Evaluating necroptosis competency in malignant melanoma

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Biswajit Podder

Stuttgart, 10th of July 2020

This thesis is dedicated to three million freedom fighters who sacrifice their lives for my beloved country Bangladesh

"There will be obstacles. There will be doubters. There will be mistakes. But with hard work, there are no limits."

—Michael Phelps

Research outputs

Journal Article:

Podder B., Guttà C., Rožanc J., Gerlach E., Feoktistova M., Panayotova-Dimitrova D, Alexopoulos LG., Leverkus M., Rehm M., TAK1 suppresses RIPK1-dependent cell death and is associated with disease progression in melanoma
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Conference presentations:

Podder B., Gerlach E., Feoktistova M., Panayotova-Dimitrova D., Rehm M., Leverkus M; Studying necroptosis competency in malignant melanoma
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The 25th European Cell Death Organization (ECDO), September 2017, Leuven, Belgium

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List of Abbreviations

BCL-2	B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
BSA	Bovine Serum Albumin
C3	Caspase-3
C7	Caspase-7
C8	Caspase-8
cFLIP	Cellular FLICE-like inhibitory protein
CHX	Cycloheximide
cIAPs	Cellular inhibitor of apoptosis proteins
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Control
CYLD	Cylindromatosis (Protein)
DCFDA	Dichlorohydrofluorescein
DD	Death domain
ddH2O	Double-distilled water
DED	Death effector domain
DISC	Death-inducing signaling complex
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Etylene Diamine Tetraacetate
ERK	extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain protein
FasL	Fas Ligand
FBS	Fetal bovine serum
FLICE	FADD-like-1β-converting enzyme
GAPDH	Glyceraldehyde 3-phosophate dehydrogenase

HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HOIL-1	Heme-Oxidized IRP2 Ubiquitin Ligase 1 homolog
HOIP	HOIL-1 Interacting Protein
HRP	Horseradish peroxidase
IAP	Inhibitor of Apoptosis
IFN-γ	Interferon-γ
IκB	Inhibitor of κB
IKK	IκB-kinase
IL	Interleukin
IP	Immunoprecipitation
JNK	c-Jun N-Terminal kinase
KD	Kinase dead
KD	Knockdown
KO	Knockout
LPS	Lipopolysaccharide
LUBAC	Linear Ubiquitin Chain Assembly Complex
Mab	Monoclonal Antibody
MAP3K7	Mitogen-activated protein kinase kinase kinase-7
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCL-1	Myeloid leukemia cell differentiation protein
MEF	Murine Embryoinc Fibroblasts
MLKL	Mixed-lineage kinase domain like
MOMP	Mitochondrial outer membrane permeabilization
NEC-1	Necrostatin-1
NEMO	Nuclear Factor-kappa B essential modulator

NF-κB	Nuclear Factor kappa light polypeptide gene enhancer in B-cells
NIK	NF-κB inducing kinase
OXO	(5z)-7-Oxozeaenol
PARP-1	Poly (ADP-ribose)-polymerase 1
PI	Propidium iodide
PTM	Post-translational modification
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Real-Time PCR
RHIM	Receptor (TNFRSF)- Interacting Protein Homotypic Interaction Motif
RIPK1	Receptor (TNFRSF)- Interacting Serine-Threonine Protein Kinase 1
RIPK3	Receptor (TNFRSF)- Interacting Serine-Threonine Protein Kinase 3
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Room temperature
rTRAIL	Recombinant human TRAIL
siRNA	Small interfering RNA
SMAC	Second mitochondria derived activator of Caspases
TAB	Tak1-associated Binding Protein
TAB1	TAK1-Binding protein 1
TAB2	TAK1-Binding protein 2
TAK1	TGF-β-Activated Kinase 1
tBid	Truncated Bid
TGF-β	Transforming growth factors beta
TL	Total cell lysate
TLR	Toll-Like Receptor
TNF	Tumor necrosis factor
TNFR1/2	TNF Receptor 1/2

TRADD	TNF receptor type 1-associated death domain protein
TRAF2	TNF Receptor-Associated Factor 2
TRAIL	TNF-related apoptosis inducing ligand
UV	Ultraviolet
WT	Wildtype
XIAP	X-linked inhibitor of apoptosis protein
zVAD	z-Val-Ala-DL-Asp-fluoromethylketone

Abbreviations of units

%	Percentage
°C	Degree celsius
Bp	Base pair
cm	Centimetre
g	Gram
h, min, s	Hours, Minutes, Seconds
kDa	Kilodalton
l	Litre
M	Molar
mA	Milliampere
mg	Milligram
ml	Millimetre
mM	Millimolar
mol	Mol
ng	Nanogram
nm	Nanometre
nM	Nanomolar
rpm	Revolutions per minute
V	Volts
W	Watt
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

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Abstract

Melanoma cells are highly resistant to conventional genotoxic agents, and BRAFV600/MEK-targeted therapies, as well as immunotherapies, frequently remain inefficient. Therefore, alternative means to treat melanoma, particularly through the induction of programmed cell death modalities such as apoptosis or necroptosis, still need to be explored. Apoptotic and necroptotic cell death depends on cysteine aspartate-specific proteases (caspases) and receptor-interacting serine/threonine kinase (RIPK) 1/3, respectively. RIPK1/3 and caspases can directly interact with the TNFR signalling complex, thereby inducing programmed cell death. Thus, these cell death regulations need to be explored in melanoma cancer. In the course of this thesis, a panel of melanoma cell lines was studied for cell death susceptibility in response to the activation of TNF family receptors. Apoptosis and necroptosis responsiveness, as well as cell death sensitization approaches, were tested, followed by mechanistic signal transduction studies making use of pharmacological and genetic interventions. In addition, phosphoprotein and secretome analyses were performed by xMAP (Luminex) assays. This study reports that melanoma cell lines responded heterogeneously to either single treatment of death ligands (DLs) or in combination with an IAP antagonist. Furthermore, it was observed that melanoma cell lines expressing notable amounts of RIPK1, RIPK3, and MLKL, the key players in necroptosis signal transduction, failed to execute necroptotic cell death. Interestingly, the activity of transforming growth factor β-activated kinase 1 (TAK1) appears to prevent RIPK1 from contributing to cell death induction, since TAK1 inhibition by (5z)-7-Oxozeaenol, deletion of *MAP3K7*, or the expression of inactive TAK1 were sufficient to sensitize melanoma cells to RIPK1-dependent cell death in response to TNF-α or TRAIL-based combination treatments. However, cell death was executed exclusively by apoptosis, even when RIPK3 expression was high. In addition, TAK1 inhibitor (5z)-7-Oxozeaenol suppressed intrinsic or treatment-induced pro-survival signaling, as well as the secretion of cytokines and soluble factors associated with melanoma disease progression. Collectively, these results demonstrate that TAK1 suppresses susceptibility to RIPK1-dependent cell death. These findings were in line with high expression of TAK1 indicating an increased risk for disease progression in melanoma, as shown by subsequent collaborative work.

Zusammenfassung

Aktuelle Studien zeigen, dass neu entwickelte therapeutische Ansätze das Überleben von Melanompatienten signifikant verlängern können. Jedoch wurde auch berichtet, dass Melanomzelllinien in hohem Maße resistent gegen Chemotherapeutika, zielgerichtete Inhibitoren (z.B. BRAFV600) und Antikörper-basierende Immuntherapien sind. Um die Resistenzmechanismen zu umgehen, untersucht die folgende Dissertation die Induktion des programmierten Zelltodes, genauer der Apoptose oder Nekroptose, in Melanomzelllinien. Apoptotischer oder nekroptotischer Zelltod sind zum einen abhängig von Cystein-Aspartat-spezifischen Proteasen (Caspasen) und zum anderen angewiesen auf die Rezeptor-interagierenden Serin/Threonin Kinasen 1 und 3 (RIPK1/3). RIPK1/3 und Caspasen können direkt mit dem Tumornekrosefaktorrezeptor-(TNFR)-Signalkomplex interagieren und leiten dadurch den programmierten Zelltod ein. Aufgrund dessen soll im Rahmen dieser Dissertation in Melanomzelllinien der Zusammenhang zwischen dem TNF-Signalweg, dessen Interaktionspartnern (Caspasen; RIPK1/3) sowie der Induktion von Apoptose bzw. Nekroptose untersucht werden. Dazu wurde mittels pharmakologischer Inhibition oder genetischer Modifikation die Sensibilität von Melanomzelllinien gegenüber Apoptose bzw. Nekroptose analysiert. Weiterhin wurde das Phosphoproteom bzw. das Sekretom von Melanomzelllinien mittels XMAP (Luminex) untersucht. In dieser Dissertation wird aufgezeigt, dass Melanomzelllinien sehr heterogen auf die Induktion des TNFR-Signalkomplexes mittels Todesliganden (DLs) reagieren. Eine Kombination von DLs mit Apoptose-Inhibitoren (IAPs) zeigte dieselbe Heterogenität. Weiterhin wurde beobachtet, dass Melanomzelllinien große Mengen der Nekroptose-Schlüsselproteine (RIPK1/3, MLKL) exprimieren und trotzdem nicht in der Lage sind Nekroptose auszulösen. Interessanterweise konnte gezeigt werden, dass die Aktivität der transformierenden Wachstumsfaktor β-aktivierenden Kinase 1 (TAK1) RIPK1 daran hindert den Zelltod einzuleiten. Andererseits genügte die Inhibition von TAK1 durch (5z) -7-Oxozeaenol, dessen Deletion oder die Expression einer inaktiven TAK1-Mutante, um Melanomzelllinien für RIPK1-Abhängigen Zelltod über TNFα bzw. TRAIL basierende Kombinationen zu sensitivieren. Jedoch wurde ausschließlich apoptotischer Zelltod beobachtet, auch bei erhöhter RIPK3 Expression. Zusätzlich supprimierte TAK1-Inhibition die intrinsischen oder Behandlungs-induzierten Überlebenssignale, die Sekretion von Zytokinen sowie die Sekretion von löslichen Faktoren, welche mit einem Fortschreiten von Melanom assoziiert sind. Die erhöhte

Expression von TAK1 korrelierte mit reduziertem Überleben von Patienten mit primärem Melanom in kollaborativen Analysen. Zusammenfassend verdeutlichen die Ergebnisse dieser Dissertation, dass TAK1 die Sensibilität gegenüber RIPK1-abhängigem Zelltod vermindert und dass erhöhte TAK1 Expression ein verstärktes Risiko für das Fortschreiten von Melanomkarzinomen in Patienten darstellt.

Introduction

1. Introduction

Melanoma are highly chemoresistant and, despite the success of BRAF/MEK-targeting therapeutics and immune checkpoint inhibitor-based treatments, many patients still do not benefit from these novel treatment options or experience disease relapse[1,2]. An attractive addition to the armament against malignant melanoma could be treatments that induce programmed cell death through receptors of the tumor necrosis factor (TNF) family. TNF receptor activation can trigger two signaling cascades towards cell death, namely apoptosis and necroptosis[3,4].

1.1 Overview of human skin

Human skin is the largest and most protective organ in the human body. It shields inner organs from mechanical stress factors present outside the body. The skin structure comprises three distinct layers: epidermis, dermis, and the inner subcutaneous layer (Figure 1).

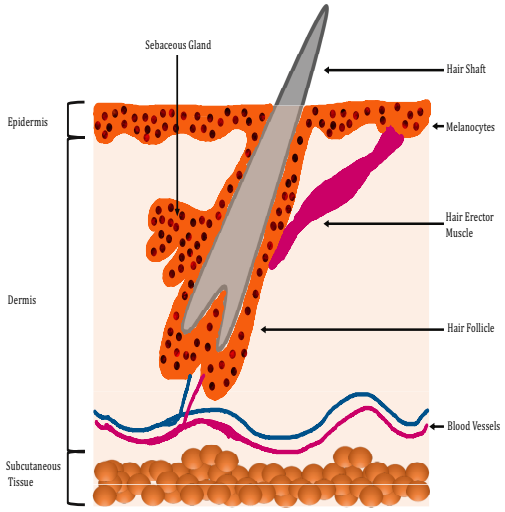


Figure 1: Structure of human skin. Skin is composed of three fundamental layers: epidermis, dermis, and subcutaneous tissue.

Pigmentation of the human skin is a major feature and is responsible for skin color diversity, even within racial groups. The appearance of skin varies from reddish to black and is determined by melanin, a pigment manufactured by dendritic cells called melanocytes. Melanocytes are located beneath the skin's epidermis.

1.2 Malignant melanoma

Malignant melanoma is the most aggressive and deadliest form of skin cancer. Melanoma results from genetic mutations in melanocytes, which can be found in the skin, eye, inner ear, and leptomeninges. In addition, metastatic melanoma has a detrimental effect[5,6] and usually occurs several months to years after the primary melanoma diagnosis. Early diagnosis of melanoma is crucial, as it can lead to a survival rate of up to 99%[7,8]. Over the last few decades, the prevalence of this disease has increased substantially, with a higher incidence rate in women[5]. Despite extensive melanoma research efforts, the overall survival rate is not promising, because tumor cells exhibit or develop resistance to available therapeutic avenues[7]. Therefore, the challenges of melanoma treatment are twofold: (1) early diagnosis and (2) overcoming resistance mechanisms.

1.3 Causes and risk factors of melanoma

Every human being is at some risk for melanoma. However, some factors can increase the risk, including prolonged exposure to the sun, moles on the skin, and genetics[9–11]. The malfunction of melanin-producing melanocytes is the main cause of melanoma. DNA damage in skin cells can be caused by a combination of factors, both environmental and genetic. DNA changes can turn on oncogenes or turn off tumor suppressor genes. Changes within multiple genes are usually required for a cell to become a cancer cell. One widely accepted finding is that exposure to ultraviolet (UV) radiation from the sun and tanning lamps and beds is the leading cause of melanoma[12–15]. It is commonly known that sun exposure might not immediately result in cancer. Apart from the effects of UV radiation, inherited genetic abnormalities are the second leading cause of familial melanomas[12,16–19].

1.4 Melanoma development and progression

Clinical and histopathological evidence suggests that melanoma develops from melanocytes of skin. Initially, melanocytes start to proliferate, leading to benign nevi (Figure 2). This represents an early phase of hyperplastic melanocyte lesions followed by aberrant growth and dysplasia[14,15,19]. The radial growth is a certain malignant stage in which tumor cells proliferate intraepidermally. Malignant cells gain the ability to grow vertically and invade the dermis and subcutaneous tissue. Tumor cells can then spread to a distant organ, eventually becoming metastasized[19,20].

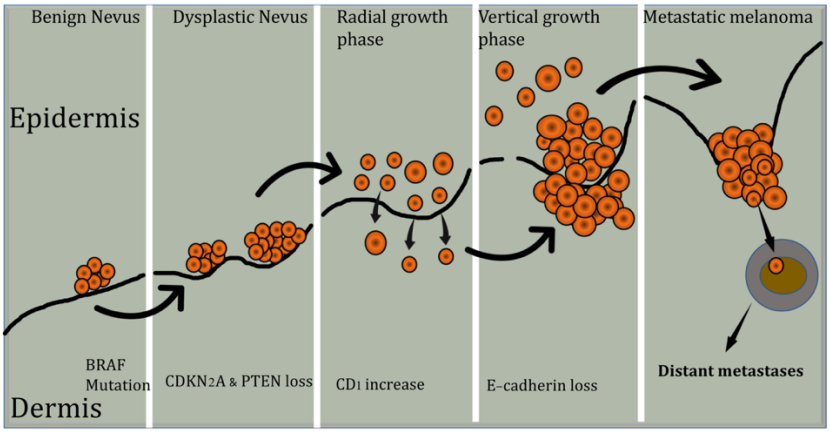


Figure 2: Melanoma tumor progression (adapted from the Clark model)

1.5 Melanoma microenvironment

The microenvironment of normal skin differs from melanoma-impacted skin. In fact, the microenvironment is an active participant in melanoma growth, proliferation, and metastasis. The melanoma microenvironment is highly heterogeneous and comprises a variety of extracellular matrix and non-carcinogenic cells, such as fibroblasts, keratinocytes, and inflammatory cells[21,22]. The communication between cancerous cells and the surrounding environment is very intricate. Melanoma cells produce various cytokines and growth factors, which are responsible for recruiting many types of stromal cells to the tumor microenvironment[23,24]. Cytokines and growth hormones can directly play a role in tumor initiation and progression. Indirect growth factors and stromal cells are responsible for melanoma initiation, progression, and subsequent metastasis. During invasive stages, melanoma cells use a specific mechanism to survive in a new and adverse environment outside their original niche and successfully establish a new residence in a new location[25–27]. During the tumor progression phase, melanomas produce numerous growth factors which allow them to survive in hostile conditions. Since the tumor microenvironment plays an important role in melanoma development, a better understanding of the tumor microenvironment may allow us to develop more effective treatments.

1.6 Overall scenario of melanoma treatment

Over recent decades, the FDA has approved several therapeutic options to treat melanoma that cannot be removed by surgery or that have metastasized. Depending on the tumor type and stage, there are several treatment options available, such as surgical resection, chemotherapy, radiotherapy, photodynamic therapy (PDT),

immunotherapy, and targeted therapy[28–30]. In 1974, Dacarbazine (DTIC), an alkylating agent, was introduced to treat melanoma as the first chemotherapeutic drug, but the overall success rates are not impressive due to the low response rate of patients to the DTIC treatment[28,31]. However, chemotherapy still remains important in the palliative treatment of refractory, progressive, and relapsed melanomas.

Efforts have continued to improve overall melanoma treatment, and the outcomes of these efforts include targeted therapy and immunotherapy[32]. In the 19th century, life scientists found that cancer and the immune system are interconnected. It is known that, in many types of cancer, complex interactions between the tumor and the immune system play a role in the cancer's metastatic spread to distant sites[33,34]. Based on the immunogenic nature of melanoma, immunotherapy is a viable option to treat melanoma, especially in its advanced stages. Since 1995, several FDA-approved immunotherapy options for melanoma have been developed (Figure 3). These include cytotoxic T-lymphocyte antigen inhibitor (Ipilimumab), programmed death-1/programmed death ligand-1 (Nivolumab, Pembrolizumab), cytokines (Aldesleukin, Interferon alfa-2b, Peginterferon alfa-2b), and the combination of any of these drugs with other available therapeutic options[32,35–39]. Targeted therapy significantly improves treatment for melanoma patients[40]. For instance, 90% of patients with BRAF mutations who received vemurafenib showed tumor regression and improved rates of overall survival[8]. Mutations of oncogenes are the main causes of melanoma's aggressiveness and poor prognosis. Approximately 50 % of all malignant melanomas harbor somatic mutations in the BRAF gene (mainly V600E and V600K)[10], a key serine-threonine kinase from the mitogen-activated protein kinase (MAPK) signaling pathway, which is associated with constitutive activation of the MAPK signaling pathway and results in increased growth and proliferation of cancer cells. Vemurafenib (a selective oral BRAF-mutant inhibitor, FDA approved in 2011) and dabrafenib (a selective oral BRAF-mutant inhibitor, FDA approved in 2013) are BRAF inhibitors specific to melanomas harboring the BRAF V600E and V600E/K mutations, respectively[41–43]. There are several ongoing clinical trials with vemurafenib and dabrafenib as monotherapy and in combination with radiotherapy, immunotherapies, and other targeted therapies. Recently, another BRAF-inhibitor, encorafenib is under clinical testing both in monotherapy (NCT01436656) and in combination with targeted therapy (NCT02159066/NCT01909453) and immunotherapy (NCT02902042/NCT03235245/NCT02631447)31.

Besides BRAF inhibitors, other targeted therapies also showed promising outcomes for melanoma patients, either in monotherapy or in combination therapy with therapeutic options such as MEK inhibitors (Trametinib, Cobimetinib), CKIT inhibitors (Imatinib, Sunitinib, Dasatinib), VEGF inhibitors (Bevacizumab), or cyclin-dependent kinase (CDK) inhibitors (Ribociclib, Abemaciclib)[43–51]. Intensive research is necessary to improve treatment of melanoma, as a comprehensive understanding of melanoma pathogenesis is crucial for the development of improved therapy options. Because tumor heterogeneity is a major cause of melanoma resistance, personalized treatment can achieve better clinical outcomes for melanoma patients.

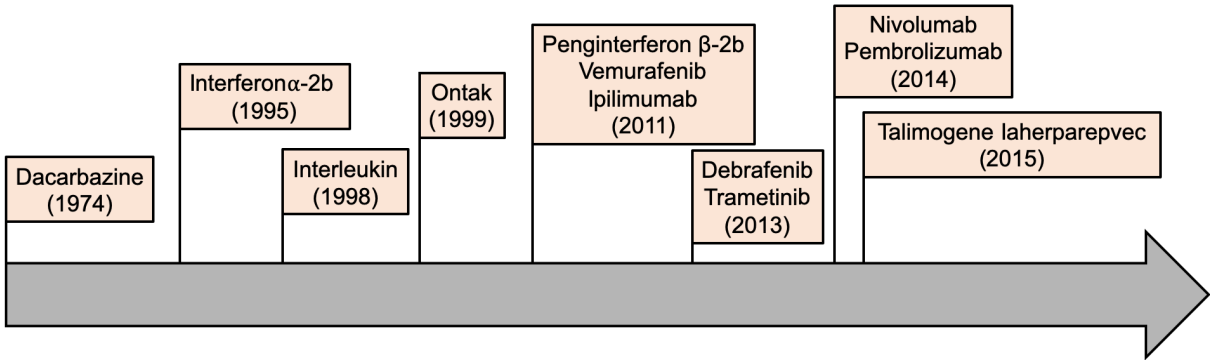


Figure 3: Current FDA-approved treatments for melanoma. Dacarbazine was the first drug approved, in 1974, followed by interferon α-2b, interleukin 2, and ontak in the 1990s. Since 2011, 10 therapies were approved, including selective inhibitors, antibodies, and targeted therapies. (adapted from Domingues, B. et al., 2018)[32]

1.7 Overcoming melanoma resistance

Like other cancers, melanoma develops resistance to traditional cancer therapies[32]. A wide range of anticancer therapies is incompetent in killing melanoma cells, which implies that the resistance mechanisms in melanoma are complex[52–54]. Melanomas display intrinsic resistance or acquire resistance to targeted therapy and immunotherapy. It is therefore necessary to create new treatment strategies. The heterogeneous nature of melanoma is also a large obstacle in attaining the optimal response from available treatment options[13]. In fact, most patients with metastatic melanoma do not obtain long-lasting clinical benefits from ipilimumab and responses to BRAF inhibitors. The study of heterogeneity suggests developing new biomarkers capable of recognizing patients who respond to available therapeutics. However, identifying new biomarkers does not guarantee long-term responses, because melanoma may develop mechanisms to blunt normal immune system responses and accumulate genetic mutations in key regulatory genes, with activation of alternative

proliferation pathways. Thus, it is necessary to identify novel therapeutic targets and continue efforts to renew lines of treatment.

1.8 Cell death

Carl Vogt first described programmed cell death in 1842, but there was no experimental evidence of regulated cell death until the mid-20th century. There are two kinds of cell death: active cell death and passive cell death. Programmed cell death is highly regulated and supports an organism's life, whereas passive cell death is uncontrolled and results in adverse effects for organisms. Cell death is an indispensable event in many biological processes, including tissue sculpting during embryogenesis, development of the immune system, and destruction of damaged cells[55]. Various abnormalities in cell death pathways have been associated with diseases, including ischemic, neurodegenerative, cancer and infectious disease[56,57]. Understanding how these pathways are triggered, signaled, and regulated may lead to new therapies for the treatment of human diseases.

1.9 Apoptosis

Apoptosis, meaning "falling off" in Greek, plays a common biological role both in embryonic development and in adult life[58]. A distinct physiological feature of apoptosis is the partitioning of cell membrane-enclosed 'apoptotic bodies', which are comprised of intracellular components[59]. A family of caspases (initiator caspases: caspase-2, -8, and -9; executioner caspases: caspase-3, -6, and -7) are essential to coordinate this programmed cell death[60]. Upon apoptotic stimuli, initiator caspases activate the pathway and subsequently activate executioner caspases, leading to a rapid feedback loop to facilitate apoptosis. Apoptosis is executed through two major and distinct signaling pathways: (1) the intrinsic pathway, gated through proteins encoded by the B-cell lymphoma 2 (BCL-2) gene family[61], and (2) the extrinsic pathway, activated by death receptor-mediated signals (Figure 4)[62]. These two pathways frequently reinforce one another.

The intrinsic, alternatively called mitochondrial, pathway is the most prevalent mechanism and is mediated through mitochondrial outer membrane permeabilization (MOMP). This permeabilization leads to the release of cytochrome c and other sequestered proteins from the intermembrane space (IMS) into the cytosol[62]. This pathway is activated by various cellular stress such as cytoskeletal disruption, DNA

damage, accumulation of unfolded proteins, hypoxia, etc. The intrinsic pathway is tightly controlled by BCL-2 proteins such as (a) single BH motif (BH3-only) proteins (e.g., death agonist: BAD, BIK, BMF, BID, PUMA, and NOXA), and (b) multi-BH motif proteins, which possess three or four BH regions and act respectively as agonists (e.g., BAX, BAK, BOK) or antagonists (e.g., BCL-2, BCL-XL, BCL-w, A1, MCL-1) of apoptotic stimulation[63]. After cytochrome c release into the cytoplasm, where it forms a complex or apoptosome with caspase-9 and the adaptor APAF-1. This complex activates downstream caspases (caspase-3 and -7), and subsequently results in cell death[64].

The extrinsic pathway, also known as the death receptor-mediated pathway, is mostly driven by plasma membrane receptors, so called death receptors[65]. Death ligands (e.g., tumor necrosis factor [TNF], TNF-related apoptosis-inducing ligand [TRAIL], CD95-ligand [CD95-L; also known as Fas-L and Apo-1L]) bind to their respective DRs (e.g., TNFR1, FAS [CD95], or DR4/5) and form a receptor-bound death-inducing signaling complex (DISC) by recruiting pro-caspase-8 to the adaptor proteins Fas-associated protein death domain (FADD) or TNF receptor-associated death domain (TRADD)[66–69]. The DISC allows caspase-8 to form homodimers and activates caspase-3 and caspase-7. Consequently, active effector caspases proteolytically cleave cellular proteins, and amplify the cell death cycle[70].

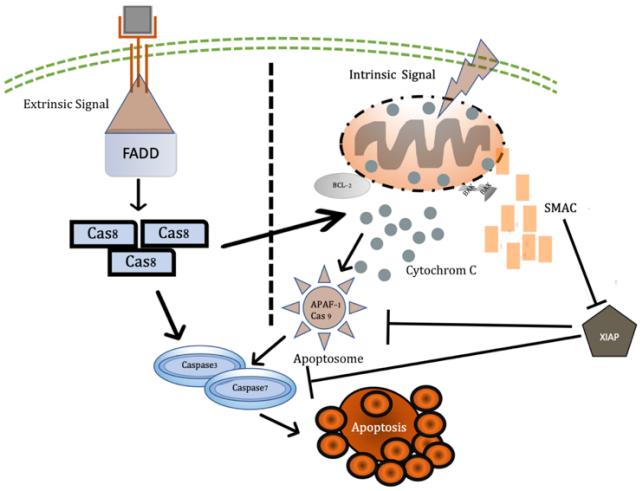


Figure 4: Molecular mechanisms of the intrinsic and extrinsic signaling pathways in apoptosis. The intrinsic pathway is induced via diverse intracellular stress signals that lead to mitochondrial outer membrane permeabilization, release of cytochrome c, formation of the apoptosome, and effector caspase activation. Death ligands induce the extrinsic pathway via caspase-8 activation, which can either directly activate caspase-3 and -7 or induce MOMP by BID cleavage.

1.10 Programmed necrosis or necroptosis

Like apoptosis, regulated necrosis or necroptosis both plays a pivotal role in normal development and has been implicated in the pathogenesis of a variety of human diseases. Necrosis was long considered accidental cell death, but it has recently been classified as programed cell death[71]. Necroptosis mostly occurs in response to viral infection, because cells execute cell death in a caspase-independent manner in the presence of a viral caspase inhibitor and eventually restrict viral replication[72]. Necroptosis also has some beneficial effects because necroptosis can induce inflammatory response in a cellular environment. Apoptosis and necroptosis are morphologically and mechanistically distinct.

Various stimuli (TNF receptor superfamily, T-cell receptors, interferon receptors, Toll-like receptors, cellular metabolic and genotoxic stresses, or various anticancer compounds) can engage necroptotic cell death signaling under the condition of caspase inhibition[73,74]. Three molecular components of necroptosis signaling have been identified; these include two receptors interacting protein kinase (RIPK1 and RIPK2) and mixed lineage kinase domain-like (MLKL)[75]. During initiation of necroptosis, an intracellular signal is mechanistically transmitted through the formation complex IIC, alternatively called the “necrosome”. Two prime components of the necrosome are RIPK1, and RIPK3[76]. These two kinases interact through RIP homology interaction motif (RHIM)[77]. The necrosome represents a platform for auto- and transphosphorylation of RIPK1 and RIPK3. The autophosphorylation of RIPK1 contributes to recruiting RIPK3[78–80]. RIPK1 and RIPK3 form fibrillar structures akin to β-amyloids, which augment necrosome signaling and necroptosis activation[81]. The transphosphorylation of RIPK1 and RIPK3 promotes the recruitment of another critical necroptosis-signaling protein: MLKL[81]. However, as a pseudokinase, MLKL does not phosphorylate any additional protein targets. Following RIPK3-mediated phosphorylation, MLKL oligomerizes and translocates to the plasma membrane, leading to its rupture (Figure 5)[82].

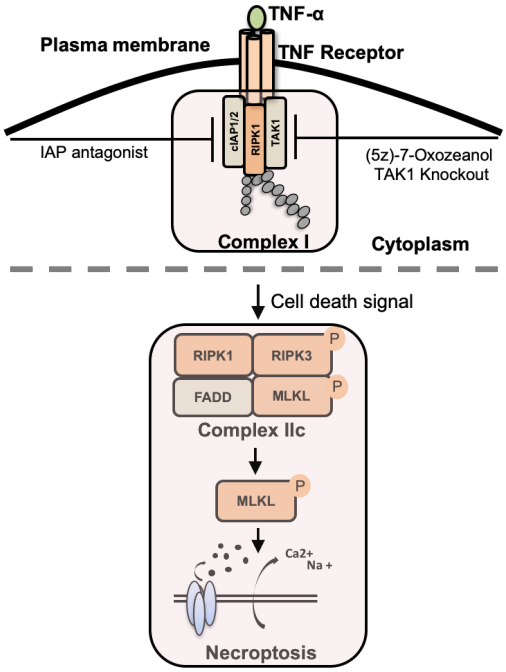


Figure 5: Necroptosis signaling pathway. TNF binding to TNF receptor 1 (TNFR1) stimulates the recruitment of TRADD, which recruits RIPK1. RIPK1 is ubiquitin-modified by the IAP1/2/TRAF2 complex; when RIPK1 ubiquitination is inhibited by SMAC mimetics and caspase 8 activity is inhibited, RIPK1 phosphorylates RIPK3, which then phosphorylates MLKL, resulting in MLKL translocation to the plasma membrane. MLKL translocation results in changes to membrane permeability, DAMP release, and induction of necroptosis.

1.11 The RIP kinase family and functions

The Receptor Interacting Protein (RIP) kinase family consists of seven serine/threonine protein kinases. These kinases contain an identical kinase domain (KD) in the N-terminus. (Figure 6)[83]. In RIPK1, the C-terminus contains a death domain and bridging intermediate domain (ID) with a RIP homotypic interaction motif (RHIM). Unlike RIPK1, RIPK2 contains an ID without RHIM and a C-terminal caspase activation and recruitment domain (CARD). In contrast, RIPK3 does not have an ID, but it has a unique C-terminal sequence that contains a RHIM. RIPK4 and RIPK5 have ankyrin domains, KD, and ID in C-terminals. Although they share identical structures, they have different functional roles. RIPK4 primarily plays a key role in keratinocyte differentiation and RIPK5 drives cell apoptosis[83]. Finally, RIPK6 and RIPK7 have major structural differences to other kinases in the RIPK kinase family. Although both contain a homologous KD, they also contain a number of additional and diverse domain structures, such as leucine-rich repeat regions[84]. The functional role of RIPK6 and

RIPK7 is not elucidated clearly. Thus far, the molecular and physiological role of RIPK1-3 has been intensively studied in inflammation and cell death[84].

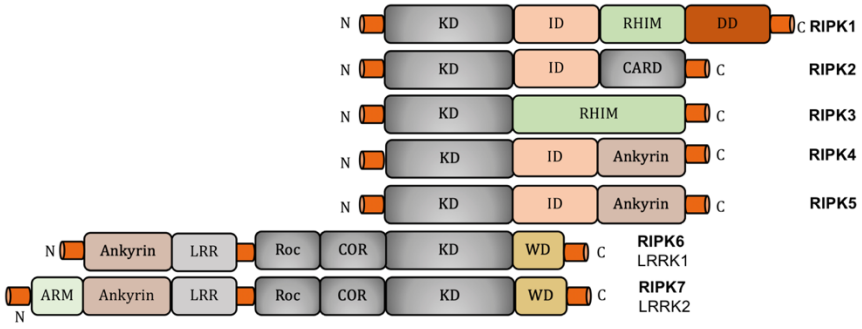


Figure 6: Domain structure of the RIP kinase family. (adapted from Zhang, Lin and Han 2010)[83]

1.12 Other forms of cell death

Pyroptosis (from the Greek word *pyro*, meaning 'fire') is inflammatory cell death and can be both beneficial (i.e., act as a savior from pathogenic organisms) and detrimental for organisms [85]. Interestingly, pyroptosis exhibits some characteristics reminiscent of apoptosis (caspase dependence, chromatin condensation, and DNA fragmentation) and necroptosis (membrane pore formation, cellular swelling, and inflammatory response). Pyroptosis is activated by inflammatory caspases (human caspase-1, -4, and -5; rodent caspase-1, -11), and It was first reported that caspase-1 could convert pro-IL-1b and pro-IL-18 into their active forms[86,87].

Ferroptosis is an iron- and ROS-dependent form of regulated cell death. In 2012, Prof. Brent R Stockwell first discovered ferroptosis at Columbia University[88]. Ferroptosis is morphologically and genetically distinct from other types of programed cell death, including apoptosis, necroptosis, and autophagic cell death[88]. Ferroptosis is a mechanistically complex process and is associated with the accumulation of lipid peroxidation and reactive oxygen species, caused by iron metabolism. Glutathione peroxidase 4, heat shock protein beta-1, and nuclear factor erythroid 2-related factor 2 act as negative regulators whereas NADPH oxidase and p53 act as positive regulators of ferroptosis[89,90].

Autophagic cell death, a catabolic process, and was reported by Clark's phenotypic description of 'Type II autophagic cell death' in 1990[91,92]. At the start of autophagy, autophagosomes are formed by engulfing cytoplasmic content, eventually fusing with

a lysosome. A set of autophagy-related genes (*Atgs*) are involved in executing autophagy cell death[93]

1.13 Necroptosis in cancer

Drug resistance is a major hindrance to the effectiveness of proapoptotic therapy (e.g., using cisplatin, carboplatin, paclitaxel, 5-fluorouracil, and gemcitabine as chemotherapy)[94]. Cancer cells develop resistance mechanisms due to impairment of the apoptosis machinery (mutation of anti-cancer genes such as p53, Apaf-1, etc.; increased expression of antiapoptotic proteins such as FLIP, Bcl-2, Bcl-xL, or Mcl-1, etc.) or a high degree of molecular heterogeneity in tumor cells[94,95]. Since the necroptotic pathway uses components that are distinct from the apoptotic pathway, it was reasoned that cancer cells with apoptotic resistance might be sensitive to necroptosis stressors[96]. Indeed, there is substantial evidence demonstrating that many currently available anticancer agents and several natural products can activate necroptotic signaling pathways and thereby elicit cell death in malignant cells[96]. A previous study revealed that Shikonin (a natural compound) stimulates necroptotic cell death in Bcl-2 or Bcl-xL-overexpressing cell lines that are resistant to proapoptotic therapies[94]. Necroptotic proteins are lost during cancer development and progression due to downregulation and functional mutations of RIPK1, RIPK3, and MLKL in several cancer cell lines, including colorectal cancer, leukemia, lung cancer, and breast cancer, among others[72]. Interestingly, among these cancer cells, only a few colorectal cancer cell lines undergo necroptotic cell death by classic necroptosis inducers (e.g., TNF+cycloheximide+zVAD, TNF+IAP inhibitors+zVAD, and TNF+5Z-7-oxozeaenol+zVAD)[95]. Several studies indicate that cancer cells (such as colorectal cancer cells HCT-11 and ovarian cancer cells OVCAR4) with low expression of RIPK1/3 and MLKL are unresponsive to necroptosis inducers[95]. Some chemotherapeutic agents are undergoing clinical trials as necroptotic death inducers, including obatoclax alone, obatoclax plus dexamethasone, 3-bromopyruvate plus chloroquine, and shikonin and its analogs[78,95].

1.14 Transforming growth factor-β activated kinase 1 (TAK1)

In 1996, TAK1, a mitogen-activated protein kinase kinase kinase (encoded by *MAP3K7* on chromosome 6 in humans)[97,98], was first discovered by a group of scientists from

Japan at Nagoya University for its role in mediating TGFβ and bone morphogenetic protein (BMP) signaling[97,98]. The serine/threonine kinase protein TAK1 is highly conserved and is found in C. elegans (MOM4), Drosophila (dTAK1), human (hTAK1), and mouse (mTAK1)[99,100]. TAK1 can be activated by various signaling molecules, including cytokines, death ligands that interact with Toll-like receptors, and exogenous stressors[101].

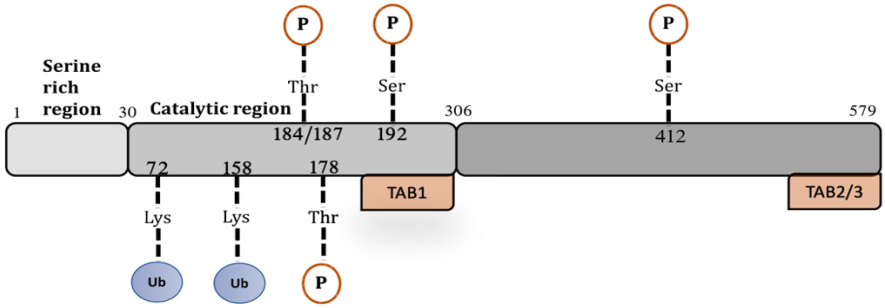


Figure 7: Domain structure of TAK1. The N-terminal region of TAK1 contains a TAB1-binding domain and kinase domain. The c-terminus contains TAB2/3 binding domain. (adapted from Roh YS, Song J, and Ekihiro 2014)[102]

1.15 Molecular mechanisms in TAK1 signaling

Though TAK1 is activated by various stimuli, the TNF-α signaling pathway has been extensively studied thus far. Once TNF-α binds to TNF receptors, complex-1-associated proteins, including adaptor molecules TRADD, TNF receptor-associated factor 2 and 5 (TRAF2 and TRAF5), cellular inhibitor of apoptosis 1 and 2 (cIAP1/2), and RIPK1 are recruited to the receptor complex of TNF receptor 1 (TNFR1)[101]. The complex platform allows RIPK1 to be ubiquitinated by E3 ligases[103]. There are three TAK1-associated binding proteins (TAB1, -2, and -3) which help activate TAK1 through the formation of complexes. TAB1 and TAB2 are structurally unidentical, whereas TAB2 and TAB3 share 48% of their amino acid sequence[104–106]. Previous studies have revealed that TAB1, TAB2, and TAB3 do not have same functional role in signaling pathways. For instance, TAB1 is indispensable for TGF-β1-induced TAK1 activation in glomerular mesangial cells, whereas TNF-α and IL-1 signaling pathway exclusively require TAB2 and TAB3, respectively, rather than TAB1[103]. In the TNF-α signaling pathway, TAK1 binds to TAK-binding protein 2 (TAB2) and is recruited to the RIPK1 polyubiquitin chain. The binding of TAB2 and the RIPK1 polyubiquitin chain leads to TAK1 phosphorylation and activation of the I kappa B kinase (IKK) complex composed of two kinases (IKKα, IKKβ), and regulatory subunit NF-κB essential modulator (NEMO; alternatively called IKKγ). This leads to phosphorylation and degradation of

IκB, eventually resulting in activation of NF-κB[107]. TAK1 also regulates Mitogen-activated protein kinases (MAPKs; such as ERK, p38, and JNK) in downstream signaling pathways. The activation of nuclear factor-κB (NF-κB) and MAPKs induces downstream expression of inflammatory cytokines and anti-apoptotic proteins[103,107].

1.16 The role of TAK1 in cancer

It has become increasingly clear that TAK1 is a vital player in cell signaling due to its role in inflammation, survival, and carcinogenesis[103]. The role of TAK1 in cancer remains a paradox because TAK1 can act as both an oncogene and a tumor suppressor gene. Despite the paradoxical role of TAK1, a substantial amount of evidence has demonstrated that TAK1 is predominantly relevant in the pathogenesis of human tumors, including prostate, thyroid, lymphoma, pancreatic, and breast cancer tumors[108]. Studies suggest that the activation of TAK1 increases production of tumor-promoting proinflammatory cytokines, chronic inflammation, and aggressive lung cancer growth[109]. On the other hand, deficiency of TAK1 can lead to an accumulation of reactive oxygen species (ROS) and reduce skin tumors size[103]. LYTAK1, an orally active TAK1 inhibitor, significantly inhibits NF-κB activity and sensitizes cancer cells to gemcitabine, oxaliplatin, and SN38[110]. Another study has demonstrated that (5z)-7-Oxozeaenol (OXO) is a selective TAK1 inhibitor that significantly inhibits proliferation and promotes apoptosis of a variety of tumor cells by downregulating the TAK1 protein[111]. This evidence indicates that TAK1 is now an attractive molecular target for the treatment of multiple types of cancer.

1.17 The role of epigenetics in cancer

In mammals, epigenetic mechanisms are indispensable for normal development and maintenance of gene expression patterns. Dysregulation of epigenetic processes can lead to altered gene function and it results malignant cellular transformation. DNA methylation, histone modification, and micro RNA modulation are the key epigenetic dysregulation events to the genesis of cancer (Figure 8)[112]. Extensive studies report that epigenetic modifications are manifested by epigenetic writers and removed by erasers in a dynamic and highly regulated manner, but not by an alteration in the DNA sequence[113]. Global hypomethylation results in genomic instability. In addition, specific promoter hypermethylation of tumor-suppressor genes mediates gene silencing[114,115]. DNA methylation, is the most common epigenetic event in human cancer, it primarily

occurs by the covalent modification of cytosine residues in CpG dinucleotides[115]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), such as DNMT1, DNMT3a, and DNMT3b and each of them have different functional roles[115]. For instances, DNMT1 participates in maintaining methylation status during cell replication, while DNMT3a and DNMT3b induce de novo methylation[116,117].

In melanoma, many tumor suppressor genes that are involved in cell cycle regulation, DNA repair, apoptosis, etc. are hypermethylated. For instance, apart from common methylation, long interspersed nucleotide element-1 (LINE-1) methylation has been discovered in Brazilian melanoma patients and results in cancer risk[118]. The loss of 5-hydroxymethylcytosine (5-hmC) is used as a biomarker to differentiate physiological melanocytes and benign melanocyte proliferation. Previous studies have revealed that DNA methylation is one of the major causes of the silenced RIPK3 gene in various cancers. Unlike genetic dysregulation, pharmacological inhibitors are able to intervene and correct epigenetic changes in cancer[119–121]. Therefore, the development of pharmacological inhibitors as epigenetic drugs has become a promising option to treat cancer. Over recent decades, a list of small molecule inhibitors has been developed through targeting of DNMTs and histone deacetylases (HDAC)[112], and the epigenetic alterations have become increasingly recognized as valuable targets for the development of cancer therapies[112]. The FDA approved two DNMT inhibitors (5-azacytidine and 5-aza-2'-deoxycytidine) and HDAC inhibitors (vorinostat, romidepsin, belinostat, and panobinostat) to treat liquid cancer[113]. However, more than a dozen clinical trials of epigenetic drugs are underway to treat hematologic malignancies. Recently, the combination of epigenetic drugs and chemotherapy has become a powerful strategy to treat cancer, including solid and liquid cancers[112,113]. Several ongoing Phase-I/II clinical trials are dedicated to investigating the effect of combining epigenetic agents with immunotherapy[112,113].

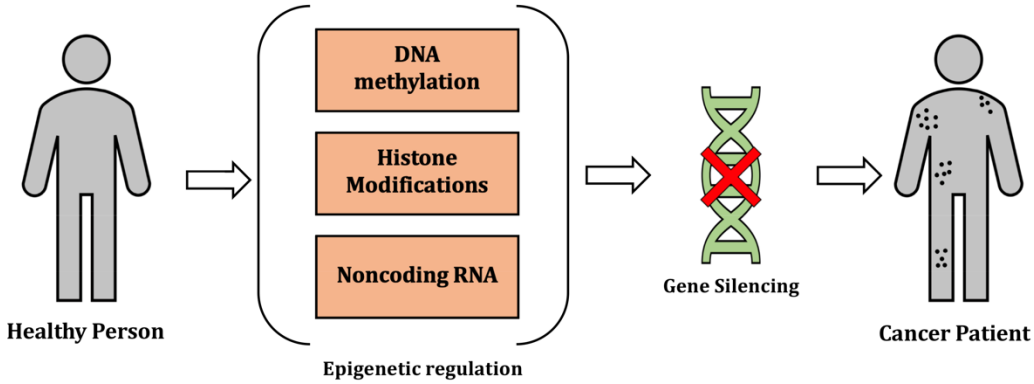


Figure 8: The role of epigenetics in cancer. Heritable gene silencing involves DNA methylation and histone covalent modifications, as well as noncoding RNA.

1.18 NF-κB signaling pathway

The nuclear factor-κB (NF-κB) family are well studied transcription factors, and they control more than 500 different gene products that are linked to inflammation, cellular transformation, tumor cell survival, proliferation, invasion, angiogenesis, and metastasis[122]. The NF-κB family consists of the submits p105/p50 (NF-κB1), p100/52 (NF-κB2), c-Rel, RelA (p65), and RelB[123,124]. The canonical and non-canonical NF-κB pathways are involved in the signal transduction. In the canonical signaling pathway, I kappa B kinase (IKK) phosphorylates IκBα at two N-terminal serines, resulting in its ubiquitination and proteasomal degradation[125]. These events lead to the nuclear translocation of NF-κB complexes, mostly the dimers of p50/RelA and p50/c-Rel. In the noncanonical signaling pathway, the inducible processing of p100 activates the RelB/p52 NF-κB complex. The processing of p100 serves to both generate p52 and induce the nuclear translocation of the RelB/p52 heterodimer.

The master regulator NF-κB controls a set of genes responsible for cell proliferation and tumorigenesis (e.g., gastrointestinal cancers, colorectal cancer, breast cancer, etc.). Constitutively activated NF-κB activates anti-apoptotic genes and subsequently protects cells from death. Therefore, cancer cells develop metastatic characters. NF-κB also inhibits regulated cell death by inducing the expression of anti-apoptotic proteins such as cIAPs, caspase-8/FADD (FAS-associated death domain)-like IL-1beta-converting enzyme (FLICE) inhibitory protein, and members of the BCL2 family[126]. Past studies have revealed that NF-κB produces chemokines, such as interleukin-8 (IL-8), that promote angiogenesis. Many cancers have aberrant NF-κB

activation; moreover, limiting NF-κB activity results in the suppression of cell proliferation[127]. Therefore, targeting of NF-κB signaling has potential therapeutic applications in cancer. Over recent decades, many natural products (Wedelolactone, Parthenolide, Honokiol, etc.) have been developed as anticancer agents inhibiting NF-κB activity[122].

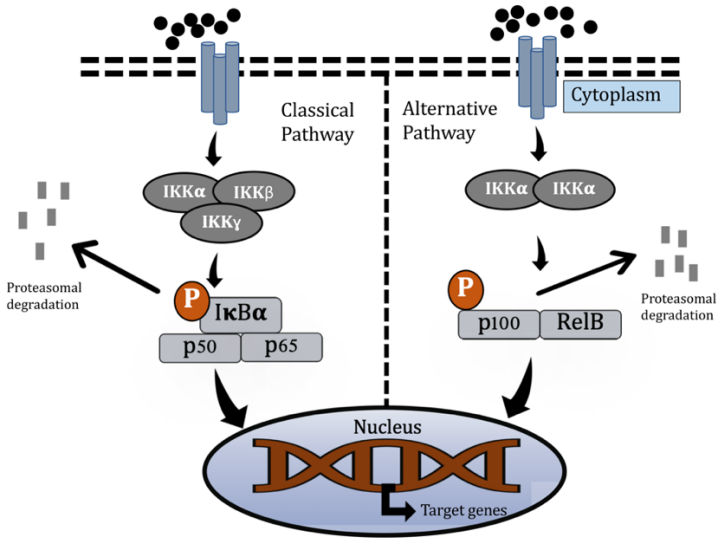


Figure 9: NF-κB signaling pathway. Canonical and alternative NF-κB pathways are represented on the left and right, of IκB-α by the IKK complex and its subsequent degradation by the proteasome. Consequently, the RelA/p50 complex translocates to the nucleus, where it activates the transcription of target genes. The non-canonical pathway is based on the activation of IKKα by the NF-κB-inducing kinase (NIK) after stimulation. In turn, the NIK-IKKα complex phosphorylates the p100 subunit. As a consequence, p100 is processed in a proteasome-dependent manner, generating the subunit p52. This event results in the activation of p52-RelB, which induces the transcription of distinct target genes.

Materials and Methods

2. Materials and methods

2.1 Materials

Table 1: Reagents and kits

Reagents and kits	Manufacturer
Annexin V binding buffer (10X)	BD Biosciences, San Diego, USA
Bolt MES SDS Running Buffer (20X)	Thermo Fisher Scientific Inc., Waltham, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, Munich, Germany
Bromophenol blue	Serva Electrophoresis GmbH, Heidelberg, Germany
cOmplete Protease Inhibitor Cocktail Tablets	Roche Diagnostics AG, Basel, Switzerland
Crystal violet powder	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DH5α-*E. coli*	Thermo Fisher Scientific Inc., Waltham, USA
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Eosin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Fetal calf serum	PAN-Biotech GmbH, Aidenbach, Germany
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hoechst 33342	Thermo Fisher Scientific, Ulm, Germany
iBlot 2 Transfer Stacks	Thermo Fisher Scientific Inc., Waltham, USA
KCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

KH_2PO_4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Na_2HPO_4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NuPAGE Antioxidant	Thermo Fisher Scientific Inc., Waltham, USA
NuPAGE MES SDS Running Buffer (20x)	Thermo Fisher Scientific Inc., Waltham, USA
NuPAGE Novex 4-12 % Bis-Tris Protein Gels	Thermo Fisher Scientific Inc., Waltham, USA
Opti-MEM®I Reduced Serum Medium	Thermo Fisher Scientific Inc., Waltham, USA
PageRuler prestained protein ladder	Thermo Fisher Scientific Inc., Waltham, USA
Pierce ECL Western Blotting substrate	Thermo Fisher Scientific Inc., Waltham, USA
Protein G agarose beads	Thermo Fisher Scientific Inc., Waltham, USA
Restore Western Blot stripping buffer	Thermo Fisher Scientific Inc., Waltham, USA
RNase-free water	QIAGEN, Hilden, Germany
RNeasy Plus Mini Kit	QIAGEN, Hilden, Germany
RPMI 1640 medium	Life Technologies, Gibco, Karlsruhe, Germany
Sodium pyruvate	Thermo Fisher Scientific Inc., Waltham, USA
SOC Medium	Sigma-Aldrich, Munich, Germany
Tris-(hydroxymethyl)-aminomethane	Carl Roth GmbH & Co. KG, Karlsruhe,
Tris-HCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Triton X-100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween-20	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Table 2: Inhibitors

Inhibitor	Manufacturer
(5z)-7-Oxozeaenol	Sigma-Aldrich, St. Louis, USA
5-Aza-2′-deoxycytidine	Sigma-Aldrich (St. Louis, USA
Compund A (IAP antagonist)	Tetralogics Corp. Philadelphia, USA
Necrostatin-1	Seleckchem, Houston, USA
NG-25	MedChem Express New Jersey, USA
zVAd-fmk	Apexbio, MA, USA

Table 3: Antibodies

Primary Antibodies	Source	Dilution	Manufacturer	#Catalog Number
Actin	mouse monoclonal	1:10000	Cell signaling	CST-3700
Caspase-3	rabbit polyclonal	1:1000	Cell signaling	CST-9662
Caspase-8	mouse monoclonal	1:1000	Cell signaling	CST-9746
cFLIP	mouse monoclonal	1:1000	Enzo Life Science	ALX-804-961-0100
cIAP1	Rat polyclonal	1:1000	provided by J. Silke	NA
cIAP2	Rat polyclonal	1:1000	provided by J. Silke	NA
DNMT1	rabbit monoclonal	1:1000	Cell signaling	CST-5032
ERK1/2	mouse monoclonal	1:1000	Cell signaling	CST-9107
FADD	mouse monoclonal	1:1000	BD Biosciences	F36620
IκBα	mouse monoclonal	1:1000	Cell signaling	CST-4814
JNK	rabbit polyclonal	1:1000	Cell signaling	CST-9252
MLKL	rabbit polyclonal	1:1000	Sigma-aldrich	M6697
NF-κB (p65)	rabbit monoclonal	1:1000	Cell signaling	CST-8242

p38	rabbit polyclonal	1:1000	Cell signaling	CST-9212
PARP	mouse monoclonal	1:1000	BD Pharmingen™	BD556494
Phospho-ERK1/2	mouse monoclonal	1:1000	Cell signaling	sc-7383
Phospho-IκBα	rabbit monoclonal	1:1000	Cell signaling	CST-2859
Phospho-JNK	rabbit polyclonal	1:1000	Cell signaling	CST-9251
Phospho-MLKL	rabbit monoclonal	1:1000	abcam	ab187091
Phospho-NF-κB (p65)	rabbit monoclonal	1:1000	Cell signaling	CST-3033
Phospho-p38	mouse monoclonal	1:1000	Cell signaling	CST-9216
Phospho-RIPK1	rabbit monoclonal	1:1000	Cell signaling	CST-65746
Phospho-RIPK3	rabbit monoclonal	1:1000	abcam	ab209384
phospho-TAK1	rabbit monoclonal	1:1000	Cell signaling	CST-4580
RIPK1	mouse monoclonal	1:1000	Transduction Laboratories,	R41220
RIPK3	rabbit polyclonal	1:1000	IMGENEX,	IMG-5846A
TAK1	rabbit monoclonal	1:1000	Cell signaling	CST-4505
TNF-R1	Mouse monoclonal	1:200	Hycult Biotech,	HM2007
TNF-R2	Mouse monoclonal	1:200	Hycult Biotech	HM2005
TRAIL-R1	Mouse	1:250	Biomol	AG-20B-0022-C100
TRAIL-R2	Mouse	1:250	Biomol	AG-20B-0023-C100
XIAP	mouse monoclonal	1:1000	BD Biosciences	H62120

Secondary Antibodies	**Source**	**Dilution**	**Manufacturer**	**#Catalog Number**
Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG+IgM	goat	1:10000	Jackson Immuno Research	115-001-003 115-035-003

HRP-conjugated goat anti-rabbit IgG+IgM	goat	1:10000	Jackson Immuno Research	ABIN102123
HRP-conjugated goat anti-mouse IgG+IgM	goat	1:10000	Antibodies-Online	

Table 4: Analysis Software

Analysis software	Source
BD FACSDIVA Software	BD Biosciences
FCS Express V3	De Novo Software
Flowing software 2.5.1	Turku Centre for Biotechnology
FlowJo 7.6.1	Tree Star Inc., Ashland, Oregon, USA
Image J	National Institute of Health (NIH)
MACSQuantify	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
Wallac 1420 WorkOut Data Analysis software	PerkinElmer

Table 5: Devices

Instruments	Manufacturer
BD FACSAriaTM III	BD Biosciences
ECL imager	FUSION SOLO S, Vilber Lourmat, Eberhardzell, Germany
ECL imager	Amersham Imager 600, GE Healthcare Europe GmbH, Freiburg, Germany
Electrophoresis power supply CM30505,	EPS 601, Amersham Pharmacia Biotech; GE Healthcare Europe GmbH, Freiburg, Germany
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific, Ulm, Germany
VICTOR3 1420 Multilable Reader	PerkinElmer

MACSQuant Analyser 10	Militneyi Biotec, Bergisch Gladbach, Germany
iBlot 2 Dry Blotting System	Thermo Fisher Scientific Inc., Waltham, USA
Incubator	Varolab GmbH, Giesen, Germany
Inverted digital microscope	EVOS FL Imaging System, Thermo Fisher Scientific, Ulm
Microplate reader microplate	SPARK Tecan, Meannedorf, Switzerland Infinite
Mini Gel Tank	Thermo Fisher Scientific Inc., Waltham, USA
Rotary microtome	RM2255 Mikrotom, Leica Biosystems Nussloch GmbH, Nussloch, Germany
Shaking platform	Sarstedt TPM-2, Nuembrecht, Germany
Nitrogen tank	K SERIES cryostorage system, tec-lab GmbH, Taunusstein, Germnay
Heat block	Eppendorf Thermomixer compact, Merck KGaA, Darmstadt, Germany
Centrifuge	Eppendorf centrifuge 5415R, Hettich lab technologies, Tuttlingen Germany
Luminex FLEXMAP 3D® platform	Luminex, Austin, USA

Table 6: Plasmids

Vectors	**Source**
pSpCas9(BB)-2A-GFP-PX458	Addgene, MA, USA
CRISPR vector for TAK1	Santa Cruz Biotechnology, Texas, USA
pCMV-TAK1-WT	Kindly provided by Prof. Jun Ninomiya-Tsuji, NC State University, Raleigh, USA
pCMV-TAK1-K63W	Kindly provided by Prof. Jun Ninomiya-Tsuji, NC State University, Raleigh, USA
Empty vector control	Kindly provided by Prof. Jun Ninomiya-Tsuji, NC State University, Raleigh, USA

Table 7: Stimulatory Cytokines

Stimulatory cytokines	Source
Human recombinant TNF-α	R&D Systems, Minneapolis, USA
Fc-scTRAIL	Kindly provided by Prof. Roland Kontermann, University of Stuttgart, Germany

Table 8: Buffer Solution

Buffer	Preparation
Phosphate buffered saline (1X PBS, pH7.4)	2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4
Lysis buffer	30 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10% (v/v) Glycerol, 1% (v/v) Triton X-100, 2 tablets Complete protease Inhibitor cocktail/100 ml
5X Laemmli	312.5 nM Tris-HCl pH 6.8, 25% (v/v) glycerine, 10% (w/v) SDS + 500 mM DTT, 0.05% (w/v) Bromophenol blue
Crystal violet dye	0.5 % (w/v) crystal violet 20 % (v/v) methanol
PBA	1XPBC, 0.05% (W/V) BSA, 0.02% (W/B) NaN_3 in ddH_2O
1 × Annexin-V binding buffer	10 mM Hepes, pH 7.4, 140 mM NaCl, 2,5 mM CaCl2

Table 9: Anti-sense siRNA sequences

siRNA	Targeted gene	Sequence	Source
#Hs_CASP8_11	Caspase8	AAGAGTCTGTGCCCAAATCAA	Qiagen, Germany
#Hs_RIPK1_5	RIPK1	TACCACTAGTCTGACGGATAA	Qiagen, Germany
#Control siRNA	None	N/A	Qiagen, Germany

Table 10: Plasmids

Vector	Inserts	Source
pSpCas9(BB)-2A-GFP-PX458	Empty Vector	Addgene, MA, USA
TAK1 CRISPR /Cas9 KO plasmid (h)	TAK1	Santa Cruz Biotechnology, Texas, USA
pCMV-TAK1-WT	Wild type TAK1	Kindly provided by Prof. Jun Ninomiya-Tsuji, Raleigh, USA
pCMV-TAK1-K63W	Mutated TAK1	kindly provided by Prof. Jun Ninomiya-Tsuji, Raleigh, USA

Table 11: Cell lines used in this study

Human Cell Line	Type of cells	Mutation status	Source
WM115	Melanoma	BRAF	ATCC, Manassas, VA, USA
SK-MEL 5	Melanoma	BRAF	ATCC, Manassas, VA, USA
WM793B	Melanoma	BRAF	ATCC, Manassas, VA, USA
Malme-3M	Melanoma	BRAF	ATCC, Manassas, VA, USA
MeWo	Melanoma	WT	ATCC, Manassas, VA, USA

SK-MEL 2	Melanoma	NRAS	ATCC, Manassas, VA, USA
WM35	Melanoma	BRAF	Wister Institute, Philadelphia, USA
WM852	Melanoma	BRAF	Wister Institute, Philadelphia, USA
WM3211	Melanoma	c-KIT	Wister Institute, Philadelphia, USA
WM1346	Melanoma	NRAS	Wister Institute, Philadelphia, USA
WM1366	Melanoma	NRAS	Wister Institute, Philadelphia, USA
WM1791C	Melanoma	CDK4	Wister Institute, Philadelphia, USA
MEL-JUSO	Melanoma	CDK4	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany
WM3248	Melanoma	BRAF	Rockland Immunochemicals Inc, Limerick, PA, USA
SK-MEL 147	Melanoma	NRAS	Memorial Sloan-Kettering Cancer Center, New York, USA
HT-29	Colon cancer	BRAF, SMAD4, PIK3CA	ATCC, Manassas, VA, USA
HaCaT	Keratinocytes	WT	Kindly provided by Petra Boukamp (DKFZ, Heidelberg, Germany)

2.2 Methods

2.2.1 Cell culture

Cells were cultured in Roswell Park Memorial Institute medium (RPMI) with 10% fetal calf serum (FCS), at 5% CO_2 at 37°C. Cell lines used in this study were tested to be free of mycoplasma and were authenticated through STR profiles.

2.2.2 Cell treatment with stimuli and inhibitors

Melanoma cells were treated with different death ligands (TNF-α, and Fc-scTRAIL) in RPMI medium for 24 h and 48 h in typical cell culture condition (5% CO_2, 37°C). Lyophilized TNF-α was dissolved in 0.1% BSA to maintain shelf life of ligands. All inhibitors unless mentioned otherwise were reconstitutes in DMSO. Cells were pre-treated for 30 minutes prior to DLs treatment. DMSO served as vehicle control and the concentration was not more than 0.1% in control medium.

2.2.3 siRNA-mediated knockdown

For transient knockdown experiments, the following siRNA duplexes were used: FlexiTube siRNA for caspase-8, and the corresponding control siRNA. All siRNA preparations were from QIAGEN. For transient transfection, $2x10^5$ cells and 5 X 10^3 cells per well were seeded in a 6-well and 96 well plate respectively and incubated overnight. Before transfection, siRNA (10 nM) and transfection reagent was diluted with Opti-MEM medium and incubated at room temperature for 5 min to form the siRNA-lipid complex, followed by transfection according to the manufacturer´s recommendations using Lipofectamine RNAiMAX and the siRNA species.

2.2.4 Transformation and DNA isolation

The plasmid DNA was incubated together with 10 µl bacteria on ice for 30 min. Following incubation, cells were heat shocked for 20 s at 42°C and kept on ice for on additional 2 minutes. Next, 200 µl of SOC-medium was added to the samples and the cells were further incubated at 37°C for 1 h with 300 rpm speed. Afterwards, 100 µl plasmid transfected bacteria were spread on Luria-Bertani (LB) agar plates containing 100 µg/ml of Ampicillin and then incubated overnight at 37°C. After overnight incubation, a single isolated colony was picked and inoculated in 5 ml LB medium with

100 µg/ml of Ampicillin and incubated overnight at 37°C and 170 rpm. Plasmid DNA was isolated using the NucleoBond® Xtra Midi kit according to the manufacturer's protocol. The constructs were verified by sequencing. Plasmids were eluted in 1 ml DNase free water and analysed with a NanoDrop 2000 spectrophotometer. Solely samples complying with the quality recommendations (260 nm/ 280 nm ratio: 2.0 +/- 0.2; 260 nm/ 230nm ratio: 2.0 +/- 0.2) were further used.

2.2.5 Generation of stable cell lines

TAK1 deficient WM1791C cells were generated by the CRISPR/Cas9 approach. Cells were transfected with empty vector pSpCas9(BB)-2A-GFP-PX458 or a vector targeting *MAP3K7*. Two days after transfection, GFP positive clones were isolated by cell sorting and plated as single clones in 96-well plates. Clones were cultured for 2-3 weeks and analysed for successful TAK1 knockout by Western blotting.

2.2.6 Cell viability (MTT assay)

For cell viability assay 0.5-2 x 10^4 cells (depending on the cell line) were seeded per well of a 96 well plate and incubated overnight at 37°C. Then the cells were stimulated with stimuli for 24 h and 48 h. Prior to experimental endpoints, cells were incubated with MTT at 5 mg/ml for 3 to 4 h and kept maintained in cell culture incubators. After incubation, medium was then aspirated, 100 µl methanol were added, followed by 30 min incubation. The optical density (OD) of each well was measured with a TECAN multi reader at 570 nm. Cellular viability was calculated as a percentage relative to the control treated samples (survival of control). All shown data are the summary of at least three independent experiments, the error bars indicate standard error of the mean from at least three independent experiments.

2.2.7 Annexin/PI assay

For detection of cell death, cells were stimulated as indicated in the figure legends. After incubation of cells for 24 hours, cells were collected and suspended in 1 × Annexin-V binding buffer containing Annexin V-EGFP (1:200) and Propidium iodide (PI, 2 µl/ml) for 15 min in the dark at room temperature. Finally, flow cytometric measurements were performed using a MACSQuant Analyzer 10 and data were analysed using Flowing Software.

2.2.8 xMAP (Luminex) assay

The cells were cultured on 96-well plates and grown to near confluence. The cells were treated with inhibitors and TNF- α, incubated for 6 h and 24 h. For cytokines and phosphoprotein assays, the supernatants were collected in separate 96-well plates for Cytokines assay and cells were lysed with ProATonce lysis buffer (ProtATonce, Athens, Greece). The proteins were collected by centrifugation at 2700 g for 20 min. The total amounts of isolated cell proteins were quantified by Bradford assay, and 250 µg/µL proteins were used to measure phosphoproteins. For cytokine measurements, the protein concentrations of supernatants were adjusted to 100 µg/µl. Antibody-coupled magnetic microspheres were added into 96-well plate (50 µl/well) and washed twice with assay buffer. Custom antibody-coupled beads were technically validated as described before [128]. Then 50µl of the sample was added to the well and the plate was shaken at maximum speed (800 rpm) for 90 minutes at room temperature. After incubation, the assay buffer was discarded using magnetic separator and washed twice again with assay buffer. 20 µL of detection antibody was added to the each well of 96 well plate and the plate was shaken at maximum speed (800 rpm) for 90 minutes at room temperature. Then, biotinylated detection antibody (50 µL) was added into the sample and xMAP assays were performed on a Luminex FLEXMAP 3D® platform.

2.2.9 Surface receptor expression staining

To check receptors expression (TNF-Rs and TRAIL-Rs) on the cell surface, I used flow cytometry to measure surface receptor expression. In brief, 1x 10^4 cells were washed with PBS and transferred into U shaped 96-well plate, followed by incubation with respective primary antibody for 30 min at RT. Then, the cells were washed with 1x PBS and stained with mouse isotype secondary antibody for 30 min. Next, the cells were washed with PBS and stained with fluorescent dye FITC Alexa 488 for 45 min at RT. Finally, the stained cells were analyzed by flow cytometry.

2.2.11 Statistical analysis

Results were plotted using Excel (Microsoft Office version 10) and statistical analysis was performed using GraphPad Prism 7 (GraphPad software version 7.04). If not stated otherwise, the mean values of three independently conducted experiments are plotted, where bars show for the standard error of mean (SEM). Data were analyzed for statistically significant differences using One-way ANOVA with Bonferroni's

correction for multiple testing. A confidence level of 95% was used for all statistical analyses (p-value = 0.05). Results were summarized as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant.

3. Principle aims of the thesis

Malignant melanoma is a highly aggressive form of cancer, and it exerts an extreme chemo-resistance to existing therapies. Significant research has been made over recent decades; however, the overall survival rate among melanoma patients remains poor or largely unchanged due to the heterogeneity of the tumor. Melanoma cells are often resistant to apoptotic drugs and often bypass extrinsic or intrinsic cell death. To overcome this problem, it is of high relevance to discover alternative programmed cell death to avoid apoptotic resistance.

Thus, the study aims to address the following questions:

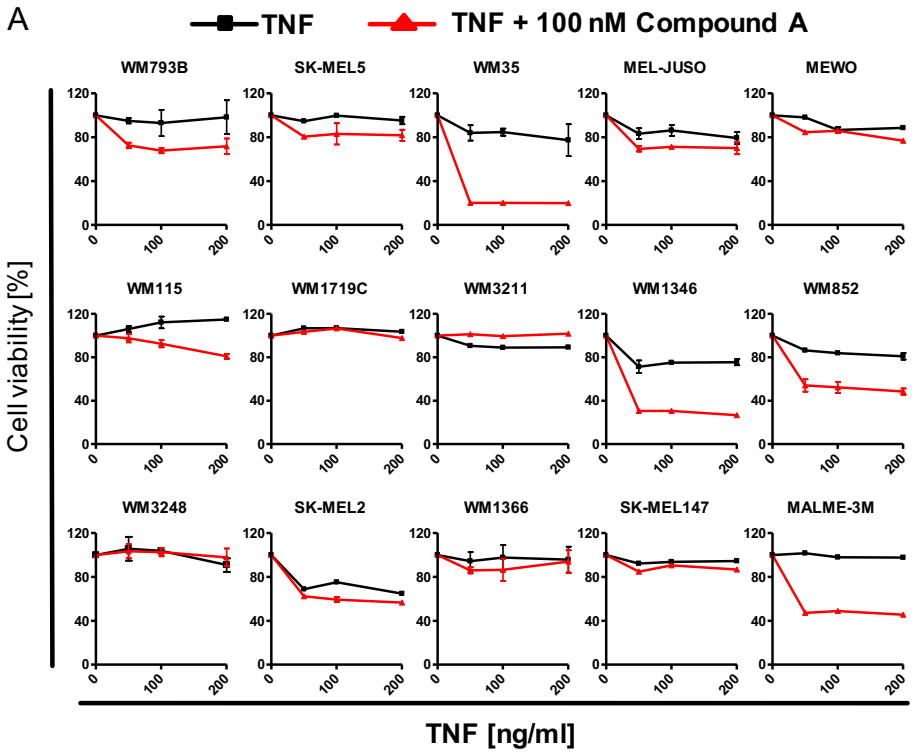
1. How do melanoma cells response to various treatments?
2. What are the cell death modalities in melanoma cells?
3. Does TAK1 loss cause sensitivity to TNF and IAP antagonist-induced cell death?

Results

4. Results

4.1 Melanoma cell lines heterogeneously respond to death ligands and IAP antagonist

This study investigated the response pattern of human malignant melanoma cell lines, which represent diverse mutation statuses and disease progression stages, to death ligands (DLs), alone or in combination with inhibitor apoptosis proteins (IAPs) antagonist (compound A). The sensitivity of various melanoma cell lines was characterized in combination with death ligands TNF-α and Fc-scTRAIL (TRAIL) and cell viability was measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay. WM793B, SK-MEL5, WM35, MEL-JUSO, WM115, WM1346, WM852, and Malme-3M, but not MeWo, WM1791C, WM3211, WM3248, SK-MEL2, WM1366, and SK-MEL147 were sensitized to TNF ligands, mediating the loss of viability (Figure 10A). Additionally, in response to TRAIL, WM793B, SK-MEL5, WM35, MEL-JUSO, SK-MEL147, WM3248, Malme-3M, and WM1791C cells were significantly or partially sensitized. In contrast, MeWo, WM115, WM1346, WM852, and WM1366 cell lines did not response at similar fashion (Figure 10B). Thus, tested melanoma cell lines responded heterogeneously to DLs in combination with compound A.



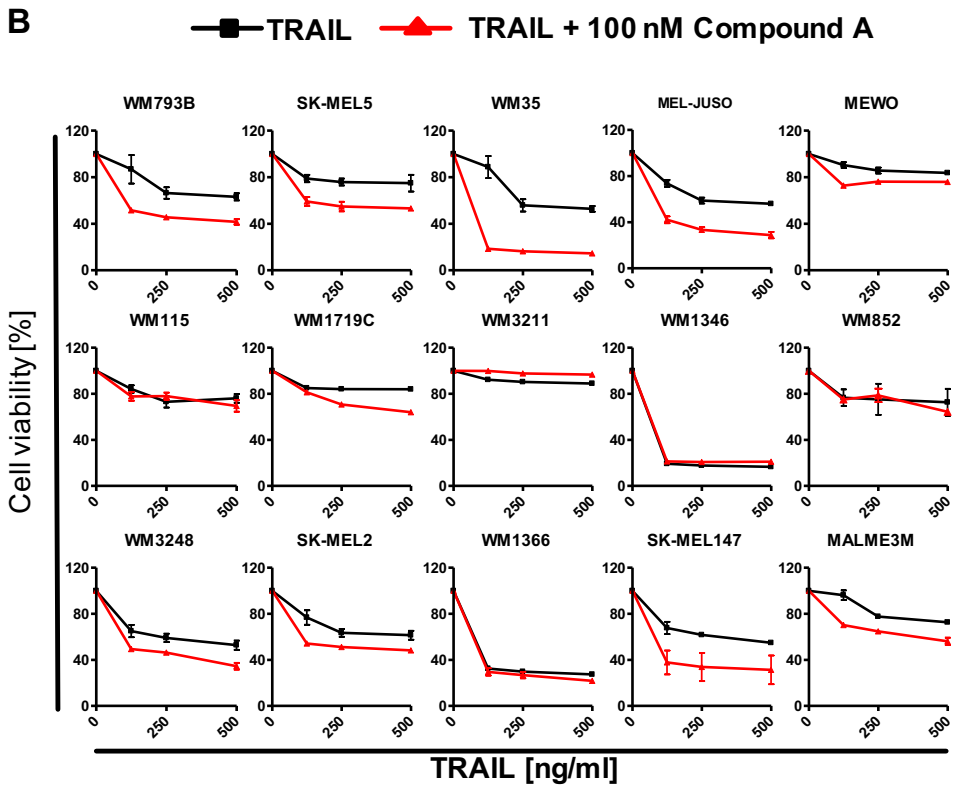


Figure 10: IAP antagonist heterogeneously sensitizes melanoma cells to death ligands. (A, B) Cell viability in response to combination treatment of TNFα or TRAIL with IAP antagonist compound A, as determined by MTT assays. Cells were treated for 48 h. Data display the mean ± S.E.M. from n = 3 independent experiments.

4.2 Melanoma cells heterogeneously express cell death-related proteins

To determine the expression of cell death surface receptors (TNF-Rs and TRAIL-Rs), surface receptor expression analysis was performed via flow cytometry. The surface receptors were heterogeneously expressed regardless of mutational status in melanoma cells (Figures 11A and 11B). Since cell death proteins and IAPs are involved in mediating cell death signaling, protein expression was analyzed and compared with the HaCaT keratinocytes. While caspase-8, $cFLIP_L$, and FADD were detected in most of the melanoma cell lines, $cFLIP_S$ was less visible or absent in the tested melanoma cell lines (Figure 12A). To investigate the IAPs in malignant melanoma, protein levels of cIAP1/2 and XIAP were examined, revealing that cIAP1 and XIAP were homogenously expressed, but cIAP2 was only detected at low levels in WM793B, WM115, WM1366, WM1346, SK-MEL147, and WM1791C (Figure 12A). As noted previously, RIPK1, RIPK3, and MLKL are required to mediate necroptotic cell death [129]. Therefore, this study investigated RIPK1, RIPK3 and MLKL protein levels in melanoma cell lines. The results revealed that RIPK1 protein was expressed in most

of the melanoma cells; however, the amount of MLKL protein expression was heterogeneous in the tested melanoma cell panel. Importantly, the expression of RIPK3 was low in most of the cells (WM35, WM793B, WM 852, WM 3248, WM 115, SK-MEL 5, WM1366, WM 1346, SK-MEL147, SK-MEL2, MEL-JUSO, and MeWo) and moderately expressed in some melanoma cells (SK-MEL 1, MALME-3M WM 1791C, and WM3211), as compared to control cells HaCaT (known to be necroptosis competent) (Figure 12A). Overall, these observations suggest that melanoma cells have heterogeneous protein expression.

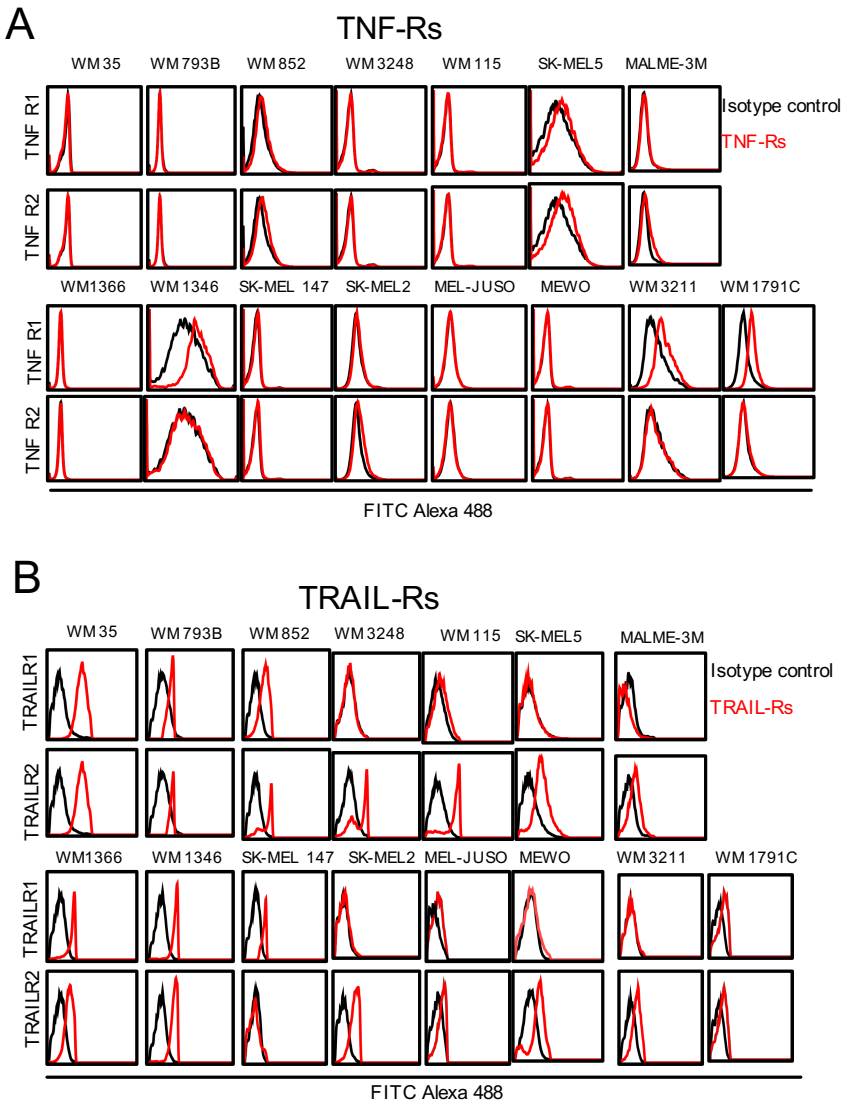


Figure 11: Cell surface receptors are heterogeneously expressed in melanoma cells. Surface receptor expression of TNF-Rs (A) and TRAIL-Rs (B) in melanoma cells were stained with respective antibodies and quantitatively analyzed via flow cytometry. Isotype control staining was used to verify the specificity of the antibodies. One representative experiment of three independently performed experiments is shown.

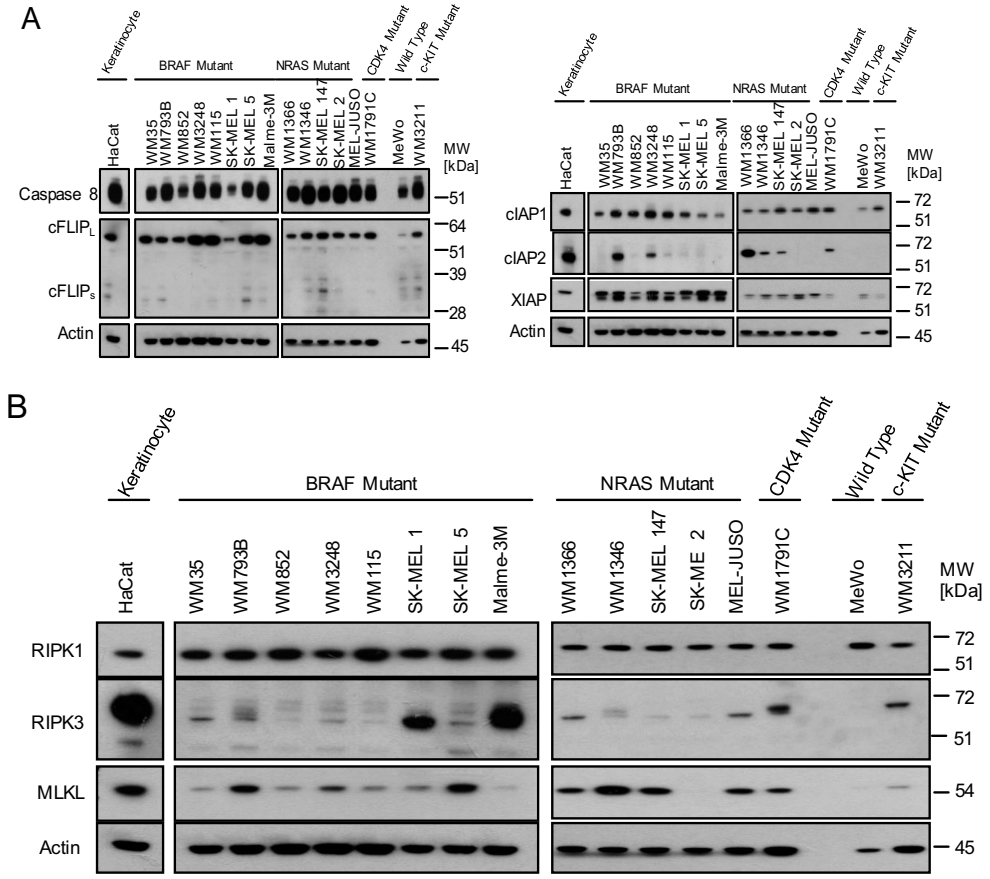


Figure 12: Heterogeneous cell death regulatory proteins expression in melanoma. (A, B) Basal expression of key cell death regulatory proteins in a set of melanoma cell lines (with BRAF, NRAS, CDK4, and c-KIT mutation status; wild type) was determined via immunoblotting. Necroptosis-competent HaCaT keratinocytes were used as a control. Actin served as a loading control. One representative experiment of three independently performed experiments is shown.

4.3 Death ligands and compound A induce caspase-8-mediated apoptotic cell death in malignant melanoma, but not necroptotic cell death

Next this study aimed to discover whether melanoma cells are necroptosis competent. To answer this question, cell lines Malme-3M, MEL-JUSO, WM3211, and WM1791C were used, since they express detectable amounts of RIPK1, RIPK3, and MLKL. In this experiment, human colon cancer cell line HT-29 was used as the control for necroptosis cell death. Necroptosis can be induced through TNF receptor pathways when combining TNF-α receptor ligands with IAP antagonists and when suppressing apoptosis signaling with caspase inhibitors like zVAD-fmk. Melanoma cells were treated with TNF-α and multivalent TRAIL variant (Fc-scTRAIL) in combination with IAP antagonist compound A for 24 h. Cell viability was measured 24 hours post-

treatment by MTT assay. The combined effect of TNF-α and compound A shows the response variability among melanoma cells. In the four melanoma cells tested, WM3211, and Malme-3M, cells lost around 50% viability after the combined treatment of TNF-α and compound A. In contrast, WM1791C and MEL-JUSO cell viability remained unchanged by the same treatment regimen (Figure 13A). These results indicate a heterogeneous response pattern of tested melanoma cells to the combined treatment.

However, Fc-scTRAIL drastically sensitizes melanoma cells in combination with compound A (Figure 13A). To test whether the viability loss was caspase-dependent apoptosis or RIPK1-dependent cell death, this study used pan-caspase inhibitor zVAD-fmk and the RIPK1 inhibitor Necrostatin-1. As expected, zVAD-fmk prevented the loss of cell viability in combination TNF-α, and Fc-scTRAIL with Compound A. However, RIPK1 inhibitor Necrostatin-1 was unsuccessful in preventing the loss of viability in melanoma cell lines (Figure 13A). To validate the inhibitor study, the gene knockdown approach was used in this study. In this approach, the caspase-8 gene was silenced by siRNA in these melanoma cells, and transfection efficacy was confirmed by immunoblotting (Figure 14A). Caspase-8 silencing in melanoma cells revealed a pattern similar to the effect of the zVAD-fmk inhibitor. Overall, the results confirmed that cell death was fully apoptotic, as zVAD-fmk mostly protected the loss of viability, whereas Nec-1 was unable to block cell death in melanoma cell lines (Figure 14B).

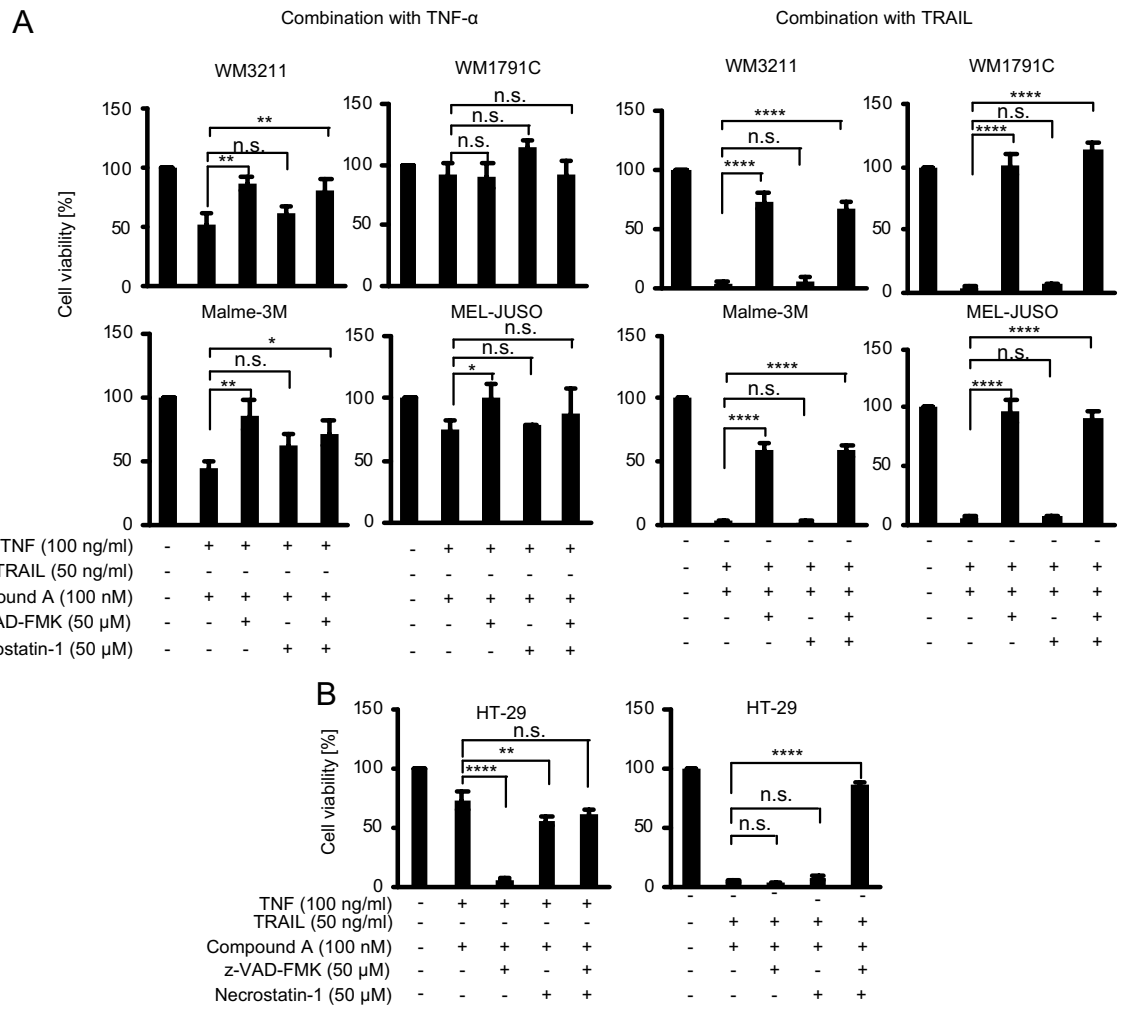


Figure 13: Melanoma cells expressing RIPK1, RIPK3, and MLKL are necroptosis resistant. (A) Cell viability in response to combination treatment of TNFα or TRAIL with IAP antagonist compound A, as determined by MTT assays. Cells were treated for 48 h. zVAD-fmk and Necrostatin-1 were added 30 min prior to treatment. (B) HT-29 cells served as necroptosis competent control. Data show mean ± S.E.M. from n = 3 independent experiments.

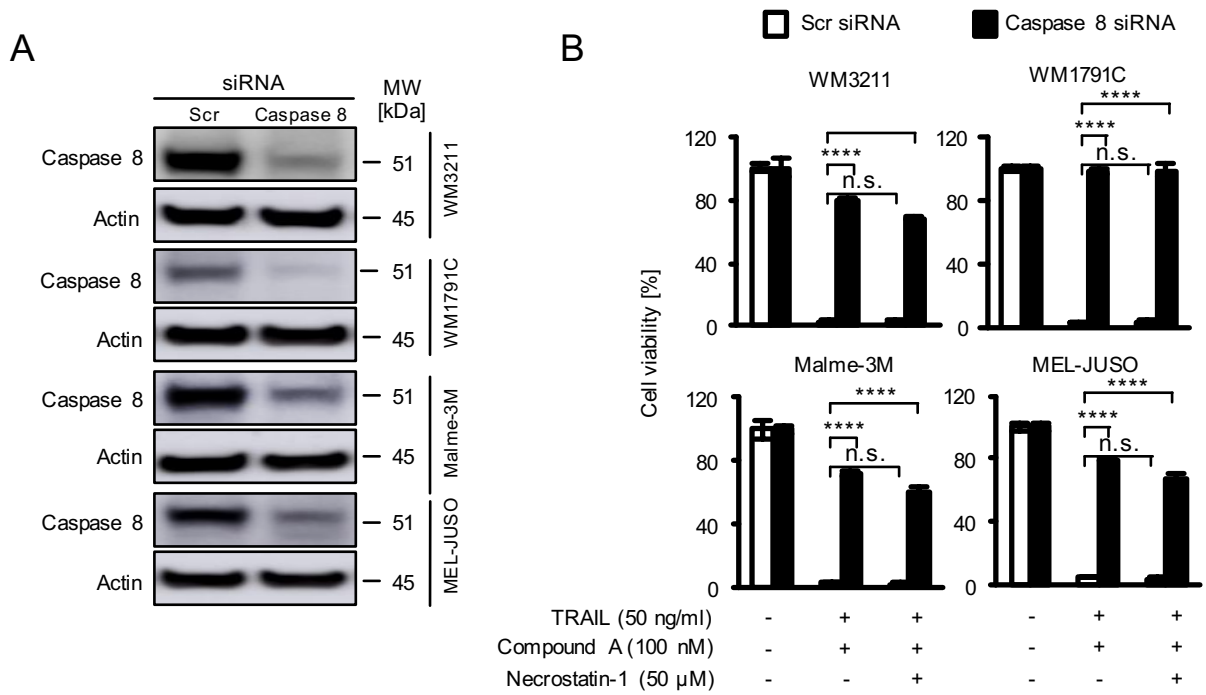


Figure 14: Cell death induced by TRAIL/Compound A is caspase-8 dependent. (A) Confirmation of caspase-8 depletion by immunoblotting. Cells were transfected with siRNA, as indicated, and whole cell extracts were generated 48 h after transfection. Actin served as loading control. (B) Melanoma cells were treated for 24 h, as indicated. Cell viability was measured using MTT assays. Data show means ± S.E.M. from n = 3 independent experiments.

4.4 Elevating intrinsic RIPK3 expression does not establish necroptosis competency in melanoma

Next, the current study reasoned that elevating the protein expression of RIPK3 could induce necroptosis, since RIPK3 protein expression was weak in melanoma cells. It was hypothesized that an increased level of the RIPK3 protein executes necroptosis in melanoma cell lines. It was previously demonstrated that DNA methyltransferase activity might silence RIPK3 expression. Therefore, it was reasoned that treatment with 5-aza-2′-deoxycytidine (5-AD)[130], a demethylating agent that induces the degradation of DNA (cytosine-5)-methyltransferase 1 (DNMT1)[131], could increase intrinsic RIPK3 expression and therefore necroptosis competency in melanoma cells. To do this, melanoma cells were treated with new 5-AD (2 µM) medium every consecutive day for up to four days. Indeed, RIPK3 protein expression was elevated in all tested melanoma cell lines upon 5-AD treatment; however, RIPK1 protein expression remained unchanged (Figure 15A). MLKL protein expression was also elevated after 5-AD treatment in Malme-3M and MEL-JUSO cells, but not in WM3211 or WM1791C cells (Figure 15A). DNMT1 catalyzes the transfer of methyl groups to

specific CpG structures in DNA[132]. Therefore, DNMT1 protein expression was also tested in melanoma cells. As expected, the DNMT1 protein was downregulated upon 5-AD treatment in melanoma cell lines (Figure 15B). It was observed that RIPK3 and MLKL protein amounts remained elevated for several days after 5-AD withdrawal (Figure 15C), indicating that 5-AD pre-treatment might represent an attractive strategy for priming cells towards RIPK3/MLKL-dependent necroptosis. However, despite reconstitution of RIPK3 by 5-AD, melanoma cells were unable to undergo necroptosis (Figure 15D). In conclusion, the results indicated that hyper-methylation could be a potential cause of the reduced expression of RIPK3 in melanoma cells. Signal transduction towards necroptosis is likely inhibited upstream of RIPK3/MLKL signaling.

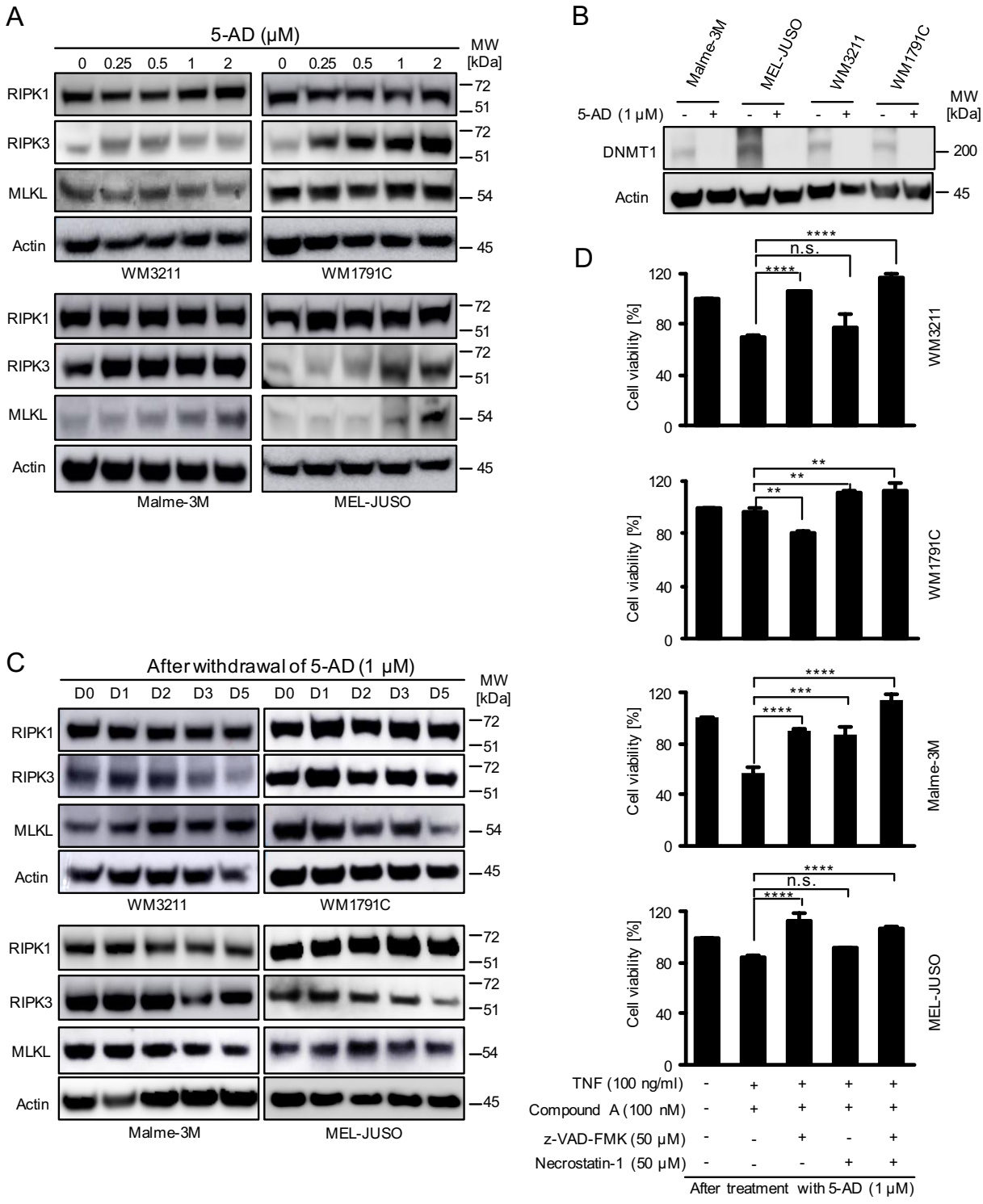


Figure 15: Elevating intrinsic RIP3 expression fails to establish necroptosis competency in melanoma. (A) Melanoma cells were treated with 5-AD for 4 days and whole cell extracts were probed for RIPK1, RIPK3, and MLKL expression by immunoblotting. Actin served as loading control. (B) DNMT1 expression was determined following 2 days of 5-AD treatment by immunoblotting. Actin served as loading control. (C) Elevated RIPK3 expression persists after 5-AD withdrawal. As in (A), protein amounts were probed in melanoma cells up to 5 days after 5-AD washout. (D) Cell viability of melanoma cells in which intrinsic RIPK3 expression was elevated

by 5-AD pre-treatment. Viability was determined by MTT assays. Data show mean ± S.E.M. from n = 3 independent experiments.

4.5 TAK1 inhibitors establish RIPK1-dependent apoptosis

It is well known that melanoma cells are often resistant to therapeutic intervention. To study the effect of TAK1 inhibition, the high-affinity inhibitor (5z)-7-oxozeaenol (OXO) was used to inhibit TAK1 activity. Melanoma cells were treated with a combination of TNFα and compound A. As a single drug or in combination with compound A, OXO was only modestly effective in reducing melanoma cell viability, whereas the triple combination with TNF-α strongly reduced cellular viability in all cell lines tested (Figure 16A). An alternative TAK1 inhibitor (NG-25) was tested in similar experimental settings. As expected, NG-25 demonstrated a similar effect in WM1791C melanoma cells (Figure 16B). Viability loss in WM3211 was completely caspase dependent, while, in WM1791C cells, and to a lesser extent in Malme-3M cells, RIPK1 inhibition by Necrostatin-1 (Nec-1) prevented viability loss (Figure 16A). In MEL-JUSO cells, Nec-1 reduced viability loss only in combination with caspase inhibitor zVAD-fmk (Figure 16A). OXO established conditions of caspase- and RIPK1-dependent cell death in TNF-α/compound A-treated cells, as prominently seen in the WM1791C cell line and colon cancer cell line HT-29 (Figure 16A and C). Disproving the argument that the effect of OXO is not limited to only the WM1791C cell line, similar findings were observed in another melanoma cell line, WM35 (Figure 16D). Interestingly, cell death remained independent of RIPK1 in TRAIL-based treatment scenarios (Figure 16E and 16F).

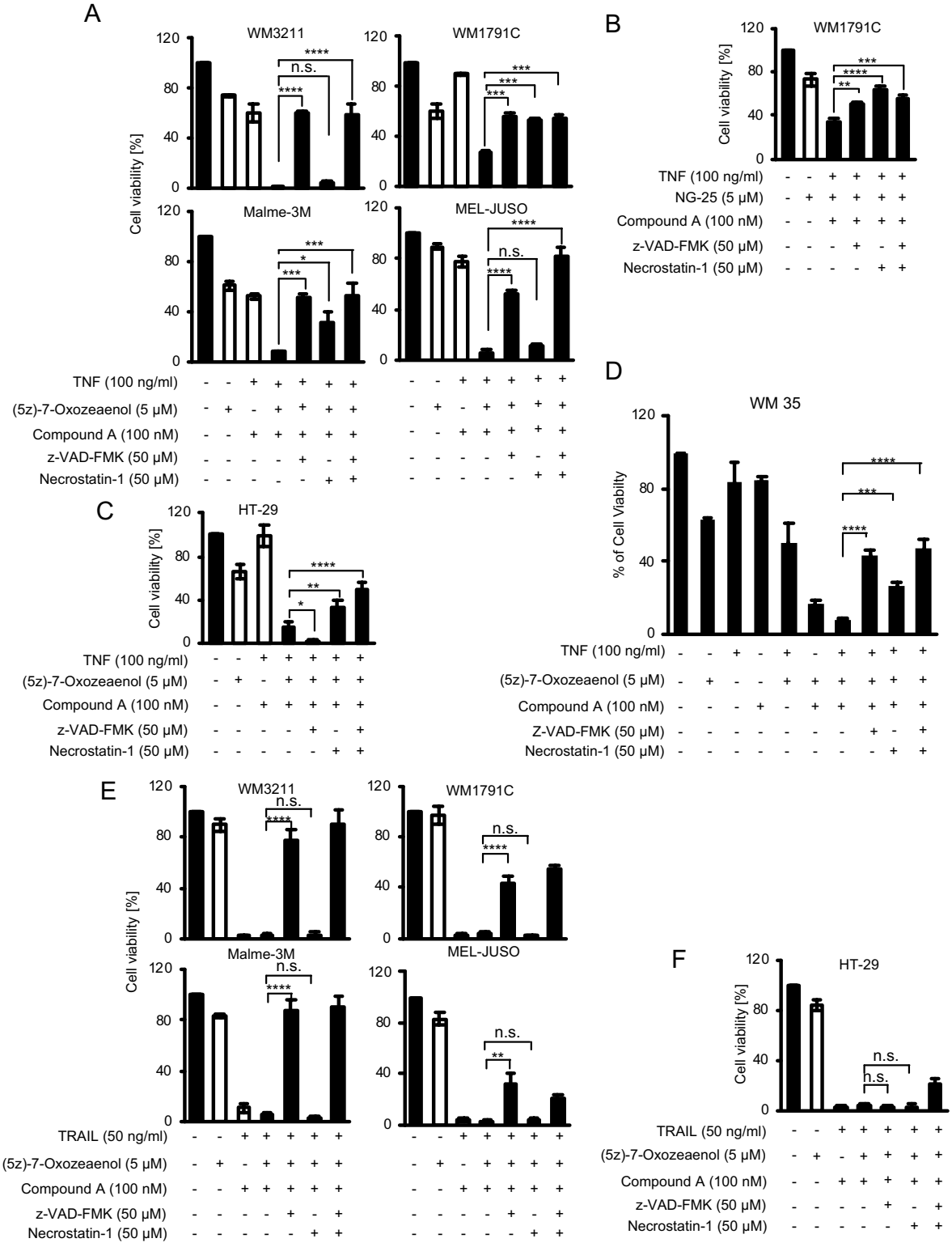


Figure 16: TAK1 inhibitors establish RIPK1-dependent apoptosis. (A, B) Cell viability of melanoma cells or (C) HT29 cells treated as indicated (D) WM35 melanoma cells were treated as indicated. Cells were pre-treated with zVAD-fmk (50 µM), Nec-1(50 µM) Compound A (100 nM), (5z)-7-oxozeaenol (5 µM), NG-25 (5 µM) alone or in combination for 30 min and subsequently stimulated with TNF (100 ng/ml) for 24 h. (E, F) Cells were stimulated with TRAIL for 24 h. Viability was determined by MTT assays

following 24 h of treatment. Data show mean ± S.E.M. from n = 3 independent experiments.

4.6 Inhibition of TAK1 induces RIPK1- and caspase-dependent cell death

To more specifically test for RIPK1 or caspase-8 dependency in this treatment scenario, RIPK1 and caspase-8 expression were depleted in WM1791C cells. In line with our previous results, viability loss required the presence of RIPK1 and caspase-8 (Figure 17A). Processing of caspase-8 and -3 as well as PARP cleavage was prominently detectable after treatment with TNF-α, Compound A and OXO, with both zVAD fully and nec-1 partially suppressing this apoptotic cascade (Figure 17B). Cell morphology and cell death measurements by PI staining further supported that cell death was executed primarily by RIPK1 dependent apoptosis (Figure 17C and 17D). Taken together, OXO appears to enhance the competency of melanoma cells to execute RIPK1 dependent apoptosis, but not necroptosis.

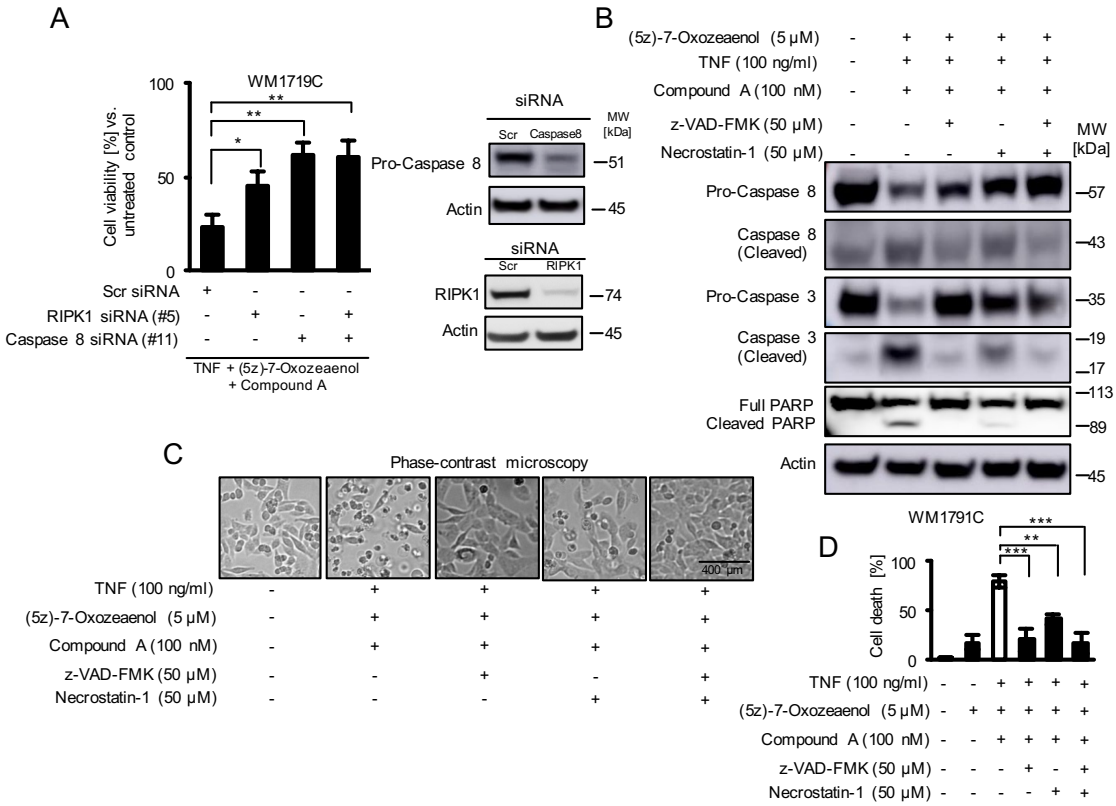


Figure 17: Cell death sensitization by (5z)-7-Oxozeaenol requires the presence of RIPK1 and caspase-8. (A) Cell viability of WM1791C cells in which RIPK1 and/or caspase-8 expression was depleted by siRNA transfection (48 h), following 24 h of treatment. Immunoblots demonstrate depletion efficiency. (B) WM1791C cells were treated as indicated and whole cell extracts were studied by immunoblotting. Actin served as loading control. (C) Cells were treated for 24 h as indicated, stained by propidium iodide (1 µg/ml) and observed by phase contrast microscopy. (D) Cell death quantification for WM1791C cells treated for 24 h as indicated. Cell death was

determined by flow cytometric measurement of propidium iodide positive cells. Data show means ± S.E.M. from n = 3 independent experiments.

4.7 Necroptosis remains blocked upon combination treatment with 5-AD and OXO

Since demethylation was sufficient to recommence RIPK3 expression in melanoma cell lines, and since OXO treatment established conditions of RIPK1-dependent apoptosis in melanoma cells, the combined effect of demethylation-mediated RIPK3 restoration and OXO on necroptosis was investigated. To explore whether combination treatments with TNF-α/compound A/OXO were now capable of inducing necroptotic cell death after RIPK3 elevation by 5-AD, melanoma cells were pre-treated with 5-AD and subsequently treated with TNF-α/compound A/OXO for 24 h. In contrast to what was expected, no tested melanoma cell lines exhibited necroptosis (Figure 18A). However, dependency on RIPK1 activity was increased among WM1791C cells and WM3211 (Figure 18A), possibly due to increased amounts of RIPK3 supporting RIPK1-dependent apoptosis in this setting[133]. To study necroptosis signal transduction through the RIPK1, RIPK3, and MLKL cascade, this study examined phosphorylation statuses in these proteins. While the conventional necroptosis-inducing treatment of TNF-α/compound A/zVAD-fmk induced strong phosphorylation of all three proteins in cell line HT-29, no RIPK1 phosphorylation was detected in WM1791C (Figure 18B). Demethylation failed to initiate RIPK1/RIPK3/MLKL phosphorylation in WM1791 cells upon TNF-α/compound A/OXO treatment (Figure 9C), which may partially explain the incompetence of demethylation of the RIPK3 gene in necroptosis induction.

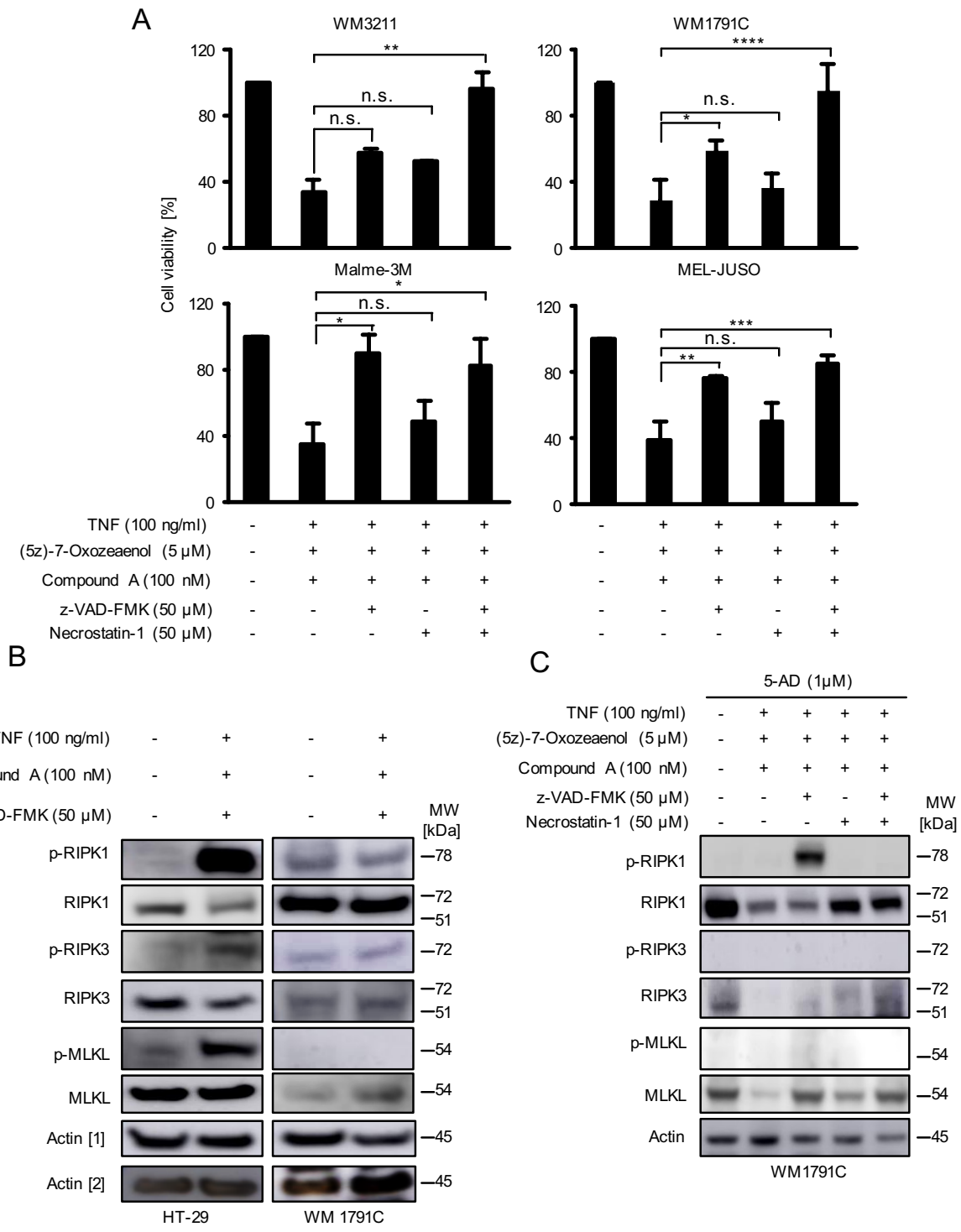


Figure 18: Necroptosis remains blocked upon combination treatment with 5-AD and (5z)-7-Oxozeaenol. (A) Cell lines in which RIPK3 expression was restored by 5-AD treatment (1 µM; 4 d) were subsequently treated as indicated. Cell viability was measured by MTT assays. Data show means ± S.E.M. from n = 3 independent experiments. (B) HT-29 and WM1791C cells were treated for 24 h as indicated and whole cell extracts were probed for the amounts of phosphorylated or overall RIPK1, RIPK3 and MLKL. Actin served as loading control. (C) WM1791C cells were treated with 5-AD for 4 days and afterwards treated for 24 h as indicated. Whole cell extracts were probed for the amounts of phosphorylated or overall RIPK1, RIPK3 and MLKL. Actin served as loading control.

4.8 TAK1 inactivation is essential for RIPK1-dependent apoptosis in melanoma

Thus far, the impact of inhibition of TAK1 by OXO in RIPK1-dependent cell death has been studied in melanoma cell lines. Even though OXO is a high affinity inhibitor of TAK1, off target effects cannot be excluded based on its selectivity profile [134]. We therefore performed genetic studies to test the relevance of TAK1 in preventing RIPK1-dependent apoptosis in melanoma more specifically. First, *MAP3K7*-knockout WM1791C (*MAP3K7*: gene name of TAK1) cells were generated using the CRISPR/Cas9 system. TAK1 expression was eliminated by CRISPR/Cas9 targeting of the *MAP3K7* gene. CRISPR knockout efficiency was satisfactory, as exemplified by western blotting (Figure 19A). The results revealed that TAK1 protein expression was completely silenced in TAK1 CRISPR knockout cells, whereas no change of TAK1 protein expression was observed in parental WM17991C cells (Figure 19A). Cell viability was studied after treatment with TNFα/Compound A. As previously observed, suppression of TAK1 activity by pharmacological inhibitors induced RIPK1-dependent cell death. WM1791C cells lacking TAK1, but not parental control cells, were substantially sensitized to treatment with TNFα/Compound A, and cell death could be completely prevented by zVAD or nec-1 (Figure 19B).

Next, this study examined cell morphology and cell death upon treatment, as previously described. The results revealed that propidium iodide uptake returned similar results, indicating apoptosis induction specifically for the TNFα/Compound A combination treatment in cells lacking TAK1. The penetration of PI into cells was higher in TAK1-deficient cells, while it was entirely blocked by zVAD-fmk and Necrostatin-1 (Figure 19C). In addition, loss of cellular integrity was observed by phase contrast microscopy. Overall, the data demonstrate that TAK1 inhibition induced RIPK1-dependent apoptotic cell death.

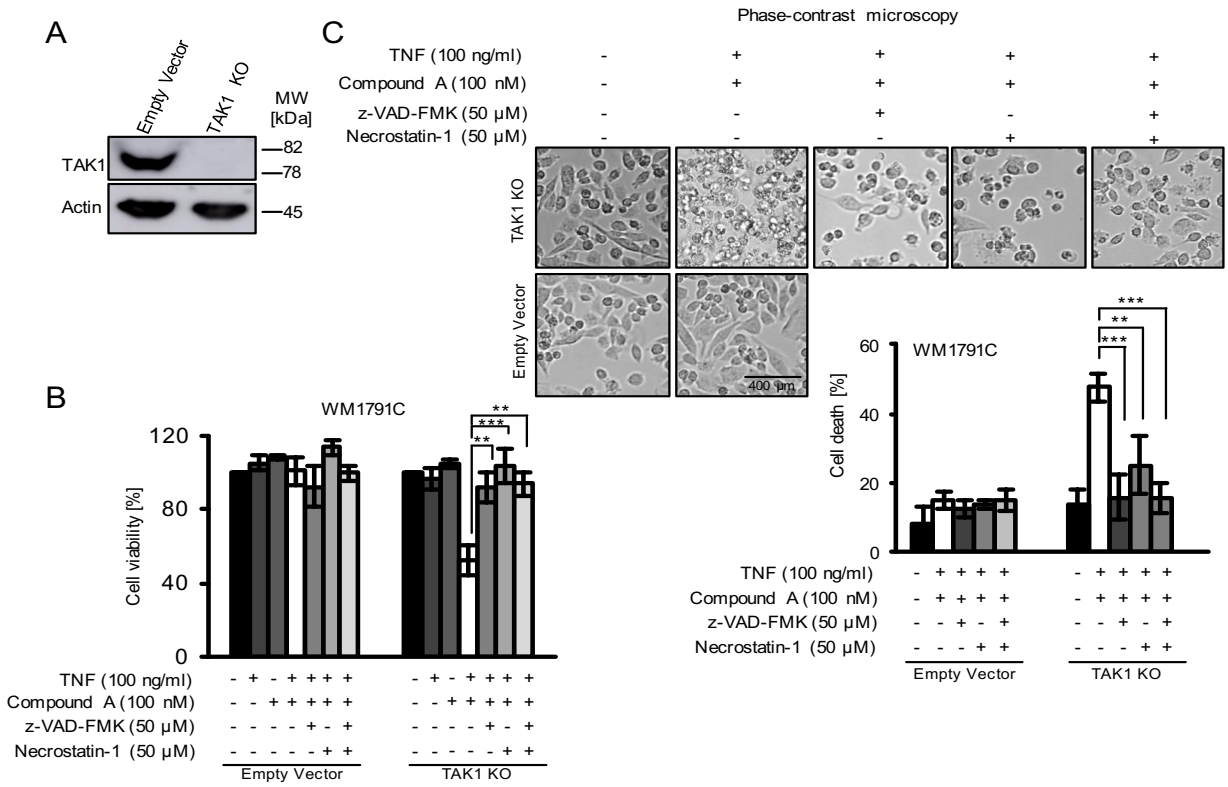


Figure 19: TAK-1 inactivation is essential for RIPK1-dependent apoptosis in melanoma. (A) Confirmation of TAK1 knockout in WM1791C cells by immunoblotting. Actin served as loading control. (B) Control and TAK1-deficient WM1719C cells were treated for 24 h, as indicated. Cell viability was determined by MTT assay. Data show means ± S.E.M. from n = 3 independent experiments. (C) Cells were treated for 24 h, as indicated, stained by propidium iodide (1 µg/ml), and observed by phase contrast and fluorescence microscopy. Cell viability was determined by MTT assay. Data show means ± S.E.M. from n = 3 independent experiments.

4.9 Kinase function of TAK1 is indispensable for melanoma survival

A series of reconstitution experiments were performed to elucidate the role of TAK1 kinase activity. TAK1 knockout WM1791C cells were reconstituted with empty vector, wild-type TAK1 (TAK1-WT), and a catalytically inactive mutant of TAK1 (TAK1-K63W) constructs, to investigate whether TAK1 kinase activity was required for melanoma cell survival. Transfection efficacy was confirmed by western blotting, and results revealed that a significant amount of TAK1 was reconstituted. Spontaneous phosphorylation TAK1 was observed upon TAK1-WT constitution (Figure 20A). In contrast, introducing kinase-dead TAK1 (TAK1-K63W) was not sufficient to induce phosphorylation of TAK1 (Figure 20A), suggesting that kinase function of TAK1 is entirely inactivate. To examine the role of kinase activity in melanoma survival, reconstituted cells were treated with

TNF-α/Compound A with or without zVAD-fmk for 24 h. The data demonstrated that reconstitution of the empty vector in TAK1 KO WM1791C cells conferred sensitivity to the treatment. Importantly, reconstitution of kinase-dead TAK1 (TAK1-K63W) was unable to initiate a response similar to empty vector and wild-type TAK1 reconstitution (Figure 20B). In summary, the results corroborate that the kinase activity of TAK1 is a critical suppressor of RIPK1-dependent apoptosis in melanoma.

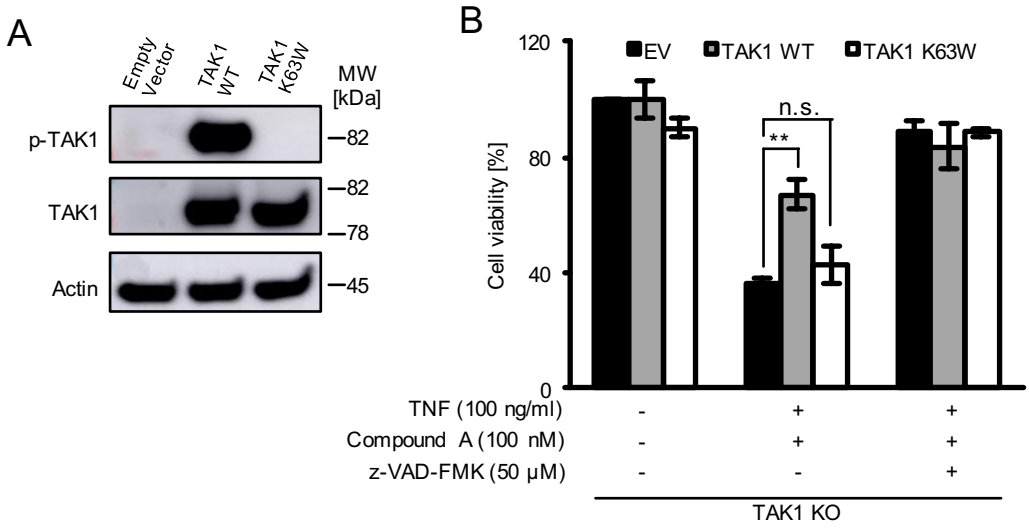


Figure 20: Kinase function of TAK1 is necessary for melanoma cell survival. (A) Confirmation of TAK1 reconstitution (wild type or K63W) by immunoblotting, following transient transfection with respective expression vectors. (B) Control and TAK1-reconstituted cells were treated for 24 h, as indicated. Cell viability was determined by MTT assay. Data show means ± S.E.M. from n = 3 independent experiments.

4.10 Inhibition of TAK1 inhibits NF-κB signaling

Since inhibition of TAK1 is associated with RIPK1-dependent apoptotic cell death, the molecular mechanisms of TAK1 action demand further investigation. Previous studies have demonstrated that the NF-κB signaling pathway is a major molecular pathway to regulate cell survival. The IKK kinase phosphorylates IκB, resulting in proteasome-mediated degradation, leading to NF-κB translocation into the nucleus[123]. Therefore, it has been hypothesized that TAK1 might activate the NF-κB signaling pathway by IκB degradation in melanoma cancer cells. To determine the function of TAK1 in NF-κB signaling pathway, WM1791C cells were treated with OXO for various time points before TNF-α treatment and immunoblotting analysis were probed with specific antibodies for p65/p50 complex signaling (Figure 21A). Consistent with the hypothesis, the results revealed that TAK1 inhibitor OXO significantly blocked phosphorylation of

p65 and the degradation of IκB (Figure 21A and B). TNF-α activated phosphorylation of p65 (Ser536) and degraded IκBα, resulting in upregulation of phospho-p65 and phospho-IκB (Ser32) (first lane of the blots).

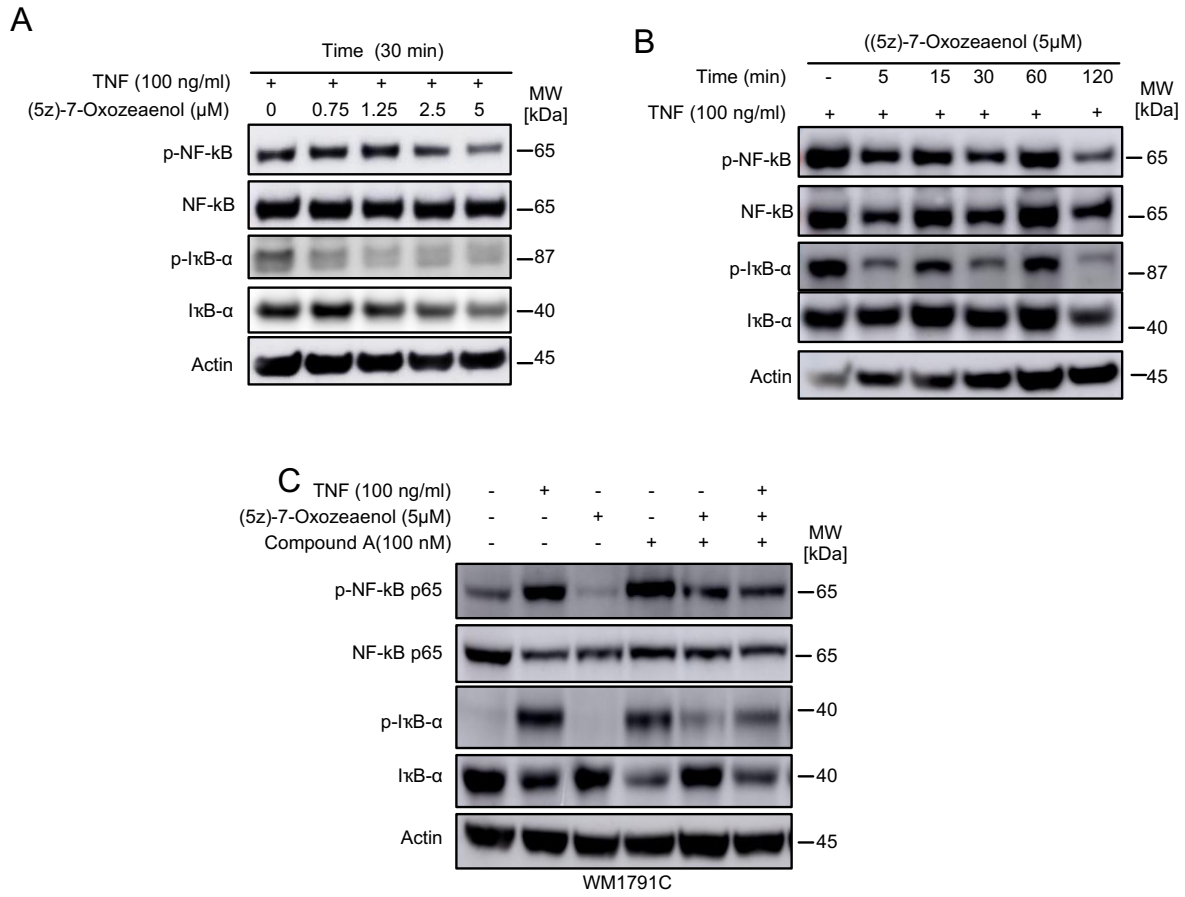


Figure 21: TAK1 inhibitor (5z)-7-Oxozeaenol blocks NF-κB signaling. (A) Melanoma cells WM1791C were treated with several concentrations of OXO before TNF-α stimulation, and the expression of NF-κB signaling molecules was analyzed. (B) Cells were treated with OXO for the indicated time points before TNF-α stimulation. (C) WM 1791C cells were treated as indicated. Whole cell extracts were analyzed 2 h after stimulation with TNF-α. Actin served as loading control. Cell viability was determined by MTT assay. Data show means ± S.E.M. from n = 3 independent experiments.

4.11 Inhibition of TAK1 suppresses MAP kinase signaling pathway in melanoma cancer

Depending on the cellular and microenvironmental context, TAK1 functionally activates MAP kinase proteins (p38, JNK, and ERK1/2) and triggers the increased production of proinflammatory cytokines (such as IL-1b, IL-6, and TNF-α). This causes an

aggressive proliferation of various solid and liquid cancers, including breast, thyroid, colon, and lymphoma cancer[107].

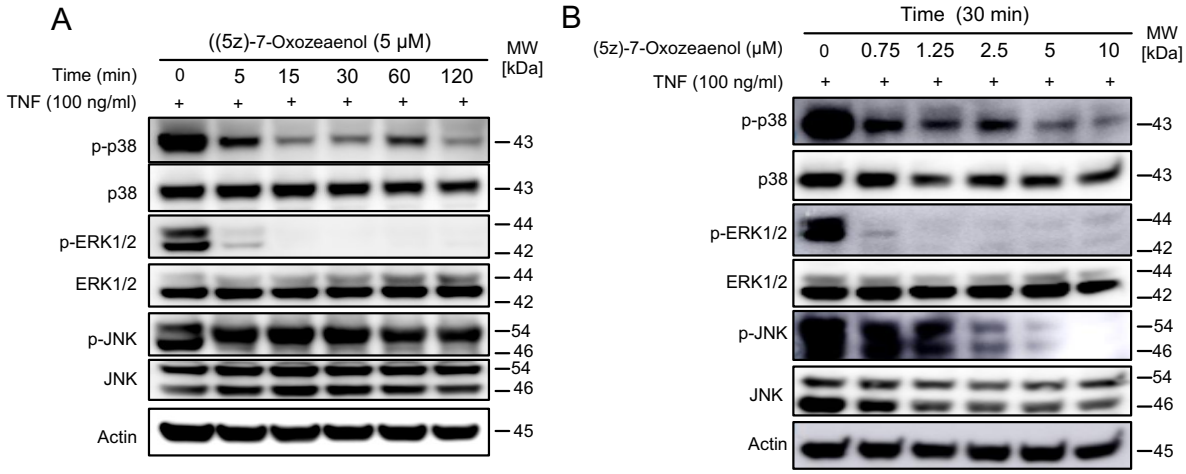


Figure 22: (5z)-7-Oxozeaenol blocks MAP kinase signaling in melanoma cells. (A) WM1791C cells were treated with 5 μM of OXO for indicated time points and the expression of MAP kinase signaling molecules was analyzed (B) WM1791C cells were treated with various concentrations of OXO prior to TNF-α stimulation and respective proteins were analyzed by western blot. Data show means ± S.E.M. from n = 3 independent experiments.

Therefore, this study aimed to investigate the role of TAK1 in the MAP kinase signaling pathway. To examine the role of the MAK kinase signaling pathway, it was examined whether OXO inhibits TNF-α induced JNK/p38/ERK activation. WM1791C cells were pre-treated with 5 μM of OXO and stimulated cells with TNF-α. Consistent with previous findings, TNF-α phosphorylated JNK/p38/ERK, whereas OXO significantly abrogated phosphorylation of those proteins in a time-dependent manner in as early at 5 minutes in WM1791C cells (Figure 22A). In addition, OXO reduced the phosphorylation of MAP kinase proteins in a dose-dependent manner, as little as 0.75 μM of OXO was sufficient for this reduction (Figure 22B).

4.12 TAK1 inhibition reduces pro-inflammatory chemokine/cytokine production and phosphoprotein levels

Melanoma is composed of the malignant cells and the supporting stroma, which includes fibroblasts, endothelial cells, immune cells, soluble molecules, and the extracellular matrix [21]. Chemokines and cytokines are secreted by malignant cells and tumor stroma in tumor microenvironment. Chemokines and cytokines bind to the cell surface through G-protein–coupled receptors and which can alter the tumor

microenvironment. In melanoma, various mutations stimulate the MAPK pathway, causing an overproduction of chemokines/cytokines which, in turn, can promote proliferation, invasion, migration, and angiogenesis[26]. Nevertheless, the role of chemokines and cytokines is not fully elucidated in melanoma cancer. The inhibition of TAK1 contributes to RIPK-dependent cell death in melanoma cells, but secretion of chemokines, cytokines, and phosphoprotein levels upon TAK inhibition remains to be analyzed.

This study sought to test the hypothesis that TAK1 inhibition might modulate chemokines, cytokines, and phosphoprotein levels. To support the hypothesis, the impact of TAK1 inhibition on chemokines/cytokines and phosphoprotein levels was characterized by Luminex assay analysis in melanoma cells. Melanoma cells were stimulated with TNF-α/compound A, and the cytokines and chemokines were analyzed on a Luminex 300 (Luminex). The results demonstrated that TNF-α/compound A stimulation substantially augmented the production of interleukin-8 (IL8), chemokines C-X-C motif ligand 1 (CXCL1), chemokine (C-C motif) ligand 5 (CCL5), follistatin (FST), and Intercellular Adhesion Molecule 1 (ICAM1) in melanoma cells (Figure 23). A previous study showed that the hypersecretion of chemokines/cytokines is highly correlated with melanoma disease progression and escape from immune surveillance. Intriguingly, OXO treatment reduced basal or TNF-α/compound A-induced secretion of cytokines and soluble factors in melanoma cells two- to threefold, with the exception of IL8 and CCXL1 secretion in MEL-JUSO and CCXL1 in Malme-3M cells. Of note, the addition of zVAD-fmk and Necrostatin-1 did not interfere with secretion patterns of chemokines/cytokines in most of the experimental settings, aside from CCXL1 expression in Malme-3M cells (Figure 23). These data provide additional confirmation that OXO treatment blocks chemokine/cytokine production in a majority of melanoma cells.

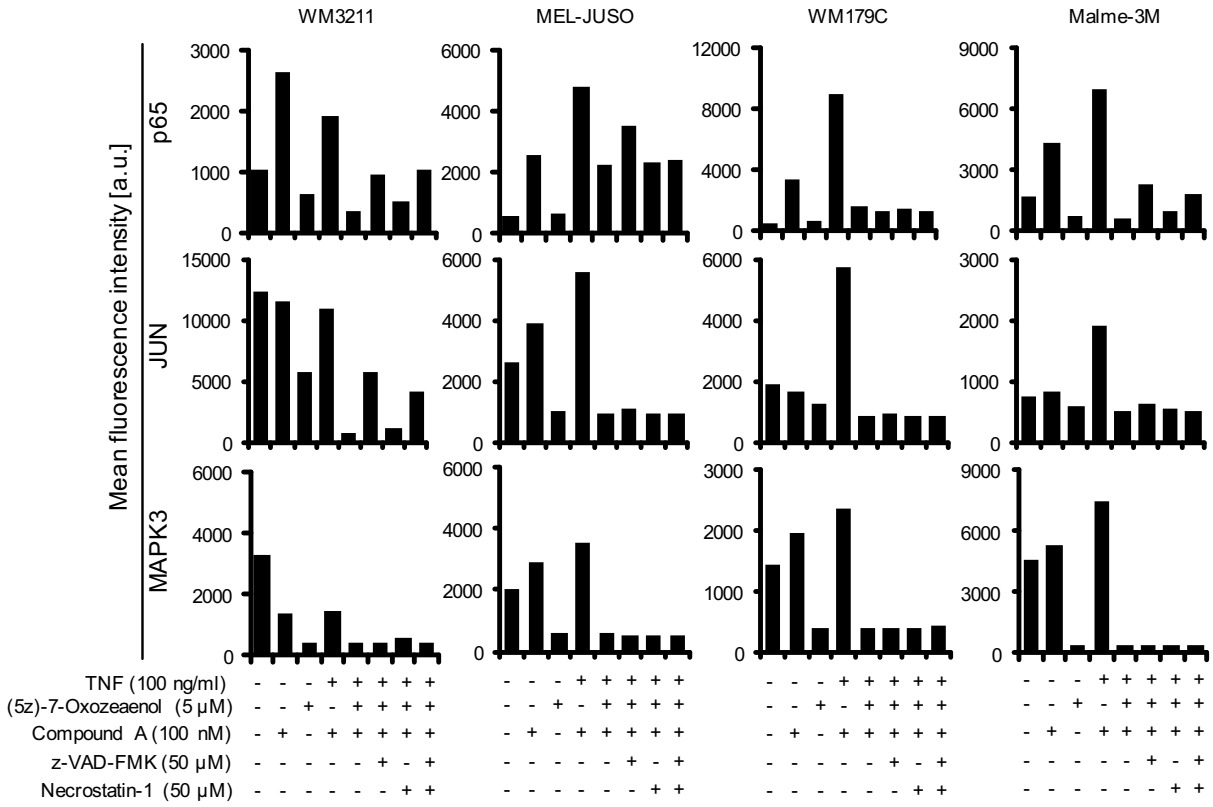


Figure 23: (5z)-7-Oxozeaenol suppresses TNF-α and/or compound A-induced phosphoproteins. Melanoma cells were treated for 6 h, as indicated, and changes in phosphoprotein amounts were detected in cell extracts using Luminex-based phosphoprotein profiling. Data are shown in arbitrary fluorescence units and n=1 experiment.

This study attempted to profile the phosphoproteins in melanoma cells. To evaluate whether TAK1 plays a role in activating these signaling pathways, melanoma cells were stimulated with TNF-α/compound A in combination with or without OXO, and respective phosphoproteins were measured by Luminex assay. TNF-α/compound A-induced phosphorylation of p65, Jun kinase (JUN), and MAP kinase 3 (MAPK3) was markedly repressed by TAK1 inhibition. (Figure 24). These data suggest that OXO prevents basal or TNFα/Compound A-induced phosphorylation of p65, JUN, and MAP kinase 3 (MAPK3) in melanoma cells.

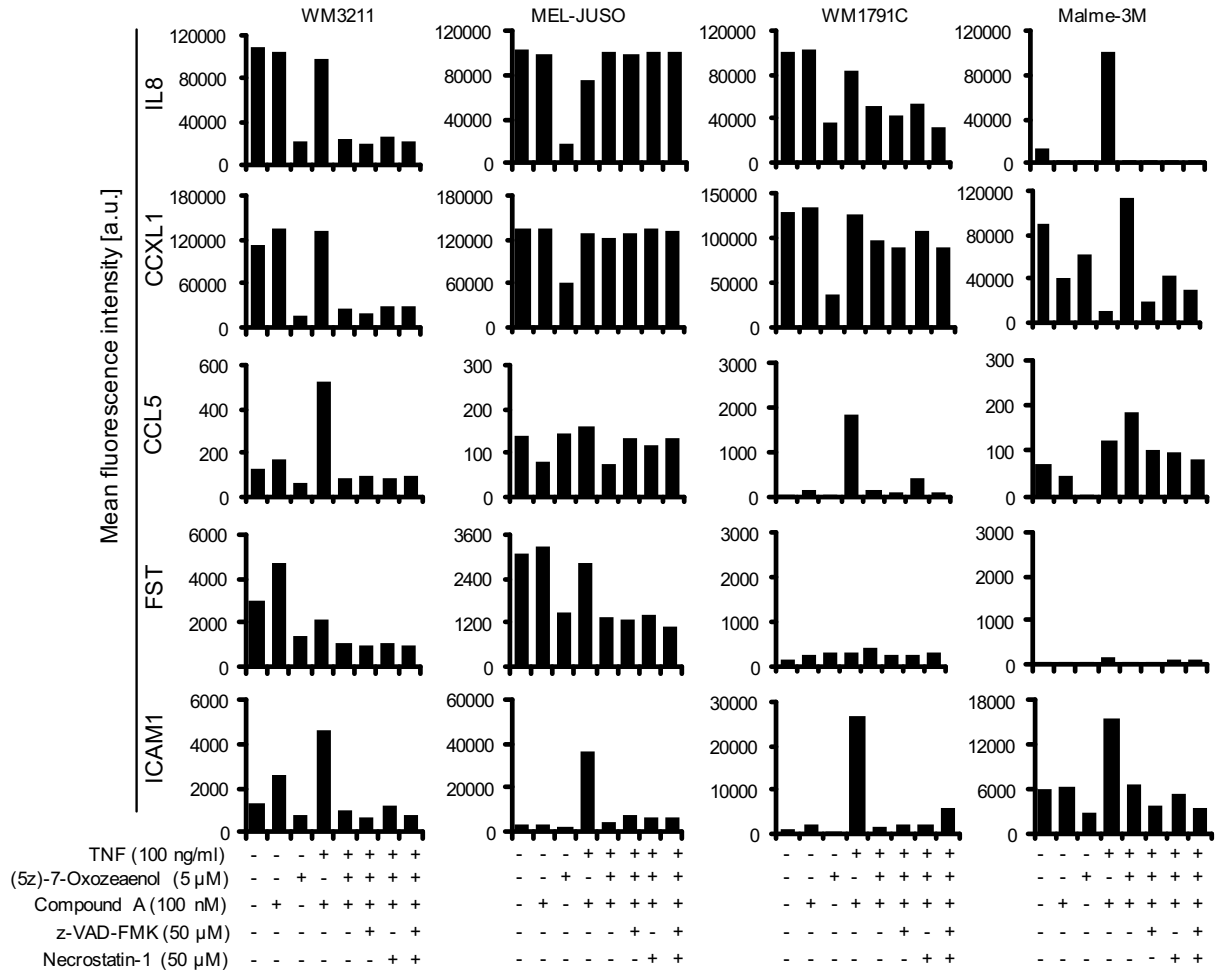


Figure 24: (5z)-7-Oxozeaenol suppresses TNFα and/or compound A-induced kinase signaling. Cells were treated for 24 h, as indicated, and supernatants were collected for Luminex-based cytokine profiling. Data are shown in arbitrary fluorescence units and n=1 experiment.

Discussion

5. Discussion

5.1 Melanoma response to treatment is heterogeneous: death ligands and IAPs antagonist

Melanoma is the most aggressive and deadliest form of skin cancer, and its treatment options are highly complex. The efficacy of the available treatments can decrease due to the development of diverse resistance mechanisms. For instance, melanoma acquires resistance to targeted therapeutics such as BRAF inhibitors alone or BRAF inhibitors in combination with MEK inhibitors[135]. Therefore, it is necessary to examine resistance mechanisms of melanomas and increase its sensitivity to therapeutics. In this study, the cell sensitivity to either TNF and TRAIL or the cell sensitivity to in combination with an IAP antagonist of human melanoma cell lines was studied. The results indicate that the response of melanomas to treatment is heterogeneous. Reportedly, TNF is a good candidate for therapeutic usage against soft tissue sarcoma, irresectable tumors of various histological types[136]. In a solid tumor, TNF-α causes hemorrhagic necrosis, which was enhanced by chemotherapeutic drugs in rat perfusion models[137]. In addition, TNF-α selectively augments the accumulation of chemotherapeutic drugs in the tumor up to three- to six-fold and results in promising antitumor activity[138,139]. Many studies have illustrated that TNF-α is present in different tumor environments and exhibits antitumor activity in various cancer models[140]. In contradiction, TNF-α propagates cancer invasion and progression in other models, such as breast cancer[140]. To unmask the paradoxical role of TNF-α in melanoma, the effect of TNF-α was investigated in a set of melanoma cells with different mutation statuses. Most melanoma cells were resistant or only partially sensitive to TNF-α treatment, suggesting that TNF-α might activate pro-survival signaling, rather than death signals.

TNF-α receptors (TNF-Rs) play a significant role in TNF-α signaling. The receptor family's TNF-R1 and TNF-R2 transmit signals for TNF-α activity. TNF-R1 and TNF-R2 structurally share four repeated cysteine-rich domains but have functionally distinct intracellular domains[141,142]. In fact, TNF-R2 lacks an intracellular death domain. Moreover, most importantly, TNF-R1 is responsible for promoting death signals, whereas TNF-R2 is accountable to activate a pro-survival signaling pathway[143]. In this

study, the expression level of TNF-Rs was low across the melanoma cells panel and melanoma cells did not respond to TNF-α treatment.

Over recent decades, TRAIL has emerged as an attractive candidate for therapy due to its ability to induce apoptosis in a wide range of human cancer cell lines without causing significant toxicity to normal cells[144–146]. Despite its preclinical success, TRAIL-based therapy has not had notable results in clinical trials due to its short half-life. In addition, it is rapidly cleared from the body by the kidney. Tumor cells expressing decoy rectors (TRAIL-R3/R4) can neutralize TRAIL activity[147,148]. Therefore, a series of research efforts are underway to optimize the efficacy of TRAIL in clinical settings. For instance, modification of TRAIL by adding peptide tags, human serum albumin, and nanoparticles enhances the stability of TRAIL and triggers its activity[149–152]. To improve TRAIL-based therapy, a second-generation TRAIL compound is currently undergoing clinical trial as monotherapy or in combination with chemotherapeutics drugs[153–155]. Often, cancer cells acquire both intrinsic and acquired resistance to first-generation TRAIL treatment through defects of the TRAIL-signaling pathway, from ligand binding to cleavage of the effector caspases[156]. In the present study, the sensitivity of the second generation of TRAIL was tested in human melanoma cell lines with various mutational statuses. Most of the melanoma cells exhibited a moderate to strong response to TRAIL, which is consistent with previous results[157,158]. Cell surface receptor expressions of TRAIL-R1 and TRAIL-R2 were measured to relate the TRAIL sensitivity to TRAIL receptors binding in this study's experimental settings. Consistent with previous data, this study found a relatively high expression of TRAIL-R2 in melanoma cell lines. In contrast TRAIL-R1 was not present in a similar fashion in melanoma[158,159]. A previous study demonstrated the synergy between a TRAIL-R2-specific antibody, AMG655, and TRAIL in ovarian cancer cells. AMG655 binds TRAIL-R2 at an epitope that does not limit the receptor's concomitant interaction with TRAIL, so that both proteins can bind TRAIL-R2 simultaneously[160]. This binding capacity results in an enhanced TRAIL-R2 crosslinking and apoptosis-inducing capacity. Since high expression of TRAIL-R2 existed in tested melanoma cell lines, it is reasoned that it could be possible to overcome the limited efficacy of currently employed TRAIL in combination with AMG655.

In many cancers, including melanoma, IAPs play a significant role in mediating cancer resistance to cell death inducers[161]. Thus, inhibition of IAPs in melanoma cells is a promising strategy to facilitate programmed cell death. In line with a previous study, TNF-α offered minimal antitumor effects in our tested melanoma cell lines during mono-therapy[162]. In combination with IAP antagonist compound A, however, TNF-α potentiated sensitivity in most melanoma cell lines. Several past studies have confirmed that the IAP antagonist can initiate an apoptotic death signal by depleting cIAP1 and XIAP, but that is incompetent in providing anti-tumor activity[162]. These results indicate that melanoma cell lines exhibit differential sensitivity to IAP antagonist and death ligands. One possible explanation for differential sensitivity to the treatment may be that differing levels of cIAP1 interfere with essential cell death signaling molecules. This study indicates that compound A synergizes with TNF-α/TRAIL in killing melanoma cell lines. To improve existing treatment regimens, there is a growing interest in increasing the level of cytokines, including TNF-α and TRAIL, in tumor microenvironments. This can lead to synergy with IAP antagonists. In head and neck cancers, for example, viruses and adjuvants stimulate cytokines and consequently result in synergism[163].

5.2 Melanoma and necroptosis: melanoma cells have limited necroptotic machinery and are incompetent to undergo necroptosis

It is established that necroptotic molecules RIPK1, RIPK3, and MLKL are required to execute necroptotic cell death in cell line models[164]. Thus, it was worth investigating the protein status and necroptosis competency in melanoma cell lines. In this study, RIPK3 and MLKL were expressed heterogeneously, whereas RIPK1 revealed a homogenous expression in protein level across the tested melanoma cell lines. Consistent with these results, another study showed that RIPK3 appears to be lost during melanoma development and is typically absent in most commonly used melanoma cell line models, as compared to melanocytes and nevus cells [130,165]. Koo and colleagues reported that RIPK3 expression was mostly lost in two-thirds of 60 cancer cell lines representing different types and stages of cancers, including glioblastoma, lung cancer, and pancreatic cancer[130]. Recently, a large-scale bioinformatics analysis identified which mutations or overexpression of genes correspond to necroptosis resistance. The study revealed that BRAF gain-of-function mutations and AXL overexpression were found among high percentages of cancer cell

lines derived from cancers of various tissue and cell types[166]. In this study, the majority of BRAF mutated melanoma cell lines expressed low or undetectable amounts of RIPK3, with the exception of SK-MEL-1 and Malme-3M, both of which expressed very high amounts of RIPK3.

Nevertheless, the dissection of how BRAF or AXL activity suppresses the expression of RIPK3 and how relevant and broadly applicable such a mechanism is in the melanoma setting warrants further investigation. It was recently discovered that a lack of RIPK3 protein expression is caused by insufficient RIPK3 mRNA expression[165]. Similar results were observed in lung and colon cancer[167,168]. In line with published data[165], it was observed that silencing RIPK3 expression in melanoma cell lines likely depends on DNA methylation, since 5-AD treatment and the associated degradation of DNMT1 are correlated with RIPK3. A decrease in the RIPK3 mRNA level can be mediated through limited promoter activation due to epigenetic DNA modification, such as DNA methylation and histone deacetylation[130,169]. Conversely, RIPK3 promoter demethylation is necessary to restore RIPK3 expression in various human cancer cell models[130]. DNMT1 methylates the RIPK3 promotor and thereby silences RIPK3 expression and increases necroptosis resistance to otherwise necroptosis competent cells, such as mouse embryonic fibroblasts, L929 cells, and human HT-29 cells[170]. Until now, melanoma cells were not studied in this context, and no treatment conditions were used that could unequivocally confirm that restoring RIPK3 expression is sufficient to enhance canonical necroptosis signal transduction. Increased intrinsic RIPK3 expression achieved by demethylating agent 5-AD was insufficient to establish necroptosis competency in melanoma cell lines, highlighting that additional regulatory processes must play a role in suppressing necroptosis competency in this setting. One apparent reason is that insufficient RIPK3 expression and reduced MLKL expression limit necroptosis in melanoma. Previous studies have confirmed that MLKL expression is correlated with necroptosis susceptibility in a large cell line-based screen[166]. In the present research, melanoma cells heterogeneously expressed MLKL but failed to undergo necroptosis, even in the presence of elevated RIPK3 expression. Although MLKL expression increased after 5-AD pre-treatment in Malme-3M and MEL-JUSO cells, these cells remained necroptosis resistant. The increase in MLKL after 5-AD treatment indicates that MLKL expression can be silenced by DNA methylation. To the researcher's knowledge, this has not yet been studied, but it would align with the hypothesis that disease progression in cancer can select for increased necroptosis

resistance[166]. Recent studies suggest that inhibition of the BRAF mutation in melanoma cancer can be a powerful way to reap the therapeutic benefits of necroptosis[166]. On the contrary, BRAF inhibitor dabrafenib interferes with RIPK3 expression and blocks necroptosis[165].

5.3 TAK1 is a key regulator of cell survival in vitro

TAK1 has emerged as an essential regulator of signal transduction and plays a critical regulatory role in stress response, inflammation, immunity, and cancer development and progression[171]. As such, regulation of TAK1-signaling is essential in maintaining tissue homeostasis. Previous *in vivo* studies have demonstrated that deficiency of TAK1 in mice induce necrosis and is often associated with disease pathologies[103]. Contrarily, inhibition of TAK1 enhances chemotherapeutic efficacy in various cancer models, including prostate and thyroid cancers[110,172–174]. The current study determined that signal transduction towards necroptosis never proceeded past RIPK1, indicating that additional regulatory processes exists. It identified TAK1 as a critical regulator of cell death susceptibility in response to activating TNF-Rs and inhibiting cellular IAPs. TAK1 is well known to modulate both apoptosis and necroptosis susceptibility[103,175,176], but the consequences of interfering with TAK1 activity appear to be complex and possibly context dependent. In necroptosis-competent fibroblasts, inhibition of TAK1 suppresses pro-survival NF-κB signaling in response to TNF-α, augments RIPK1 phosphorylation, and strengthens both apoptosis and necroptosis signaling branches[175].

Furthermore, the extent to which TAK1 directly phosphorylates RIPK1 can regulate apoptosis versus necroptosis signaling[103]. If NF-κB signaling is suppressed, transient RIPK1 Ser321 phosphorylation supports RIPK1-independent apoptosis, whereas sustained multi-site phosphorylation of RIPK1 supports necroptosis in L929 cells and MEFs[176]. Irrespective of this and regardless of whether TAK1 is inhibited, it remains to be studied why melanoma cells exclusively direct signaling towards apoptosis, rather than necroptosis, even though all key necroptosis effectors are in place and treatment scenarios otherwise sufficient to induce necroptosis are chosen. In TRAIL treatment scenarios, inhibition of TAK1 increased TRAIL induced cell death and RIPK1 activity was dispensable[177,178]. Since TAK1 also regulates interleukin and toll-like receptor

pathway signaling[171], interfering with the basal activities of these pathways may have contributed to cell death sensitization in this study.

This study discovered that high TAK1 expression correlates with a higher risk of disease recurrence in patients diagnosed with primary melanoma. Since TNF-α is found within melanoma microenvironments[179], TAK1 might contribute to suppressing cell death in situ and could thereby promote disease progression. Of note, TNF-α combined with DNA-alkylating agents can exert anti-tumor activity during the treatment of loco-regionally metastatic melanoma by isolated limb perfusion[180]. This treatment regimen is beneficial to patients but is not systemically tolerated. Suppressing TAK1 activity with specific inhibitors, however, can provide opportunities to improve these therapeutic strategies in the future.

TAK1 presents survival advantages through NF-κB and Mitogen Activated Protein (MAP) kinase signaling. Inhibition of TAK1 sensitizes melanoma cells by interfering with NF-κB and MAP kinase signaling in WM7191C cells. Many chemotherapy agents, genotoxic drugs, and death ligands are well documented to trigger transcriptional factor NF-κB and activate autocrine and paracrine feedback loops[181,182]. A previous study showed that TAK1 prevents apoptotic and necroptotic cell death through both NF-κB-independent and NF-κB-dependent checkpoints[175]. The ablation of TAK1 in melanoma cells caused the loss of TNF-α-induced NF-κB activity by blocking IκBα degradation. These results are additionally supported by earlier findings[183]. For instance, NF-κB activity and associated cytokine production were entirely abolished in TAK1-deficient MEFs[184]. Another study in the TRAIL context revealed that Bay11-7082 (a NF-κB inhibitor) sensitizes TRAIL treatment in mouse stem cells[185]. However, any positive effect of TAK1 depletion was not observed in the TRAIL treatment scenario. The same study additionally showed that MAP3K7 deletion in neutrophils increases NF-κB activity after lipopolysaccharide (LPS) stimulation[186]. A possible explanation for this discrepancy is cell-type specific, where cancer cells and immune cells behave differently. The dual role of TAK1 presents a promising therapeutic option to treat cancer by triggering cell death activity and activating immune cells in the tumor microenvironment.

In addition, TAK1 inhibitors block TNF-α-induced MAP kinase signaling. Prior studies have revealed that TAK1 activates downstream molecules, including p38, ERK, and JNK[187]. TAK1 inhibitor NG-25 and (5z)-7-Oxozeanol (OXO) partially block MAP kinase

signaling in breast cancer, colon cancer, and acute myeloid leukemia cancer cells, respectively[188–190]. In the current study, WM1791C cells were pretreated with OXO before TNF-α stimulation. OXO completely hindered TNF-α-induced phosphorylation of MAP kinase molecules p38, JNK, and ERK, which is in line with previous findings[191]. Contrary to previous findings, however, this study's results suggest that TAK1 function is largely dependent on p38, JNK, and ERK activity. Moreover, p38, JNK, and ERK inhibition by respective inhibitors phenocopied TAK1's inhibition effect on cell viability in melanoma cell lines. In triple negative breast cancer (TNBC), the TAK1-p38 axis facilitated an autocrine positive feedback loop to induce TAK1-activating cytokine expression in tumor cells[192]. Notably, other studies using mice have shown that the p38 inhibitor SB203580 robustly reduces cancer metastasis[193]. However, the TAK1-MAP kinase relation in the melanoma microenvironment context has not been tested. It is not clear whether melanoma tumor cells share a stringent connection with tumor stromal cells. Other studies also reported that stimulating ERK activation conferred tumor resistance in targeted melanoma therapy[135,189]. Like p38, the beneficial effect of TAK1 inhibitor OXO is also caused by inhibiting phosphorylation of ERK1/2. In 293-IL-1RI cell line, OXO inhibits IL-1-induced JNK activation[109]. A similar result was consistently observed in WM1791C cells. These results suggest that OXO selectively inhibits TAK1, thereby inhibiting TNF-α-induced p38/JNK/ERK activation in melanoma cell lines and confirming that TAK1 is profoundly involved in melanoma growth. On the other hand, TAK1 inhibition has a detrimental effect on melanoma proliferation using negative regulation of MAP kinase signaling. Overall, these findings lay the foundation for further investigation of the role of NF-κB and the MAP kinase signaling pathway in melanoma cancer.

5.4 Effect of TAK1 inhibition on cytokine expression

Blocking TAK1 kinase activity with an inhibitor OXO-attenuated N-formyl-methionyl-leucyl-phenylalanine (fMLF) induced cytokine and chemokine levels[194]. In a Luminex-based ELISA array, the data posed similar findings, where TNF-α-induced pro-inflammatory cytokines and chemokines were downregulated to avoid systemic inflammation in melanoma cancer. As previously described, OXO profoundly suppresses NF-κB activity and, as expected, the tested cytokines and chemokines in our experimental settings. In a TAK1-deficient mouse model, however, circulating

levels of cytokines and chemokines were up-regulated with no stimulation[195]. These results conflict with the present findings and allude to differential behavior of *in vitro* versus *in vivo* models. A nearly decade-old study observed that depletion of TAK1 in normal skin causes hyperinflammation[196,197]. In the present findings, it is concluded that the regulation of cytokine and chemokine secretion patterns differs based on cell lines. Of note, caspase inhibitor zVAD-fmk and RIPK1 kinase inhibitor Necrostatin-1 did not change phosphoprotein levels. In conclusion, inhibition of TAK1 may play a role in the control of systemic inflammation in melanoma.

Since TNFα can be found within melanoma microenvironments[179], elevated expression of TAK1 in melanoma cells could contribute to preventing TNFα-induced apoptotic signaling and may additionally promote migration and invasion through other signaling pathways, as illustrated in breast and colon cancers[198]. One of my colleagues, Cristiano Guttà, therefore studied how the mRNA expression of *MAP3K7* related to disease progression in melanoma in a cohort of n = 102 patients diagnosed with primary melanoma, for which dates of sample procurement and sufficient follow-up information were available. The study discovered that high TAK1 expression correlates with a higher risk of disease recurrence in patients diagnosed with primary melanoma. Consequently, low MAP3K7 mRNA expression could be a marker of better prognosis for primary melanoma patients (Podder et al., 2019, Cell Death and Differentiation)[199]. Prior studies have reported that *MAP3K7* is deleted in pediatric T-lymphoblastic leukemia and accelerated cell proliferation[200]. In addition, a study reported that co-inhibition of PAK7, MAP3K7, and CK2α kinases reduced the growth of MiaPaCa2 pancreatic cancer cell xenografts[201]. In contrast, TAK1 (the protein name of *MAP3K7*) is lost in high-grade human prostate cancer. Using immunohistochemistry staining, researchers have found that TAK1 expression is progressively lost with increasing Gleason grade during hypermethylation of the MAP3K7 promoter[185]. The opposite results were found in a metastatic melanoma patient cohort, illustrating that TAK1 follows distinct mechanisms in primary versus metastatic cancer.

In summary, this study reveals that inhibition of TAK1 expression induces RIPK1-dependent cell death in melanoma. Thus, suppressing TAK1 activity with specific inhibitors could provide opportunities to improve anti-tumor therapeutic strategies in the future.

6. Concluding remarks and future perspective

Collectively, the current study discovered that IAPs antagonist potentiates the beneficial effect of TNF-α and TRAIL-based therapy in melanoma cancer. Although TRAIL has capability to induce apoptosis in tumor cells, however, the results from TRAIL using clinical trials have been disappointing, showing little antitumor efficacy. This study suggests that, on a subset of melanoma cell lines, a small molecule IAP antagonist could sensitizes TNF-α and TRAIL responsiveness. Recently, various IAPs inhibitors have undergone clinical investigation either as monotherapy or in combination with therapeutic candidates. The success of a clinical trial could open a new avenue of melanoma treatment and overall cancer treatment.

Additionally, while melanoma cell lines do not undergo necroptotic cell death, some are potentially able to with a moderate amount of RIPK1/RIPK3/MLKL. These findings highlight the point that there may be candidates responsible for regulating necroptosis other than the most commonly studied necroptotic molecules. Therefore, based on the present research, this study proposes more intense research to discover new regulators of necroptosis. It also suggests the development of a novel necroptotic inducer that can directly target MLKL and is able to induce necroptosis.

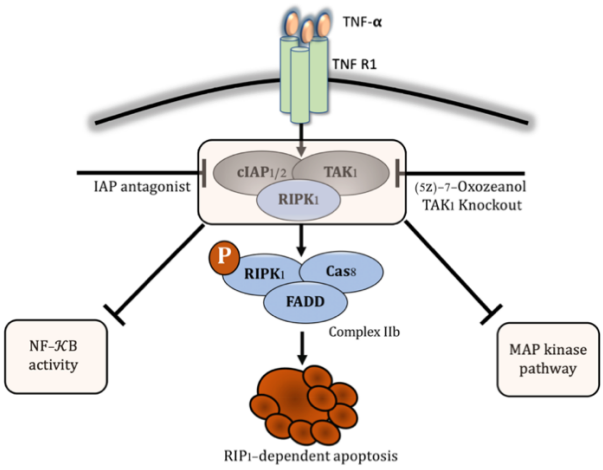


Figure 25: Proposed working model of the role of TAK1 inhibition in melanoma cancer cells. Inactive cIAPs and TAK1 block (i) NF-κB activity and (ii) activation of the MAP kinase signaling pathway. Consequently, inhibition of cIAP1/2 and TAK1 induces RIPK1-dependent apoptosis through assembly of the cytosolic death complex IIb.

The results of this study demonstrate that TAK1 exclusively suppresses susceptibility to RIPK1-dependent cell death (Figure 25) and that high expression of TAK1 indicates an increased risk for disease progression in melanoma. Since TAK1 inhibition leads to

TNF-α/IAPs antagonist-induced cell death, this could provide a way for medical practitioners to stratify melanoma cancer patients based on TAK1 status. However, this study only reports that the inhibition of TAK1 showed a predominant effect *in vitro*. The observation of the same findings *in vivo* will help provide further understanding of TAK1's role in melanoma cancer. Additionally, this study did not test how TAK1 contributes to tumor microenvironment modulating immune cells. Though various TAK1 inhibitors have been developed over the years, none of them underwent clinical trials, due to systemic toxicity [202]. It is also important to avoid undesirable effects, considering the pathological impact of TAK1 inhibition in the liver, which can include hepatic injury, inflammation, and fibrosis [202]. Therefore, it is imperative to establish clinically tolerable and effective small molecules to recap the benefits of this preclinical outcome. Information on how TAK1 regulates cancer tissue is lacking. Thus, the establishment of immunohistochemistry for activated TAK1 in the tissue would offer new insight into its pathophysiological functions in cancer. Addressing the role of TAK1 in human disease remains a significant challenge and requires the establishment of selective and sensitive molecular markers in TAK1-based therapy. Ultimately, the current data give rise to the hope to develop direct therapeutic targets of TAK1 in combination with the TNF-α/IAP antagonist for the treatment of melanoma cancer.

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7. References

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