-fmk did not reduce tumor growth in RAG KO mice, MyD88 KO mice and mice treated with Apyrase, suggesting that HMGB1 and nucleotides like ATP mediate the anti-tumor immune reaction by zVAD-fmk and that cells of the adaptive immune systems, especially T cells, are the main effector cells. We assume that melanoma cell death modulation with zVAD-fmk results in an enhanced release of the danger signals ATP and HMGB1 by inducing necroptosis. These danger signals activate especially DCs which then activate CD8+ T cells that are capable of killing the tumor cells. We conclude that HMGB1, nucleotides and CD8+ T cells mediate zVAD-fmk induced anti-melanoma immune reactions in multimodal therapy settings.
Zusammenfassung


Zellen, vor allem CD8+ T Zellen, die daraufhin die Tumorzellen effektiv zerstören können. HMGB1, Nukleotide und CD8+ T Zellen sind somit für die, durch zVAD-fmk induzierten, Immunreaktionen in multimodalen Therapien verantwortlich.
1 Introduction

1.1 Metastatic Melanoma

Metastatic melanoma is the most aggressive form of skin cancer with an overall survival of 8 to 18 months. The incidence rate of metastatic melanoma has increased over the past decades [1]. Every year, more than 132,000 patients were diagnosed with melanoma internationally. The rates have increased by 28% in men and 21% in women [2, 3]. The highest incidences are within the white population, especially in Australia and New Zealand. Here, more than 60 cases per 100,000 inhabitants are reported every year [4]. The highest risk factors for melanoma are the genetic background and environmental factors. Above all, excessively exposure to sunlight is the main risk factor. People with green or blue eye color, red or blond hair color and the presence of freckles have a higher risk to get a melanoma [5]. About 20% of all patients develop a metastasis and have therefore very poor prognosis [6]. The usual treatment scheme is the surgical resection. Until 2011 only two drugs were approved for metastatic melanoma, the chemotherapeutic agent dacarbazine (DTIC) and high dose IL-2. But both therapies cannot dramatically increase the overall survival. The response rate of DTIC is only 10-15% and leads to an overall survival of eight months [2]. In comparison, high dose IL-2 induces T cell activation and proliferation and has response rates of 15% but only 6-10% of the patients have complete remissions. But high dose IL-2 induces severe toxicity [3, 7]. Both the toxicity and the absence of randomized phase III trials are the reasons that high dose IL-2 is not a standard form of therapy for metastatic melanoma [1].

In 2011, the Food and Drug Administration (FDA) approved the immunotherapeutic agent ipilimumab, an anti-CTLA4 antibody and also vemurafenib, a v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor, as treatment against melanoma. This form of cancer is a very heterogenous disease but 40-60% of the tumors have mutations in the gene coding for BRAF and 90% of these mutations results in the substitution of valine for glutamine (V600E) [8, 9]. Due to the mutated form of BRAF, mitogen-activated protein kinase pathway (MAPK) is constitutive activated and leads to
an increased cellular proliferation and oncogenic activity. The BRAF inhibitor vemurafenib is selective for BRAF with the V600E mutation. A phase III trial compared patients with vemurafenib to dacarbazine and demonstrated improvements in response rates (48% to 5%) and in progression free survival (5.3 to 1.6 month) [9]. But the therapy is limited by a short duration that averages only 6 month [1].

The melanoma is considered as “immunogenic” since it has the ability for spontaneous regressions [10-12]. For that reason the immune therapy is an innovative and promising approach for treatment of melanoma. Due to the immunogenicity of melanoma, they are often associated with infiltration of lymphocytes. The infiltration correlates with areas of histologic regression [11].

CTLA4 is a negative regulator of the immune system. Up-regulation of CTLA4 on the surface of cytotoxic T cells (CTLs) results in termination of proliferation and activation of these cells. Owing to the fact that CTLs are the key players in the immune response against the tumor, suppression of CTLA4 can be an effective treatment against melanomas [13, 14]. Different clinical trials could show that response rates of the anti-CTLA4 antibody ipilimumab are between 5 and 20%. Studies with higher concentrations of ipilimumab could show better response rates but higher toxicities have been observed [15-17].

1.2 Anti-tumor immune response

Efficient anti-tumor therapies aim to stop the proliferation of the tumor cells, kill them and induce a systemic anti-tumor immunity. This leads not only to the deletion of the primary tumor but also to the prevention of metastasis and recurrences. For an immune response against the tumor, the immune cells have to recognize the tumor cells. Activated immune cells, which recognized the tumor as foreign, can fight the tumor. An immune memory forms which can protect against metastasis and recurrences. However, tumors can also efficiently escape immune surveillance when being established and during development.

1.2.1 Immunosurveillance and immunoevasion

Tumor immune escape consists of three steps. The first step is the elimination phase and the surveillance by immune cells [18]. It starts with recruitment of
innate immune cells such as NK cells, NKT cells, γδ T cells, macrophages and dendritic cells (DCs) [19]. Especially NK cells kill tumor cells, which results in the release of tumor specific antigens. The antigens are phagocytosed and processed by DCs. The DCs migrate to the next lymph node while presenting the tumor antigen via MHCI or cross present them via MHCI. This leads to activation of naïve CD4+ and CD8+ T cells [20]. The activated T cells migrate back to the tumor and attack and kill tumor cells [21]. Immunosurveillance results in destroying of premalignant cells so that tumors do not develop. But the anti-tumor immune response might be too weak to prevent cancerogenesis. The cells enter the equilibrium phase which can last for years. In this phase, cells and mediators of the adaptive immune system prevent the outgrowth of tumor cells but cannot destroy them completely. The main actors are CD4+ and CD8+ T cells and the cytokines INFγ and IL-2. In the escape phase, tumor cells begin to expand in an uncontrolled manner [19]. The escape may result from the enrichment of immunosuppressive immune cells, mediators and cytokines in the tumor microenvironment. Responsible are production of mediators like galectin-1 or indoleamine 1,2-dioxygenase (IDO) or the immunosuppressive cytokines IL-10 or TGFβ as well as the recruitment of regulatory immune cells as regulatory T cells (Tregs) or myeloid derived suppressor cells (MDSCs) [22]. The secreted products of the tumor can also obviate the differentiation, maturation and migration of DCs and therefore the effector function of these cells [23]. Tumor cells do not only escape from innate and adaptive immune system but also change processing and presentation of tumor associated antigens (TAA) by altering expression of MHCI or HLA molecules [24-26]. Additionally, vesicles with tumor antigens – tumor exosomes – are able to induce tolerance against the tumor [27]. The type of macrophages in the tumor has also effects on the tumor microenvironment. Tumor associated macrophages (TAM) mainly are M2 macrophages which results in T\textsubscript{H2} responses through the production of prostaglandin E2 (PGE2), TGFβ and IL-10. In contrast, M1 macrophages results in T\textsubscript{H1} responses and therefore in anti-tumor immunity by production of IL-12 (summarized in [18, 28]). Different mediators induce the type of macrophages. TNFα, LPS or INFγ lead to a shift to M1. In contrast IL-4, IL-10 or TGFβ favor M2 macrophages [29]. A chronic inflammatory environment and hypoxic conditions promote tumor development.
It decreases the secretion of IL-12 and TNFα from macrophages and increases IL-10 secretion by these cells [30, 31]. Therefore tumor development can be a consequence of a chronic inflammation [32, 33]. But also the form of tumor cell death impacts on induction of anti-tumor immunity.

1.3 Cell death

It has become evident that cancer cells can be rendered visible to the immune system by standard therapies such as chemotherapy (CT) or radiotherapy (RT), either alone or in combination with (further) immune stimulation (e.g. with hyperthermia (HT)). The resulting tumor microenvironment determines which immune cells get recruited and triggers the activation or suppression of DCs. It has become more and more evident that besides the induction of tumor cell apoptosis by RT, CT and/or immune therapy, other tumor cell death forms exist that bear high immunogenic potential.

1.3.1 Apoptosis

It was thought for long time that apoptosis is the only programmed death mechanism in animals and therefore absolutely essential for development and tissue homeostasis. In addition, apoptosis impacts on immune reactions. Cells express Fas or TNF receptors via that immune cells can induce apoptosis which is characterized by distinct morphological changes. During the early phase of apoptosis, cell shrinkage and chromatin condensation (pyknosis) takes place [34]. Because of cell shrinkage, the cells become smaller which is in combination with chromatin condensation one of the most characteristic feature of apoptosis. In later apoptosis, membrane blebbing and destructive fragmentation of the nucleus (karyorrhexis) occurs. This is followed by separation of cell fragments into apoptotic bodies which is called “budding” (summarized in [35]). Mainly apoptotic cell death is non- or even anti-inflammatory since apoptotic cells do not release their cellular constituents into the surrounding tissue. Immune cells, especially macrophages, quickly phagocytose most apoptotic cells which prevent secondary necrosis. The engulfing cells do not produce inflammatory cytokines [36, 37]. But apoptosis is not non- or even anti-inflammatory in general. Under specific conditions, apoptosis can be immunogenic. Mostly immunogenic apoptosis is characterized
by expression of calreticulin, especially on early apoptotic cells [38], [39]. Additionally, apoptotic cell death is necessary for induction of an efficient anti-tumor immune response since apoptotic cells recruit immune cells to the tumor [13]. However, mice with defects in key elements of apoptosis have an almost normal development. One possible and perhaps the only explanation for this fact is the existence of other programmed death mechanisms which can balance defects of apoptosis [40]. Those death forms are of main interest in cancer therapy, since often resistances to apoptosis are observed [41].

1.3.2 Necrosis

![Diagram of immune response to cell death forms](image_url)

**Figure 1:** Radio(chemo)therapies are capable of inducing various cell death forms that differ in their immunogenicity.

The tumor cells die after treatment with chemotherapeutics, hyperthermia and ionizing irradiation. Dependent on the treatment the tumor cells can die apoptotic which results in a non- or even an anti-inflammatory response. In contrast cells can die necrotic which results in release of DAMPs and therefore in an activation of both, the innate and adaptive immune system. Modified after [42].

In contrast to apoptosis, necrosis was considered as a passive and uncontrolled cell death form. It is induced by extremely cellular stress like heat stress or by toxic agents.
However, these stimuli can also induce apoptosis. Mostly the energy level determines the cell death form. It could be shown that low levels of ATP lead to necrotic cell death [43, 44]. Necrotic cells are morphological clearly different from apoptotic ones. The plasma membrane becomes permeable, organelles can dilate and ribosomes dissociate from the endoplasmic reticulum. In the late necrosis, the nucleus also disintegrates [45].

1.3.3 Necroptosis

Nowadays, evidences have come up that also a programmed form of necrosis exist, the so called necroptosis [46]. It is characterized by similar changes as they occur in classical necrosis like rounding of the cell, cytoplasmic swelling, lack of DNA fragmentation and plasma membrane rupture. Necroptosis can be induced by activation of pattern recognition receptors (PRRs) like Toll like receptors (TLR). The PRRs are expressed on almost all cells of the innate immune system and recognize pathogen associated molecular patterns (PAMPs) [47]. PAMPs have been shown to induce necroptosis in some cell types. For example, Lipopolysaccharide (LPS) can induce necroptosis in macrophages when caspase 8 activity is inhibited [48]. Necroptosis can be further activated by TNFα, Fas Ligand, TRAIL, respectively by ligation of the death receptors (CD95, TNFR1, TNFR2, TRAIL1 or TRAILR2). Of note is that the same triggers can also induce apoptosis [49]. However, necroptosis is independent of caspases and mainly occurs when the caspases (especially caspase 8) are not activated or inhibited [50]. It has to be regarded as a cell death backup mechanism when apoptosis is inhibited [51]. The best characterized pathway for the induction of necroptosis is the ligation of TNFR1. Of note is that addition of TNFα can cause cell survival, apoptosis and necroptosis (Figure 2). If the so called complex I is formed, cell survival results. Complex I consists of TNF receptor associated death domain (TRADD), receptor interacting protein1 (RIP1), TNF receptor associated factor (TRAF2) and TRAF5. The apoptotic pathway starts after formation of complex Ila. This complex consists of caspase 8, FADD and RIP1. The deubiquitinilation of RIP1 by CYLD results in the activation of the RIP1 and RIP3. Caspase 8 activates the classical caspase cascade and can further inactivate RIP1 and RIP3 by cleaving. If caspase 8 is inhibited or deleted, the apoptotic pathway is disturbed resulting in activation of the necroptotic pathway [52]. RIP1 and RIP3 are
therefore the key factors for necroptosis. They interact via their RIP homotypic interaction motif (RHIM).

**Figure 2: Necroptosis as an alternative form of programmed cell death.**
After binding of TNFα to the receptor, complex I is building. This can result in three ways, in activation of NF-κB, in apoptosis or necroptosis. Thereby necroptosis is inhabitable with necrostatin-1 (nec-1) and can be induced by blocking apoptosis with zVAD-fmk. Modified after [40].

RIP1 is a serine-threonine kinase and exists in two forms, the open and the closed form. The catalytically cleft is blocked in the closed form and the protein is inactive. Due to the autophosphorylation on Ser161 in the activation loop, the protein is in the open form and the catalytically cleft is accessible for the substrate [53]. This can be inhibited by necrostatin-1 (nec-1). Therefore, nec-1 is an effective inhibitor of necroptosis.

1.3.4 zVAD-fmk
Another possibility to induce necroptosis is inhibition of apoptosis by zVAD-fmk. It has been shown that zVAD-fmk is capable of inducing this programmed form of cell death in a selected group of cells, e.g. in mouse fibrosarcoma L929 cells [54]. This pan-caspase inhibitor blocks apoptosis by inhibiting caspase 8 and therefore may foster necroptosis by triggering the induction of the autocrine production of TNFα and simultaneously by inhibiting the apoptotic pathway.
Moretti et al. showed that inhibition of caspase 8 by zVAD-fmk enhances the efficacy of RT in solid tumors. It decreases the tumor outgrowth and increase extracellular HMGB1 in the tumor microenvironment [55].

1.3.5 Immunogenicity of dying and dead cells

Independent of the way of necrosis induction, this cell death form is generally more immunogenic compared to apoptotic cell death. Specialized phagocytes, especially macrophages, swiftly take up apoptotic cells. This results in non- or even anti-inflammatory events. The activated macrophages secret anti-inflammatory cytokines like IL-10 or TGFβ after contact with apoptotic cells [56]. In contrast, necrotic cells have immune stimulatory potential. Due to the necrotic cell death, the plasma membrane of the cells becomes permeable. This leads to the release of damage associated molecular pattern (DAMPs) [57].

1.4 Danger signals

Most danger signals are present in every cell and are necessary for survival of the cells. They are required intracellular but act as danger signals when being extracellular. The presence of DAMPs outside the cell is a sign for danger that activates the immune system [58].

1.4.1 HMGB1

One of the most prominent danger signals is HMGB1. It is ubiquitously expressed in the nucleus of mammalian cells and highly conserved between different species. It act as a non-histone chromatin-associated protein, it binds to DNA and facilitates the binding of transcription factors [59]. Another function is it’s the role in recognition of DNA damages in the process of mismatch repair [60]. Since necrosis leads to plasma membrane rupture, intracellular HMGB1 gets passively released [61] [62], but inflammatory cells even secrete HMGB1 actively [63]. Standard tumor therapies like RT or CT have been shown to induce the release of HMGB1 [64], which then can act as immune activating DAMP. Such therapies induce both, apoptosis and necrosis. HMGB1 can bind several receptors but it mostly binds to the receptor for advanced glycation end products (RAGE) and to toll like receptors (TLR), especially TLR2 and TLR4.
HMGB1 thereby represents a strong activator of DCs [66]. It fosters antigen cross presentation by DCs and consecutive activation of naïve T cells.

1.4.2 HSP70

Other important DAMPs are heat shock proteins (HSPs), especially HSP70 [67]. Inside the cell, HSPs protect the cells of cellular stress. They act as chaperones and thereby stabilize proteins or can ubiquitinate damaged proteins which leads to their degradation in the proteasome. Of note is that outside the cell, HSPs can efficiently activate the immune system. Many HSPs like HSP70 chaperon tumor proteins. When HSP70 gets released, it delivers the bound antigens to APCs. The latter internalize the HSPs and thereby also the antigens by receptor mediated endocytosis. Finally, the tumor antigens get cross-presented via MHCI molecules and can stimulate the CD8+ T cell response (cytotoxic T cells; CTL) in this way [68]. Another immune stimulatory effect of HSPs is the enhanced secretion of proinflammatory cytokines by APCs like DCs when binding to PPR. Taken together, extracellular HSPs act as danger signals resulting in maturation and activation of APCs. But HSPs do not only act on APCs they also stimulate and activate NK cells. HSP70 in presence of proinflammatory cytokines stimulate the cytotoxic activity of NK cells [69].

1.4.3 ATP

Another DAMP is Adenosin-5’-triphosphate (ATP). In contrast to the other DAMPs that are mostly recognized by PRRs, there is another sensor for ATP, the inflammasome. The NOD-like receptor family pryrin domain containing-3 protein (NLRP3) inflammasome detects bacterial products or endogenous damage signals like ATP, uric acid kristals or alum [70]. Usually, the intracellular concentration of ATP is relatively high (3-10mM) but the extracellular concentration is pretty low (400-700nM) [71]. Different sorts of stress induce the release of ATP by the cells. ATP acts on purinergic receptors especially on P2RX7 [72]. ATP binds the P2RX7 receptor on DCs which leads to the activation of the NLRP3 inflammasome [73]. ATP stimulation results in the aggregation of NLRP3 with apoptosis-associated speck-like protein (ASC) and caspase-1. The mature caspase-1 cleaved pro-IL-1β and IL-1β gets released. Active IL-1β is important for priming of CD8+ T cells. Therefore, the activation of the inflammasome establishes a link between the innate and the adaptive
immune system and is an important part of the anticancer immunity [74]. The release of ATP is stress-induced and controlled by different mechanisms. There are several mechanisms known like maxo-anion channel [75], volume sensitive chloride channel [76] and P2X7 receptor channels dependent ones [77]. Some studies already have shown that ionizing radiation also influences the ATP release. Gamma irradiation induced P2X7 receptor dependent ATP release from B16-F10 melanoma cells [78]. A big challenge is to identify multimodal tumor therapies that lead to activation of the immune systems by triggering the release of DAMPs.

1.5 Radiotherapy

Radiotherapy (RT) is defined as the medical use of ionizing radiation and is used for treatment of acute and chronic inflammatory diseases with low doses (single dose ≤1Gy) or for local tumor control in cancer patients with high dose (single dose >1Gy) [79]. RT in general is an important tool in cancer treatment and applied in over 50% of all tumor therapies. The exposure to ionizing radiation induces DNA damage. Chemical bounds within the nucleic acid are disturbed and DNA double strand breaks may result. Many, but not all, double strand DNA breaks can be fixed by repair systems of the cell. The photons of the ionizing radiation can directly or indirectly ionize atoms and break covalent molecular bonds in this way. The indirect effects of ionizing radiation are mainly induced by free oxygen radicals that induce ionizations and DNA damage [80, 81]. Besides induction of DNA double strand breaks, also modifications of bases and DNA cross linking occurs. The radiation-induced damage of the cell is partly dependent of the phase of its cell cycle [82]. Thereby the G2 phase is the most radio-sensitive cell cycle phase [83].

Unfortunately, melanomas are very radioresistant so that standard radiotherapies are not an effective treatment option for melanoma. But in the last years, the radiation techniques have become better and better. Consequently, there is the possibility to use higher doses of single radiation fractions. Hypofractionation is coming more in the focus for innovative RT. It has been shown that hypofractionated RT is a possibility to treat melanoma [84]. Nonetheless, RT is not a common treatment for melanoma. Only unresectable
and metastatic melanomas are treated with a combination of radiotherapy and immune therapy with ipilimumab. Radiotherapy can increase the anti-tumor effects of ipilimumab [85, 86]. Also in a preclinical murine model it was shown that CTLA4 blockade alone does not affect the tumor growth or survival of the animals at all. Only the combination with radiotherapy improved the overall survival significantly. The effect is dependent on CD8+ T cells and NK cells [85]. This gives first hints that radiotherapy can modulate and increase the anti-tumor immune response in melanoma and that multimodal therapies consisting of standard tumor therapies in combination with immunotherapy are important tools in the cancer therapy of this tumor entity.

1.6 Chemotherapy

Chemotherapy is one of the most used cancer therapies and is often combined with radiotherapy, surgery, and/or hyperthermia. Chemotherapeutic agents are cytotoxic. They kill cells that divide very rapid, which is a characteristic of cancer cells. It can work by different mechanism. There are alkylating agents, antimetabolites, anti-microtubule agents, topoisomerase inhibitors and cytotoxic antibiotics (summarized in [87]).

Dacarbazine (DTIC) is an alkylating agent and the only chemotherapeutic agent which is approved by the United States Food and Drug Administration (FDA) for the treatment of melanoma. The response rate of patients with monotherapy is very poor. Only 1-2% of patients have complete response rates and less than 2% are alive after 6 years [88]. The half-life of dacarbazine is only 19 minutes and it has a terminal half-life of 5h [89]. As all alkylating agents, DTIC destroys cells and especially cancer cells by adding an alkyl group in their DNA. This prevents the growing of tumor cells. Additionally DTIC cause apoptosis as cell death form [90].

1.7 Hyperthermia

As additive to RT and CT, hyperthermia (HT) is more and more used for heatable tumors. Mild HT is defined as heating of the tumor up to 40°C-44°C. There are various techniques used such as local, interstitial or deep regional HT
It cannot replace other standard treatments but can sensitize tumor cells for them. Therefore the main aim of HT is to enhance the efficiency of RT and CT [67]. Tumors are more heat sensitive than healthy tissue which leads to a higher damage in the tumor cells [91]. There are two main forms of HT, whole body HT or regional HT. For whole body HT, the temperature of large areas or even the whole body is increased. Especially the whole body hyperthermia is used to treat metastatic cancer that has spread. Like fever, whole body hyperthermia has general immune stimulatory effects. As already seen in the 19th century by Busch and later by Coley fever induced by bacteria can be an effective tool in cancer therapy [92, 93]. Higher temperatures increase the activity of different immune cells [94]. The local/regional HT is used as an adjuvant for treatment with chemotherapeutics or radiation. Local HT acts synergistic with chemotherapeutics since it increases the blood flow and thereby delivers the chemotherapeutics more efficiently into the tumor [95]. HT can also boost the effectiveness of RT. It inhibits the DNA repair of irradiation-induced double strand breaks by e.g. denaturation of DNA protein kinases that repair DNA damage [96]. HT can sensitize the cells for irradiation when it is delivered before RT or it can aggravate the irradiation induced cellular stress when it is applied after RT. Another effect of HT relevant for the induction of anti-tumor immunity is its capability to increase the expression and especially the release of HSPs. This is correlated with the tumor’s immunogenicity [67]. The effectiveness of HT in addition to CT or RT has been proven in several clinical studies. In most therapies, HT is performed one or two times per week. It was shown to be effective against various cancer entities like breast [97], bladder [98] but also against malignant melanoma [99]. Another positive effect is that HT does not induce severe side effects or toxicities [2]. For melanoma it is known that human melanoma cells are sensitive for heat [100]. A randomized trial of RT with or without HT could show that the addition of hyperthermia to RT increased the complete response rate from 35 to 62 percent and two-year local control rates from 28 to 46 percent in this tumor entity [99].
1.8 Scope of this thesis

The activation of the immune system is essential for an efficient cancer therapy, especially for attacking small tumor masses, recurrent tumors or metastases. Standard tumor therapies such as RT and CT aim of inducing proliferation stop of the tumor cells and consecutively tumor cell death, thereby modifying the tumor cells phenotype. Apoptosis is a non- or even an anti-inflammatory cell death form, while necrotic cells activate the immune system by the release of danger signals like HMGB1, HSPs or ATP. Especially for melanoma systemic anti-tumor responses are clinically observed after combination of radiochemotherapy with distinct immune therapies. Nevertheless, few preclinical data exist about melanoma cell death forms that are induced by combinations of standard tumor therapies alone or especially in addition with further immune stimulation by e.g. hyperthermia and how these cell death forms modulate the immune activation against the tumor. Especially the knowledge whether immunogenic necrosis or necroptosis is inducible by distinct combinations of RT, CT and HT and how this might be fostered by blocking tumor cell apoptosis by pan caspase inhibitors such as zVAD-fmk is scarce.

Therefore, the first aim of this thesis was to analyze melanoma tumor cell death forms which were induced by distinct clinically relevant treatment schemes containing RT, CT and HT and to examine how these cell death forms can be modulated with zVAD-fmk. Especially melanoma is susceptible for immune tumor therapies in multimodal settings, as recent clinical data have proven. The main focus of this work was therefore set on how the therapy-modulated melanoma cells and their supernatants impact on activation and functionality of immune cells like macrophages, DCs, T cells and NK cells as well as on the immune system related mechanisms of therapy-induced tumor growth retardation in vivo, analyzed with the syngeneic B16/C57/BL6 mouse model.
2  Material and Methods

2.1  Reagents and laboratory materials

Table 1: Cell lines

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Table 2: Laboratory materials

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<td>F4/80-e450</td>
<td>eBioscience</td>
<td>Frankfurt, Germany</td>
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<tr>
<td>FoxP3-APC</td>
<td>Miltenyi Biotec</td>
<td>Bergisch Gladbach, GER</td>
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<tr>
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<td>NK1.1-APC</td>
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<td>OVA257-264 (SIINFEKL)</td>
<td>eBioscience</td>
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2.2 Cell culture

Mouse melanoma cells (B16-F10 and B16-OVA) derived from C57/BL6 mice were cultured in RPMI 1640 medium containing stable Glutamin and supplemented with 10% heat inactivated (30min at 56°C) fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin. Both cell lines were tested negatively for mycoplasma sc. by PCR detection kit and were maintained under conditions of 37°C and 95% relative humidity in a 5% CO₂ atmosphere. The cells were used when they reached 90% confluence.

2.3 Treatment of B16-F10 cells

The B16-F10 cells were irradiated with a clinically relevant single dose of 2Gy with a X-Ray Generator (120 kV, 12.2mA, 0.5min). The only FDA-approved chemotherapeutic drug for metastatic melanoma and mostly used therapeutic agent dacarbazine (DTIC) was used in the concentration of 250µM and added immediately after the irradiation. For hyperthermia (HT), the cells were treated with an in-house developed chamber which was placed in a cell incubator as described previously [101]. The temperature was controlled all the time during the experiments. Its variation was less than 0.2°C. The cells remained at 41.5°C for 1h. For combined applications, the tumor cells were stored at 37°C for 4h between RT and HT. A 4h break between irradiation and hyperthermia is the maximum time allowed in clinical settings. The pan-caspase inhibitor zVAD-fmk was used in the concentration of 50µM and the RIP1 kinase inhibitor necrostatin-1 (nec-1) in the concentration of 10µM. Both inhibitors were solved in dimethyl sulfoxide and were added directly after HT. For degradation of extracellular ATP and other nucleotides, the cells were incubated with 10U/ml medium Apyrase right after HT.
2.4 Cell death determination

![Figure 3: Dot plot of an Anx5-FITC/PI staining of B16 melanoma cells 24h after treatment with 2Gy.](image)

An exemplary dot plot of the staining is shown and the following cell populations can be distinguished: Anx5- PI-: viable; Anx5+ PI-: apoptotic; Anx5+ PI+: necrotic (PI+ cells are referred to secondary necrotic cells, while PI++ cells are primary necrotic ones with still complete intact DNA).

The FITC labelled Anx5 binds Ca\(^{2+}\) dependent to phosphatidylserine (PS) which resides in the inner membrane leaflet within viable cells and is exposed on the surface of apoptotic cells. For this reason, Anx5 is a marker for apoptosis. However, Anx5 can also bind necrotic cells since necrotic cells lose their membrane integrity. Therefore a second marker is needed to discriminate between apoptosis and necrosis. To determine between these two cell deaths forms, PI was used. It intercalates into DNA and can pass the membrane of necrotic cells since it is disturbed. With this staining viable cells (Anx5-, PI-), apoptotic cells (Anx5+, PI-) and necrotic cells (Anx5+, PI+) can be distinguished. Further necrosis can be divided in primary and secondary necrosis (Figure 3). Secondary necrosis follows after apoptosis which results in a slight degradation of DNA. In contrast primary necrotic cells have a complete intact DNA. For the analysis, 1*10^5 cells were solved in 400µl Ringer’s solution containing 0.2µg Anx5-FITC and 0.4µg PI and incubated for 30min at 4°C in the dark. The cells were then analyzed by flow cytometry using Flow cytometer Epics XL MCL.
2.5 Analysis of cell cycle

Figure 4: Histogram of the cell cycle phase of B16 melanoma cells 24h after treatment with 2Gy.
An exemplary histogram of a cell cycle analysis of B16 melanoma cells is shown. Because of different DNA contents subG1, G1, S and G2 phases can be distinguished.

To analyze the phases of cell cycle, the cells were stained with PI in the presence of detergent and then also analyzed by flow cytometry. As detergent, Triton-X100 was used to facilitate the entering of PI into the cells. PI intercalates into the DNA and enables conclusions regarding the DNA content of a cell. The DNA duplicates during S Phase of the cell cycle. Therefore cells in the S phase have higher DNA contents than cells in the G1 phase. Cells in the G2 phase have a double content of DNA as shown in (Figure 4) with the doubled PI signal monitored in the FL3 channel. The staining was performed according to the method described by Riccardi and Nicoletti et al [102]. Shortly, a maximum of 5*10^5 cells were fixed in 70% ethanol for a minimum of 20min at -20°C. The cells were permeabilized with a solution containing 192 ml 0.2 M Na₂HPO₄ and 8 ml 0.1%(v/v) Triton X-100 at pH 7.8 and incubated at room temperature for 5min. After that the cells were stained with 2µg/ml PI and 200µg/ml RNase for 30min. The cells were analyzed by flow cytometry using Flow cytometer Epics XL MCL.

2.6 Detection of danger signals

To analyze the presence of extracellular danger signals, supernatants (SN) of B16-F10 cells were collected 24h after treatment (1ml). SN were frozen at -80°C and thawed once for analysis of HMGB1 and HSP70. The ELISAs were performed according to the manufacturer’s instructions.
2.7 Isolation of peritoneal macrophages

To isolate peritoneal macrophages of C57/M6 or MyD88 KO mice, 2.5ml of 4% (w/v) Brewer’s thioglycollate broth were injected into the peritoneum of older mice (minimum 20 weeks) as described previously by Schleicher and Bogdan [103]. 4 days after injection, the mouse was killed and the peritoneum was washed with 10ml PBS. The macrophages were cultured in RPMI 1640 medium containing 10% heat inactivated FBS (30min at 56°C), stable Glutamin, 100U/ml penicillin and 100μg/ml streptomycin (Gibco, Carlsbad, USA). The macrophages were characterized by F4/80 staining and used for activation analysis or functional assays.

2.8 Generation of bone marrow-derived dendritic cells

Generation of bone marrow-derived dendritic cells with GM-CSF was performed as described before [104]. In brief, bone marrow cells were isolated from femur and tibiae of C57/M6 or MyD88 KO mice which are between eight and ten weeks old by flushing the bones with RPMI 1640 medium. The bone marrow cells were then cultured in DCs medium consisting of RPMI 1640 medium containing 10% heat inactivated FBS (30min at 56°C), 100U/ml penicillin and 100μg/ml streptomycin, 0.1% β-mercaptoethanol (50mM) and freshly added 200U/ml mouse GM-CSF. 2 x 10^6 cells were suspended in 10ml DCs medium and seeded in 100mm petri dishes. At day three, 10ml of DCs medium containing fresh GM-CSF were added. At day six, half of the SN was collected and centrifuged (350 g, 5 min, and room temperature). Thereafter, the cell pellet was re-suspended in 10ml fresh DCs medium and returned to the plate. The DCs were harvested at day eight and characterized by CD11c staining and used for activation analysis or functional assays.
2.9 Analysis of expression of activation markers on dendritic cells and macrophages

To analyze the expression of the activation markers MHCII and CD86 on DCs and macrophages, 5 x 10⁵ isolated cells were co-incubated in 6-Well suspension cell plates with 1ml SN obtained from mock-treated or treated B16-F10 tumor cells for 16h. The SN was collected 24h after the respective treatment. After solving the cells with accutase they were incubated for 10 min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. Both DCs and macrophages were then stained for 30min at 4°C with the fluorescence antibodies MHCII-e450, CD11c-FITC and CD86-Alexa Fluor®700. DCs were additional stained with CD40-APC, CD80-PE, CD83-PE and CD11b-PE. Incubation of the cells with medium, medium with LPS, medium with DTIC, or zVAD-fmk served as controls. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.10 Detection of inflammatory cytokines secreted by DCs and macrophages

Various mouse cytokines secreted by activated macrophages or DCs were analyzed with specific ELISA kits according to the manufacturer’s instructions. For this, SN of DCs or macrophages were collected 16h after coincubation with SN of treated B16-F10 cells. SN was tested for secreted IL-1β, TNFα and IL-6.
2.11 Phagocytosis assay

For the phagocytosis assay, B16-F10 melanoma cells were labeled with carboxy-fluorescein succinimidyl ester (CFSE) 24h before the respective treatments. For this, the cells were adjusted to $2 \times 10^6$ cells per ml in PBS and 1µg CFSE was added per ml cell suspension. The cells were incubated for 20min at 37°C, washed with RPMI 1640 and consecutively sown. The peritoneal macrophages or DCs ($10^5$) were then co-incubated with the CFSE labelled tumor cells for 1h at 37°C at a ratio of 1 to 5. Afterwards, the macrophages or DCs were solved with accutase and incubated for 10min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors and then stained with F4/80-e450 (macrophages) or MHCII-e450 (DC). After that the cells were analyzed by two-color flow cytometry. As previously described [105] and again verified by confocal microscopy, the double positive cells are macrophages or DCs that have phagocytosed the tumor cells (Figure 5).

2.12 Migration assay

For transwell migration assays the ThinCert™ Tissue culture inserts system with pore sizes of 3 µm was used. The isolated peritoneal macrophages or bone marrow-derived DCs were re-suspended in medium and 800µl of the cell suspension containing 500.000 cells were placed on each of the 6-well inserts. Supernatants of mock-treated or treated B16-F10 cells were placed in the 6-well

Figure 5: Dot plot of a CFSE/ F4/80 staining of Macrophages and B16 melanoma cells after phagocytosis assay. An exemplary dot plot of the staining of a phagocytosis assay is shown. B16 melanoma cells are positive for CFSE (FL1) and macrophages for F4/80 (FL9). Macrophages which have phagocytosed B16 cells are double positive.
plates. After incubation for 16h at 37°C, the migrated cells were collected and the number was determined by flow cytometry using the Gallios® Flow Cytometer.

2.13 Analysis of presentation of the model tumor antigen OVA

For analysis of presentation of the tumor model antigen OVA, bone marrow derived DCs were co-incubated with treated B16-OVA cells for 16h. After solving the cells with accutase they were incubated for 10min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. The DCs was then stained for 30min at 4°C with the fluorescence antibodies CD11c-FITC and OVA257-264 (SIINFEKL)-PE. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.14 Cultivation of murine NK cells

NK cells were isolated from spleens of C57/BL6 mice by using the NK cell isolation kit. The isolation was performed according to the manufacturer’s instructions. Afterwards, the cells were cultured in 12 well cell culture plates. 5*10^5 cells per well were seeded in RPMI 1640 containing 10% heat inactivated FBS (30min at 56°C), 100U/ml penicillin, 100μg/ml streptomycin, 0.1% β-mercaptoethanol (50mM) and 10μg/ml IL-2.

2.15 Analysis of the maturation status of NK cells

For analysis of the maturation status, the isolated NK cells were cultured in 12 well culture cell plates. After 24h, the cells were co-incubated with 500μl SN of treated melanoma cells. Further 24h later, the cells were incubated for 10min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. The NK cells were then stained for 30min at 4°C with the fluorescence antibodies NK1.1-APC, CD3e-V450, CD11b-FITC and CD27-PE-Cy7. The detected mean fluorescence intensity values (MFI) after antibody staining was relativized to mock-treated NK cells.
2.16 Kill of B16-F10 cells by NK cells

Isolated NK cells were cultured in 12 well cell culture plates for 24h. B16-F10 cells were treated as described in 2.3. 24h after treatment the B16-F10 cells were harvested and co-incubated with NK cells for 24h. The cells were then incubated for 10 min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. To exclude NK cells, they were stained for 30min at 4°C with the fluorescence antibodies NK1.1-APC and to analyze the cell death of B16-F10 cells, they were stained with Anx5-FITC/PI. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.17 Detection of secreted INFγ by NK cells after activation with treated melanoma cells

NK cells were isolated of from spleens of C57/BL6 mice and cultured in 12 well cell culture plates for 24h. B16-F10 cells were treated as described in 2.3. 24h after treatment the B16-F10 cells were harvested and co-incubated with the isolated NK cells. SN were collected after 24h. INFγ secreted by activated NK cells was analyzed with specific ELISA kits, according to the manufacturer’s instructions.
2.18 Mixed lymphocyte reaction with CD4+ or CD8+ T cells

Dendritic cells were generated of bone marrow of C57/BL6 mice and incubation of the isolated cells with GM-CSF. Afterwards, DCs were activated with SN of treated B16-F10 as described in 2.8 and 2.9. CD4+ or CD8+ T cells were isolated of spleens of Balb/c mice to obtain allogeneic T cells. The T cells were isolated using CD4 or CD8 isolation kits, according to the manufacturer’s instructions. The isolated T cells were stained with CFSE (1.5µg/ml) for 10min at RT. The activated DCs (2.5*10^4) were co-incubated with the CFSE stained T cells (5*10^5) in 96 well round bottom plates. After 5 days at 37°C, the cells were solved and then incubated for 10min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. After that the T cells were stained for 30min at 4°C with the fluorescence antibodies CD3-e450, CD4-PerCP-Cy5.5, CD8-APC and CD25-Pe-Cy7. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.19 Analysis of proliferation of OVA-specific CD8+ T cells

Dendritic cells were generated of bone marrow of C57/BL6 mice with GM-CSF and then activated with SN of treated B16-OVA cells as described in 2.8. and 2.9. CD8+ T cells were isolated of spleens of OT1 mice. The CD8 T cells were isolated using a CD8 T cell isolation kit according to the manufacturer’s instructions. CD8+ T cells of OT1 mice are specific for OVA257-264 grouped with MHCI of C57/BL6 mice (SIINFEKL). Isolated T cells were stained with
CFSE (1.5µg/ml) for 10min at RT. The activated DCs (2.5*10^4) were co-incubated with the CFSE stained T cells (5*10^5) in 96 well round bottom plates. After 5 days at 37°C, the cells were solved and then incubated for 10 min at 4°C with Fc-blocking reagent to avoid unspecific binding of the staining antibodies to Fc-receptors. After that the T cells were stained for 30min at 4°C with the fluorescence antibodies CD3-e450, CD4-PerCP-Cy5.5, CD8-APC and CD25-Pe-Cy7. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.20 Detection of various cytokines secreted by T cells after activation

Different cytokines were analyzed in SN of isolated CD4+ or CD8+ T cells after co-incubation with activated allogeneic DCs by using a bead based assay of eBioscience which was performed according to the manufacturer’s instructions and measured with multicolor flow cytometry with the Gallios® Flow Cytometer.

2.21 Induction of B16-F10 melanomas in C57/BL6, MyD88 KO or RAG KO mice

For in vivo analysis of tumor growth after the respective treatments, eight to ten week old female C57/BL6, MyD88 KO or RAG KO mice were used. 10^6 B16-F10 cells, solved in 200µl Ringer’s solution, were injected at day zero into the right, shaved flank of the mice. The tumor volumes were followed every day. For this, width and length were measured using a digital caliper and tumor volume was calculated according to following formula: volume (mm³) = 0.5 × width^2 (mm²) × length (mm) [106].
2.22 Treatment of B16-F10 melanomas with ionizing irradiation, hyperthermia, dacarbazine, zVAD-fmk, Apyrase and NK cell depleting antibody

At day 8, 9 and 10 after tumor induction, the tumors were locally irradiated with a clinically relevant single dose of 2Gy. To irradiate the tumor bearing mice, we manufactured a Plexiglas® box which allows the irradiation of three mice at once. The mice were anesthetized before placing them into the box. For the irradiation procedure, the mice were kept under Isoflurane anesthesia. The tumors were locally irradiated at the indicated days with a clinically relevant single dose of 2Gy using a linear accelerator. The planning of the irradiation was conducted using a computer tomography image of the irradiation box and tumor bearing mice with Philips pinnacle software to obtain an optimal target volume. To further protect normal tissue, the gantry of the 6 MV linear accelerator was drifted to 340 degree. Two hours after the irradiation, dacarbazine (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. Apyrase was injected i.v. one hour after irradiation (25U at day 8). Hyperthermia was performed 4 hours after irradiation at day 8 and 10. For hyperthermia, the tumors were heated locally to 41.5°C for 30min.
using BSD50 hyperthermia system that was adjusted to the mouse experiments. Heating of the tumor is performed with a microwave probe, comparable with clinical settings. This heats the tumor locally but takes care for normal tissue. With temperature sensors we control the temperature of the tumor but also of the body temperature of the mice. During the whole treatment the mice are narcotized with isoflurane and are lying on a heat plate to prevent a cool down.

2.23 Analysis of immune cell infiltration into the tumor by flow cytometry

Figure 8: A schematic diagram and pictures of the local hyperthermia of a local hyperthermia treatment of a C57/B6 mouse with a B16-F10 tumor. For hyperthermia the mice were narcotized with isoflurane and positioned on a heat plate to control the body temperature. Heating of the tumor is performed with a microwave probe. During the whole time the temperature of the tumor is controlled. The tumor is heated to 41.5°C for 30min.

For analysis of immune cell infiltration into the tumor, the tumor was prepared with a tumor dissociation kit at day 15 according to the manufacturer’s instructions. After the dissociation, the tumor cells were centrifuged with
easycol separating solution to discard dead cells. The cells were incubated for 10 min at 4°C with Fc-blocking reagent and then stained for 30min at 4°C with the following fluorescence labeled antibodies: CD4-PCC5.5, CD8-PE, CD3-V450, CD11c-PE, NK1.1-APC, B220- PCC5.5, F4/80-AF647 and CD45.2-PCC5.5. Determination of Tregs was performed with FoxP3 Staining Buffer Set and the antibodies CD4-Vioblue, CD25-AF488 and FoxP3-APC. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.24 Analysis of immune cell infiltration into draining lymph nodes by flow cytometry

For analysis of immune cell infiltration into the draining lymph nodes, the axillary lymph nodes were prepared. The cells were incubated for 10 min at 4°C with Fc-blocking reagent and then stained for 30min at 4°C with the following fluorescence labeled antibodies: CD4-PCC5.5, CD8-PE, CD3-V450, CD11c-PE, NK1.1-APC, CD45.2-PCC5.5 and OVA257-264 (SIINFEKL)-PE. Multicolor flow cytometry was performed with the Gallios® Flow Cytometer.

2.25 Analysis of OVA-specific T cell activation by using OT1 mice and B16-OVA melanoma cells

10^6 B16-OVA cells were solved in 200µl Ringer's solution and injected into the right shaved flank of the mice at day zero. At day 8, 9 and 10 the irradiation was performed. The tumors were locally irradiated with a single dose of 2Gy using a linear accelerator (PRIMART). Two hours after the irradiation, dacarbazine (day 8 and 10) and zVAD-fmk (day 8, 9 and 10) were injected i.p.. Hyperthermia was performed 4 hours after irradiation at day 8 and 10. The tumors were heated locally to 41.5°C for 30min. At day 14, draining lymph nodes (axillary) were removed. T cell activation was measured by analyzing intracellular INFγ and intracellular IL-2 after restimulation. Therefore 2*10^6 cells were restimulated with OVA peptide (10^{-7}M) and Golgi Plug for 5 hours. Extracellular markers were stained with CD3-V450 and CD8-PE by incubating for 30min at 4°C. After washing, the cells were permeabilized by addition of Cytofix/Cytoperm and incubated for 20min at 4°C. For intracellular staining, the antibody INFγ-Pe-Cy7
was added and the cells were incubated for another 30 min at 4°C. The cells were washed and analyzed by multicolor flow cytometry with the Gallios® Flow Cytometer.

2.26 Analysis of in vivo proliferation of OVA-specific CD8+ T cells

10^6 B16-OVA cells were solved in 200µl Ringer`s solution and injected into the right, shaved flank of the C57/BL6 mice at day zero. At day 8, 9 and 10 the irradiation was performed. The tumors were irradiated with a single dose of 2Gy using a linear accelerator (PRIMART). Two hours after the irradiation, dacarbazine (day 8 and 10) and zVAD-fmk (day 8, 9 and 10) were injected i.p.. Hyperthermia was performed 4 hours after irradiation at day 8 and 10. The tumors were heated locally to 41.5°C for 30 min. At day 11 OVA specific CD8+ T cells of OT1 mice were isolated using a CD8 T cell isolation kit. These T cells were stained with CFSE (1.5µg/ml) for 10 min at room temperature. After washing of the cells they were solved in Ringer’s solution and injected i.v. (10^6 in 200µl) in tumor bearing mice. 5 days after injection, the tumors were prepared with a tumor dissociation kit. After the dissociation, the tumor cells were centrifuged with easycoll separating solution to discard death cells. The cells were incubated for 10 min at 4°C with Fc-blocking reagent and then stained for 30 min at 4°C with the following fluorescence labeled antibodies: CD4-PCC5.5, CD8-PE, CD3-V450. The cells were analyzed by multicolor flow cytometry with the Gallios® Flow Cytometer.

2.27 Statistical analysis

For all experiments Graph pad prism (Version 5.04) was applied for statistical analysis with the Mann-Whitney-U test. Results were considered statistically significant for P < 0.05 (*) or (#) and highly significant for P < 0.01 (** or ##).
3 Results

3.1 In vitro immunogenic potential of melanoma cells after treatment with RT, DTIC and/or HT in absence or presence of zVAD-fmk

3.1.1 Melanoma cell apoptosis after combination of DTIC with HT
The form of tumor cell death is important for activation of the immune system and therefore for an efficient anti-tumor treatment (summarized in [107]). Because of this we first analyzed the B16 melanoma death in vitro, induced by different single and combinatory treatments.

Figure 9: Apoptosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT.
The tumor cell death forms were analyzed with flow cytometry after staining of the cell suspension with Anx5-FITC and PI. Anx5-FITC+ and PI- cells were defined as apoptotic ones. Shown are different time points after the respective treatment(s) (A: 24h, B: 48h, C: 72h, D: 96h). Representative data of one out of four experiments, each performed in triplicates, are presented as mean ± S.D. * P < 0.05; ** P < 0.01 related to untreated (mock) cells. DTIC: dacarbazine at a concentration of 250µM; Gy: Gray; HT: hyperthermia with 41.5°C for 1h; zVAD-fmk: pan-caspase inhibitor. The order of irradiation with 2Gy and HT indicated on the x-axis determines the chronology of the treatment. mock: untreated tumor cells.
Single treatment of melanoma cells by RT, DTIC or HT did not induce higher tumor cell apoptosis, both at early and late time points after treatment (Figure 9). It is already known that melanoma cells are very radio- and chemoresistant [108]. HT has been shown to be capable to boost the efficacy of CT and RT treatments, even in melanomas [109, 110]. For that reason, we analyzed whether combination of RT and/or DTIC with HT can enhance apoptosis induction in melanoma cells. Combination of HT and the chemotherapeutic agent DTIC increased apoptosis in melanoma cells. Further addition of RT resulted in even 15% of apoptotic melanoma cells 96h after the treatment, but in dependence on the order of RT and HT application. More cells died apoptotic when HT was performed before RT. To summarize, only combinatorial treatments including HT significantly induced apoptotic cell death in B16 melanoma cells (Figure 9).

3.1.2 Melanoma cell necrosis especially after RT and HT

![Graph showing necrosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT.]

Figure 10: Necrosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT.
The tumor cell death forms were analyzed with flow cytometry after staining of the cell suspension with Anx5-FITC and PI. Anx5-FITC+ and PI+ cells were defined as necrotic ones. Shown are different time points after the respective treatment (A: 24h, B: 48h, C: 72h, D: 96h). Representative data of one out of four experiments, each performed in triplicates, are presented as mean ± S.D. * P < 0.05; ** P < 0.01 related to untreated (mock) control samples. DTIC: dacarbazine at a concentration of 250µM; Gy: Gray; HT: hyperthermia with 41.5°C for 1h; zVAD-fmk: pan-caspase inhibitor. The order of irradiation with 2Gy and HT indicated on the x-axis determines the chronology of the treatment. mock: untreated tumor cells. mock: untreated tumor cells.
Single treatment of B16 cells with DTIC did also not induce necrotic cell death in melanoma cells, even 96h after treatment. However, single treatment with 2Gy of RT was capable of inducing necrosis. This was seen 24h and 48h after irradiation. Not only irradiation alone but above all, combinatory treatments induced necrotic cell death. The highest rates are detectable 48h after treatment. Here, more than 40% of necrotic B16 cells were present after treatment with RT, DTIC and HT (Figure 10). Again, the order of RT and HT application is important. Here, more cells died necrotic when RT was performed before HT.

3.1.3 Necroptosis is inducible in B16 melanoma cells

![Figure 11: Necrosis of B16 melanoma cells after single and combined treatment with RT, DTIC and/or HT in the absence or presence of nec-1 or zVAD-fmk.](image)

The cell death forms were analyzed with flow cytometry after staining of the cell suspension with AnxA5-FITC and PI, 72 h (A) or 24 h (B) after the respective treatment. Anx5-FITC+ and PI+ cells were defined as necrotic ones. The cells were further incubated with the necroptosis inhibitor nec-1 (A) or zVAD-fmk (B). Representative data of one out of four experiments, each performed in triplicates, are presented as mean ± S.D. * P < 0.05; ** P < 0.01 related samples without (w/o) inhibitor. DTIC: dacarbazine at a concentration of 250µM; Gy: Gray; HT: hyperthermia with 41.5°C for 1h; zVAD-fmk: pan-caspase inhibitor. The order of irradiation with 2Gy and HT indicated on the x-axis determines the chronology of the treatment. mock: untreated tumor cells.
We then analyzed whether necroptosis is inducible in B16-F10 melanoma cells. The treatment with the potent small-molecule inhibitor of necroptosis, necrostatin-1 (nec-1), reduced the amounts of necrotic tumor cells. This was independent of the death stimuli (Figure 11A).

Figure 12: Impact of necrostatin-1 on zVAD-fmk-induced B16 necrosis. Necrosis of B16 mouse melanoma cells was analyzed with two color flow cytometry by staining the cells with AnxA5-FITC and PI 24h after irradiation with 2Gy in the presence or absence of zVAD-fmk or zVAD-fmk plus nec-1. Representative data of one out of three experiments, each performed in duplicates, are presented as mean ± S.D. ** P < 0.01; nec-1: necrostatin-1, Gy: Gray

Further, the pan caspase inhibitor zVAD-fmk (50 µM) was capable of inducing necrotic B16-F10 melanoma cell death. The addition of zVAD-fmk increased the percentage necrotic cells in combination with all treatments (Figure 11B). Mostly, zVAD-fmk doubles the percentage of necrotic cells. To further prove that zVAD-fmk induced necroptosis, we additionally added nec-1. The addition of this necroptosis inhibitor blocked the zVAD-fmk induced B16 tumor cell necrosis (Figure 12).
3.1.4 zVAD-fmk induces a timely-restricted G2 cell cycle arrest

Figure 13: G2 cell cycle arrest of B16 cells after single and combined treatment with RT, DTIC and/or HT in the absence or presence of zVAD-fmk.

The cell cycle phases were analyzed by PI staining after incubation of the cells with the detergent Triton-X100. The G2 arrest was determined 24h (A), 48h (B) and 72h (C) after the indicated treatment(s). Representative data of one out of four experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. DTIC: dacarbazine at a concentration of 250µM; Gy: Gray; HT: hyperthermia with 41.5°C for 1h; zVAD-fmk: pan-caspase inhibitor. mock: untreated tumor cells

The cell cycle of melanoma cells was not influenced neither 24h nor 48h or 72h after the respective treatment(s). The amount of cells in the more radiosensitive
G2 phase was always around 15% (Figure 13A-C). However, addition of zVAD-fmk induced a timely restricted G2 arrest independent of the death stimulus (Figure 13B).

3.2 Analysis of the in vitro immunogenic potential of B16 cells

3.2.1 The release of HMGB1 but not that of Hsp70 is enhanced by zVAD-fmk

![Graphs showing the release of HMGB1 and HSP70](image)

Figure 14: Release of the dangers signals HMGB1 and HSP70 of melanoma cells after single or multimodal treatments with RT, DTIC and/or HT in the absence and presence of zVAD-fmk.

The concentration of HMGB1 (A, B) and of HSP70 (C, D) in the supernatants of B16-F10 mouse melanoma cells was determined by ELISA technique 24h hours after the indicated treatments. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence (w/o) or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) Representative data of one out of three experiments, each performed in duplicates, are presented as mean ± S.D.* P < 0.05; ** P < 0.01 related to mock (A, C) or samples without (w/o) inhibitor (B, D). Gy: Gray; zVAD-fmk: pan-caspase inhibitor. mock: untreated tumor cells.

To get first hints about the immunogenic potential of the treated melanoma cells the release of the danger signals HSP70 and HMGB1 was analyzed. HT alone and in combination with RT or DTIC enhanced the release of both danger signals HMGB1 and HSP70. RT or DTIC neither alone nor in combination impacted on the release of one of these danger signals (Figure 14A and 14C).
But fostering necrosis with the cell death modulator zVAD-fmk increased the amount of HMGB1 in the SN of the tumor cells significantly. The increased release correlated with the induction of necrosis by zVAD-fmk, but was independent of the treatment with RT, DTIC and/or HT (Figure 14B). In contrast, the pan caspase inhibitor zVAD-fmk did not increase the release of HSP70 by B16 melanoma cells (Figure 14D).

3.2.2 Increased surface expression of MHCII and CD86 on macrophages
induced by supernatants of melanoma cells whose cell death was
modulated with zVAD-fmk

Figure 15: Representative histograms of the expression of MHCII or CD86 on macrophages after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of peritoneal mouse macrophages of C57/BL6 mice was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after treatment with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence (grey) or presence of the pan-caspase inhibitor zVAD-fmk (50 µM; black)
Because of the inhibition of caspases and consecutive induction of programmed necrosis, zVAD-fmk has been suggested to be a promising therapeutic strategy

![Graph A: MHCII expression](image1)

![Graph B: CD86 expression](image2)

Figure 16: Surface expression of activation markers on macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of peritoneal mouse macrophages was analyzed by multicolour flow cytometry after contact with supernatants of the B16-F10 mouse melanoma cells obtained 24 h after the respective treatments. The tumor cells were treated with ionizing irradiation with RT (2 Gy) alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence (w/o) or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) (A, B). Representative data of one out of 3 experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. MFI: mean fluorescence intensity. Gy: Gray. mock: SN of untreated tumor cells.

to enhance the efficacy of RT in solid tumors [55]. Therefore we next investigated whether SN of melanoma cells that have been exposed to zVAD-fmk in addition to RT, DTIC, and/or HT influence the activation and function of macrophages and dendritic cells (DCs). SN of neither mock treated melanoma cells nor of treated ones altered the expression of the activation markers MHCII
Figure 17: Representative histograms of the expression of MHCII or CD86 on macrophages of WT mice in the presence of apyrase or of MyD88 KO mice after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A, C) and CD86 (B, D) on the surface of peritoneal mouse macrophages of C57/BL6 mice (A, B) or MyD88 ko mice (C, D) was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after treatment with ionizing irradiation with 2Gy in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in the absence (grey) or presence of the pan-caspase inhibitor zVAD-fmk (50 µM; black). SN of treated melanoma cells were additionally incubated with Apyrase (10U/ml) (A, B).

and CD86 on macrophages (Figure 16). However, the modulation of melanoma cell death by zVAD-fmk resulted in tumor cell SN that induced a significant increased expression of these activation markers on macrophages (Figure 15 and 16). This was seen in every case when zVAD-fmk was present, even in untreated cells (Figure 16B). To prove that this is not a direct effect of zVAD-fmk on the immune cells, zVAD-fmk was directly added to macrophages or DCs. It thereby did not impact on the expression of the activation markers (Figure 16 and 21). Because the upregulation of MHCII and CD86 is not directly induced by zVAD-fmk, other molecules which were released by the
tumor cells are responsible for the upregulation of these activation markers on macrophages and DCs.

Figure 18: Surface expression of activation markers of macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A, C) and CD86 (B, D) on the surface of peritoneal macrophages of C57/BL6 mice (A, B) or MyD88 KO mice (C, D) was analyzed by multicolor flow cytometry, after contact with the supernatants (SN) of the B16-F10 mouse melanoma cells obtained 24 h after the respective treatments. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) SN of melanoma cells were additionally incubated with Apyrase (10U/ml)(A, B). Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. MFI: mean fluorescence intensity. DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. mock: SN of untreated tumor cells.

To analyze two possible effector molecules which may induce the upregulation of MHCII or CD86 on peritoneal macrophages, Apyrase was added to the SN or macrophages of MyD88 KO mice were used for the analyses. Apyrase is a calcium-activated plasma membrane-bound enzyme that degrades nucleotides. It catalyzes the hydrolysis of ATP to AMP and inorganic phosphate. MyD88 KO mice have defects in TLR signaling. The increased expression of MHCII and CD86 was is dependent on nucleotides and ligands such as HMGB1 for TLR
signaling, since no upregulation was observed when using macrophages of MyD88 KO mice or incubating the SN of the treated tumor cells with Apyrase (Figure 17 and Figure 18).

3.2.3 zVAD-fmk increased the secretion of certain inflammatory cytokines by peritoneal macrophages

![Graphs showing secretion of inflammatory cytokines](image)

**Figure 19:** Secretion of inflammatory cytokines by macrophages after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.

Supernatants of the B16-F10 mouse melanoma cells were obtained 24 h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). The peritoneal macrophages were coincubated with the obtained SN for 16h. Supernatants of these macrophages were then analyzed for TNFα (A), IL-6 (B) or IL-1β (C) by ELISA. The fold increase of the cytokine release of macrophages after incubation with SN of treated B16 melanoma cells related to macrophages after incubation with media is displayed. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. **P < 0.01 related to samples without (w/o) inhibitor. MFI: mean fluorescence intensity. Gy: Gray; mock: SN of untreated tumor cells.

Activated macrophages increase the expression of activation markers on their surface but also produce and secrete inflammatory cytokines. Incubation of macrophages with SN of irradiated tumor cells whose cell death was modulated with zVAD-fmk resulted in a significant increased secretion of TNFα, IL-6 and IL-1β (Figure 19). Neither the single treatment with RT nor the combinatory
treatment containing RT, DTIC and HT increased the release of one of these inflammatory cytokines significantly.

3.2.4 Increased surface expression of MHCII and CD86 on DCs induced by supernatants of RT, DTIC and HT treated melanoma cells whose cell death was modulated with zVAD-fmk

Figure 20: Representative histograms of the expression of MHCII or CD86 on DCs after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of bone marrow derived dendritic cells of C57/BL6 mice was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after treatment with ionizing irradiation with 2Gy in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in the absence (grey) or presence of the pan-caspase inhibitor zVAD-fmk (50 µM; black).

In contrast to macrophages, the expression of the activation markers MHCII and CD86 on the surface of bone marrow derived DCs was not generally increased by incubation with SN of tumor cells whose cell death had been modulated with zVAD-fmk. Only the cell death induction with RT in combination with DTIC and HT together with zVAD-fmk generated SN that consecutively increased the expression of both activation markers on DCs significantly (Figures 20 and 21). As already observed for macrophages, cell death induction with only RT or the combination of RT, DTIC and HT did not increase the expression of these activation markers.
Figure 21: Surface expression of activation markers of dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of bone marrow derived DCs was analyzed by multicolour flow cytometry, after contact with the supernatants of the B16-F10 mouse melanoma cells obtained 24 h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) Representative data of one out of 3 experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. MFI: mean fluorescence intensity. Gy; Gray; mock: SN of untreated tumor cells.

Figure 22: Representative histograms of the expression of MHCII or CD86 on DCs of MyD88KO mice after contact with supernatants of treated melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of bone marrow derived DCs of MyD88 ko mice was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after treatment with ionizing irradiation with 2Gy in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour) in the absence (grey) or presence (black) of the pan-caspase inhibitor zVAD-fmk (50 µM).
Figure 23: Representative histograms of the expression of MHCII or CD86 on DCs of C57/BL6 mice after contact with Apyrase-treated supernatants of treated melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of bone marrow derived DCs of C57/BL6 mice was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after treatment with ionizing irradiation with 2Gy in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour) in the absence (grey) or presence (black) of the pan-caspase inhibitor zVAD-fmk (50 µM). SN of treated melanoma cells were additionally incubated with Apyrase (10U/ml).
Figure 24: Surface expression of activation markers of dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.

The expression of the activation markers MHCII (A, C) and CD86 (B, D) on the surface bone marrow derived DCs (C57/BL6; A, B) or MyD88 KO mice (C, D) was analyzed by multicolour flow cytometry, after contact with the supernatants of the B16-F10 mouse melanoma cells obtained 24 h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) SN of treated melanoma cells were additionally incubated with Apyrase (10U/ml) (A, B). Data of three experiments, each performed in triplicates, are presented as mean ± S.D. MFI: mean fluorescence intensity. Gy: Gray; zVAD-fmk: pan-caspase inhibitor. mock: SN of untreated tumor cells.

Again, the increased expression of MHCII and CD86 is dependent on TLR signaling since bone marrow derived DCs of MyD88 KO mice did not respond to the tumor cell SN (Figure 22C, D and 24C, D). HMGB1 acts through TLR2 and TLR4, but there are other molecules which act through these receptors. To confirm that these effects are mostly dependent on HMGB1, the SN of the treated tumor cells were incubated with a HMGB1 depleting antibody before adding them to DCs of C57/BL6 mice (Figure 25). No increased expression of MHCII or CD86 on DCs was detectable anymore when incubating the SN with the anti-HMGB1 antibody. As performed for macrophages, we also analyzed the effects of nucleotides on the expression of the activation markers and revealed that their upregulation is dependent on it since no increased expression of MHCII and CD86 was detectable when incubating the SN with Apyrase (Figure 23A, B and Figure 24A, B).
Figure 25: Impact of anti-HMGB1 antibody on the surface expression of activation markers on DCs after contact with supernatants of treated melanoma cells in the presence of zVAD-fmk.

The expression of the activation markers MHCII (A) and CD86 (B) on the surface of bone marrow derived dendritic cells (DCs) of C57/BL6 wild type mice was analyzed by multicolor flow cytometry after contact with supernatants (SN) of B16 mouse melanoma cells obtained 24h after the treatments. The tumor cells were treated with ionizing irradiation with 2Gy in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM), hyperthermia (HT; 41.5°C for 1 hour), and the pan-caspase inhibitor zVAD-fmk (50 µM) in the absence or presence of a neutralizing antibody against HMGB1 (A, B). Representative data of one out of 3 experiments, each performed in duplicates, are presented as mean ± S.D. * P < 0.05 related to treated tumor cells in the absence of anti-HMGB1 antibody; MFI: mean fluorescence intensity. Gy: Gray; mock: SN of untreated tumor cells.

3.2.5 zVAD-fmk increased the secretion of certain inflammatory cytokines by bone marrow derived DCs

As performed for macrophages the activation status of DCs was also determined by analyzing the released cytokines by DCs after incubation with SN of treated melanoma cells. Neither a single treatment with RT nor the combination RT, DTIC and HT impacted on the release of TNFα, IL-6 or IL-1β. But contact of DCs with SN of melanoma cells whose cell death was modulated with zVAD-fmk resulted in an increased release of TNFα and IL-6, but not of IL-1β (Figure 26).
Figure 26: Secretion of inflammatory cytokines by dendritic cells after contact with supernatants of melanoma cells in the absence or presence of zVAD-fmk.
Supernatants of the B16-F10 mouse melanoma cells were obtained 24 h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) DCs were coinubcated with the obtained SN for 16h. Supernatants of these DCs were then analyzed for TNFα (A), IL-6 (B) or IL-1β (C) by ELISA. The fold increase of the cytokine release of DCs after incubation with SN of treated B16 melanoma cells related to DCs after incubation with media is displayed. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. Gy: Gray; mock: SN of untreated tumor cells.

To analyze the effects of the danger signals HMGB1 and nucleotides such as ATP on the secretion of inflammatory cytokines by DCs, bone marrow derived DCs of MyD88 KO mice were used or SN that had been incubated with Apyrase to degrade extracellular nucleotides. Both prevented the enhanced secretion of TNFα by DCs (Figure 27).
Figure 27: Impact of nucleotides and Toll-like receptor signaling on the secretion of the inflammatory cytokine TNFα by dendritic cells after contact with supernatants of melanoma cells.

Supernatants of the B16-F10 mouse melanoma cells were obtained 24 h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) DCs were coincubated with the obtained SN for 16h. Supernatants of these DCs were analyzed for TNFα by ELISA. The SN of melanoma cells were treated with Apyrase (10U/ml) (A) or DCs were obtained of MyD88 KO mice (B). The fold increase of the cytokine release of DCs after incubation with treated B16 melanoma cells related to DCs after incubation with media is displayed. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. MFI: mean fluorescence intensity. Gy: Gray; mock: SN of untreated tumor cells.

3.2.6 Single and multimodal melanoma treatments in the absence or presence of zVAD-fmk do not impair phagocytosis of the tumor cells and migration of macrophages and DCs towards SN of the tumor cells

One prerequisite for starting an adaptive immune response against cancer cells is the presentation of tumor derived antigens by antigen presenting cells (APCs) such as macrophages and DCs. Before antigen presentation it has to be taken up by the immune cells. Therefore, the capability of macrophages and DCs to migrate towards SN of treated B16 cells was analyzed as well as the phagocytosis of the tumor cells. Compared to mock treated tumor cells all treatments and cell death modulation with zVAD-fmk did not impact on phagocytosis of the tumor cells by macrophages or DCs (Figure 28A and C). The percentage of DCs that had phagocytosed melanoma cells was higher compared to that of macrophages.
Figure 28: Phagocytosis of melanoma cells by macrophages and dendritic cells and the migration of those towards supernatants of the tumor cells.

Phagocytosis of CFSE-labelled B16-F10 mouse melanoma cells by F4/80+ peritoneal mouse macrophages (A) or MHCII+ dendritic cells (C) was analyzed by two-colour flow cytometry. The migration of macrophages (B) or dendritic cells (D) towards supernatants of the melanoma cells was also analyzed by flow cytometry. The B16-F10 mouse melanoma cells used for the phagocytosis assays (A, C) were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). For the migration assays (B, D), supernatants of the respective treated tumour cells were used. The fold increase of migration of DCs to SN of treated B16 melanoma cells related to migration of DCs to media is displayed. Representative data of one out of three experiments, each performed in triplicates, are presented as mean ± S.D. * P < 0.05 related to samples without (w/o) inhibitor. Gy: Gray; mock: SN of untreated tumor cells.

After acquisition of tumor antigens by immature DC, the migration of the immune cells to lymph nodes and appropriate co-activation is mandatory. As outlined above, especially SN of melanoma cells whose cell death was modulated with zVAD-fmk induced an up-regulation of activation markers on APCs. We then tested if the migration of macrophages and DCs to SN of tumor cells is dependent on distinct treatment combinations and/or cell death modulation by zVAD-fmk. The migration of the immune cells was not significantly influenced by irradiation of the tumor cells or combined treatment with RT, DTIC and HT (Figure 28B and D). However, even cell death modulation of melanoma cells with zVAD-fmk did not impact on the migration of macrophages (Figure 28B), an increased migration of DCs to SN of melanoma...
cells which were treated with RT, DTIC and HT and zVAD-fmk was observed (Figure 28D).

3.2.7 Presentation of tumor antigen by DCs is not influenced by SN of treated melanoma cells

To start an adaptive immune response, DCs have to (cross-)present tumor antigens after migration to the tumor and phagocytosis of tumor cells. We therefor next analyzed the presentation of the model tumor antigen OVA by DCs after phagocytosis of B16-OVA cells which overexpress the OVA peptide. Analysis of the model antigen presentation with flow cytometry revealed that irradiation slightly, but not significantly increased it. Cell death modulation with zVAD-fmk had no impact on it (Figure 29).

![Figure 29: Presentation of the model tumor antigen OVA by dendritic cells after contact with B16-OVA melanoma cells.](image)

Presentation of SIINFEKL (OVA+MHCII) by bone marrow derived DCs was analyzed by multicolour flow cytometry, after co-incubation of DCs with B16-OVA mouse melanoma cells 24h after the respective treatment(s). The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. Gy: Gray; mock: untreated tumor cells.
3.2.8 SN of treated melanoma cells do not impair activation status, maturation status, or kill activity of NK cells

Figure 30: Phenotype of NK cells after contact with SN of treated melanoma cells in absence or presence of zVAD-fmk.

24h after isolation, murine NK cells were coincubated with SN of treated melanoma cells. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). The maturation status of NK cells was analyzed 16 hours afterwards by multicolor flow cytometry. Four maturation states were distinguished: CD11b and CD27 negative (A), CD27 single positive (B), CD11b and CD27 double positive (C) and CD11b single positive (D). The fold increase of NK cells after incubation with SNs of B16 melanoma cells related to NK cells after incubation with media is displayed. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. Gy: Gray; mock: SN of untreated tumor cells.

For anti-tumor immune responses, NK cells play an important role as cells of the innate immune system. Mature NK cells can directly induce tumor cell death or can release proinflammatory cytokines, especially INFγ, and therefore enhance the immune response against the tumor. Only mature NK cells initiate anti-tumor responses. We therefore analyzed the maturation status of NK cells after contact with SN of treated B16 cells. Immature NK cells neither express CD11b nor CD27 on their surface. With ongoing maturation, NK cells express first CD27 then both CD11b and CD27 and fully mature NK cells express CD11b but no CD27 anymore [111]. Neither the irradiation nor the combinatory combined treatment with RT, DTIC and HT, nor the modulation of cell death with zVAD-fmk had effects on the maturation status of NK cells (Figure 30).
Figure 31: Impact of treatment of melanoma cells on killing activity of NK cells.
24h after isolation, murine NK cells were coincubated with pre-treated melanoma cells. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). The kill of the B16-F10 melanoma cells by NK cells was then analyzed by multicolor flow cytometry. The cell death is relativized to treated B16-F10 cells without NK cells. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. Gy: Gray; mock: untreated tumor cells.

Figure 32: Release of INFγ by NK cells after contact with SN of treated melanoma cells.
24h after isolation, murine NK cells were coincubated with SN of treated melanoma cells. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). 16 hours later, the SN of the NK cells was analyzed for INFγ by ELISA-Data of three experiments, each performed in triplicates, are presented as mean ± S.D. Gy: Gray; mock: untreated tumor cells.

SN of treated melanoma cells did not change the expression of activation markers in NK cells that were already expressed directly after isolation.
However, the age of mice impacted on the maturation status of the isolated NK cells. The younger the mice the more mature NK cells were present. Similar effects were seen when NK cells were not incubated with SN of treated melanoma cells but directly with the treated cells. The functionality of NK cells is as important as their maturation and activation status for anti-tumor response. Therefore we next analyzed the ability to kill untreated or treated B16 cells and measured the release of the immune stimulatory factor INFγ in the SN of NK cells after contact with the melanoma cells. Treated melanoma cells were coincubated with NK cells for 16h and the cell death of B16-F10 cells was then analyzed with Anx5-FITC and PI staining. The cell death was relativized to treated B16-F10 cells without NK cells. The NK cells were all functional and killed B16-F10 cells. They killed almost 30% of the melanoma cells. However, the amount of killed cells was not dependent on the treatment of the B16 cells (Figure 31). Combination of RT, DTIC and HT decreased the release of INFγ by NK cells, but this could be reversed by zVAD-fmk (Figure 32).

3.2.9 SN of melanoma cells do not impact on DC-induced T cell proliferation, but on cytokine secretion when gathered from melanoma cells treated with RT, DTIC and HT in combination with zVAD-fmk

T cells and especially CD8+ T cells are the most effective cells in killing tumor cells when they are activated. One result of their activation is induction of proliferation. This can be measured in vitro with a mixed lymphocyte reaction (MLR). This method assumes that T cells are activated by allogeneic DCs comparable as after specific activation which results in proliferation of the T cells. Only immune suppressive factors inhibit proliferation after coincubation of T cells with allogeneic DCs. CD4+ or CD8+ T cells were isolated from spleens of BalbC mice, stained with CFSE and consecutively coincubated with allogeneic bone marrow derived DCs of C57/BL6 mice which had been activated with SN of treated melanoma cells before.
Figure 33: Proliferation of T cells after co-incubation with allogeneic or syngeneic DCs pre-incubated with SN of treated melanoma cells.

T cells were isolated of spleens of Balb/C (A, B) or C57/BL6 (C) mice and co-incubated with bone marrow derived dendritic cells of C57/BL6 mice after activation of the latter with SN of treated B16-F10 (A, B) or B16-OVA (C) cells. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). Proliferation of CFSE-stained T cells was analyzed by multicolor flow cytometry. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. Gy: Gray; mock: SN of untreated tumor cells.

When performing the experiments with CD4+ T cells, almost 50% of the T cells had proliferated after contact with allogeneic DCs. No significant changes in proliferation rates were induced by pre-incubation of the DCs with the SN of treated melanoma cells (Figure 33A). Similar effects were observed when using CD8+ T cells. In general, the proliferation rate of CD8+ T cells was higher than that of CD4+ T cells (Figure 33B). T cells proliferate not only after incubation with allogeneic DCs, but also after incubation with syngeneic DCs that present a specific antigen. Bone marrow derived DCs of C57/BL6 mice were co-incubated with treated B16-OVA cells to get activated. Afterwards, these DCs were coincubated with CD8+ T cells origination from spleen of OT-1 mice. CD8+ T cells of OT-1 mice specifically recognize DCs of C57/BL6 mice presenting the model antigen OVA257-264. A lower basal proliferation rate in comparison to the allogeneic situation was observed (Figure 33C). But again, no impact of melanoma cell treatment with by RT or RT, DTIC and HT was observed. Of note is that combination treatment of B16-OVA cells in the
presence of zVAD-fmk slightly increased the proliferation of specific CD8+ T cells (Figure 33C).

Figure 34: Secretion of cytokines by T cells after contact with dendritic cells after activation by SN of treated melanoma cells.
T cells were isolated of spleens of C57/BL6 mice and coincubated with bone marrow derived dendritic cells of C57/BL6 mice after activation with SN of treated B16-OVA cells. The tumor cells were treated with ionizing irradiation with 2Gy alone or in combination with the chemotherapeutic agent dacarbazine (DTIC; 250 µM) and hyperthermia (HT; 41.5°C for 1 hour), in each case in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM). Supernatants of T cells were analyzed for IL6 and INFγ by a bead based assay of eBioscience and flow cytometry. The fold increase of the respective cytokine related to SNs of T cells after incubation with media is displayed. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to samples without (w/o) inhibitor. Gy: Gray; mock: SN of untreated tumor cells.

Activated T cells do not only proliferate and are capable of killing tumor cells, but also secrete immune stimulatory cytokines to further enhance the anti-tumor immune response. Therefore, we next analyzed SN of activated T cells after coincubation with DC that had been pre-incubated with SN of treated melanoma cells for IL-6 and INFγ. An enhanced secretion of IL-6 and INFγ was only observed after combined treatment of B16 cells with RT, DTIC and HT with zVAD-fmk (Figure 34).

3.3 In vivo impact of zVAD-fmk on tumor growth retardation induced by RT or combination of RT, DTIC and HT

3.3.1 Combination of fractionated RT DTIC and HT reduces tumor growth significantly and addition of zVAD-fmk further retards it
Melanomas are highly radio- and chemoresistant. Therefore it is important to identify multimodal tumor treatments that result in efficient and long lasting tumor control. To prove the effectiveness of RT in combination with DTIC and
HT and further cell death modulation with zVAD-fmk against the melanomas also in vivo, $1 \times 10^6$ B16-F10 cells were injected into the right, shaved flank of approximately eight week old C57/BL6 mice. After one week, a tumor had established and the treatment was started. One group of mice stayed untreated, one got zVAD-fmk, one was fractionated irradiated with a single dose of 2Gy, one was fractionated irradiated and got zVAD-fmk, one group was treated with fractionated RT, DTIC and HT and the last group was treated with combination of fractionated RT, DTIC, HT and zVAD-fmk.

**Figure 35**: Timetable of treatment and in vivo growth of B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk.

The tumor growth of syngeneic B16-F10 tumors in wild type C57/BL6 mice after treatment is displayed (B). The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation, DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. For this, the mice were anesthetized and the tumors were heated locally under temperature control to 41.5°C for 30min using the BSD50 hyperthermia system (A). The tumor volume was monitored with an electronic caliper. Joint data of three independent experiments, each with 3 mice per group, are presented as mean ± S.D. * P < 0.05 ; ** P < 0.01 related to tumors treated with 2Gy+DTIC+HT; # P < 0.05 ; ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor.

Treatment of tumor bearing mice with fractionated RT with a single dose of 2Gy did not affect the tumor growth significantly. The same was seen when the mice
were treated with zVAD-fmk or combination of RT with zVAD-fmk. Combination of fractionated RT with DTIC and HT reduced the tumor growth significantly. Addition of zVAD-fmk to it further decreased the tumor growth significantly and resulted in the most pronounced tumor growth inhibition (Figure 35B).

Figure 36: Infiltration of cells of the innate immune system into B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence and presence of zVAD-fmk. The infiltration of immune cells [Mphs (A), DCs (B), NK cells (C) and MDSCs (D)] was analyzed by multicolor flow cytometry, 96h after the last treatment of the tumors. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. * P < 0.05 ; ** P < 0.01 related to tumors treated without (w/o) zVAD-fmk; # P < 0.05 ; ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor.

An efficient tumor therapy does not only kill tumor cells but also activates the immune system to prevent metastasis and recurrences. One indication for activation of the immune system is the infiltration of immune cells into the tumor. Important immune cells for tumor control are DCs, NK cells and CD8+ T cells.
Figure 37: Infiltration of cells of the adaptive immune system into B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence and presence of zVAD-fmk.

The infiltration of immune cells [CD8+ T cells (A), CD4+ T cells (B), and Tregs (C)] was analyzed by multicolor flow cytometry, 96h after the last treatment of the tumors. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to tumors treated without (w/o) zVAD-fmk; # P < 0.05 ; ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor.

Negative influences on the tumor treatment have the immune suppressive Tregs and MDSCs. Fractionated RT alone or in combination with DTIC and HT increased the infiltration of NK cells into the tumor. zVAD-fmk negatively impacted on it when combined with fractionated RT. In contrast, in combination with fractionated RT, DTIC and HT, it further slightly enhanced the infiltration of NK cells (Figure 36C). DCs infiltrated into the tumor only after combined treatments with zVAD-fmk (Figure 35B). All treatments decreased the infiltration of macrophages and MDSCs into the tumor (Figure 36A and D).

Figure 36D).

Fractionated RT alone or in combinatory treatments increased the infiltration of CD4+ T cells (Figure 37B). The same was seen for CD8+ T cells. Additional injection of zVAD-fmk boosted the infiltration of CD8+ T cells (Figure 37A). In comparison, the number of immune suppressive cells like Tregs was decreased by single treatment with zVAD-fmk alone and by the combination of fractionated
RT and zVAD-fmk slightly. It was significantly decreased after fractionated RT and the combination of fractionated RT, DTIC and HT in absence or presence of zVAD-fmk (Figure 37C).

3.3.2 Combination of fractionated RT, DTIC and HT with zVAD-fmk impacts on immune cell infiltration into the draining lymph nodes and the presentation of tumor antigen

The immune cells have to be present, but also activated for destroying tumor cells. For activation of cells of the adaptive immune system, the antigen has to be presented or cross presented by DCs. Here not only the number but also the phenotype and activation status of the DCs are important. Cross presentation is mainly performed by CD8+ DCs [112, 113]. This takes place in the draining lymph nodes. After capturing of antigens, DCs migrate to the lymph node and present the antigens to T cells. We therefore also analyzed the infiltration of the immune cells into the draining lymph nodes. Fractionated RT alone or combination of fractionated RT, DTIC and HT did not influence the number of CD4+ T cells in the lymph nodes significantly (Figure 38A). However, combination of fractionated RT, DTIC and HT in presence zVAD-fmk even decreased the number of CD4+ T cells significantly. The infiltration of CD8+ T cells into the lymph nodes was not influenced by any of the treatments (Figure 38B). Fractionated RT or combination of fractionated RT, DTIC and HT did further not impact on NK cell infiltration, but this was significantly enhanced by adding zVAD-fmk (Figure 38C). Similar effects were seen when analyzing the infiltration of DCs into the lymph nodes (Figure 39). Combination of fractionated RT, DTIC and HT with zVAD-fmk increased the infiltration of DCs significantly and especially that of cross presenting CD8+ DCs (Figure 39A, B).
Figure 38: Immune cell infiltration into draining lymph nodes of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk.

The infiltration of immune cells [CD4+ T cells (A), CD8+ T cells (B) and NK cells (C)] was analyzed by multicolor flow cytometry, 96h after the last treatment. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in triplicates, are presented as mean ± S.D. ** P < 0.01 related to tumors treated without (w/o) zVAD-fmk. DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. Mock: untreated tumors.

Although zVAD-fmk only enhanced the infiltration of cross presenting CD8+ DCs, all treatment schemes in presence or absence of zVAD-fmk enhanced the cross presentation of a model tumor antigen. This was shown with an enhanced presentation of OVA peptide by DCs in draining lymph nodes of OT-1 mice bearing a B16-OVA tumor. Already zVAD-fmk or fractionated RT alone enhanced the presentation of the model antigen by DCs (Figure 39C).
Figure 39: Immune cell infiltration into draining lymph nodes of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk. The infiltration of immune cells into the draining lymph nodes [DCs (A, B)] and presentation of SIINFEKL (C) was analyzed by multicolor flow cytometry, 96h after the last treatment. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in duplicates, are presented as mean ± S.D. # P < 0.05 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. mock: untreated mice.

3.3.3 Combination of fractionated RT, DTIC and HT with zVAD-fmk do not influence the proliferation, but affects the expression of immune stimulatory cytokines of T cells

Activated T cells proliferate and produce and secrete immune stimulatory cytokines such as INFγ and the T cell growth factor IL-2. B16-OVA tumor bearing mice were treated as described above and CFSE stained CD8+ T cells which were isolated of spleens of OT-1 mice were injected i.v.. 96h afterwards, the in vivo proliferation of these CD8+ T cells and the expression of intracellular INFγ and IL-2 after ex vivo re-stimulation was analyzed. Neither the
combination treatment with fractionated RT, DTIC and HT nor the combination

**in vivo Proliferation of OVA-specific CD8+ T cells**

![Graph showing in vivo Proliferation of OVA-specific CD8+ T cells](image)

**Figure 40:** *In vivo* proliferation of specific CD8+ T cells in B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk.

CFSE stained CD8+ T cells were isolated of draining lymph nodes from OT-1 mice and analyzed by multicolor flow cytometry, 96h after the last treatment of the tumors. The latter were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in duplicates, are presented as mean ± S.D. DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. mock: untreated mice.

**Figure 41:** Intracellular expression of INFγ and IL2 of CD8+ T cell of B16-OVA tumor bearing OT-1 mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk and ex vivo restimulation with OVA peptide.

Intracellular INFγ (A) and IL-2 (B) of CD8+ T cells of B16-OVA tumor bearing OT-1 mice after treatment and restimulation with OVA peptide was analyzed by multicolor flow cytometry, 96h after the last treatment. The latter were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT (41.5°C for 30min) was performed 4 hours after irradiation at day 8 and 10. Data of three experiments, each performed in duplicates, are presented as mean ± S.D. * P < 0.05 related to tumors in the absence (w/o) of zVAD-fmk. DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. mock: untreated mice.
with zVAD-fmk affected the proliferation of CD8+ T cells in vivo. Almost 90% of the CD8+ T cells had proliferated, even in mock treated mice (Figure 40). Additionally, none of the treatment(s) influenced the intracellular expression of IL-2 after ex vivo restimulation of the T cells (Figure 41B). However, T cells of mice whose tumor was treated with fractionated RT, DTIC and HT in combination with zVAD-fmk expressed higher amounts of INFγ after restimulation (Figure 41A).

3.3.4 Combination of fractionated RT, DTIC and HT with zVAD-fmk retards tumor growth in a T cell dependent manner

The enhanced infiltration of CD8+ T cells after fractionated RT, DTIC and HT and cell death modulation with zVAD-fmk gave first hints that the therapy-induced tumor growth retardation is dependent on T cells. To prove this, analogue experiments were performed with tumor bearing RAG-2-deficient (RAG KO) mice. zVAD-fmk in combination with fractionated RT, DTIC and HT did not significantly further decrease the tumor growth induced by fractionated RT, DTIC and HT (Figure 42). The latter treatment kills tumor cells directly or activates other immune cells against the tumor, since it was not significantly dependent on T cells
Figure 42: In vivo growth of B16-F10 tumors in RAG KO mice after fractionated RT, DTIC and HT in the absence or presence of zVAD-fmk.

The tumor growth of syngeneic B16-F10 tumors in RAG KO mice is displayed. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. HT was performed 4 hours after irradiation at day 8 and 10. For this, the mice were anesthetized and the tumors were heated locally under temperature control to 41.5°C for 30min using the BSD50 hyperthermia system. The tumor volume was monitored with an electronic caliper. Joint data of three independent experiments, each with 3 mice per group, are presented as mean ± S.D. # P < 0.05 ; ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor.
3.3.5 Nucleotides and HMGB1 contribute to anti-tumor effects induced by zVAD-fmk in combination with fractionated RT, DTIC and HT

**Figure 43**: In vivo growth of B16-F10 tumors in C57/BL6 mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk and in presence of Apyrase. The tumor growth of syngeneic B16-F10 tumors in wild type C57/BL6 mice is displayed. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p.. Apyrase was injected i.v. one hour after irradiation (25U/mouse at day 8). HT was performed 4 hours after irradiation at day 8 and 10. For this, the mice were anesthetized and the tumors were heated locally under temperature control to 41.5°C for 30min using the BSD50 hyperthermia system. The tumor volume was monitored with an electronic caliper. Joint data of three independent experiments, each with 3 mice per group, are presented as mean ± S.D. * P < 0.05 related to tumors treated with 2Gy+DTIC+HT; ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor. mock: untreated mice.

To analyze the influence of the danger signals ATP as nucleotide and HMGB1 on the therapy-induced tumor growth retardation in vivo, the experiments were additionally performed by injecting Apyrase (Figure 43) or with MyD88 KO mice (Figure 44). B16-F10 tumor bearing C57/BL6 mice, were treated with fractionated RT, DTIC, HT and zVAD-fmk and additional i.v. injection of Apyrase after the multimodal treatment to induce degradation of extracellular present nucleotides in vivo. Nucleotides seem not to be the main players in therapy-induced tumor growth retardation, since still significant further tumor growth retardation was observed when adding zVAD-fmk to treatment with fractionated RT, DTIC and HT in the presence of Apyrase (Figure 43). Nevertheless, the
tumor growth was still stronger compared to an environment with nucleotides being present (Figure 3).

Regarding the impact of TLR signaling, zVAD-fmk did not further impact on therapy-induced tumor growth retardation in MyD88 KO mice (Figure 4). The tumor growth retardation after treatment with fractionated RT, DTIC and HT was similar in the absence or presence of zVAD-fmk (Figure 4).

**MyD88 ko**

![Graph](image)

**Figure 4**: *In vivo* growth of B16-F10 tumors in MyD88 KO mice after fractionated RT, DTIC and HT in absence or presence of zVAD-fmk.

The tumor growth of syngeneic B16-F10 tumors in MyD88 KO mice is displayed. The tumors were locally irradiated at days 8, 9 and 10 with a clinically relevant single dose of 2Gy using a linear accelerator. Two hours after the irradiation DTIC (2mg/mouse at day 8 and 10) and zVAD-fmk (2mg/kg at day 8, 9 and 10) were injected i.p. HT was performed 4 hours after irradiation at day 8 and 10. For this, the mice were anesthetized and the tumors were heated locally under temperature control to 41.5°C for 30min using the BSD50 hyperthermia system. The tumor volume was monitored with an electronic caliper. Joint data of three independent experiments, each with 3 mice per group, are presented as mean ± S.D. # P < 0.05, ## P < 0.01 related to untreated tumors (mock). DTIC: dacarbazine; Gy: Gray; HT: hyperthermia; zVAD-fmk: pan-caspase inhibitor.
4 Discussion

4.1 B16-F10 cells as mouse model for malignant melanoma

The B16 mouse melanoma cell line is one of the most used tumor models for melanoma over the last half century and is a spontaneous C57/BL6-derived melanoma [114]. The B16 melanomas are defined as poorly immunogenic since they express rather low levels of MHCI molecules [115]. Nevertheless tumor regression can be induced by immunotherapeutic treatments which demonstrate the immunogenic potential of these cells [15]. Therefore, we decided to use the B16 melanoma cells as model system for melanoma to test the efficacy and mode of action of combined therapies in absence or presence of further cell death modulation with zVAD-fmk.

4.2 Immune therapy as a treatment option against melanoma

Melanomas are often highly radio- and chemoresistant [108]. Because of this, RT or CT alone is not an efficient treatment option for most of the melanomas. Primary tumors are often removed by surgical resection. Since this therapy form does not affect the immune system it cannot prevent recurrences or metastasis. Furthermore, metastasis often grew in places where a surgical resection is not possible. For this an additional immune therapy is necessary. Due to the fact that existing immune therapies against melanomas cause high side effects or does only lead to responses for short time [12], there is a strong need for additional and/or alternative immune therapies or immune modulating drugs. Ipilimumab, an anti-CTLA4 antibody is the most used immune modulating drug for melanoma. Blocking CTLA4, a negative regulator of the immune system and terminator of T cell activation and proliferation, leads to an enhanced and long lasting activation of T cells. Clinical trials could show that response rates of the anti-CTLA4 antibody ipilimumab are between 5 and 20%. Studies with higher concentrations of ipilimumab could show better response rates, but also higher toxicities have been observed [15-17]. New blocking antibodies for melanoma treatments are anti-PD-1 or anti-PD-1L. These antibodies aim to enhance the T cell immune response in a tumor specific manner by blocking the interaction of the inhibitory receptor PD-1 on T cells with PD-L1 expressed on tumor cells.
First results of clinical trials are promising. The response rate of nivolumab is over 2 years. The antibody is well tolerated and toxicities were mild, less frequent and less severe than those observed with ipilimumab [13, 14]. Another immune activating strategy for melanoma treatment is the use of vemurafenib, a v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor. 40-60% of the tumors have mutations in the gene coding for BRAF and 90% of these mutations results in the substitution of valine for glutamine (V600E) [8, 9]. The mutated form of BRAF results in a constitutive activated mitogen-activated protein kinase pathway (MAPK) constitutive activated which leads to an increased cellular proliferation and oncogenic activity. The BRAF inhibitor vemurafenib is selective for BRAF with the V600E mutation. A phase III trial demonstrated improvements in response rates and in progression free survival [9]. But the limiting factor of this therapy form is the short duration that in average is only approximately 6 month [1].

Beside the importance of an immune therapy, the standard therapies should not be neglected. Under certain clinical conditions, RT, especially in multimodal settings, should be considered as treatment option. New approaches of radiotherapy like stereotactic radiosurgery or brachytherapy have been shown to be an effective treatment option [116]. Hyperthermia acts as an adjuvant for RT and has been proven in clinical trials to improve local control of malignant melanoma [117]. Further, chemoresistance of malignant melanoma can be overcome in a tumor cell-selective manner by interfering with anti-apoptotic Bcl-2 family members [118]. Therefore, cell death modulators might be beneficial in multimodal settings. Targeting apoptotic pathways and modulation of apoptotic cell death are therefore promising strategies to combat cancer and inflammatory diseases in general. Here caspases represent key targets for drug development since they are central in initiation and execution of cell death and in maturation of inflammatory cytokines [4].

### 4.3 Immunogenic cell death

Innovative melanoma therapies should therefore be multimodal ones and aim to induce immunogenic tumor cell death forms. The latter modify the tumor microenvironment by releasing danger signals (DAMPs). Immune cells might
get recruited and activated. Tumor cell death is defined as immunogenic when the cells express calreticulin on their surface and/or release DAMPs like HMGB1 and ATP [119]. Calreticulin is mostly expressed on pre- or early apoptotic cells. In contrast, DAMPs are mainly released by necrotic cells. Necrosis in general is the more immunogenic cell death form. One possible new therapeutic approach is to manipulate the cell death to be more immunogenic. The tumor and its microenvironment should be modified to release danger signals which results in recruiting and activation of immune cells [16].

4.4 Necrosis and necroptosis in melanoma cells

4.4.1 Impact of multimodal treatments on melanoma cell death forms

Since malignant melanoma is resistant to standard therapies, only combinatorial therapies might result in sufficient tumor cell death. The \textit{in vitro} experiments revealed that combination of HT with DTIC leads to apoptosis and in combination with RT to necrosis. The triple combination consisting of HT, RT and DTIC resulted to the highest percentages of apoptotic and necrotic melanoma cell death. This mixture is beneficial for induction of anti-tumor immune responses, since cells dying via apoptosis release chemotactic factors to attract immune cells [13] and also together with the necrotic ones release DAMPs to activate them [17]. In this context it is also important to consider the order of therapies. Hyperthermia can sensitize the cells for irradiation when it is delivered before RT or it can aggravate the irradiation induced cellular stress when it is applied after RT [96]. Irradiation first and HT afterwards induced significantly more cell death than the other way around (\textbf{Figure 9 and 10}). However, in the clinics mostly HT is performed before RT due to logistic reasons. To improve the positive effect of HT on melanoma cell death induction primary triggered by RT, it should be considered to change the order of RT and HT also in the clinics. One possibility to manipulate the cell death in an additional way is to combine the treatments with the pan caspase inhibitor zVAD-fmk. Pan caspase inhibitors like zVAD-fmk or IDN-6556 were proven in preclinical studies and currently also in phase I/II trials for inflammatory diseases and show manageable side effects [5, 120].
4.4.2 Induction of melanoma necroptosis by zVAD-fmk

It has been shown before for the mouse fibrosarcoma L929 cells that this pan caspase inhibitor can induce necroptosis by blocking apoptosis [121]. Since the characteristics of programmed necrosis like rounding of the cell, cytoplasmic swelling, lack of DNA fragmentation and plasma membrane rupture are the same as of accidental necrosis, necroptosis must be also considered as immunogenic cell death form. How zVAD-fmk modulates cell death of malignant melanoma and how it impacts on the immunogenicity of these cells is hardly investigated. We revealed that necroptosis is inducible in B16-F10 melanoma cells, since necrosis could be inhibited with the RIP-1 kinase inhibitor necrostatin-1 (nec1). Necroptosis was inducible even in the natural dying cells being present in cell culture (Figure 11A). Furthermore necrotic melanoma cell death was fostered by blocking apoptosis with zVAD-fmk (Figure 11B). In organisms necroptosis is seen as a backup mechanism for conditions when apoptosis is not possible like after infection with certain viruses [84]. Many viruses encode Bcl-2 homologs or caspase inhibitors to block apoptosis [122]. zVAD-fmk induced necrosis could be blocked by adding nec-1 (Figure 12). This gave us a further hint that necroptosis is inducible in melanoma cells (Figure 11B).

4.5 Effects of zVAD-fmk on the immunogenic potential of B16 melanoma cells in vitro

4.5.1 Effects of zVAD-fmk on the cell cycle

Of note is that zVAD-fmk did not only modulate melanoma cell death, but also the cell cycle. 48h after every treatment (even in mock treated cells), higher amounts of cells were in G2 phase of the cell cycle when zVAD-fmk was present in treatment (Figure 13B). The G2 phase of the cell cycle is more chemo- and also radiosensitive. Cells stop in this phase of cell cycle after stress factors like RT. This fact is used for planning fractionation schemes. In the second or later fractions more and more cells are in the G2 phase and therefore more sensitive for the following RT [83].
This suggests that a time window of 48h should be considered between the treatments with RT, DTIC, HT and zVAD-fmk. We therefore applied the following treatment schemes and time schedule in the in vivo experiments: RT and injection of zVAD-fmk was performed every 24h but the second treatment with HT and injection of DTIC was performed after 48h.

4.5.2 Effects of zVAD-fmk on the secretion of DAMPs

Normally higher amounts of necrosis correlates with an enhanced passively release of DAMPs like ATP, HMGB1 and HSP70 (summarized in [123]). We saw that additional incubation with zVAD-fmk results in higher amounts of HMGB1 but not of HSP70 in the supernatants (SN) of the treated tumor cells (Figure 14). This indicates that necroptosis induced by zVAD-fmk does not increase the release of DAMPs in general. Only the release of HMGB1 correlated with the necrosis induction. Danger signals like HMGB1 enhance maturation and activation of immune cells like macrophages and DCs [85].

4.5.3 Effects of zVAD-fmk on macrophages and DCs

To analyze the impact of zVAD-fmk as cell death modulator on the immunogenicity of B16 melanoma cells in detail, we explored the impact of tumor cell SN after the respective treatments on cells of the innate and adaptive immune system.

Macrophages and DCs are the first cells which are recruited to the tumor and therefore essential for the regulation and activation of the adaptive immune system. The activation of these cells is crucial for success of an immune based tumor therapy [85, 86]. After incubation with SN of tumor cells macrophages increased the expression of MHCII and the activation marker CD86 on their surface when zVAD-fmk was used for cell death modulation of B16 cells. This was not a direct effect of the inhibitor on the immune cells, since the half- life of zVAD-fmk is short (< 40 minutes) [124] and an incubation with the inhibitor alone did not have any effects on macrophages and DCs (Figure 16 and 21). The enhanced amounts of HMGB1 and ATP in the SN were in part responsible for the increased expression of the activation markers on macrophages, since the increased expression of these markers was not observed after incubation of the SN with Apyrase or using peritoneal macrophages which were isolated from MyD88 KO mice. Apyrase is a calcium-activated plasma membrane-bound
enzyme that catalyzes the hydrolysis of ATP to AMP and inorganic phosphate. But Apyrase does not only degrade ATP but all purine and pyrimidine nucleoside 5'-di- and 5'-triphosphates [125]. Therefore the experiments with Apyrase only show that nucleotides and not specifically ATP influence the activation of macrophages.

MyD88 is an adaptor protein for TLR signaling and is used by all TLRs except TLR3, therefore MyD88 KO mice have defects in TLR signaling [126] and as a result also in signaling of HMGB1 which acts through TLR2 and TLR4 [127]. Because of the existence of other ligands for TLRs than HMGB1 it is only a hind that HMGB1 is responsible for the activation of macrophages. Other possible ligands are for example fibrinogen, fibronectin, heparan sulfate and hyaluronan which are all components of the tumor extracellular matrix [128].

An increased expression of MHCII and CD86 was also seen on DCs, but here only when zVAD-fmk had been combined with RT, DTIC and HT. Again, these effects are dependent on TLR signaling and nucleotides since the increased expression was not detectable when using MyD88 KO mice or incubation of the melanoma cell SN with Apyrase (Figure 24). MyD88 KO mice have defects in TLR signaling. HMGB1 but also other molecules like fibrinogen or fibronectin act through this pathway as mentioned above. Because of this we performed additional experiments with a HMGB1 depleting antibody to prove that the effects are especially dependent on HMGB1. We observed that the increased expression of these activation markers was not present when incubating the SN of treated tumor cells with this antibody before adding to DCs (Figure 25).

Since HMGB1 was released in the SN of melanoma cells in every treatment when zVAD-fmk was present this indicates that not only the amount but also the redox state of it and the combination with other molecules [12] are important for the activation of DCs. A reductive environment is important for HMGB1 to maintain the bioactivity [11]. We could not reconstitute the activation of DCs and macrophages by addition of recombinant HMGB1 (data not shown). But by addition of recombinant HMGB1 we did not adapt the milieu of the tumor cells, therefore we could not define the redox state of HMGB1.

Danger signals enhance maturation and antigen presentation of APC [129]. Those innate immune cells are the first ones which are recruited to the tumor by different soluble factors of the tumor microenvironment. They regulate the
activation or suppression of cells of the adaptive immune system and are therefore jointly responsible for the success of an immune-based tumor therapy (summarized in [130]). Beside the activation we analyzed the functionality of DCs and macrophages. There are reports that soluble factors of tumors impair the functionality of immune cells especially of DCs. By releasing factors like IL-10, M-CSF, vascular endothelial growth factor (VEGF), gangliosides or prostanoids tumors can prevent the differentiation and function of DCs (summarized in [131]). We observed that both APCs were still functional after contact with the treated tumor cells. They phagocytosed and migrated properly. Only the migration of DCs to SN of tumor cells which were treated with RT, DTIC and HT and whose cell death was modulated with zVAD-fmk was slightly increased (Figure 28). This suggests that this special treatment might result in an enhanced migration into the tumor of DCs also in vivo. After migration into the tumor, DCs have to be activated since activated DCs are then able to activate naïve T cells [132].

4.5.4 Effects of zVAD-fmk on T cells

Activation of especially CD8+ T cells is essential for an effective anti-tumor immune response [14]. Therefore we investigated the ability of the activated DCs, characterized by upregulation of the activation markers MHCII and CD86 and increased release of the inflammatory cytokines TNFα and IL-1β, to activate T cells. A slightly enhanced proliferation after specific activation of OT-1 CD8+ T cells with OVA presenting DCs which had been activated with SN of B16-OVA cells after treatment with RT, DTIC, HT and zVAD-fmk (Figure 33C) was observed. However, we could not detect an enhanced proliferation by MLR, but we detected an enhanced secretion of IL-6 and INFγ after coincubation with DCs (Figure 34A,B). It was increased by the combinatory treatment of RT, CT and HT and additional modulation with zVAD-fmk. IL-6 has two contrary roles in anti-tumor immunity. On the one side IL-6 can act intrinsically on tumor cells. In this way it supports the proliferation and survival of tumor cells. On the other side IL-6 can induce an anti-tumor immune response by leading to activation, proliferation and survival of lymphocytes and by supporting the T cells immune response (summarized in [114]). INFγ is an important cytokine in the initiation and execution of the anti-tumor immunity. The efficacy of tumor infiltrating lymphocytes is associated with their ability to secrete INFγ [115]. It leads to
upregulation of MHCI and therefore improves the presentation of tumor antigens by APCs [133]. Additionally it regulates the differentiation and functionality some immune cells. In this way it promotes a Th1 mediated immune response and recruit CD8+ T cells [15, 17]. Furthermore it activates macrophages to produce chemokines which results in recruitment of more immune cells [15] (summarized in [134]).

Cell death modulation with zVAD-fmk did not only activate cells of the innate immune system but also of the adaptive immune system in vitro.

4.5.5 Effects of zVAD-fmk on NK cells

Other effective immune cells against tumor cells are NK cells. These innate immune cells can kill tumor cells by different ways. In contrast to T cells NK cells can kill tumor cells independent of MHC molecules (summarized in [122]). One possibility is the kill by binding on death receptors like Fas (CD95). They can increase the expression of these receptors on the surface of tumor cells by secretion of INFγ [135]. Only mature NK cell can kill tumor cells efficiently. Treatment of tumor cells did not impact on the maturation status of NK cells (Figure 30) or the kill of tumor cells by these cells directly (Figure 31). However, tumor cells, which were treated with DTIC, decreased the release of INFγ by NK cells after coincubation. So this CT agent acts immune suppressive in this view. In contrast, cell death modulation with zVAD-fmk restored the release of INFγ when combined with RT and HT (Figure 32). Therefore the expression of the death receptor Fas on the surface of the tumor cells might be increased after this multimodal treatment since release of INFγ by NK cells can enhance Fas expression. Future experiments should examine this in more detail.

4.6 zVAD-fmk induced B16-F10 tumor growth retardation in vivo

In our preclinical in vitro systems, the combinatory treatment of RT, DTIC and HT in the presence of zVAD-fmk resulted in cell death of B16-F10 melanoma cells with high immunogenic potential. To prove the immunogenic potential of zVAD-fmk in multimodal tumor treatments, we performed mouse in vivo studies with treatments closely resembling the human situation. For this, we established both a local irradiation and a local hyperthermia application for mice with ectopic
B16-F10 tumors (**Figure 7 and 8**). The *in vivo* studies revealed that fractionated irradiation with a clinically relevant single dose of 2Gy alone did not affect the tumor growth. Similar results were obtained with the *in vitro* experiments. Together this proves that the melanoma cells are highly radioresistant, as most of the melanoma cells in patients [108]. Only the triple combination treatment with fractionated RT, DTIC and HT reduced the tumor growth significantly. Addition of zVAD-fmk further retarded the tumor growth significantly (**Figure 35**). The reduced tumor growth induced by zVAD-fmk is not a direct cytotoxic effect of the inhibitor but rather dependent on the immune system (see below).

### 4.6.1 Immune stimulatory effects of zVAD-fmk *in vivo*

The combination treatment consisting of fractionated RT, DTIC and HT had direct effects on the tumor cells but additionally, this treatment scheme changes the tumor micromilieu. It influenced the infiltration of various immune cells into the tumor and therefore potentially also the efficiency of the treatment. zVAD-fmk modulated the therapy-induced infiltration of distinct immune cells. Different studies showed that an increased infiltration of lymphocytes is associated with an improved survival [136-138].

### 4.6.2 Infiltration of immune cells into the tumor

The infiltration of innate and adaptive immune cells into the tumor is affected by the examined treatment schemes. Fractionated irradiation alone led to an enhanced infiltration of NK cells into the tumor, but in the presence of zVAD-fmk only as few NK cells as in untreated tumors were observed. In contrast, the combination treatment with RT, DTIC and HT also increased the infiltration of NK cells and addition of zVAD-fmk further slightly enhanced this (**Figure 36C**). Future work should focus on whether this is also dependent on activation of DCs after the multimodal treatment in the presence of zVAD-fmk [84]. The infiltration of DCs into the tumor was only increased in the presence of RT and zVAD-fmk (**Figure 36B**). This highlights again that RT in multimodal settings exerts its anti-tumor effects via activation of DCs. NK cells and cytotoxic CD8+ T cells are the key immune cell population that mediates the cytotoxic effects induced by RT in combination with immunotherapeutic agents [83].

Regarding immune suppressive cells such as Treg and MDSCs, combination treatment with fractionated RT, DTIC and HT significantly reduced the
percentage of these cells in the tumor, as also zVAD-fmk did alone (Figures 36 and 37).

To summarize these effects of zVAD-fmk on the infiltration of innate immune cells into the tumor, one can assert that more inflammatory or effector cells like DCs and NK cells migrate into the tumor concomitantly with less immune suppressive cells like MDSCs, Tregs and macrophages. Further, zVAD-fmk also modulated the infiltration of adaptive immune cells into the tumor. All treatments reduced the infiltration of immune suppressive Tregs (Figure 37C).

We revealed that already fractionated RT alone in combination with zVAD-fmk modulates the tumor milieu and therefore the infiltration of immune cells into the tumor. More inflammatory or effector cells and less immune suppressive cells were present. Nevertheless, RT alone or in combination with zVAD-fmk could not inhibit the tumor growth significantly. We conclude that immune modulation alone cannot be an option for a solo therapy in this case. There must be a combination with a more locally tumor damaging therapy. Only when enough dying cells are generated, cell death modulation by zVAD-fmk modulates the cell death form and might cause an immune reaction via activation of DCs and CTLs also against still viable tumor cells [139].

Since the activation of cytotoxic T cells takes place in the draining lymph nodes, we analyzed the infiltration of NK cells, DCs and T cells into them. An enhanced infiltration of NK cells into the lymph nodes after a combination treatment with fractionated RT, DTIC, HT and zVAD-fmk was observed. But there were not only more NK cells detectable, but also more DCs which are capable of activating naïve T cells (Figure 39A). Of special note is that most of the infiltrating DCs were positive for CD8 (Figure 39B). This DC phenotype is especially known to efficiently cross-present antigens [112, 113]. The percentage of CD8+ T cells in the LN was similar after all treatments, but most likely these T cells become more activated, when more CD8+ DCs are present. Especially after treatment of the tumor with fractionated RT, DTIC, HT and zVAD-fmk, ex vivo re-stimulated T cells expressed more INFγ (Figure 41).

4.6.3 Tumor growth retardation in immune deficient mice

This indicates that T cells are main effector cells of this multimodal tumor treatment scheme, which was further proven by performing similar experiments with RAG KO mice. These mice have no functional B and T cells [140]. Here,
zVAD-fmk did not have any additional effects in combination treatments in these mice (Figure 42). Nevertheless, T cells were not directly activated by the combinatory treatment but by DCs. Treatment of tumors with RT, DTIC, HT and zVAD-fmk results in higher amounts of necrotic cells and therefore in an enhanced release of the danger signals ATP and HMGB1. These danger signals activate DCs and thereby T cells. To prove the effects of the danger signals we additionally injected Apyrase [141] or used MyD88 KO mice [142] for the experiments. Apyrase degradates extracellular ATP and nucleotides and MyD88 KO mice have defects in TLR signaling. There are no additional effects of zVAD-fmk detectable when using MyD88 KO mice. Apyrase also reduced the effects of zVAD-fmk. This is a strong evidence that HMGB1 and ATP are responsible for the activation of DCs also in vivo (Figure 43 and 44).

4.7 Conclusion

Figure 45: Combination of RT, DTIC, HT with zVAD-fmk is capable of activating both innate and adaptive immune cells against the tumor cells.
The combinatory treatment consisting of fractioned RT, DTIC, HT and zVAD-fmk results in an immunogenic B16 tumor cell death: the tumor cells die more necrotic and release the danger signals HMGB1 and most likely ATP as prominent nucleotide. This results in an activation of NK cells and DCs. The latter in turn activate CD8+ T cells against the tumor. In contrast, the combination treatment without zVAD-fmk results in dying and dead tumor cells with low immunogenicity and thereby in fact acts locally but less systemically.

The detailed mechanism how zVAD-fmk in combination with RT with a single dose of 2Gy, DTIC and HT induce an anti-tumor immune response is still not clear. But we revealed that zVAD-fmk as cell death modulator of therapy-induced dying cells by RT, DTIC and HT fosters melanoma cell necroptosis as an immunogenic cell death form which then activates APCs such as DCs by the
release or secretion of danger signals (HMGB1 and nucleotides such as ATP) DCs and macrophages enhanced the expression of the activation markers MHCII and CD86 and secreted higher amounts of the proinflammatory cytokines TNFα and IL-1β. While the maturation and activation status of NK cells is not influenced by this treatment, the secretion of INFγ by these innate immune cells was enhanced.

In vivo, the tumor growth was already significantly decreased by the combination treatment of fractionated RT, DTIC and HT, but zVAD-fmk decreased the tumor growth further by enhancing the immunogenicity of the treated tumor cells. This results in an increased infiltration of DCs and T cells into the tumor and lymph nodes and also in an increased activation of CD8+ T cells. These effects are dependent on the danger signals HMGB1 and ATP and most likely also on the redox status of HMGB1. We conclude that HMGB1 in a distinct tumor microenvironment is the main effector molecule for induction of anti-tumor immunity by zVAD-fmk in multimodal therapies. But ATP and/or other nucleotides further enhance these anti-tumor immune responses (Figure 43).

zVAD-fmk should be considered in the future as an additional drug for anti-melanoma therapy. Moretti and colleagues already demonstrated that zVAD-fmk acts as radiosensitizer for tumor cells, significantly slows xenograft tumor growth retardation induced by RT and increases the expression of HMGB1 [55]. Melanoma is a tumor with a per se “immunogenic potential” because there are cases of spontaneous regression [143] and case reports about abscopal effects [144-146]. The latter are a phenomenon in which local RT induces a regression of (metastatic) cancer at a distance from the irradiated site (summarized in [147]). This is especially observed in patients with melanoma. Postow et al postulated a case report of an abscopal effect in a patient with melanoma treated with ipilimumab and RT [148]. The patient showed a systemic response to localized radiotherapy after receiving Ipilimumab. Additionally, there was a systemic increase of activated CD4+ T cells and a decrease of MDSCs in the serum of the patient detectable.

This raises hope that multimodal treatment settings that induce activation of immune cells will increase survival of melanoma patients in the future. Our data suggest including zVAD-fmk in multimodal melanoma treatments to foster the induction of immunogenic tumor cell death and therefore anti-melanoma
immunity. As zVAD-fmk showed some side effects in phase I clinical studies as liver toxicities [120], other caspase inhibitors with lower toxicities were developed such as IDN-6556 (PF-03491390) and should be tested also in combination with RT, DTIC and HT in the future. In general, caspase inhibitors have been proven in preclinical and current phase I/II clinical trials to be suitable drugs for inflammatory diseases and tissue damages with manageable side effects [5, 120]. Therefore, the way into the clinics of caspase inhibitors for the treatment of melanoma should be feasible.

4.8 Outlook

zVAD-fmk should be tested in long time experiment to even better simulate the clinical situation. Different fractionation schemes of RT should be analyzed and the most beneficial chronology of combining RT with zVAD-fmk and further agents should be uncovered [63]. To prove that zVAD-fmk in multimodal settings leads to a long lasting immune memory against the tumor, challenge experiments have to be performed. After that zVAD-fmk or new less toxic caspase inhibitors such as IDN-6556 could be tested in clinical phase I/II studies for treatment of melanoma. Additionally, zVAD-fmk should be tested as immune-stimulating molecule in combination with distinct fractionation schemes of RT and chemotherapeutic agents for other tumor entities than melanoma.
References


Publications

Original articles:


Reviews:


Short Publications:


- Werthmöller N, Frey B, Fietkau R, Gaipl US. Modulation of radiochemoimmunotherapy-induced B16 melanoma cell death by zVAD-fmk has immunostimulant anti-tumor effects. STRAHLENTHERAPIE UND ONKOLOGIE Volume: 190 Supplement: 1 Pages: 76-76 Published: JUL 2014


- Werthmöller N, Frey B; Fietkau R; Gaipl US. The influence of standard combinatory tumor treatments and the apoptosis inhibitor zVAD-fmk on B16 melanoma cell death forms and consecutively on macrophages and dendritic cells. STRAHLENTHERAPIE UND ONKOLOGIE Volume: 189 Supplement: 1 Pages: 120-120 Published: MAY 2013

Manuscript in preparation:

Patrick Finkel, Benjamin Frey, Friederike Mayer, Karina Bösl, Nina Werthmöller, Andreas Mackensen, Udo Gaipl*, Evelyn Ullrich*. Bivalent role of NK cells in antitumor reactions triggered by ionizing radiation in combination with hyperthermia. Submission to Oncoimmunology