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The role of RIPK1-dependent cell death in the regulation of the immune response

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1.Introduction

In recent years, several apoptosis-independent cell death pathways have been described. It is known that the immunological outcome of cell death processes may be different. In addition to generally tolerogenic apoptosis, cell death pathways that induce inflammation but also activate the adaptive immune response are known. In this t, we studied receptor interacting protein kinase-1 (RIPK1)-dependent cell death processes, inflammation-inducing necroptosis, and apoptosis.

The initial steps of innate immunity are primarily coordinated by macrophages and dendritic cells. The differentiation of macrophages is determined by their tissue microenvironment, which can lead to the formation of pro-inflammatory (M1) and anti-inflammatory (M2) subtypes. The balance of cell populations can be upset in the pathogenesis of diseases such as infections, tumors, chronic inflammatory or autoimmune reactions. Several clinical trials in these conditions aim to change the M1/M2 ratio. In the doctoral work, we compared the susceptibility of human monocyte-derived M1 and M2 macrophages to necroptosis. Necroptosis occurs via the RIPK1, RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL) signaling pathways, which are inhibited by both ubiquitination and phosphorylation of RIPK1. In order to induce necroptosis, we used inhibitor of apoptosis (IAP) antagonist, which inhibits ubiquitiantion of RIPK1, or transforming growth factor-β-activated kinase inhibitor, which inhibits the phosphorylation of RIPK1. Our results suggest that M2 cells are significantly more sensitive than M1 cells to TAK1 inhibitor-induced necroptosis, allowing the balance of the two subpopulations to be regulated.

Another significant cell population for innate immunity is made up of dendritic cells (DCs). Professional antigen presenters that continuously scan their microenvironment and collect samples from dead, tumor, or virus-infected cells by phagocytosis. Antigens are delivered to the lymph nodes and presented to naive T cells. In addition to antigen presenting and T cell activating properties, DCs are also known to have cell killing function. In our doctoral work, we investigated how the cytotoxic ability of human monocyte-derived DCs (moDC) at pattern recognition receptors is altered by PRR stimuli. We have shown that cytotoxicity is generally associated with DCs found in immature peripheral tissues. The supernatant of activated DC induces RIPK1-dependent immunogenic cell death, thus allowing DCs to monitor the tissue environment and facilitate the presentation of intracellular pathogens as well as tumor antigens.

1.1 The role of DC cytotoxicity in the immune response

DCs provide the connection between the two functional units of the immune system, the natural and the adaptive immune system. Immature DCs, which migrate continuously between tissues, are professional phagocytes and antigen-presenting cells, and as elements of the innate immune system, are able to recognize various microbes primarily through PRRs. Following antigen uptake, they migrate from peripheral tissues to the lymph node, while they differentiate into mature DCs, and present the processed antigens to T cells. Accordingly, the main function of immature DCs is phagocytosis and that of mature DCs is the presentation of antigens. In mature DCs, the expression of the cell surface costimulator required to activate T lymphocytes and the molecules involved in antigen presentation is enhanced compared to immature cells. By phagocytosis of dead cells, antigens from various cells can also enter the DCs, so that proteins from microbes released from tumor cells or from microbes incapable of infecting professional antigen-presenting cells can be important sources of both major histocompatibility complex II (MHC-II) and for presentation on MHC-I molecules.

DCs are known in the literature for their cytotoxic effects in addition to their antigen presenting and T cell activating functions. The cytotoxic ability of DC is mainly related to the immature phenotype localized in tissues, whereas mature cells migrating to the lymph node are less cytotoxic, which was confirmed by testing both blood-isolated and in vitro differentiated DCs. However, the molecular background underlying DC cytotoxicity and the exact physiological function of their cell killing ability are not yet clear. This cytotoxic effect is particularly unexpected given that mature DCs that are activated upon recognition of the pathogen migrate to the lymph node, so they may not be able to kill large amounts of infected cells at the periphery. The cytotoxic effect can be induced by DCs via soluble factors such as tumor necrosis factor α (TNF), Limphotoxin α1ß2, FasL, or TNF-related apoptosis inducing ligand (TRAIL). This function of DCs has been described as a collective effect of soluble ligands as well as through direct cell-cell contact.

The cytotoxic properties of DCs allow them to act as regulators of the immune response. On the one hand, they are able to inhibit the expansion of certain lymphocytes in aged or autoreactive lymphocytes. DCs can be considered as effector cells of innate immunity and have also been shown to kill tumor target cells. Furthermore, for immature DCs generated in vitro or

circulating in the blood, killing of target cells may allow rapid phagocytosis and cross-presentation of antigens from apoptotic cells to cytotoxic T cells.

1.2 Heterogeneity of macrophages

Macrophages play a key role in defense against infection, removal of damaged or apoptotic cells, and regeneration following tissue injury. In addition to their well-known phagocyte function, they are involved in maintaining homeostasis and regulating the immune response; through the presentation of antigens, the production of cytokines and other biologically active molecules, and through the regulation of T cells. Macrophages can play different roles in space and time, with their functional properties depending not only on their origin but also on molecular signals from the tissue environment. They may undergo reversible changes in different tissue environments driven by local cytokine and PRR stimuli. The plasticity of macrophages driven by appropriate stimuli ensures their dynamic role in the formation and elimination of inflammatory processes.

1.3 The role of M1 and M2 macrophage subpopulations in the immune response

Two well-defined polarization states of macrophages are known, the group of macrophages with the classically activated M1 and alternatively activated M2 phenotypes, which produce different factors, have different biological functions, and have cell surface markers. M1 cells provide the first line of defense for the immune system, activating both innate and adaptive immunity. M2 macrophages are responsible for the regulation of tissue regeneration, are involved in the clearance of apoptotic bodies, and also contribute to the development of immunosuppression. Recent research, however, suggests that the integration of different microenvironmental conditions is able to generate the full spectrum of polarization states between M1 and M2.

1.4 Tumor-associated macrophages

One of the largest populations of immune cells infiltrating the tumor microenvironment is comprised of monocytes/macrophages. Solid tumors are characterized by the recruitment and reprogramming of monocytes or macrophages, resulting in the formation of tumor-associated macrophages (TAMs). TAMs promote tumor growth and progression by enhancing angiogenesis

and inducing growth factor production and immunosuppression. The weak antigen presenting ability of TAMs, the secretion of IL-10 and TGFβ, and their ability to activate regulatory T cells (Treg) also contribute to the development of an immunosuppressive environment.

1.5 The effect of cell death on the immune system

In the human body, billions of cells die every day through various cell death pathways to which the immune system can respond in a number of ways. Based on their effect on the immune system, we can distinguish between tolerogenic, inflammatory and immunogenic types. The course of these cell death processes is controlled by different molecular pathways that affect the functioning of the natural and adaptive immune systems differently.

1.6 Apoptosis

Several pathways of apoptosis have been described, each of which is a function of activation of the initiator and executive caspases. Morphologically, apoptotic cells perform a characteristic sequence: the dying cell moves away from the surrounding cells, terminates its contact with the extracellular matrix, and then rounds off. Protrusions form on the cell membrane, chromatin condenses, and then DNA is fragmented and disrupted into 200-250 base pair sections. Eventually, apoptotic bodies are formed that contain cellular components flanked by a membrane. Cell content is not excreted into the extracellular space, accordingly, apoptosis is a regulated cell death (RCD) that usually has a tolerogenic outcome. However, contrary to our prior knowledge, apoptosis can be immunogenic under certain conditions, inducing the adaptive immune system. According to some publications, the active mechanisms of apoptotic cell death may contribute to the immunogenicity of dying cells. Such a mechanism could be, for example, the formation of neoepitopes revealed by the active function of caspases, which are known to be "preferred" during cross-presentation.

1.7 Possible outcomes of TNFα signaling

TNFα, TRAIL, Fas ligand are all members of the tumor necrosis superfamily, and their receptors consist of an extracellular ligand binder, a transmembrane, and an intracellularly located DD. The activation of DRs is triggered by receptor oligomerization following specific

ligand binding, which initiates the signaling cascade. The signaling of the TNF receptor (TNFR) is best known in the literature.

Upon binding to the TNFα receptor, TNFR trimerizes and changes its conformation, resulting in the release of its inhibitory protein, the silencer of death domain (SODD), from the DD. TRADD binds to a receptor released from inhibition, which recruits RIPK1, TNF receptor associated factor 2 (TRAF2), and TRAF5 proteins. TRAF2 binds the ubiquitin ligases IAP1 and IAP2 to the complex and also the LUBAC protein complex (HOIL-1, HOIP, and Sharpin). IAP1 and IAP2 ubiquitinate (forming a K63 polyubiquitin chain that facilitates protein-protein interactions) RIPK1 at its lysine 377. In addition, LUBAC attaches additional linear ubiquitin chains to K63 polyubiquitin, to which the NEMO adapter protein can bind. Polyubiquitin chains and NEMO, in turn, allow the recruitment of the IKK complex (IKKα / β, TBK1 / IKKε) and TAK1, TAB2, and TAB3 molecules, which activate the phosphorylation steps of downstream signaling pathways (NF-κB and MAPK). The resulting protein complex (type I complex) mediates several survival signals in addition to cell activation. On the one hand, by activating transcription factors (NF-κB and MAPK), it results in the expression of anti-apoptotic proteins (FLIP, IAP), and on the other hand, TAK1 kinase - and its downstream substrates - inhibit RIPK1-mediated cell death signals through phosphorylation of RIPK1. Thus, RIPK1, as an enzymatically inactive member of the complex, contributes to TNFα-induced cell activation as a scaffold protein.

Another possible consequence of TNFR activation is the occurrence of cell death processes. If TNFα-induced survival signals are not inhibited, Complex I dissociates from TNFR1 and the long isoform of TRADD, FADD, FLICE-like inhibitor protein (FLIPL) and pro-caspase-8 proteins in the cytosol. II.a complex (TRADD, FADD, RIPK1, caspase-8, FLIPL) is formed. In this complex, procaspase-8 is oligomerized, cleaved, and activated, leading to apoptotic cell death. Furthermore, FLIPL and pro-caspase-8 heterodimers formed during complex assembly, or active caspase-8 homodimers, cleave RIPK1 and RIPK3. Thus, they inhibit the onset of necroptosis and ensure the dominance of tolerogenic apoptosis over necroptotic cell death.

When RIPK1 enters the deubiquitinated state, the composition of complex II changes, it is called complex IIb. Deubiquitination of RIPK1 can occur on the one hand by activation of CYLD or A20 deubiquitinase, or by inhibition or absence of IAP proteins that ubiquitinate RIPK1. In

complex IIb, instead of TRADD, RIPK1 binds FADD and pro-caspase-8 proteins, at which point RIPK1-dependent apoptosis occurs. Another possible outcome of TNF signaling is the development of necroptosis, a prerequisite for which is the inactive, inhibited state of caspase-8. In TNF-induced necroptosis, RIPK1 forms a complex with RIPK3 (complex IIc) as described in the previous chapters.

In addition to ubiquitination, RIPK1 activity is also modified by phosphorylation / post-translational changes. In addition to phosphorylation events that promote necroptosis, several phosphorylation steps that inhibit RIPK1-dependent cell death are known, such as RIPK1 phosphorylation by TAK1, TBK1, and IKKε.

1.8 Inhibitor of apoptosis proteins (IAPs)

The IAP family of proteins is involved in the regulation of various cellular processes, including cell death, immune and inflammatory responses, cell proliferation, differentiation, and motility. To perform these processes, IAPs interact with caspases, regulators of the NFκB pathway, elements of the actin cytoskeleton, and also transcription factors.

Members of the IAP family may bind / recognize the UBA domain of K63 or, in some cases, polyubiquitin chains linked at position K48 (IAP1). When ubiquitins bind to each other through the K48 position, degradation of the target protein occurs. However, when ubiquitins at position K63 are linked, various processes, protein-protein interactions, cytoplasmic transport, translation, or DNA repair may be activated instead of proteasomic degradation.

The RING domain at the C-terminus of IAPs functions as E3 ubiquitin ligase (E3, the last member of the ubiquitin conjugating enzyme family). The RING domain allows the association of ubiquitin-carrying E2, previously activated by E1, with the target protein and promotes the transfer of ubiquitin to the target protein. Due to their ubiquitin ligase properties, IAPs are able to poly-ubiquitinate proteins (e.g., Second mitocondria-derived activator of caspase (SMAC), TRAF, caspase enzymes) at position K63 and other IAP proteins and themselves at position K48. Auto-ubiquitination thus induces their prosthetic degradation, which ensures a relatively constant level of IAPs in the cells.

1.9 SMAC mimetics, Birinapant

Artificially produced variants of mitochondrial SMAC proteins are SMAC mimetics, also known as IAP antagonists.

Birinapant is a bivalent SMAC mimetic that, in addition to binding to the XIAP-BIR domain, is able to displace activated caspases from interaction with XIAP. Among the members of the IAP family of proteins, it induces the degradation of IAP1 and IAP2 in cells. Birinapant treatment deubiquitinates RIPK1 to form the RIPK1-caspase-8 protein complex, which allows activation of caspase-8.

1.10 The role of TAK1 in TNFR-induced signaling

TGF-β activated kinase 1 (TAK1), a member of the MAPKKK family, is involved in the regulation of the natural and adaptive immune systems and in the control of inflammatory processes.

TAK1 has an inhibitory effect on caspases and the initiation of apoptosis. Following TNF stimulation, TAK1 is known to reduce complex II formation and/or activation. Recent studies have described several mechanisms through which TAK1 may protect cells from TNFα-induced apoptosis. TAK1 is an upstream kinase of NF-κB that induces the transcription of anti-apoptotic proteins such as FLIP and IAPs in this transcriptional pathway. In addition, TAK1 phosphorylates RIPK1 directly or indirectly through TAK1-activated kinases. This phosphorylation leads to inhibition of RIPK1.

1.11 Immunogenic cell death

Immunogenic cell death (ICD) can elicit an inflammatory response to stress that results in the activation of adaptive immunity and the development of long-term immunological memory.

Phagocytosis of dead cells by DCs and cross-presentation of ingested antigens are also required for cytotoxic T cell activation. The type of cell death greatly influences the cross-representation ability of DCs. The adjuvant ability and antigenicity of dying cells depends on the properties of cell death and stress-inducing stimuli, whereas the immunogenicity of RCD is ultimately a function of the microenvironment of dying cells.

Regardless of the inducing stimulus, a unique feature of the ICD cell death form is its ability to activate the adaptive immune response. In the process, the antigens can be exogenous

released by dead cells or endogenous antigens from a dying cell. ICD is characterized by the release of different types of DAMPs and the development of antigen-specific immunological memory.

Through these DAMPs, ICD eliminates the immunosuppressive tumor microenvironment by enhancing antigen presentation and phagocytosis, costimulatory expression, and cytokine production in both tumor and antigen presenting cells after tumor cell death. As a result, it is able to induce activation of tumor antigen-specific T cells.

1.12 Immunogenic effect of RIPK1-dependent cell death on the immune system

The process of cross-representation allows DCs to display exogenous, phagocytic antigens, such as tumor-derived or viral proteins, through MHC-I. In initiating an anti-tumor immune response, immunogenic cell death is the most effective form of cell death that generates antigens.

RIPK1-dependent signaling triggers both cell death and NF-κB pathways. This, together with the inflammatory processes in dying cells, mobilizes the immune system against tumor cells. Dead cells release DAMPs, chemokines, and also cytokines, which activate immune cells recruited into the microenvironment of the dying cell. DCs present peptides from the dead cell through a cross-presentation pathway as a result of cytokine stimuli. However, little is known about exactly how immunogenic cell death affects the cross-representation ability of DCs to trigger CD8 + T cell responses.

2. Objectives

More accurate knowledge and regulation of transitions between cell death types with different immunological roles may contribute to the development of new therapies to help eliminate tumors or treat inflammatory diseases.

Our objectives included, among others, examining the following:

• When different stimuli are applied, does cell death occur in the same manner in M1 and M2 cell populations?

• What type of cell death is induced in M1 and M2 macrophages by TAK1 inhibitor and SMAC mimetic?

• What type of cell death is induced in TAM-like cells by TAK1 inhibitor and SMAC mimetic?

DCs are known to be cytotoxic under certain conditions[6]. Our objectives included examining the following:

• Can the supernatant of DCs induce cytotoxic effects on different cell lines?

• Is the cytotoxic effect of the supernatant of mature and immature DCs different?

• What cell death receptors mediate the cytotoxic effect?

• To what extent does the presence or absence of RIPK1 protein affect cell death induced by the supernatant?

3. Materials and methods

3.1 Monocyte separation from human buffy coat

In our experiments, we worked with human monocyte-derived dendritic cells (moDC) and monocyte-derived macrophages. The buffy coat was diluted with physiological saline and after that was layered on a 1:2 Ficoll-Paque Plus solution and subjected to a gradient-based centrifugation to separate peripheral mononuclear cells. After the washing steps, $CD14^+$ monocytes were separated by a positive separation method. For this separation the paramagnetic bead-conjugated anti-CD14 antibody (Miltenyi Biotec) was used. The labeled cells adhered to the special separation column in the strong magnetic field, from which the monocytes were eluted with a syringe into a sterile centrifuge tube.

3.2 Differentiation of human monocytes to dendritic cells

Isolated monocytes differentiated to resting DCs for 5 days (37 °C, 5%, CO2) in serum-free AIM-V medium in the presence of 100 ng/ml IL-4 (PeproTech) and 80 ng/ml GM-CSF (Gentaur Molecular Products*)* at a cell density of 2.0 x 10^6 cells/ml in 6-well tissue culture plate in AIM-V media (Thermo Fischer Scientific). GM-CSF and IL-4 required for differentiation were replaced on day 3.

3.3 Differentiation of human monocytes to IFN dendritic cells

Isolated monocytes differentiated to resting DCs for 5 days (37 °C, 5%, CO2) in serum-free AIM-V medium in the presence of 40 ng / ml GM-CSF and 100 U / ml rhIFN-α (Roferon-A) at a cell density of 2.0x10^6 cells /ml in 6-well tissue culture plate. GM-CSF and rhIFN-α required for differentiation were replaced on day 3.

3.4 Differentiation of human monocytes to dexamethasone dendritic cells

Isolated monocytes differentiated to resting dendritic cells in serum-free AIM-V medium for 5 days (37 ^{0}C, 5%, CO2) in the presence of 100 ng /ml GM-CSF, 20 ng/ml IL-4, and 0.25 μM dexamethasone (Sigma-Aldrich) at a cell density of 2.0 x 106 cells/ml in 6-well tissue culture plates. GM-CSF, IL-4, and dexamethasone required for differentiation were replaced on day 3.

3.5 Collection of dendritic cell supernatants (moDC, IFN DC, dexa DC)

On day 5 of differentiation, DCs were treated with CL075 (1 µg /ml, Sigma) or LPS (0.5 µg/ml, InvivoGen) for 30 minutes. To remove TLR ligands, cells were washed three times (RPMI 1640) and incubated at 4.0×10^6 cells /ml DC for 2, 8, or 24 hours in serum-free RPMI 1640 medium. Supernatants produced by DCs (moDC, IFN DC, dexa DC) were collected by centrifugation at 9500 relative centrifugation force (rcf)/minutes for 10 minutes at 4 °C.

3.6 Differentiation of human monocytes to mature dendritic cells

On day 5 of differentiation, DCs were treated with LPS (100 ng/ml). After 24 hours, LPS pretreated DCs (LPS-DC) were conditioned with TLR ligands for 30 minutes, and after three washing steps, 4.0×10^6 cells/ml DC were incubated for 2 hours in serum-free RPMI 1640 medium. The supernatant produced by the DCs was collected by centrifugation at 9500 rcf/minutes for 10 minutes at 4 °C.

3.7 Generation of monocyte-derived M1, M2 macrophages and TAM-like cells

Isolated monocytes were cultured for 5 days in 6-well tissue culture plates at a density of 2.0×10^6 cells/ml in Gibco's serum-free AIM-V medium (Thermo Fischer Scientific) supplemented with 50 ng/ml M-CSF (PeproTech). In order to acquire the M1 and M2 types, cells were stimulated on the fifth day of differentiation for 24 hours with lipopolysaccharide (50 ng/ml ultrapure LPS, InvivoGen), IFNγ (20 ng/ml, PeproTech) to M1 and IL-4 (20 ng/ml, PeproTech), IL-10 (20 ng/ml, PeproTech) and TGFß (20 ng/ml, PeproTech) to M2 phenotype. For the differentiation of TAM-like cells, isolated monocytes were cultured for 5 days in 6-well tissue culture plates at a density of 2.0×10^6 cells/ml in Thp-1 supernatant supplemented with IL-4 (20 ng/ml), IL-10 (20 ng/ml) and TGFß (20 ng/ml). On the fifth day TAM-like cells were treated again with Thp-1 supernatant for 24 hours.

3.8 Cell lines

SVT35 Jurkat and HT-29 cell lines were cultured in RPMI 1640 medium in the presence of 10% FCS (Gibco), 2 mM L-glutamine (Biosera) and 40 mg/l Gentamicin (Sigma-Aldrich) (37 °C, 5%, CO2). The THP-1 cell line was cultured in serum-free AIM-V medium (37 °C, 5%, CO2). HUVECs cells were cultured in M199 (Gibco) medium containing 20% FCS, 2 mM L-

glutamine, 1% penicillin/streptomycin (Gibco), EGMTM-2 Endothelial Single QuotsTM Kit (Lonza) (37 ºC, 5%, CO2).

3.9 Production of THP-1 supernatant

To generate THP-1 supernatant, cells were cultured at a density of 2×10^5 cells/ml in Gibco's serum-free AIM-V medium (Thermo Fischer Scientific) for 2 days and the supernatant was collected at 2000 rcf/minute for 5 minutes.

3.10 Co-cultures of M1-M2 cells

To test the functional importance of differences in cell death intensity between M1 and M2 cells, a macrophage co-culture was created. An equal number of M1 and M2 macrophages, 4×10^5 cells/ 0.5 ml were co-cultured for 4 hours in 24-well tissue culture plates. Mixture of M1 and M2 cells were treated with necroptotic stimuli. The percentage of surviving macrophages was labeled with anti-CD209-PE and anti-CD80-FITC antibodies in 24 hours. To distinguish between M1 and M2 cell death, M2 macrophages were stained using CellTracker™ Green CMFDA Dye (Thermo Fischer Scientific). M2 cells were loaded with 10 ng/ml CellTracker™ Green CMFDA Dye at 37 ^{o}C for 30 minutes. After intense washing, $4x10^5$ cells in 0,5 ml volume were co-cultured with unlabelled M1, in the same concentration in 24-well tissue culture plates. M1 and M2 co-cultures were treated with necroptotic stimuli and the cell death of the different populations was determined after 24 hours by PI staining.

3.11 ELISA (Enzyme-linked immunosorbent assay)

The supernatants of M1 and M2 macrophages were harvested on the sixth day of differentiation. The supernatant of DCs were collected on days 5 of differentiation and measured by ELISA. The concentration of TNFα, IL-6, IL-12p70 and IL-10 cytokines were measured using OptEIA kits (BD Biosciences) following the manufacturer's instructions.

3.12 Measurement of caspase-3/7 enzyme activity

SVT35 Jurkat cells were incubated with supernatants of moDC or in the presence of 20 ng/ml TNFα for 24 hours at 37 °C. Caspase 3/7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega) following the manufacturer's instructions.

3.13 Immunoprecipitation

SVT35 Jurkat cells were incubated with supernatants of moDCs or in the presence of TNFα and SMAC mimetic (Birinapant) for 1 hour (37 ° C), followed by 1% Triton-X 100 (Sigma-Aldrich) and 1% protease inhibitor cocktail (Sigma -Aldrich) was suspended in lysis buffer and incubated for 30 minutes (4 °C). The cell lysate was collected after centrifugation (12.350 rcf, 10 minutes, 4 °C) and mixed with anti-caspase-8 antibody-coated Protein G beads (GE Healtcare) (overnight, 4 °C).

The next day, the beads were washed four times (lysis buffer) and resuspended in 2X Laemli buffer. Protein samples were linearized (5 minutes, 95 °C) and run on SDS-PAGE gel. The RIPK-1-caspase-8 interaction was detected by anti-RIPK1 western blot. The development of the interaction was also confirmed by anti-caspase-8 western blot following immunoprecipitation with Protein G beads coated with anti-RIPK1 antibody.

3.14 Flow cytometry

Fluorescent intensity was measured by flow cytometry using FACS Calibur (BD Biosciences), and data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

3.14.1 Investigation of the cell surface molecules

The following cell surface labels were measured: CD209-PE (DC-SIGN, BioLegend), CD206-phycoerythrin cyanine^{TM5} (Pe-Cy^{TM5}, BD Pharmingen), CD80-FITC (SONY Biotechnology), CD14-PE (BioLegend), HLA-DR- peridine chlorophyll protein (PercP, BD Pharmingen), PD-1/CD279-PercP (BioLegend), CD163-PE (Biosciences).

Labeling with antibodies were performed in each case as follows: the directly labeled antibody was added to the cell pellet and incubated for 20 minutes at 4 ° C in the dark. The cells were then washed with FACS buffer (5 minutes, 1500 rpm, 4 °C). Cell surface protein expression was measured on 10000 cells.

3.14.2 Cell death measurement

3.14.2.1 Sub-G1 measurement

DNA fragmentation was determined by sub-G1 measurement. After activation, cells were fixed in 70% alcohol at 4 °C for 1 hour and then washed with citrate buffer (38 mM, pH 7.4). Cells were labeled in citrate buffer containing 50 μg/ml propidium iodide (PI, Sigma-Aldrich) for 15 minutes at 37 °C.

3.14.2.2 Membrane integrity measurement

Total cell death was quantified based on the loss of membrane integrity and the uptake of propidium iodide (PI, Sigma-Aldrich). Cells were stained with PI (10 μg/ml) before analysis by flow cytometry.

3.15 Western blot

Protein extraction was performed by lysing the cells in 2X Laemmli sample buffer. Proteins were separated by SDS gel electrophoresis using 10% polyacrylamide gels and transferred onto nitrocellulose membranes ER (Bio-Rad Laboratories). Nonspecific binding was blocked by TBS-Tween with 5% non-fat dry milk. Transfer membranes were immunoblotted with the indicated antibodies: RIPK1 (BD Biosciences), RIPK3 (Cell Signaling), MLKL (Sigma-Aldrich), pMLKL (Cell Signaling), TAK1 (Cell Signaling), p38 (Thermo Fischer Scientific), pErk (Thermo Fischer Scientific), pJNK (Thermo Fischer Scientific), pIκß (R&D Systems) and β-actin (Sigma-Aldrich) all diluted 1:1000. Anti-rabbit (GE Healthcare), anti-mouse (GE Healthcare) or anti-rat (Sigma-Aldrich) antibodies conjugated to horseradish peroxidase were used as the secondary antibodies.

3.16 Statistics

Two-way ANOVA or one-way ANOVA, followed by *Sidak's multiple comparisons test* was used for multiple comparisons. The results are expressed as mean +SD. All analyses were performed by using GraphPad Prism software, version 6.0. Differences were considered to be statistically significant at $P < 0.05$. Significance is indicated by *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

4. Results

In the first phase of the doctoral dissertation, we compared the susceptibility of human monocyte-derived M1 and M2 macrophages to necroptosis.

4.1 M2 macrophages, but not M1 cells are sensitive to TAK1 inhibitor-induced necroptosis

To confirm the polarization of subtypes, we analyzed cell surface marker expression on the differentiated macrophages. We detected the induction of CD80 on M1 cells, and higher expression level of protypical M2 markers CD206 and CD209 on M2 cells. We also checked the functionality of the two cell populations by measuring the production of M1 and M2 related cytokines. According to widely used protocols, M1 cells released significantly higher amounts of IL-12 than the M2 population, but IL-10 production was more relevant in M2 macrophages.

To check the susceptibility to cell death stimuli of polarized M1 and M2 macrophages, we treated these cell types with various apoptotic and necroptotic activators. We used LPS, TNF, SMAC mimetic (birinapant), TAK1 inhibitor (5Z-7-oxozeaenol; 5Z-7) and the combinations of these treatments as apoptotic triggers. In the presence of a caspase inhibitor (Z-VAD), all these stimulants are well known activators of necroptosis in macrophages. All the investigated stimuli induced cell death at the same level in both cell populations, but TAK1 inhibitor induced necroptosis was significantly higher in M2 cells To confirm this result, we studied the dose dependence of birinapant- and 5Z-7-mediated cell death. 5Z-7 induced cell death was more intense in M2 cells than in M1 macrophages under caspase compromised conditions at all investigated doses. Under the same experimental settings birinapant treatment did not result in higher cell death intensity in M2 than in M1 macrophages at any dose. Birinapant-induced cell death was even slightly, but non-significantly, more intense in M1 cells.

We checked whether the detected cell death in caspase inhibited conditions is due to necroptosis. For this, we pretreated the polarized macrophages with a specific RIPK1 inhibitor Necrostatin-1 (Nec-1) and RIPK3 inhibitor (GSK′872), we observed that Nec-1 and GSK′872 and blocked birinapant/Z-VAD (BZ)-induced cell death equally in the two cell types. In case of M2 cells, Nec-1 and GSK′872 also inhibited the 5Z-7/Z-VAD-induced cytotoxicity..

We also analyzed the phosphorylation of MLKL following BZ and 5Z-7/Z-VAD activation as a characteristic marker of necroptosis. We detected phospho-MLKL upon BZ activation in

both M1 and M2 cells, but its phosphorylation occurred only in M2 macrophages following 5Z-7/Z-VAD stimulation.

The question arose as to whether the autocrine TNFα production of functionally different M1 and M2 macrophages may underlie the different sensitivity of cell death in the two populations. TNF-R1: Fc fusion protein only partially, but not significantly inhibited 5Z-7+Z-VAD or SM+Z-VAD -induced necroptosis, indicating that other mechanisms than autocrine TNF production may also play a role in 5Z-7+Z-VAD or SM+Z-VAD induced necroptosis in human macrophages. Based on this result, we hypothesize that in addition to autocrine TNFα production, other mechanisms may play a role in OZ/BZ-mediated cell death.

4.2 Co-culturing M1 and M2 cells does not sensitize M1 cells to TAK1 inhibitor-induced necroptosis

The two different macrophage phenotypes are present simultaneously at the site of chronic inflammation or in the tumor microenvironment. We tested the ability of the two cell types to regulate each other's sensitivity to necroptosis by co-culturing M1 and M2 cells. We loaded M2 macrophages with CellTracker™ Green CMFDA Dye and co-cultured cells were treated with BZ or 5Z-7/Z-VAD for 24 hours. We determined the intensity of cell death in both CellTracker positive and negative populations. Consistent with the results observed with the separately treated cell types, M2 cells were sensitive, but M1 cells remained resistant to 5Z-7/Z-VAD-induced necroptosis. In contrast, BZ treatment effectively killed both macrophage populations in the co-culture. We confirmed this result by measuring the cell surface markers of the surviving cell populations before and after the induction of cell death. 5Z-7/Z-VAD treatment reduced the amount of CD209 positive cells, and consequently pushed the balance of M1/M2 cells toward M1 excess, whilst BZ treatment had no significant effect on the M1/M2 ratio. Overall, the treatment of co-cultured M1 and M2 macrophages with TAK1 inhibitor shifted the balance of surviving cells towards M1 dominance. Based on these results, we can conclude that the difference in the sensitivity of the two macrophage subtypes to necroptosis does not depend on M2-derived cytotoxic or M1-derived survival factors, but is regulated by the intrinsic properties of the two cell subtypes.

4.3 The inhibition of the downstream components of TAK1 signaling induces necroptosis in M2 cells

We attempted to find which downstream component of TAK1 signaling could be responsible for TAK1 inhibitor-induced necroptosis. For this purpose, molecules in TAK1 regulated pathways were inhibited one by SP600125 (JNK inhibitor), SB203508 (p38MAPK inhibitor), U0126 (ERK inhibitor) and TPCA1 (IKK inhibitor). Under caspase compromised conditions all these inhibitors induced cell death in M2 macrophages. When we used suboptimal doses of these inhibitors, we detected more intense cell death in M2 than in M1 cells. All observed differences were significant, except for p38 kinase inhibitors. Caspase activity protected both macrophage subtypes from MAPK or IKK inhibition-induced necroptosis. Combinations of these inhibitors also induced more intense cell death in M2 than in M1 macrophages. Phorbol 12-myristate 13-acetate (PMA) treatments resulted in comparable intense phosphorylation of MAPKs and IκB in M1 and M2 cells, indicating that the observed differences in cell death appear to be due to alterations in the necroptotic pathway, and not due to the availability of MAPK signaling. According to our observations, we concluded that the absence of any survival signals results in the necroptosis of M2 cells under caspase compromised conditions, but presumably M1 macrophages utilize additional survival signals to block TAK1 inhibition-mediated necroptosis

To explore the molecular background behind the different sensitivity of M1 and M2 cells to necroptosis, we checked the protein expression of necrosome components in M1 and M2 cells. However, we could not detect considerable differences in RIPK1, RIPK3 or MLKL expression

4.4 TAM-like macrophages are sensitive to TAK1-inhibitor-induced necroptosis

Because the M2-like TAMs show similar functional properties to M2 macrophages, TAMs were *in vitro* differentiated and the cell death intensity of this TAM-like cells were compared to M1 cells. Isolated monocytes were plated in medium complemented with M-CSF and IL-10, IL-4 and TGFβ and the supernatant of THP-1 cells. *In vitro* differentiated TAM-like cells were characterized by flow cytometry. In good accordance with published data, these cells were CD206 and CD163 positive, but expressed low amounts of CD14 while the appearance of MCHII and PD-1 were more intense on the surface of TAM-like cells compared to M1 and M2 macrophages. The differentiated TAM-like cells were treated with BZ and 5Z-7/Z-VAD to induce necroptosis. We found that TAM-like cells were as sensitive to BZ-induced necroptosis as

M1 cells, but significantly more susceptible to 5Z-7/Z-VAD treatments than M1 macrophages. Sensitivity of TAM-like macrophages to TAK1-inhibitor-induced necroptosis promises to be an effective therapeutic strategy to eliminate immunosuppressive macrophages, while preserving the inflammatory M1 cells in the tumor microenvironment.

In the second phase of the dissertation, we investigated how the cytotoxic capacity of human monocyte-derived DCs (moDC) is altered by PRR stimuli.

4.5 The supernatant of moDCs induce cell death

To demonstrate the ability of DCs to induce cell death, moDCs were activated with ligands of intracellular and cell surface PRRs, namely with CL075 or LPS for 30 minutes. Following activation, ligands were washed out completely and the cells were incubated for an additional 2 hours in fresh serum-free medium. SVT35 Jurkat cells were then activated with the collected supernatants (DCsup) of these short term activated DCs (actDC) for 24 hours. While the supernatants of untreated DCs exhibited minimal cytotoxic activity, supernatants collected after activation with either CL075 or LPS induced intense cell death of SVT35 Jurkat cells as we detected by subG1 peak or caspase 3/7 activity assay .

To investigate whether the supernatants had cell-killing effects on other cells, the cytotoxic effect was also tested on the HT-29 colon-derived adenocarcinoma cell line and on primary HUVECs cells. DCsup-mediated cytotoxicity was not limited to the Jurkat cells, as DCsup also induced significant cell death in the HT-29 cell line and HUVEC. However, the DCs themselves were resistant to the cytotoxic effect induced by DCsup.

Our results suggest that the cytotoxic function of DC is not T cell/Jurkat selective, both HT-29 and HUVECs cells were significantly destroyed by LPS or CL075-treated DC supernatant.

Next, we compared the effects of supernatants of actDCs and LPS preconditioned DCs to determine whether the cytotoxic ability of DCsup is specific to the short term activated DC phenotype alone or also to LPS preconditioned DC. The supernatant of LPS preconditioned DCs induced less efficient killing of Jurkat cells than that of actDCs. These results indicate that DCs acquire cytotoxic ability after PRR-induced short term activation.

4.6 Supernatant of short term activated DCs induces cell death in a TNF-dependent manner

We monitored the role of each DR in the cytotoxic effect of DCsup using soluble death ligand antagonists. The actDC supernatant was pre-treated with TNF:Fc, TRAIL:Fc or Fas:Fc to neutralize the possible ligands in the supernatant. While the TNFR1:Fc fusion protein was able to block DCsup-induced killing, the specific inhibitors against FasL or TRAIL did not prevent cell death at the concentration at which they were able to block recombinant FasL or TRAIL-induced killing. MoDCs were activated with CL075 or LPS for 30 min, and supernatants of DCs were collected after 2 and 8 or 24 h, respectively. All these supernatants were cytotoxic to Jurkat cells and TNF-α alone inhibited this effect. FasL or TRAIL antagonists were unable to block cell death regardless of how long the supernatant was produced. Supporting the observed TNF-dependent cytotoxicity, we detected significant TNF-α production by DCs after CL075 or LPS pre-activation. These observations suggest that PRR activation-induced TNF-α production is responsible for short-term activated DC-mediated cytotoxicity.

4.7 RIPK1 is required for DCsup-induced cell death

We examined whether RIPK1 was also required for DCsup-induced cell death. Therefore, we compared the susceptibility of Jurkat cells and their RIPK1 deficient subclones to DCsup-induced cytotoxicity. Treatment with the supernatants of both CL075- and LPS-activated DCs resulted in more intensive cell death in wild-type cells than in their RIPK1-deficient counterparts. Based on cell death measurements, the TLR-treated DC supernatant induced significantly greater cell death in RIPK1+/+ wild-type cells than in the RIPK1-negative cell line.

To analyse the interaction between RIPK1 and pro-caspase-8 in our system, RIPK1 was immunoprecipitated from Jurkat cells after DCsup-induced activation. We found that caspase-8 is associated with RIPK1 after treatment of cells with the supernatants of CL075- or LPS-activated DCs, but not in the presence of the supernatant of unstimulated moDC. The intensity of RIPK1/caspase-8 association was similar in DCsup-treated and TNF plus SMAC mimetic-treated samples, the latter is a frequently used stimulus to activate RIPK1-dependent cell death. We confirmed these results by immunoprecipitating caspase-8 from Jurkat cells after DCsup-induced activation. RIPK1 phosphorylation is a widely accepted marker to characterize RIPK1-mediated cell death pathways. We detected increased phosphorylation of RIPK1 in Jurkat cells treated with either CL075 or LPS-activated DCsup but not after treatment with unstimulated DC supernatant.

Altogether our results indicate that RIPK1-driven signaling contribute to DCsup-induced cell death.

4.8 The supernatant of dendritic cells activates apoptosis

Various inhibitors were used to determine whether DC supernatant-induced cell death was considered apoptosis or necroptosis. Pre-treatment of Jurkat cells with 10 μM Z-VAD inhibited DCsup-induced apoptosis. In addition, Z-VAD pre-treatment almost completely reduced cell death detected by PI-uptake. Necrostatin-1, however, did not have any effect on cell death nor was it additive to the Z-VAD-mediated inhibition. These results indicate that DCsup induces caspase-dependent apoptotic but not necroptotic cell death.

4.9 Tolerogenic microenvironment reduces the cytotoxic capacity of DCs

To check whether controlling the availability of intracellular antigens is a constitutive function of DCs or it is also regulated by the surrounding tissues, we modelled the effects of tolerogenic and also immunogenic conditions. DCs were pre-treated with dexamethasone to induce the differentiation of tolerogen DCs, or with type I IFNs to mimic intracellular infection. To confirm the differentiation of these cells, we examined the production of inflammatory and tolerogenic cytokines in each differentiated DC population following LPS or CL075 activation. IFN-treated DCs produced significantly higher level of IL-6 than the tolerogen DCs, but dexamethasone-pre-treated DCs secreted more IL-10.

We checked the cytotoxic capacity of polarized DCs, by treating all these subpopulations with LPS or CL075 and examined the effect of their supernatants on Jurkat cells. The supernatant of dexamethasone-pre-treated DCs induced significantly less intense cell death than that of normal DCs, while cell death increased slightly, but not significantly after exposure of Jurkat cells to the supernatant of IFN-pre-treated DCs.

To determine whether the immunomodulatory environment also affects the susceptibility of target cells to DC-induced cytotoxicity, we treated the Jurkat cells were with type I IFN or with tolerogenic cytokines such as IL-10 and TGFβ. Regardless of pre-treatment of the target cells, the DC supernatant induced comparable cell death. However, IFN pre-treatment slightly, though not significantly, elevated cell death intensity. We can conclude that the tolerogenic

microenvironment reduces the DC-induced cytotoxicity, and this effect is due to the reduced killing capacity of DCs rather than an acquired resistance of target cells.

Taken together, these observations suggest that PRR stimulation-induced TNF production is responsible for moDC-mediated cytotoxicity, which is at least in part RIPK1-mediated apoptosis. Based on the results, we concluded that the tolerogenic microenvironment reduces the DC-induced cytotoxicity. This effect is mediated through DC-mediated cell killing and does not alter the sensitivity of target cells.

5. Discussion

The innate immune system is our body's first line of defense. Its main cellular constituents are macrophages and DCs, which are the first to detect incoming pathogens and, through their immediate response, ensure the occurrence of early defense mechanisms. In their operation, they recognize conserved molecular patterns that are found in many pathogens. Upon recognition of PAMPs, an intracellular signaling cascade is initiated in the defense cells. The goal is to generate an immediate, general, pro-inflammatory response and ultimately eliminate pathogens. Congenital immune cells are able to recognize a wide range of environmental and endogenous danger signals through PRRs. In this way, they can detect not only PAMPs from pathogens, but also endogenous signals, DAMPs, indicating damage to their own cells. PRRs initiate a significant phenotypic and functional change in cells of the innate immune system following both PAMP and DAMP stimuli, which are activated by this state.

Immune system activation and cell death processes are linked at several points. The effect of cell death types on the immune system depends on how effectively they detect signs of danger or antigens from dying cells. DAMPs released in addition to antigens bind to PRRs and initiate gene expression changes that enhance the phagocytic capacity of innate immune cells and the expression of cell surface MHC and costimulatory molecules. Thereby, innate immune cells are activated in such a way as to initiate inflammation and activation of T cells specific for antigens from dying cells. The relationship between cell death and activation of the immune system can have both positive and unfavorable consequences. By generating an inflammatory response, on the one hand, the defective cells of the innate immune system reach the site of the injury and signal to the adaptive immune system the potential problem that led to cell death. On the other

hand, the initiation of an immune response is accompanied by the release of inflammatory mediators that cause tissue damage.

Two polarization states of macrophages are known, the class of classically activated M1 and alternatively activated M2 macrophages. The two subpopulations are characterized by the production of different factors, different biological functions, and cell surface markers. Targeted regulation of the M1/M2 transition is a promising strategy in the treatment of various diseases. The aim of ongoing clinical trials is to modify the proportion of M1 and M2 macrophages in, for example, cancer, atherosclerosis, multiple sclerosis and endometriosis. The therapies used aim, on the one hand, to regulate the differentiation of cells and, on the other hand, to change the function of already polarized cells. Selective killing of different macrophage populations may provide an obvious therapeutic option to modify the M1-M2 ratio. However, in the therapeutic induction of cell death, it should be borne in mind that macrophages are resistant to most apoptotic stimuli but are particularly sensitive to newly known inflammatory forms of regulated cell death, such as necroptosis.

In our research, we compared the sensitivity of M1 and M2 cells to different cell death stimuli. Human monocyte-derived M1 and M2 macrophages were used in the experiments, which subpopulations showed similar sensitivity to most of the apoptotic or necroptotic inductions studied. The latter was induced by two necroptosis-inducing treatments accepted in the literature. Antagonists of apoptosis-inhibiting proteins block RIPK1 ubiquitination, whereas TAK1 inhibitors prevent RIPK1 phosphorylation. When used in the presence of a caspase inhibitor, both agents can also induce necroptosis in macrophages. In the case where cell death was induced by TAK1 inhibitor treatment, M2 cells were observed to be more sensitive to necroptosis than M1 cells. Consistent with classical necroptosis, administration of RIPK3 and RIPK1 inhibitors prevented induced cell death.

Because TAMs generally have similar functional properties to M2 macrophages, the question arose as to whether similar cell death pathways occur in these cell populations as in alternatively activated macrophages. In vitro TAM-like cells were differentiated in the presence of THP1 supernatant and M2-polarizing cytokines (IL-4, IL-10, TGFβ) and the susceptibility of the cells to necroptosis was examined. We have shown that TAM-like cells, such as M2 macrophages, are equally sensitive to necroptosis induced in the presence of the TAK1 inhibitor. Macrophage subpopulations M1 and M2 occur simultaneously at the site of chronic inflammation

or in the microenvironment of tumors. This raises the question of whether co-culture of M1 and M2 cells modifies susceptibility to cell death.

To investigate this, M1 and M2 cells treated with the TAK1 inhibitor were co-cultured. M2 cells were also more sensitive to cell death induced in the presence of the TAK1 inhibitor when treated with co-cultured macrophage populations. The inhibitor shifted the balance of the two cell populations toward M1 dominance. This also rules out the possibility that the difference in cell death is due to the autocrine cytotoxic produced by M2 or the survival factor produced by M1. This result justifies the use of the TAK1 inhibitor in tumor therapy, as opposed to the use of SMAC mimetics, by inducing necroptosis in both M1-M2 and TAM-like cells. However, the therapeutic use of necroptosis-inducing agents requires further studies to verify the effect of induced cell death on other cell types in the tissue microenvironment. We hypothesized that increased resistance to cell death may suggest that a specific survival signal may function in M1 macrophages that overrides TAK1 inhibitor-induced necroptosis.

We found that at least two different necroptotic pathways function in macrophages. This observation underlines the importance of further investigation and more specific understanding of necroptotic pathways. In the course of the doctoral work, we identified conditions when M1 or M2 macrophages have a different tendency for cell death, thus being suitable for regulating macrophage ratios. Our results represent a new approach in diseases where the ratio of M1 and M2 macrophages can influence the course of the disease, such as in the treatment of tumors, chronic inflammatory diseases and chronic infections.

In the doctoral work, we investigated how the cytotoxic capacity of human monocyte-derived DCs (moDC) is altered by PRR stimuli.

DCs provide the connection between the two functional units of the immune system, the inherited and the adaptive immune system. DCs that migrate continuously between tissues phagocytose antigens from dead, infected, or tumor cells in their environment and may alternatively cross-represent them. This allows the activation of $CD8^+$ naive T cells specific for intracellular pathogens or antigens from tumor cells. Cross-representation of intracellular antigens is essential for naive cytotoxic T cell activation, a process that most efficiently occurs after immunogenic cell death of antigen-containing cells.

The immunological outcome of cell death can be classified as: (1) tolerogenic, primarily after apoptosis, (2) inflammatory (necrosis or necroptosis), and (3) RIPK1-dependent immunogenic

cell death outputs that may be beneficial by increasing cross-representation. the likelihood of its occurrence.

In addition to phagocytosis and antigen presentation, a newly discovered function of DCs is cytotoxicity, the immunological role of which is not yet fully elucidated. Because DCs migrate from the site of infection to the lymph nodes within a short period of time after activation, they are unable to kill large amounts of infected or tumor cells. Based on these, we hypothesized that the cytotoxic property of DCs is not a cytotoxic effector function to eliminate pathogens in the classical sense, characteristic of T and NK cells. We hypothesize that the cell-killing ability of DCs serves to allow phagocytosis of surrounding cells, making intracellular antigens available, with the goal of activating specific CD8 + T cells through a cross-representation pathway. This idea is supported by the fact that the cytotoxic and phagocyte function of DCs patrolling between tissues is more intense than that of mature DCs.

The experiments were performed using human moDC, which were hypothesized to be induced by the presence/detection of pathogens. We observed that the supernatant of short-term DCs conditioned with PRR ligands (LPS or CL075) induced cell death in different target cells (SVT35 Jurkat, HT-29, HUVECs). Thus, in our experiments, we found that short-term PRR activation is required to induce DC cytotoxicity. From this we can conclude that DC cytotoxicity cannot be considered as a constitutive process, in the absence of infection, DCs do not control the tissue environment in this way. However, the conditioned supernatant collected from LPSs preactivated with LPS for a long time is less cytotoxic. 24 hours after activation, while DC maturation also occurs, the differentiated cells are less cytotoxic. In vivo, these DCs already typically leave peripheral tissues and migrate to secondary lymphoid organs as antigen-presenting cells, so they are no longer tasked with detecting infections.

We hypothesized that the tissue environment also regulates the cytotoxic property of DCs. DCs are cross in particular, it is essential for initiating an adaptive immune response specific for antigens from intracellular pathogens and tumors. DCs may be regulated by the immunosuppressive effect of most tumors and by the detection of type I interferons produced by infected cells. We compared the cytotoxic ability of DCs differentiated in the presence of a tolerogenic stimulus, dexamethasone, or type I interferon. DCs developed in medium containing dexamethasone induced less cell death following PRR stimulation than DCs cultured in the absence of tolerogenic stimulus. From the results, we can conclude that the tolerogenic

microenvironment may reduce the cell killing ability of DC. Surprisingly, the presence of immunomodulatory interferon type I, although somewhat enhanced, did not significantly alter DC supernatant-induced cytotoxicity compared to DCs cultured in the absence of IFN-α. The RIPK1-mediated cell death process is known to be a prerequisite for efficient cross-representation. To support our theory that the cell killing ability of DCs is to detect antigens for cross-presentation, we examined the RIPK1 dependence of DC supernatant-induced cell death. We compared the effect of DC supernatants on wild-type SVT35 and RIPK1-deficient Jurkat target cells. Based on our results, we concluded that DC cytotoxicity is at least in part a RIPK1-dependent process, as RIPK1 +/+ target cells induced higher cell death than supernatants than the RIPK1 -/- cell line. In addition, increased phosphorylation of RIPK1 and increased RIPK1-caspase-8 interaction in target cells also suggest that RIPK1-dependent signaling pathways are also involved in DC supernatant-induced cell death. This observation confirms our hypothesis that DCs induce immunogenic/RIPK1-dependent cell death to facilitate access to intracellular antigen fragments from surrounding cells.

The cell killing ability of DCs is mostly linked to soluble ligands in the literature. In our experimental system, we investigated the signaling processes of DC-induced cell death. We observed that TNF: Fc inhibited the cytotoxic activity of DC, in contrast to Fas and TRAIL antagonists. Based on this, it can be concluded that the cytotoxic ability of DCs is mediated by TNFα secretion, in which FasL or TRAIL cell death ligands have no detectable role. TNF receptor signaling can also cause RIPK1-dependent apoptosis or RIPK1-dependent necroptosis, depending on the composition of the cell death-inducing complex. Using inhibitors specific for apoptotic and necropotic processes, we investigated the type of cell death induced by DC supernatant on target cells. We found that supernatant-induced cell death was apoptosis because it could be inhibited by a caspase inhibitor. In contrast, administration of the necrostatin-1 necroptosis inhibitor did not alter the toxic effect of the supernatant.

In our research, we have shown that the supernatant of DCs conditioned with short-term PRR ligands induces apoptosis of target cells in a RIPK1-dependent manner. These results provide an opportunity to expand the cytotoxic therapeutic mechanisms used against tumors.

6. Summary

Nowadays, it has become apparent that the activation of immune system and certain cell death processes are connected. The major cellular components of the innate immune system are macrophages and dendritic cells (DCs). These cells are able to generate a pro-inflammatory reaction in response to the presence of pathogens. However, excessive activation of immune cells can result in tissue damage and lead to conditions such as autoimmune or tumor diseases. In our work, we studied the inflammatory-inducing necroptosis and the RIPK1-dependent form of apoptosis. Our experiments helped us to have a more detailed view on the molecular mechanism of these RIPK1-dependent cell death processes and their effect on the immune response.

Targeted elimination of M1 and M2 may provide outstanding therapeutic benefits in cardiovascular and chronic inflammatory diseases, infections, or tumors. Based on literature data, macrophages are known to resist to most apoptotic stimuli but sensitive to newly discovered inflammatory forms of regulated cell death. In our work, we compared the sensitivity of human monocyte-derived M1 and M2 cells to different cell death signals. In the present study, we observed that macrophage cell types show different sensitivity to TAK1 inhibitor treatment, which induced more intense necroptosis in M2 and TAM-like cells. Based on our results, it can be concluded that the different necroptosis susceptibility is not the effect of the autocrine cytotoxic produced by M2 or the survival factor produced by M1. A possible explanation for this phenomenon might be that hitherto unknown signaling mechanisms may provide a surviving signal in M1 cells. Our results show that there are at least two different necroptotic pathways in macrophages. Furthermore, conditions suitable for the regulation of macrophage functions have been identified when M1 and M2 have different tendencies to cell death using the TAK1 inhibitor.

We studied the molecular mechanism of RIPK1-dependent cell death processes in moDCs. Preliminary studies have shown that DCs also have cytotoxic function, thus being able to induce apoptosis of surrounding cells. This function provides the professional antigen presenters with the ability to release antigens from surrounding cells and to monitor their microenvironment. In our work, we hypothesized that the cell-damaging effect is induced by the detection of pathogens. The results of our experiments show that short-term, PRR agonist-conditioned supernatants of DCs can induce cell death in different cell lines. Human moDCs occur throughout the body, significantly contributing to the communication between the immune

system and the tissue microenvironment, thereby fine-tuning immune responses under both normal and pathological conditions. We observed that the dexamethasone-induced tolerogenic microenvironment may reduce the cell killing ability of DC, whereas the supernatant of immunogenic DCs differentiated in the presence of IFN-α did not affect the rate of cell death. In our work, we showed that the supernatant of short-term PRR stimulated DCs induces apoptosis of the target cells in a RIPK1-dependent manner.

In summary, we have successfully described new mechanisms in the regulation of macrophage and DC functions those we hope to be useful as immunotherapeutic targets for various inflammatory processes or tumor diseases in the future.

7. Publications

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Registry number: DEENK/11/2021.PL
Subject: PhD Publication List

Candidate: Zsófia Varga
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Varga, Z.**, Rácz, E., Türk-Mázló, A., Korodi, M., Szabó, A., Molnár, T., Szöőr, Á., Veréb, Z., Bácsi, A., Koncz, G.: Cytotoxic activity of human dendritic cells induces RIPK1-dependent cell death.
   Immunobiology. 226 (1), 1-7, 2021.
   DOI: http://dx.doi.org/10.1016/j.imbio.2020.152032
   IF: 2.788 (2019)
2. **Varga, Z.**, Molnár, T., Türk-Mázló, A., Kovács, R., Jenei, V., Kerekes, K., Bácsi, A., Koncz, G.: Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis.
   Cancer Immunol. Immunother. 8, 1-15, 2020.
   DOI: http://dx.doi.org/10.1007/s00262-020-02623-7
   IF: 5.442 (2019)

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List of other publications

3. Hancz, D., Szabó, A., Molnár, T., **Varga, Z.**, Hancz, A., Gregus, A., Hueber, A. O., Rajnavölgyi, É., Koncz, G.: Flagellin increases death receptor-mediated cell death in a RIP1-dependent manner.
   Immunol. Lett. 193, 42-50, 2018.
   DOI: http://dx.doi.org/10.1016/j.imlet.2017.11.007
   IF: 2.552
4. Bene, K., **Varga, Z.**, Petrov, V. O., Boyko, N. V., Rajnavölgyi, É.: Gut Microbiota Species Can Provoke both Inflammatory and Tolerogenic Immune Responses in Human Dendritic Cells Mediated by Retinoic Acid Receptor Alpha Ligation.
   Front. Immunol. 8 (427), 1-17, 2017.
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8. Key words

necroptosis; macrophage; tumor; inflammation, dendritic cell; cytotoxicity; immunogenic cell death; RIPK1; immunotolerance

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