The role of necroptosis in bladder cancer

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UNIVERSITY OF SPLIT

SCHOOL OF MEDICINE

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LIST OF ABBREVIATIONS

BBN - N-Butyl-N-(4- hydroxybutyl) nitrosamine

Bcl2 - B-cell lymphoma 2

BSA - Bovine Serum Albumin

cIAP - Cellular Inhibitor of Apoptosis Protein

ClCas3 - Cleaved caspase 3

CXCl1 - Chemokine (C-X-C motif) ligand 1

DAMPs - Damage associated molecular patterns

ddH2O - Double distilled water

EtOH -Ethanol

FADD - Fas-associated protein with death domain

FoxP3 - Forkhead box P3

IL - Interleukin

MIBC - Muscle invasive bladder cancer

MLKL - Mixed lineage kinase domain-like protein

Nec1s - Necrostatin-1s

NF-kB- Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NMIBC - Non muscle invasive bladder cancer

PAMPs - Pathogen associated molecular patterns

p-MLKL - Phosphorylated mixed lineage kinase domain-like protein

qPCR - Quantitative polymerase chain reaction

RIPK - Receptor-interacting serine/threonine-protein kinase

SDS - Sodium dodecyl sulfate

SMAC - Second mitochondria-derived activator of caspases

TNF - Tumor necrosis factor

TRADD - Tumor necrosis factor receptor type 1-associated DEATH domain protein

TRAF2 - TNF receptor-associated factor 2

TRAIL - Tumor Necrosis Factor Related Apoptosis Inducing Ligand

TURBT - Transurethral removal of bladder tumor

Ub - Ubiquitin

zVAD - Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

1. INTRODUCTION

1.1 Bladder cancer

1.1.1 Epidemiology

Bladder cancer is the 9th most common cancer in the world. Every year there are around 430 000 new cases, from which more than half occur in less developed regions of the world. 165 000 people die each year from bladder cancer and 50% of these deaths are occurring in less developed regions of the world (1). In 2014, the Republic of Croatia had a bladder cancer incidence of 19.8/100 000 (2). Men comprise 75% of bladder cancer patients, making it a strongly male-predominant type of cancer (1,2).

1.1.2 Etiology

The major risk factor for developing bladder cancer is tobacco smoking (1). Much of the geographical and sex disparities seen worldwide can be explained by the differences in the prevalence of smoking (1). The high burden of disease in parts of Northern and sub-Saharan Africa can be explained by infections with a parasitic worm known as Schistosoma (1,3). Aromatic amines, azo-dyes, bladder stones and certain medications like cyclophosphamide and phenacetin are some of the additional risk factors (2).

1.1.3 Patient presentation

Patients with bladder cancer usually present with painless hematuria i.e. blood in the urine. If the blood is visible, it is termed macrohematuria. Blood (red blood cells) can also be detected by urine microscopy, which is then termed microhematuria (2,4). Chronic urinary tract infections can be another presenting symptom. Disease progression is associated with symptoms of urgency and frequency, mainly because of the decreased luminal capacity of the bladder. Hydronephrosis may be present if the tumor is large enough. Late stage bladder cancer tends to metastasize to the abdominal lymph nodes, liver, lungs and bones (2).

1.1.4 Diagnosis and tumor classification

Patients are usually men with a mean age of 75 presenting with painless hematuria. Such patients are further investigated with cystoscopy and intravenous urography (2,5). If a tumor is detected during cystoscopy, transurethral resection of the mass with a subsequent histological analysis is performed. MRI, CT scan and chest x-ray are useful for evaluating the extent of the disease, including potential metastases (2). As previously stated, late stage bladder cancer tends

to metastasize to bones. Therefore, it is worth performing alkaline phosphatase levels and bone scintigraphy in patients who present with complaints related to the skeletal system. In 90% of cases, bladder tumors originate in a specialized epithelium, called the urothelium. The urothelium constitutes the luminal layer of the urinary tract and lines the renal pelvis, ureters, bladder and the proximal parts of the urethra. Although urothelial carcinomas can originate in all parts of the urinary tract, urothelial neoplasms are 50-100 times more common in the bladder (2). Less common forms of bladder cancer are adenocarcinomas, small cell carcinomas and squamous cell carcinomas (2). The latter, although rare in more developed regions of the world, is commonly found in regions where *Schistosoma haematobium* is endemic (5). After a histological sample has been obtained via transurethral resection and all necessary imaging has been performed, the tumor is classified by the TNM classification from the American Joint Committee on Cancer (AJCC) (2). This step is crucial for both treatment and prognosis. The TNM classification and the pathohistological progression of bladder cancer are summarized in Table 1 and Figure 1, respectively. Based on this classification, bladder cancer is generally divided into two major subtypes – muscle invasive and non-muscle invasive bladder cancer. Non-muscle invasive tumors can be benign or malignant, whereas muscle invasive tumors are always malignant. Within the TNM classification, non-muscle invasive tumors are Ta, Tis and T1. Tumor growth beyond T2 is considered muscle invasive. With around 70% of all cases, non-muscle invasive bladder cancer is much more common than its invasive counterpart (2). Out of these, approximately 70-75% are non-invasive papillary (Ta) lesions (6). Non-invasive papillary urothelial carcinoma (Ta) is defined by the WHO as a papillary urothelial neoplastic proliferation. It has some level of cytological and architectural disorder visible at low to intermediate magnification, with no invasion beyond the basement membrane (Figure 1B) (6). These lesions have characteristically high risk of recurrence, with 10-70% recurrence within 5 years (2,6). However, only a small percentage of patients develop the invasive disease (6). Another non-invasive urothelial lesion is the urothelial carcinoma in situ (CIS) or TIS (Figure 1C). It is defined as a flat lesion of variable thickness, devoid of papillary structures containing cytologically malignant cells (6). In contrast to Ta lesions, TIS lesions are exceedingly rare in their pure form. However, they tend to occur adjacent to or subsequent to invasive tumors (5). When a lesion breaks through the basement membrane, it is by definition an infiltrating urothelial carcinoma (6). If the basement membrane is breached and the tumor is infiltrating the subepithelial connective tissue, it is considered a T1 lesion. Further invasion into the muscularis propria of the tumor is classified as a T2 lesion (muscle invasive bladder cancer) (6).

Histological features of invasive lesions include irregular nests, single cell infiltration and tentacular finger-like projections (Figure 1D). As the tumor progresses, it gradually invades the perivesical tissue, making it a T3 lesion. Invasion of the surrounding structures such as the prostate, seminal vesicles, uterus or vagina and finally the abdominal or pelvic wall, is classified as a T4 lesion (6). Generally, all infiltrating urothelial carcinomas (from T1-T4) have a high propensity for divergent differentiation (6). After evaluating lymph nodes and distant metastases, the TNM classification can be summarized in one of six stages (Table 1).

Table 1. TNM classification of carcinomas of the urinary bladder. Adapted from Moch *et al.* (6)

TNM classification	
T - Primary tumor	**N - Regional lymph nodes**
TX Primary tumor cannot be assessed **T0** No evidence of primary tumor **Ta** Non-invasive papillary carcinoma **Tis** Carcinoma in situ/ flat tumor **T1** Tumor invades subepithelial connective tissue **T2** Tumor invades muscularis propria **T2a** Tumor invades superficial muscularis propria **T2b** Tumor invades deep muscularis propria **T3** Tumor invades perivesical tissue **T3a** Microscopically **T3b** Macroscopically (extravesical mass) **T4** Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall **T4a** Tumor invades the prostatic stroma, seminal vesicles, uterus or vagina **T4b** Tumor invades the pelvic wall or abdominal wall	**NX** Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Metastasis to a single lymph node in the true pelvis (a hypogastric, obturator, external iliac, or presacral lymph node) **N2** Metastasis to multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node) **N3** Metastasis to common iliac lymph node(s)
	M - Distant metastasis
	M0 No distant metastasis **M1** Distant metastasis
	Stage grouping
	Stage 0a Ta, N0, M0 **Stage 0is** Tis, N0, M0 **Stage I** T1, N0, M0 **Stage II** T2a-b, N0, M0 **Stage III** T3a-b, N0, M0/ T4a N0 M0 **Stage IV** T4b, N0, M0 / Any T, N1-3, M0 / Any T, Any N, M1

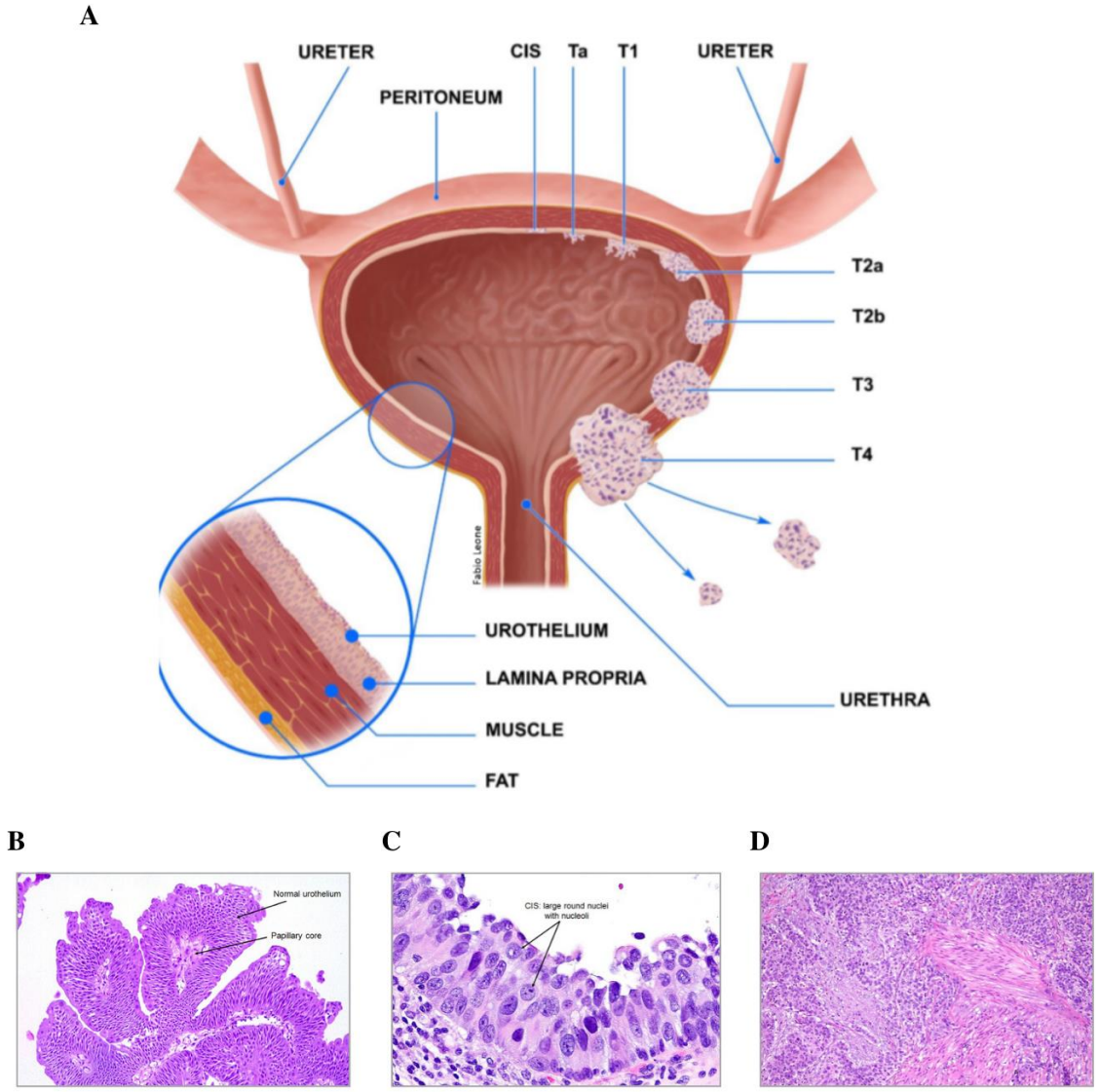


Figure 1. Pathohistological characteristics of bladder cancer. (A) Tumor progression through the layers of the bladder wall. (B) Non-invasive papillary lesion (Ta). Papillae with fibrovascular core lined by normal appearing urothelium can be seen (7). (C) *Carcinoma in situ* (Tis). The flat lesion is composed of malignant urothelial cells and confined to the basement membrane with full or partial thickness involvement of urothelium (7). (D) Muscle invasive bladder cancer (T2). The lesion demonstrates irregular nests, single cell infiltration or tentacular finger-like projections (7). Figure 1A: Fabio Leone. Figure 1B-D: American urological society.

1.1.5 Treatment

In general, non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) and metastatic disease are all therapeutically distinct entities. The transurethral resection of the bladder tumor (TURBT) is usually the first treatment option for NMIBC (2,5). Usually, right after the TURBT procedure, intravesical adjuvant immunotherapy with BCG (Bacillus Calmette-Guerin) or chemotherapy with mitomycin C is administered. BCG immunotherapy evidently elicits a strong adaptive immune response against the tumor (2,5). The decision for adjuvant immuno- or chemotherapy is based on the risk of cancer progression or recurrence (5). As previously mentioned, the most common tumors found in the urinary bladder are non-invasive papillary (Ta) lesions, which tend to have a very high recurrence rate. This also is why every patient who underwent TURBT needs to be followed up with cystoscopy every 3 months (2). Due to the aggressive nature of MIBC, almost all patients with resectable nonmetastatic muscle-invasive bladder cancer are treated with radical cystectomy with bilateral pelvic lymphadenectomy and cisplatin-based neoadjuvant chemotherapy (2,5). In selected patients, a bladder preserving approach can be chosen instead. In that case, concomitant radiotherapy is the treatment of choice (2). Metastatic disease is treated with chemotherapy and immunotherapy. First line therapy is based on the CG-protocol, which is a combination of cisplatin and gemcitabine. For patients who do not tolerate cisplatin, one can replace it with atezolizumab (anti-PD-L1) or pembrolizumab (anti-PD-1) (2). Immune checkpoint inhibitors like these are also used as a second line therapy, which has been proven to be especially effective in bladder cancer patients (2).

1.1.6 Prognosis and follow up

Despite its tendency to recur, NMIBC is a treatable disease, with 5-year survival rates of up to 96% (5). MIBC, on the other hand, has a 5-year survival rate of only 35-55%, even after radical cystectomy with bilateral pelvic lymphadenectomy (2). Adding neoadjuvant cisplatin-based chemotherapy results in an absolute 5-year survival benefit of 5-8% (5). Primary chemoradiation has a 5-year survival rate of 20-40%. Overall, around 50% of patients have disease recurrence (2). Follow up depends on the clinical stage and ranges from cystoscopic control exams to regular CT scans (5).

1.1.7 Molecular subtypes of bladder cancer

Bladder cancer can not only be differentiated based on its histological appearance, but also by its molecular features. Bladder cancer presents with an impressive degree of molecular heterogeneity, as is the case with all human cancers. Therefore, it can be concluded that every cancer harbors a unique pattern of subclonal heterogeneity, DNA methylation, mutations, copy number variations, and RNA expression patterns (8). But even with this level of heterogeneity, bladder cancers can still be grouped into two major molecular subtypes with distinct biological and clinical features. Those are *basal* and *luminal* molecular subtypes (8). The more aggressive basal cancers are characterized by an advanced stage and metastatic disease at presentation. They tend to be enriched with squamous and small cell/neuroendocrine features and inactivating mutations, as well as deletions of TP53 and RB1. Basal cancers can be further divided into "epithelial" and "mesenchymal" subsets. Mesenchymal subsets are also known also "claudin-low" basal cancers. A portion of the mesenchymal subset forms a "neuroendocrine/neuronal" subset that is associated with particularly poor survival (8). On the other hand, luminal cancers have a more favorable prognosis and are often enriched with papillary histopathological features and activating mutations in FGFR3. A further subdivision can be made according to the infiltration of stromal cells, relative genomic instability, and the expression of carcinoma in situ (CIS) gene signatures (8). When further subdivided, 5 individual molecular subtypes can be identified, which are luminal papillary/uroA, luminal infiltrated/p53- like, luminal/GU, basal squamous, and neuroendocrine. Overall, these subtypes have prognostic significance and also appear to be associated with different responses to systemic therapies (8). Basal squamous, claudin-low, and neuroendocrine tumors all appear to be intrinsically aggressive, but they derive the most benefit from chemotherapy, and in these patients, aggressive clinical management can dramatically improve prognosis. A possible explanation for their aggressive nature might be a higher level of EMT (epithelial-mesenchymal transition) biomarkers than in the luminal tumors, which make them more prone to invasion and metastasis. Although patients with small cell/neuroendocrine tumors are also initially highly chemosensitive, responses tend to be very short-lived. Fortunately, they seem to be attractive targets for immune checkpoint blockade, and preclinical data suggest that they may also be sensitive to PARP inhibitors (8). Luminal papillary/uroA tumors are associated with the best survival and approximately half of these tumors appear to be downstaged by neoadjuvant chemotherapy. Luminal papillary tumors display an "immune desert" phenotype, and patients with luminal papillary tumors seem to have the least benefit from immune checkpoint blockade.

However, activating FGFR3 mutations and fusions are greatly enriched in these tumors, so local or systemic therapy with FGF receptor inhibitors (with or without immunotherapy) is an attractive therapeutic approach (8).

1.2 Necroptosis

1.2.1 General aspects

Programmed cell death, also known as cellular suicide, describes a regulated process by which a cell dies following an underlying signaling program. By definition, "programmed" refers to a form of cell death that is regulated and occurs as a part of tissue homeostasis (9). It is of great importance in many physiological processes such as organismal development, physiological homeostasis, epithelial cell renewal and lymphocyte selection (10). Like apoptosis, necroptosis is one of the many forms of programmed cell death (10). This insight is fairly new because for a long time it was believed that tissue necrosis is an accidental, unprogrammed and unregulated event that occurs when a cell is damaged by noxious stimuli beyond a threshold of reasonable repair (9). Today we know that there are several forms of cell death that are morphologically similar to necrosis but are in fact orchestrated by a set of signaling pathways (9). In that case, we call this form of necrosis controlled or intended necrosis, as opposed to accidental necrosis (9).

1.2.2 Signaling pathway

The intracellular machinery controlling the execution of necroptosis has been well described in recent years. Necroptosis is most intensely studied upon stimulation of death receptors (DRs) (9). The death receptors from the TNF superfamily such as TLR3 or TLR4 have been studied in particular. There are also other receptors like CD95, TRAIL-R1/R2 that have been linked to necroptosis induction (9,10). However, by far the most extensively studied inducer of necroptosis is the TNF receptor TNFR1 (9,10). A graphic representation of this signaling pathway is depicted in Figure 2. After ligation of TNF to its receptor TNFR1, the subsequent signaling cascade results in one of two major outcomes – cell survival or cell death (9,10). The first commonality between these two pathways is the formation of a protein complex, known as complex I. Complex I consists of TRADD, RIPK1, TRAF2, and cIAP1/cIAP2. The molecules cIAP1/ cIAP2 are responsible for the polyubiquitination of RIPK1. The polyubiquitination of RIPK1 is essential for the activation of the NF-kB signaling

pathway that is ultimately responsible for the activation of pro-survival pathways. When cIAP1/cIAP2 are inhibited, for instance by the molecule SMAC or by SMAC mimetics, the deubiquitination of RIPK1 is favored, which switches the cell signaling in favor of cell death i.e. apoptosis necroptosis (10). When RIPK1 is deubiquitinated, complex I is released into the cytosol and recruits FADD. The resulting complex is now called complex II (complex IIa). Whether the death signal will be switched to apoptosis or necroptosis depends on the activity of caspase-8. When caspase-8 is active, it not only allows for apoptosis to happen but also inhibits necroptosis by cleaving essential mediators of necroptosis like RIPK1 and RIPK3 (10). Necroptosis is a caspase-independent type of cell death, so when caspase-8 is inhibited, for instance by zVAD, recruitment of RIPK3 to complex II is allowed, thereby forming complex IIb/necrosome. The death signal is now switched to necroptotic (10). After RIPK1 and RIPK3 are combined, RIPK1 gets phosphorylated by RIPK3. Intramolecular auto- and trans-phosphorylation of RIPK1/RIPK3 promotes recruitment of another key necroptosis-signaling protein to complex IIb, the mixed lineage kinase domain-like protein (MLKL) (11). MLKL is phosphorylated by RIPK3, which leads to its oligomerization and localization to the plasma membrane (10). Although not fully understood, it is known that the membrane translocation of p-MLKL leads to membrane disruption and subsequently cell death. One proposed mechanism is that p-MLKL activates sodium and calcium channels, which leads to a change in the osmotic pressure rupturing the plasma membrane. Another proposed mechanism is that p-MLKL creates pore structures in the plasma membrane which can lead to a change in the osmotic pressure. One feature of necroptosis that distinguishes it from apoptosis is that upon membrane disruption, there is a release of DAMPs (Damage associated molecular patterns) and intracellular contents. Examples of DAMPs include monosodium urate (MSU), high mobility group box 1 protein (HMGB1) and annexin A (10). These molecules attract immune cells and induce an inflammatory response (10). Therefore, necroptosis is an inflammatory type of cell death, explaining its importance in various inflammatory, infectious and degenerative diseases (10).

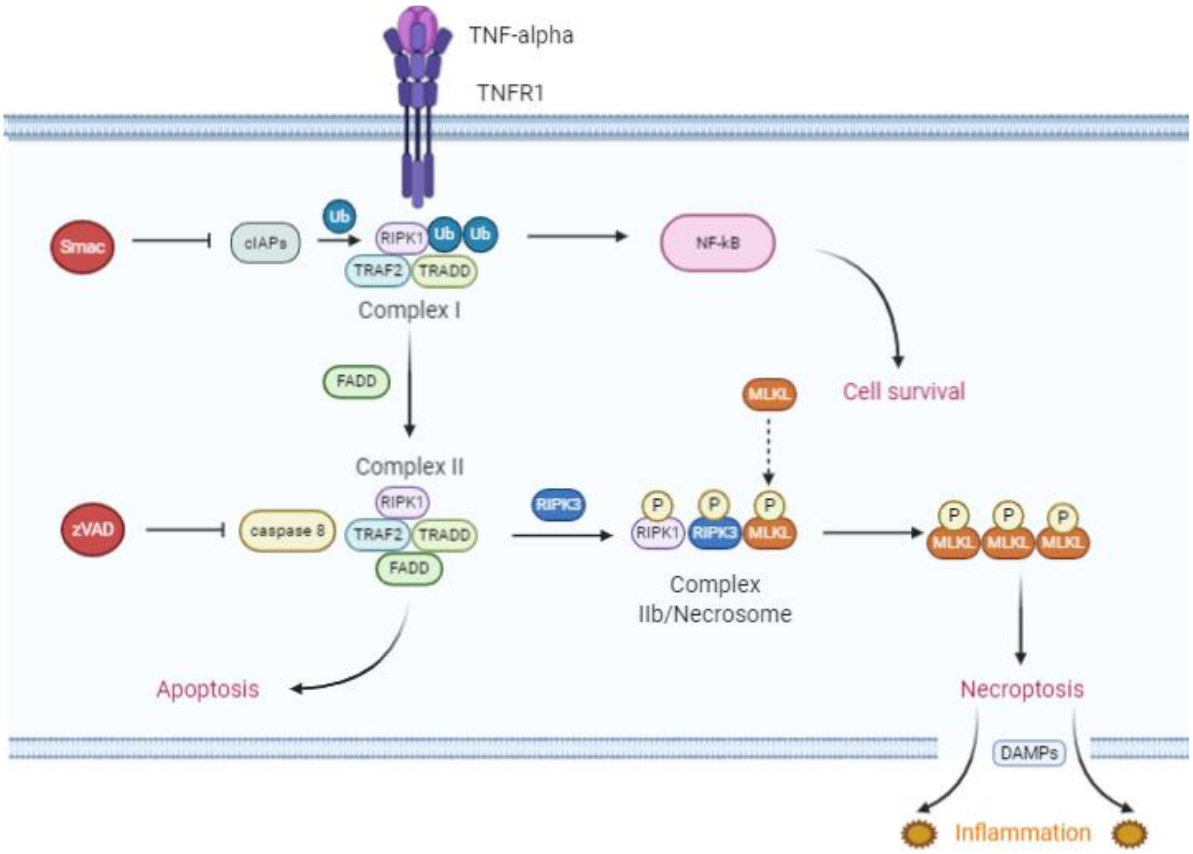


Figure 2. Necroptosis signaling pathway. Ligation of TNFα to TNFR1 leads to the formation of complex I (TRADD, RIPK1, TRAF2, cIAP1/cIAP2). Polyubiquitination of RIPK1 is essential for the activation of the NF-kB signaling pathway that is ultimately responsible for the activation of pro-survival pathways. When RIPK1 is deubiquitinated, complex I is released into the cytosol and recruits FADD. The resulting complex is now called complex II. Active caspase-8 allows for apoptosis to happen and at the same time inhibits necroptosis. Inhibition of caspase-8 activity allows the recruitment of RIPK3 to complex IIa, after which MLKL is recruited as well. This finally forms the necrosome (RIPK1, RIPK3, MLKL). MLKL is phosphorylated by RIPK3, which leads to its oligomerization and localization to the plasma membrane where it causes it to rupture. Upon plasma membrane disruption, there is a release of DAMPs (Damage associated molecular patterns) and intracellular contents which then induce an inflammatory response. Adapted from Qin *et al*.

1.2.3 Necroptosis core proteins

From the previous discussion, it becomes clear that the serine/threonine kinases RIPK1, RIPK3 and MLKL are essential for TNF induced necroptosis (10). In the following paragraphs these three molecules, otherwise known as necroptosis core proteins, are going to be discussed in more detail.

1.2.3.1 RIPK1

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is a protein that in humans is encoded by the *RIPK1* gene, which is located on chromosome 6 (12). It is an enzyme that belongs to the Receptor Interacting Protein kinases (RIPK) family, and it is the first member of this group of seven enzymes (13,14). To reiterate, RIPK1 plays an important role in both cell survival pathways like NF-kB, Akt, JNK, and within cell death pathways during apoptosis and necroptosis (9,10,15). RIPK1 contains 671 amino acids which together make three distinctive domains. A kinase domain can be found on the N-terminus (11). The importance of this kinase domain during the execution of necroptosis is demonstrated by its inhibition with an allosteric kinase inhibitor called Necrostatin-1. It inhibits necrotic cell death and is used in many studies, including our own, to examine the effects of necroptosis on different diseases (11,16). Being a multifunctional adaptor protein, RIPK1's C-terminal Death Domain (DD) allows for the interaction with homologous DD on Death receptors such as TNFR1, Fas and TNF-related apoptosis inducing ligand (TRAIL). This domain mediates cell survival via NF-kB pathways, but also apoptosis and necroptosis. Between the kinase domains and the death domain sits the intermediate domain, which contains a RIP homotypic interaction motif (RHIM) domain that allows for the interaction between RIPK1 and RIPK3 (11). The three domains and their respective interacting molecules are depicted in Figure 3.

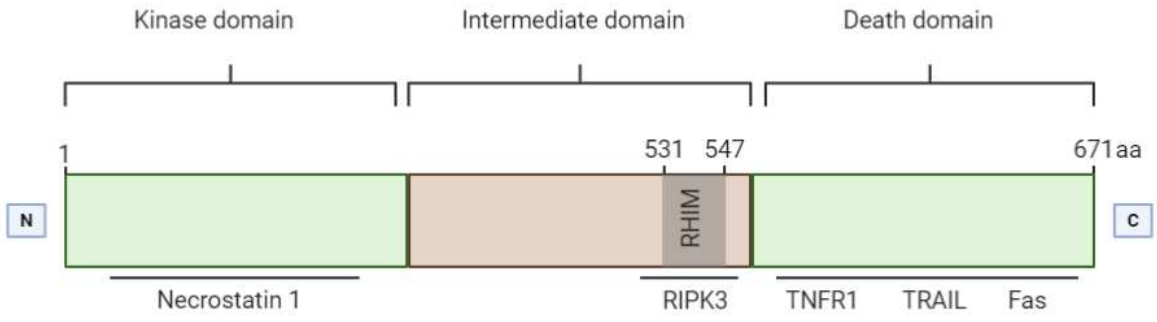


Figure 3. Schematic representation of RIPK1 protein. RIPK1 protein with its three characteristic domains and respective interacting molecules. Adapted from Ofengeim *et al.*

1.2.3.2 RIPK3

Receptor-interacting serine/threonine-protein kinase 3 (RIPK3) is a protein that in humans is encoded by the *RIPK3* gene, which is located on chromosome 14 (17). Just as RIPK1, RIPK3 is a member of the receptor-interacting protein (RIPK) family of serine/threonine protein kinases, and although it shares nearly half of its 518 amino acids with RIPK1 and has a very similar topology, it contains a unique c-terminus without a DD, through which it inhibits RIPK1 and TNFR1-mediated NF-κB activation (11). When caspase-8 is inhibited or deficient, RIPK1 combines with RIPK3 via its RIP homotypic interaction motif (RHIM) domain to form the RIPK1/RIPK3 complex (Figure 3). RIPK3/RIPK3 homo-oligomerization leads to RIPK3 autophosphorylation. Phosphorylated RIPK3 recruits and phosphorylates MLKL, which promotes p-MLKL oligomer-executed necroptosis (11).

1.2.3.3 MLKL

Mixed lineage kinase domain-like pseudokinase (MLKL) is a protein that is encoded by the *MLKL* gene and is located on chromosome (18). MLKL belongs to the protein kinase superfamily. Although it contains a protein kinase-like domain, it is thought to be inactive because it lacks several residues required for such activity. As stated before, MLKL plays a pivotal role in the execution of necroptosis. The intramolecular auto- and trans-phosphorylation of RIPK1/RIPK3 (complex IIb) promotes the recruitment of MLKL, which is then phosphorylated by RIPK3 (11). The phosphorylated form of MLKL (p-MLKL) moves to the plasma membrane to oligomerize and induce, among other proposed mechanisms (see previous description), membrane pore formation. Finally, this results in the destruction of membrane integrity and eventually leads to necrotic cell death (11). It is worth mentioning that p-MLKL serves as a useful marker for cells that are undergoing necroptosis. Therefore, antibodies against p-MLKL can be used in immunohistochemical staining and immunoblotting to detect necroptotic cell death.

1.3 The role of necroptosis in cancer-associated inflammation

In 1863, Rudolf Virchow already hypothesized that the origin of cancer was at sites of chronic inflammation. Today it is very well established that chronic states of inflammation indeed can potentiate neoplastic tissue growth. Chronic inflammation caused by infections agents accounts for approximately 15% off all malignancies worldwide (19). Examples include Hepatitis C, HPV and Helicobacter infection that can cause liver cancer, cervical cancer and stomach cancer, respectively (19). In addition to the mutagenic potential of certain infectious

agents, another possible explanation is that immune cells induce DNA damage in proliferating cells by generating reactive oxygen and nitrogen species during the response to the infection itself (19). Another striking example is that inflammatory bowel disease, more precisely, ulcerative colitis, greatly increases the risk for colorectal cancer (19). But inflammation does not only induce malignant growth, it can actually sustain it and promote it. This was demonstrated in a paper by Grivennikov *et al.*, where the IL6-STAT3 cascade was shown to be required for survival of intestinal epithelial cells and the development of Colitis-Associated Cancer (20). Furthermore, our group recently showed that STAT3 is also important for bladder cancer induction and promotion (21). On the other hand, the immune system has a crucial role in preventing malignancy from developing in the first place, as demonstrated by the concept of immune surveillance (22). How important the immune system is in preventing malignancy can be seen preclinically in immunodeficient mice that are more susceptible to transplanted or carcinogen-induced tumors, and clinically in immunocompromised patients, where the failure to clear human herpes virus (HHV) is one of the factors involved in the pathogenesis of Kaposi sarcoma. However, cancer cells can develop strategies which help them escape immune surveillance (22). Not only are cancer cells of autologous origin and therefore have intrinsically poor immunogenicity, but even when the tumor is infiltrated with immune cells, the tumor microenvironment is typically dominated by immunosuppressive mediators(23). By boosting or rescuing the antitumor immune response, modern immunotherapies try to address the aforementioned issue (23). A very popular example used nowadays is the immune checkpoint inhibitors like Atezolizumab (anti-PD-L1) and Ipilimumab (anti-CTLA-4). They work by blocking the co-inhibitory signals during the immune response, and by doing so effectively disinhibit the immune system, allowing for anti-tumor immunity to occur (23). For instance, in metastatic bladder cancer, immune checkpoint inhibitors seem to be very effective and improve overall survival, and due to their good safety profile are even suitable for patients that do not tolerate standard chemotherapy (2,24). Another approach in boosting the anti-tumor immune response lies in the activation of pattern recognition receptors (PRRs) that can detect pathogen associated molecular pattern (PAMPs) and damage associated molecular patterns (DAMPSs) (23). PAMPs and DAMPs are associated with microbial pathogens and with components of host cells that are released during cell damage or death, respectively (25). PRRs are germ-line encoded receptors (in contrast to antigen receptors found on lymphocytes that are produced by somatic recombination of genes) and are mainly expressed by cells of the innate immune system, such as dendritic cells, phagocytes but also endothelial and epithelial cells. Upon their

activation with DAMPs and PAMPs, they mediate the release of inflammatory cytokines and activate the antigen-specific adaptive immune response (25–27). As pointed out previously, DAMPs like monosodium urate (MSU), high mobility group box 1 protein (HMGB1) and annexin A, are released upon necroptotic cell death. It is thought that the crosstalk between tumor infiltrating cells and necroptotic cells, via death associated molecular patterns, could reawaken the immune surveillance (23). One could hypothesize that the iatrogenic initiation of necroptotic cell death could boost antitumor immunity and therefore act as a form of immunotherapy. The main mechanisms by which necrotic cell death could promote anti-tumor immunity is by inducing the release of pro-inflammatory cytokines and chemokines, upregulation of stimulatory molecules and enhanced cross-presentation, and eventual trigger of adaptive immune responses (28). Phagocytic cells, like dendritic cells, macrophages, monocytes, and neutrophils are attracted to necroptotic cells by the massive release of DAMPs (28). After reaching the necroptotic cell, phagocytic cells identify it by "eat me" signals like annexin A1, phosphatidylserine (PS), and the C-type lectin Clec9A, that are presented on the cell surface of the dying cell. The engulfment of necrotic cells by phagocytes triggers the production of pro-inflammatory cytokines (IL1a) and chemokines and eventually triggers adaptive immunity by the process of cross-presentation (28). Cross-presentation is a process by which antigen-presenting cells take up, process and present antigens of a cell (*here* tumor cell) via MHC class I molecules to CD8 T-cells. This way naïve cytotoxic T-cells (CD8+T cells) become activated (primed) cytotoxic T-cells, thereby inducing specific anti-tumor immunity (25). Although tumor necrosis clinically correlates with rapidly growing malignant tumors and a poor prognosis, in moderate amounts, tumor necrosis as an immunogenic cell death could induce specific antitumor immunity by previously described mechanisms (29).

In accordance with that, a study by Aaes *et al.* demonstrated how vaccination with necroptotic cancer cells induces cross priming of T-cells and efficient anti-tumor immunity, significantly protecting against tumor growth in a subcutaneous tumor model using a colon cancer cell line (30). Furthermore, even the success of some cancer chemotherapies could be attributed to immunostimulating effects that take place upon drug induced necroptotic cell death (28). In patients with squamous cervical carcinoma, it was shown that increased expression of necroptosis core proteins like MLKL correlates with improved survival (10). In contrast to these findings, there is opposing evidence that shows how necroptosis might be pro-tumorigenic in certain forms of cancer. Seifert *et al.* demonstrated how the necrosome (RIPK1, RIPK3, MLKL) activates downstream effectors like CXCL1 and SAP130 which promote pancreatic

oncogenesis by creating an immunosuppressive tumor microenvironment (31). Additionally, a paper by Jiao *et al.* suggests that MLKL-mediated necroptosis in tumor cells promoted metastasis of breast cancer (32). It appears that necroptosis, in the context of cancer, can modulate the immune response into different directions depending on the type and stage of the malignancy. Furthermore, it is possible that not all effects of necroptosis are a direct result of the actual process of cell death, but that some of them might be mediated by the necroptosis core proteins and their downstream effectors.

1.4 Animal models

Animal models provide a bridge between the preclinical evaluation and the implementation of new treatment modalities into clinical practice. In order to make this process accurate and efficient, animal models of bladder cancer need to represent the human counterpart as closely possible. There are several approaches to *in vivo* bladder cancer models. One can use bladder cancer cells and implant them into the organ of their origin (i.e bladder), which is called orthotopic, or one can implant the cancer cells outside the organ of origin, usually subcutaneous, which is called heterotopic (33). This approach can be further distinguished based on the origin of the cancer cells that are used for implantation. It is possible to use human bladder cancer cells (xenogeneic) or cancer cells of the same species as the host animal (syngeneic). It is worth mentioning that in the xenogeneic model the host animal needs to be immunodeficient. This is due to the introduction of biological material from a distinct species and the stronger immune response that comes with it (33). Another method for inducing bladder cancer in animals is the administration of chemical carcinogens. The most commonly used carcinogen for that purpose is N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) (33,34). It is found in tobacco smoke, which is one of the most important risk factors for the development of bladder cancer (34). BBN displays remarkable specificity for bladder carcinogenesis. The genomic analysis of BBN-induced cancer in mice revealed a genomic mutational landscape similar to muscle invasive bladder cancer in human patients (34). Finally, there is also the possibility to use genetically modified (transgenic) animals that develop tumors through the activation of oncogenes such as Ha-ras or alteration in the tumor suppressor genes RB1 and p53 (33). All these techniques are mostly conducted on rodents, more specifically on mice, because their urinary tract closely resembles that of humans. Additionally, they reproduce within a short time and are easily maintained at a low cost (33). The mice for this study were obtained from the Jackson Laboratory (USA) and are formerly known as C57BL/6J.

1.4.1 Syngeneic heterotopic model with MB49 cells

One of the models used in this study is a syngeneic subcutaneous bladder cancer model with MB49 cells. Inoculation of cancer cells into the subcutaneous tissue of laboratory animals is performed when the original site is not convenient for inoculation, as was the case here with the urinary bladder. In this model, murine bladder cancer cells (syngeneic) called MB49, are injected into the subcutaneous tissue of C57BL/6J male mice (heterotopic). Subcutaneous cancer models are easy to establish, easy to manage and cost-effective (33). They are widely used in mechanistic studies, as well as for evaluating the efficacy of novel therapeutic agents (33). MB49 cells are a murine bladder cancer cell line, that was induced by feeding 7,12-Dimethylbenzanthracene to the C57BL/6 mouse strain. They are very similar to human bladder cancer cells, which makes them a good model to study novel therapies (33). After the tumor mass reaches a certain size, the mice are treated with either Shikonin or Nec1s. These chemicals should have the exact opposite effects. Shikonin as an inducer and Nec1s as an inhibitor of necroptotic cell death. Nec-1s inhibits RIPK1 and therefore the formation of the necrosome. It has been described as a potent and specific inhibitor of necroptosis (35,36). Naphthoquinone, also known as Shikonin, is isolated from the plant Lithospermum erythrorhizon (37). Shikonin inhibits the pyruvate kinase isozyme M2 (PKM2), an enzyme which is important for the shift in energy production from the Krebs cycle to glycolysis. Thus, PKM2 is important in the energy metabolism of many cancers, including bladder cancer. Shikonin was described to induce both, necroptosis and apoptosis (38–40). Which of these effects dominates in our model has to be investigated. The tumor volume will be assessed at different time points during tumor growth. After sacrificing the animals, the tumor tissue will be analyzed with immunohistochemistry and immunoblotting.

1.4.2 Autochthonous BBN induced bladder cancer model

Since bladder cancer is often caused by continuous exposure to carcinogens such as the one found in tobacco smoke, autochthonous bladder cancer models try to mimic that by exposing rodents to chemical carcinogens with aromatic amine compounds. As mention earlier, the most commonly used compound from that group is BBN. The gene expression profile and the protein levels in BBN-induced rodent bladder cancers mimic those of muscle invasive cancer in humans. This is mainly why it is considered to be a reliable model for studying bladder cancer in vivo (34,41). A recent publication by our group has shown that the BBN model in mice displays an immunological profile similar to the one in human muscle invasive bladder

cancer, and thus represents an optimal model for preclinical studies (34). The analysis will be performed using gene expression studies and immunohistochemical staining.

1.5 Study design

Although great progress has been achieved in terms of bladder cancer management, survival rates for muscle invasive bladder cancer are still dismal, which warrants the search for new therapeutic approaches. As already described, the role of necroptosis in carcinogenesis varies with different types of cancer and so it must be examined on an individual basis. Understanding how it behaves in different cancer types allows one to find the tumors that are sensitive to either necroptosis induction or inhibition, which in turn could carry great therapeutic potential.

The first model that is being used is a carcinogen-induced bladder cancer model (for more information see method section under "autochthonous BBN induced bladder cancer model"). Since this model closely mimics human muscle invasive bladder cancer on both genomic and proteomic levels, the goal was to investigate the presence of necroptosis under (patho)physiological conditions at different time points during oncogenesis (34). Because of this resemblance, obtained results could be extrapolated to the human counterpart to some degree. Therefore, the amount of necroptotic cell death will be evaluated in different time points during carcinogenesis in a BBN induced bladder cancer model. Additionally, apoptotic cell death will be investigated in the same time points and compared to necroptosis. In terms of methods, immunohistochemistry and gene expression studies will be used.

The second model that will be used is a subcutaneous bladder cancer model with murine bladder cancer cells (for more information see method section under "Syngeneic heterotopic model with MB49 cells"). This model is relatively quickly obtained and allows for objective and comparable tumor volume tracking over time. It is also good for assessing the effect of different chemicals on carcinogenesis. Since the effects of necroptosis induction and inhibition on bladder cancer carcinogenesis are largely unknown, both an inhibitor and an inducer of necroptosis will be used in this study. Necrostatin1s (Nec1s) is going to be used as an inhibitor of necroptosis. This compound has been described as a potent and specific inhibitor of necroptosis (35,36). Nec1s was not used in this exact model before, which is why its specificity and effectiveness as necroptosis inhibitor will be assessed. Although TNF-alpha is the major inducer of necroptosis, it cannot be used *in vivo* since it can lead to endotoxic shock and

therefore death of the experimental animal (42). That is why naphthoquinone, also known as Shikonin, will be used as an inducer of necroptosis. Shikonin was also described as an apoptosis inducer, which is why its specificity as a necroptosis inducer will be assessed at the end of the experiment (40). The influence on the tumor volume of these compounds is going to be measured at multiple time points during the experiment. As already outlined, immunogenic cell death has great therapeutic potential. Therefore, the immune infiltrates of the subcutaneous tumors will be analyzed, to see if they show differences and if these differences could be correlated to potential effects on tumor growth. The analysis is going to be performed using immunohistochemistry, immunoblotting and gene expressing studies.

2. OBJECTIVES

The goal of this study is to investigate the role of necroptosis in bladder cancer using two distinct murine bladder cancer models.

In the BBN-induced bladder cancer model the amount of necroptotic cell death will be evaluated in different time points during carcinogenesis. Additionally, apoptotic cell death will be investigated in the same time points and compared to necroptosis.

In the subcutaneous bladder cancer model, mice are going to be treated with Necrostatin-1s and Shikonin. These two compounds should have the opposite effect of inhibition and induction of necroptosis, respectively. It is going to be investigated if these two compounds influence tumor growth. Additionally, the immune infiltrates of the subcutaneous tumors will be analyzed.

In summary, the objective of this study is to assess the prevalence of necroptosis at different time points during tumor progression and investigate if the induction or inhibition of necroptosis influences tumor growth and the immune infiltrate.

3. METHODS

3.1 Animal work

Experiments were performed on 6 to 8-weeks-old C57BL/6J male mice. All animal experimental procedures were in accordance with the guidelines provided by the Animal Health Protection Act and the Instructions for Granting Permits for Animal Experimentation for Scientific Purposes and were approved by the Ethics Committee, University of Split, School of Medicine (permit no. 2181-198-03-04-14-0036). Mice were maintained under standard conditions with a 12 h light/dark cycles and provided with free access to tap water and food pellets *ad libitum*.

3.2 Syngeneic heterotopic model with MB49 cells

C57BL/6J immunocompetent male mice were subcutaneously injected with a total of $1,5x10^5$ MB49 cells into the right flank (n=30). After two weeks, the tumor size has reached $62,5mm^3$. At this point, mice are divided into three groups of ten. One group was treated with Nec1s (5mg/kg), one with Shikonin (5mg/kg) and one group with 5mg/kg of DMSO (control). The respective treatments started at day 14 and were administered every three days via intraperitoneal injection (i.p injection). In total, every treatment was administered four times. At day 28 the mice were sacrificed. Measurements were taken multiple times until the tumor reached the appropriate volume of $62,5mm^3$, and after that at days 14, 21 and 28 of the experiment. Tumor size was assessed using a caliper and tumor volume was calculated using the following formula: (length x $width^2$) x 0.5. At day 28 the tumors were removed and divided into equal portions. One of them was conserved in 4% paraformaldehyde for paraffin embedding and further histological analysis. The other portion was immediately stored in liquid nitrogen in order to be used in gene expression studies and for protein analysis. The experimental outline is summarized in Figure 4.

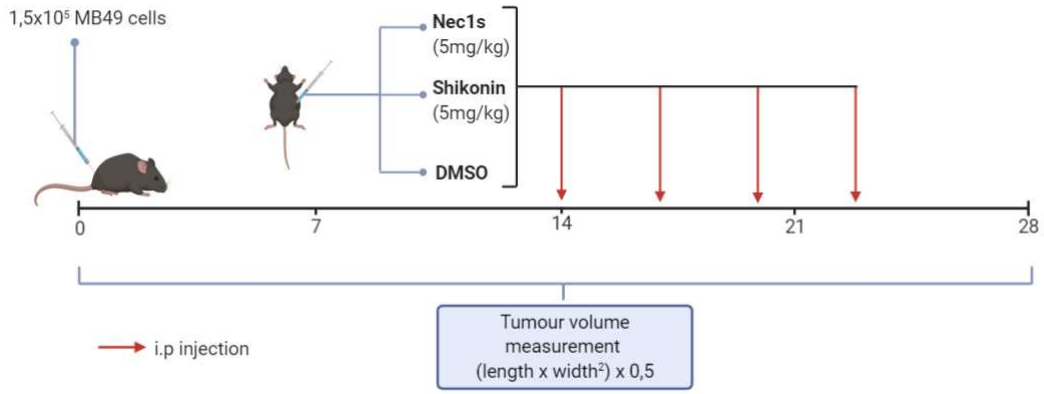


Figure 4. Syngeneic heterotopic model with MB49 cells. Mice were subcutaneously injected with $1{,}5x10^5$ MB49 cells. After the tumors reached a volume of $62{,}5mm^3$ on day 14, treatment with Nec1s, Shikonin and DMSO was initiated and repeated every three days, with a total of four treatments in every group. Tumor volume was assessed at days 14,21 and 28 of the experiment.

3.3 Autochthonous BBN induced bladder cancer model

Mice were given tap water containing 0.05% BBN (TCI Europe) for a maximum of 12 weeks, after which it was replaced by normal tap water until the end of the respective experiment. The control group was given normal tap water throughout the experiment. Mice were sacrificed at 1, 2, 4, 14, 20 weeks after the beginning of the BBN treatment. After the mice were sacrificed the urinary bladder was removed and cut sagittally into two parts. The part designated for RNA and protein isolation was stored immediately in liquid nitrogen and the second part was stored in 4% paraformaldehyde for paraffin embedding and histological examination. Mice used in this diploma thesis were the same mice which were used in our previous publication by Degoricija *et al.* following the principles of the 3Rs (43).

3.4 Immunohistochemistry

Paraffin sections were deparaffinized in xylol and hydrated in decreasing EtOH concentrations. Antigen retrieval was achieved using EDTA buffer and a steamer (pH 8). The endogenous peroxidase activity was blocked with 3% H_2O_2 in ddH_2O for 10 min and non-specific staining was blocked with 1-15% bovine serum albumin (BSA) for one hour at room temperature. Sections were incubated overnight at 4 °C with the following primary antibodies: anti-p-MLKL (Abcam, ab196436, 1:2000), anti-cleaved caspase 3 (Cell Signaling,

9661S, 1:200), anti-FoxP3 (Abcam, ab54501, 1:1000) and anti-Ki67 (Abcam, 16667, 1:200). For secondary antibodies, HRP-conjugated anti-rabbit (Dako, P0448, 1:200) were applied for 1 h at room temperature, followed by standard 3,3′-diaminobenzidine (DAB) staining procedure. Sections were counterstained with haematoxylin and dehydrated in increasing EtOH concentrations. Microscopy images were acquired using an Olympus BX43 (Olympus Corporation)

3.5 Western Blot Analysis

Tissue samples were directly lysed with RIPA buffer (50mM Tris, 1mM EDTA, 150mM NaCl, 1% Triton, 0.5% Triton, 0.5% DOC, 0.1% SDS, pH 7.4, 50mM NaF, 1mM activated NaOv, Roche protease inhibitors and Roche Phospho STOP) for 10 min on ice. The lysates were centrifuged at 12000×g for 20 min at 4 °C to obtain the supernatant. Equal amounts of protein were subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membranes were blocked in TBS-5% BSA with 0.1% Na-azid for one hour at room temperature, washed and incubated overnight at 4 °C with antibodies against anti-p-MLKL (Abcam, ab196436, 1:2000), anti-cleaved caspase 3 (Cell Signaling, 9661S, 1:200) and anti-vinculin (Sigma-Aldrich, V9131, 1:1000). After washing the membranes in TBS-Tween 0,05%, they were incubated in HRP-conjugated anti-rabbit (Dako, P0448, 1:1000) antibody and HRP-conjugated anti-mouse (Bio Rad,170-6516, 1:6000) for one hour at room temperature. The blots were washed and the immunoreactive proteins were visualized using luminol and a protein gel imager (ChemiDoc, BioRad). Signal intensity was normalized against vinculin.

3.6 RNA isolation, qPCR, and RNA sequencing

RNA was extracted using QIAzol (Qiagen) from bladder tissue according to the manufacturer's instruction. In the final step, RNA was dissolved in water. One microgram of total RNA was used for cDNA preparation which was performed following the High- Capacity cDNA Reverse Transcription Kit manufacturer's instructions (Applied Biosystems). Real-time PCR was carried out with Power SYBR Green master mix (Applied Biosystems) using the 7500 Real-Time PCR System (Applied Biosystems). Primers that were used in this study are shown in Table 2. Actin was used to normalize gene expression.

For the RNA-seq gene expression analysis of necroptosis relevant genes, we used RNA sequencing data published in Degoricija *et al.* (34). The RNA from 9 male mouse bladders at

20 weeks, 8 weeks post-BBN treatment, and 9 control, not treated mice that were matched in sex and age and were pooled into 6 samples (3 RNAs per sample). Library was prepared using TruSeq Stranded mRNA LT (Illumina), size distribution of the amplified DNA was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies) and KAPA SYBR® FAST qPCR Kit (KAPA Bio Systems) was used for quantitation of library concentration. RNA sequencing and FASTQC analysis were performed by Applied Biological Materials Inc. (Richmond, B.C. Canada), single-end 40 million reads were requested and sequenced on Illumina NextSeq500.

Table 2. Real time PCR Primer Sequences. Key: F= forward primer, R= reverse primer

Gene	Gene ID	Primer sequence	
Ripk1	NM_009068	F	GGAAGGATAATCGTGGAGGC
		R	AAGGAAGCCACACCAAGATC
Ripk3	NM_019955	F	AAGTTATGGCCTACTGGT
		R	TCCGAACCCTCCTTTACC
Mlkl	NM_029005	F	ACTGTGAACTTGGAACCCTG
		R	TGCTGATGTTTCTGTGGAGTG
Il33	NM_133775	F	TTCCAACTCCAAGATTTCCCC
		R	CAGAACGGAGTCTCATGCAG
Cxcl1	NM_008176	F	AACCGAAGTCATAGCCACAC
		R	CAGACGGTGCCATCAGAG
Bcl2	NM_009741	F	CAGAGACAGCCAGGAGAAATC
		R	GATGACTGAGTACCTGAA
Actin	NM_009609	F	GCTGATCCACATATGCTGGA
		R	CATTGCTGACAGGATGCAGAA

3.7 Statistical analysis

All the data were statistically analyzed using GraphPad Prism 8. The Shapiro-Wilk normality test was performed and all the data showed normal distribution. Two-sided Student's t-test and the one-way ANOVA test with post-hoc Tukey analysis were used to assess differences in percentage of positive cells in immunohistochemical staining, western blot normalized signal intensity, expression of genes and differences in tumor size. Individual statistical tests are shown for each experiment in the figure legend. All data are presented as mean±standard deviation. The data from whole genome sequencing is presented as a heatmap displaying median based z-scores of genes.

4. RESULTS

4.1 Necroptosis and apoptosis occur in separate clusters and their prevalence increases towards later tumor stages

The prevalence of necroptosis and apoptosis was assessed and compared in different time points during carcinogenesis. Degoricija *et al.* recently showed that major histological changes in mice at 1, 2, 4 and 14 weeks of BBN exposure include reactive atypia, dysplasia, and carcinoma in situ (CIS) (34). In these time points one can observe sporadically appearing apoptotic (ClCas3 positive) and necroptotic (p-MLKL positive) cells. Representative images of a 2-week BBN treatment are shown Figure 5 A and Figure 5 B.

Mice treated with BBN for 20 weeks are in more advanced stages of carcinogenesis and therefore present with invasive tumors (34). In contrast to earlier tumor stages, invasive tumors show an increased prevalence of necroptosis and apoptosis (Figure 5C, D). It is also evident that apoptotic and necroptotic cells group within distinct clusters, known as hotspots (Figure 5C, D). These hotspots occur in separate locations and so do not overlap. Although the number of apoptotic and necroptotic cells increases, neither of these two types of cell death seems to dominate in invasive tumors i.e. the ratio of necroptotic to apoptotic cells is not significantly different (Figure 5E).

The notion that necroptosis becomes a significant phenomenon only in more advanced stages of tumor development is further supported by qPCR results (Figure 5F). These show that the expression of necroptosis core proteins (*Ripk1, Ripk3, Mlkl*) is significantly upregulated after 20 weeks of BBN treatment. *Bcl2,* a negative regulator of apoptosis*,* is downregulated after 20 weeks of BBN treatment as well (Figure 5F). This suggests that the prevalence of apoptosis, similar to necroptosis, is higher in more advanced tumor stages (Figure 1F).

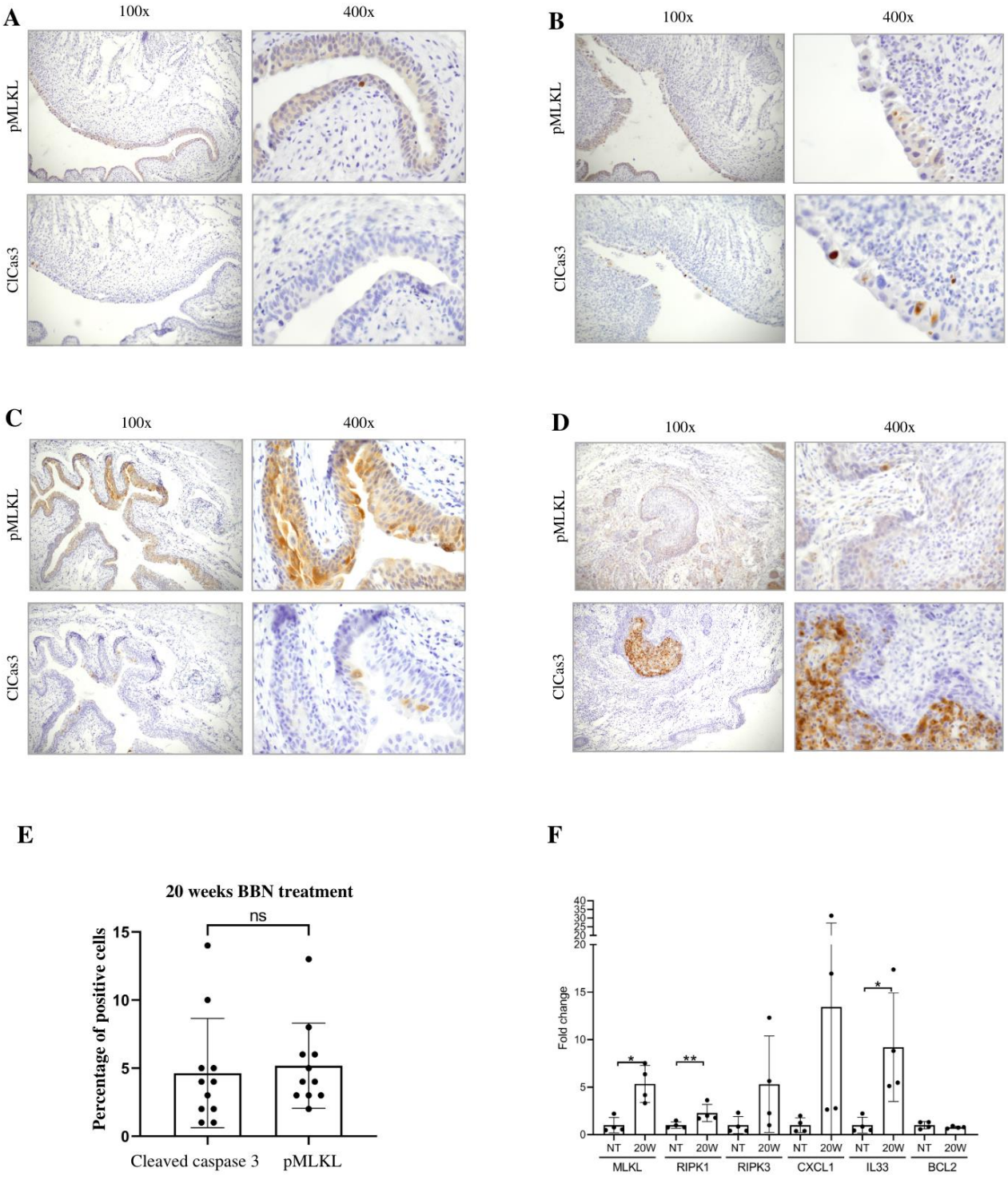


Figure 5. Necroptosis and apoptosis occur in separate clusters of cells and their prevalence increases towards later tumor stages. (A, B) Immunohistochemistry of a bladder section stained for anti-p-MLKL and anti-cleaved Caspase 3 (ClCas3) on 2 weeks BBN treated mice. Left panel 100x magnification, right panel 400x magnification. (C, D) Immunohistochemistry

of a bladder section stained for p-MLKL and cleaved Caspase 3 (ClCas3) on 20 weeks BBN treated mice. Left panel 100x magnification; right panel 400x magnification. (E) Percentage of positive cells within apoptotic and necroptotic hotspots in 20 weeks BBN treated mice. *p<0.05, **p<0.005, Student's *t*-test. Data are presented as mean±SD. (F) Expression of necroptosis core proteins (*Mlkl, Ripk1, Ripk3*), their downstream effectors (*Cxcl1, Il33*) and *Bcl2* in 20 weeks BBN treated mice. *p<0.05, ** p<0.005, Student's *t*-test. Data are presented as mean±SD.

As shown by the heatmap in Figure 6, almost all genes that are involved in necroptosis signaling are upregulated after 20 weeks of BBN treatment in comparison to the non-treated control. Interestingly, there is also a significant increase in the expression of genes that are downstream of the necrosome such as *Cxcl1* and *Il33* (Figure 5E, Figure 6).

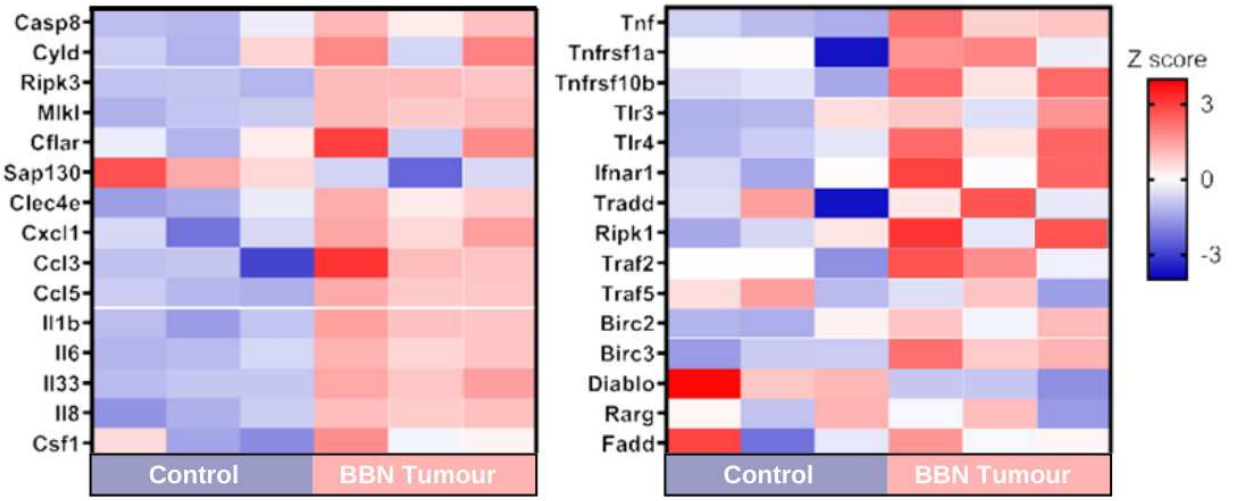


Figure 6. Genes that are involved in necroptosis signaling are upregulated after 20 weeks of BBN treatment. Heatmap displaying median based z-scores of genes that are involved in the necroptosis signaling pathway.

4.2 Necrostatin-1s and Shikonin change the tumor volume significantly but at different time points

Shikonin treated mice did not show any observable effect on tumor volume up to day 21 of the experiment. From day 21 onwards Shikonin seems to slow down tumor growth so that a significant difference in tumor volume can be seen by the end of the experiment at day 28, with tumors being smaller in the Shikonin group than in the control group. Mice treated with Necrostatin-1s present with consistently higher tumor volumes from the start of the experiment when compared to control, with a significantly higher tumor volume at day 21 of the experiment. Interestingly, this effect tapers off from day 21 onwards and can only be slightly, but not statistically significantly, observed at the end of the experiment (Figure 7).

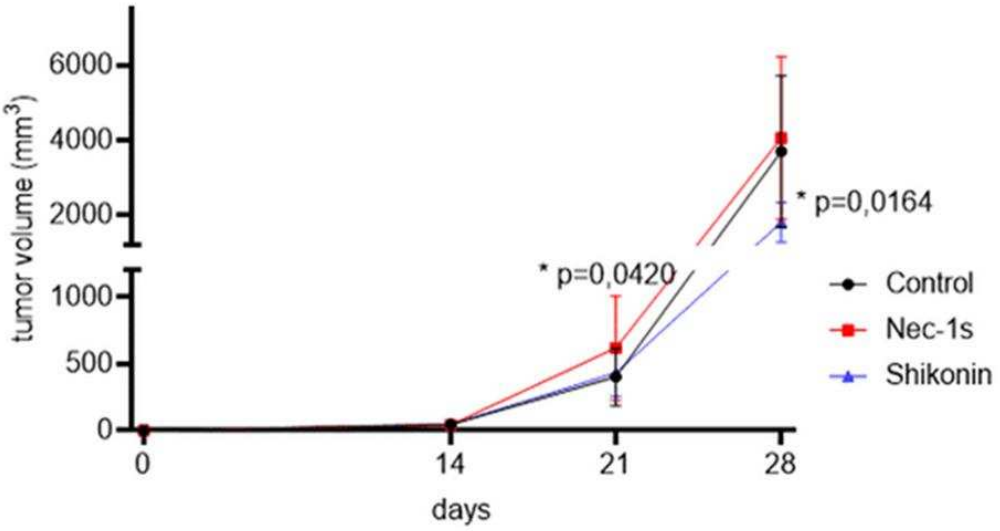


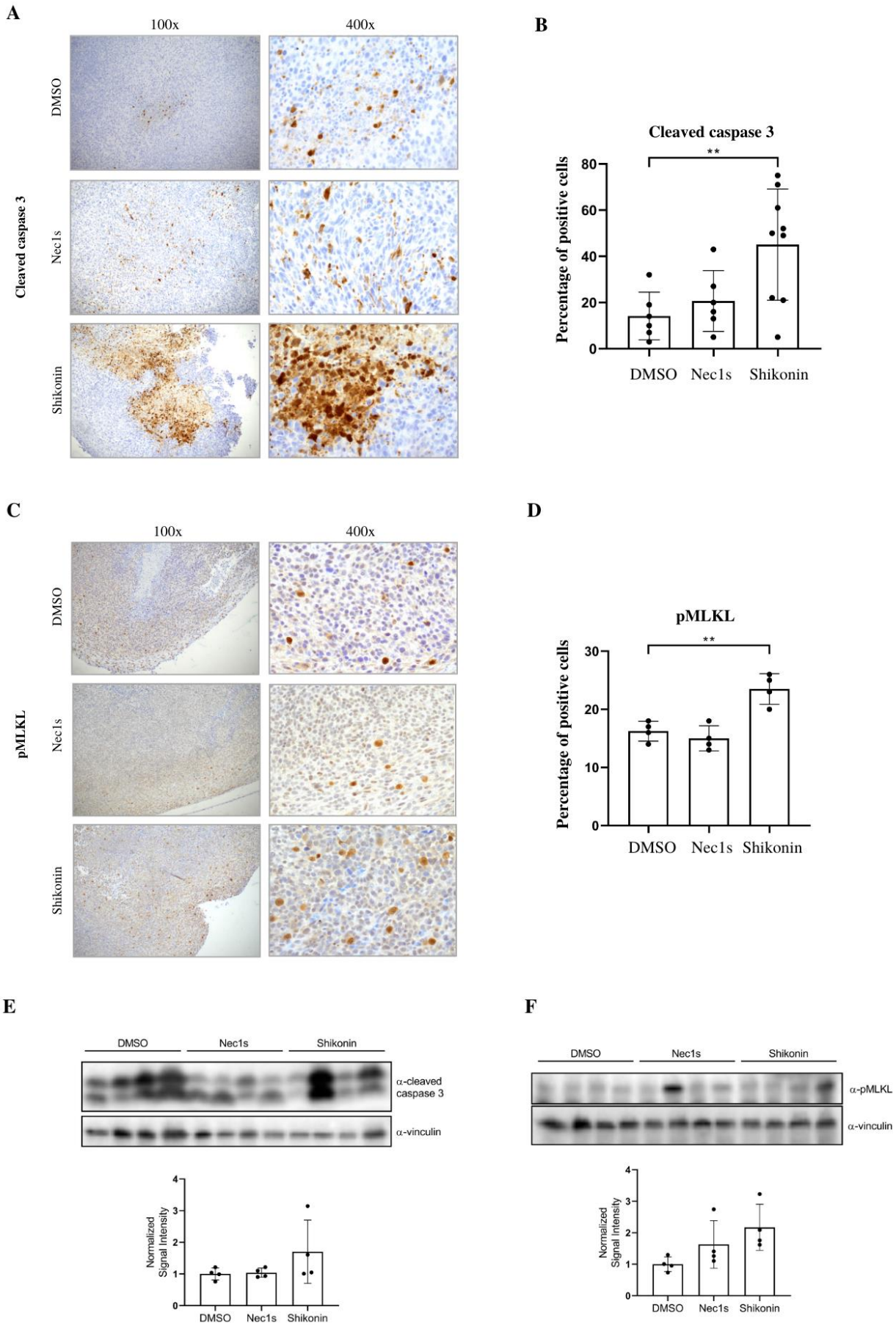
Figure 7. Necrostatin-1s and Shikonin influence subcutaneous MB49 tumor growth. Tumor volume over time for Necrostatn-1s (Nec-1s), Shikonin and DMSO (control) treated groups. n=10 per group, * $p<0.05$, Student's *t*-test.

4.3 Shikonin induces both apoptosis and necroptosis and additionally increases the percentage of T-regulatory cells

In the control group (DMSO), the number of apoptotic and necroptotic cells is similar. Shikonin increases the number of apoptotic cells (Cleaved caspase 3 positive) by more than 3 times and the number of necroptotic cells (p-MLKL positive) by 0.5 times, showing that Shikonin primarily induces apoptosis (Figure 8A-D).

Noteworthy is that the percentage of necroptotic cells (p-MLKL positive) does not differ significantly between the control and the Necrostain-1s (Nec-1s) treated group, suggesting that Nec1s does not have the expected inhibitory effect on necroptosis on day 28 of the experiment (Figure 8C, D). In contrast, Shikonin significantly increases the percentage of necroptotic cells (Figure 8C, D).

Although not statistically significant, western blot results support the notion that Shikonin induces apoptosis and necroptosis (Figure 8E, F). One can also observe a paradoxical increase of necroptosis in the Nec1s treated group (Figure 8F). Additionally, when looking at the histological sections of the subcutaneous tumor model, one can see how necroptotic cells usually localize close to the capsule of the tumor (Figure 8C).


A
100x
400x
DMSO
Nec1s
Shikonin
Cleaved caspase 3
B
Cleaved caspase 3
**
Percentage of positive cells
80
60
40
20
0
DMSO
Nec1s
Shikonin
C
100x
400x
DMSO
Nec1s
Shikonin
pMLKL
D
pMLKL
**
Percentage of positive cells
30
20
10
0
DMSO
Nec1s
Shikonin
E
DMSO
Nec1s
Shikonin
α-cleaved caspase 3
α-vinculin
Normalized Signal Intensity
4
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Nec1s
Shikonin
α-pMLKL
α-vinculin
Normalized Signal Intensity
4
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2
1
0
DMSO
Nec1s
Shikonin

Figure 8. **Shikonin induces both necroptosis and apoptosis** (A) Immunohistochemistry stained for anti-cleaved caspase 3 (ClCas3) on subcutaneous bladder cancers treated with DMSO, Nec1s and Shikonin. Left panel 100x magnification, right panel 400x magnification. (B) Percentage of positive apoptotic cells (ClCas3). *p<0.05, **p<0.005, ANOVA with post-hoc Tukey analysis. Data are presented as mean±SD. (C) Immunohistochemistry stained for necroptotic cells using anti-p-MLKL on subcutaneous bladder cancers treated with DMSO, Nec1s and Shikonin. Left panel 100x magnification, right panel 400x. (D) Percentage of positive necroptotic cells (p-MLKL). *p<0.05, **p<0.005, ANOVA with post-hoc Tukey analysis. Data are presented as mean±SD. (E) Subcutaneous tumors were analyzed for apoptosis with anti-cleaved caspase-3 (ClCas3) using Western blot. Equal loading was controlled by using anti-vinculin. Results are presented as normalized signal intensities. p = ns, ANOVA with post-hoc Tukey analysis. Data are presented as mean±SD. (F) Subcutaneous tumors were analyzed for necroptosis with anti-p-MLKL using Western blot. Equal loading was controlled by using anti-vinculin. Results are presented as normalized signal intensities. p = ns, ANOVA with post-hoc Tukey analysis. Data are presented as mean±SD.

The tumors of mice that were treated with Shikonin show a significant increase in the percentage of T-regulatory cells (FoxP3 positive cells) (Figure 9A, B). Finally, immunohistochemical staining for anti-Ki67 showed how the three groups did not seem to differ in terms of mitotic activity (Figure 9C).

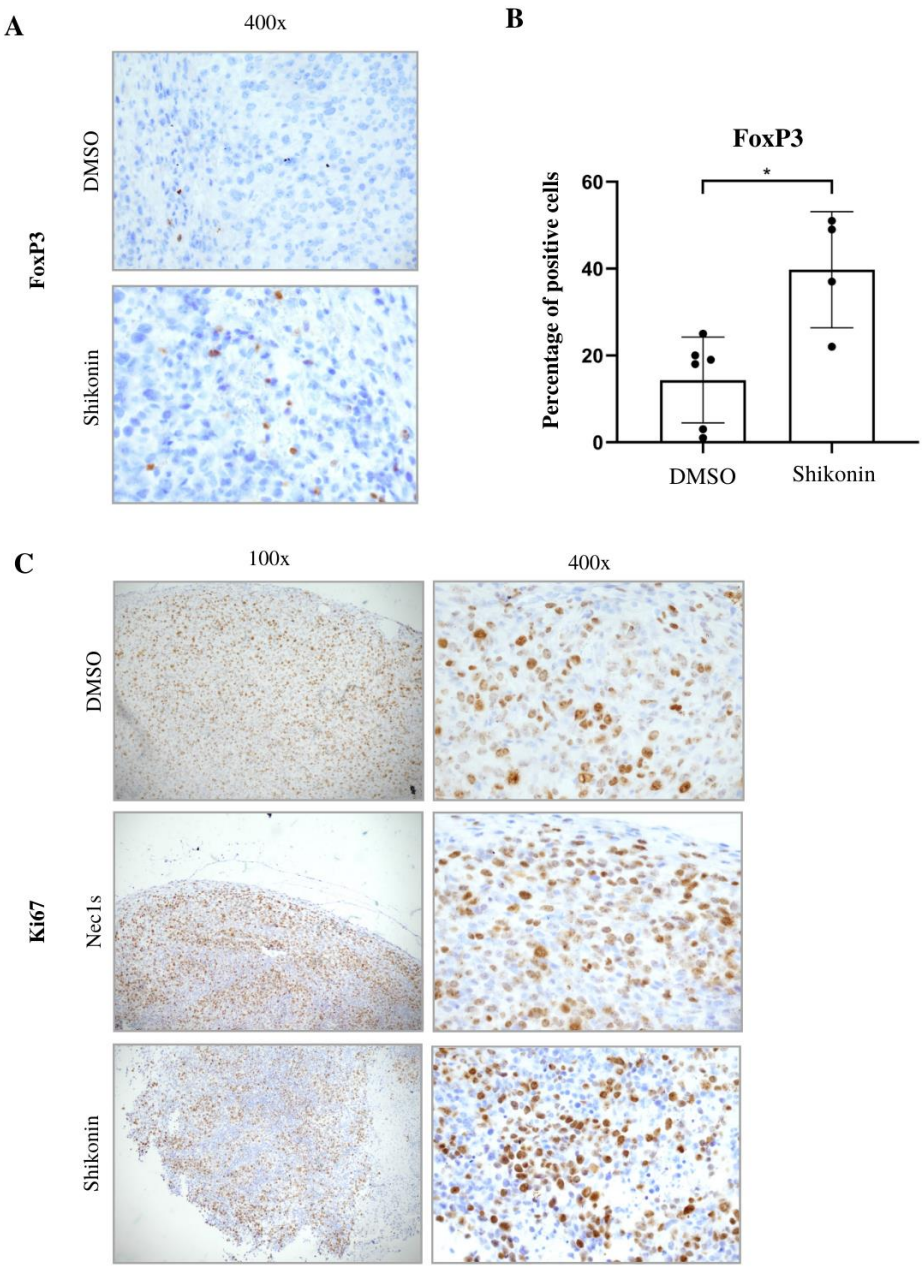

Figure 9. **Shikonin increases the percentage of T-regulatory cells but does not influence mitotic activity** (A) Immunohistochemistry stained for anti-FoxP3 (T-regulatory cells) on subcutaneous bladder cancers treated with DMSO, Nec1s and Shikonin. 400x magnification. (B) Percentage of T-regulatory cells (FoxP3). *p<0.05, Student's *t*-test. Data are presented as mean±SD. (C) Immunohistochemistry stained for anti-Ki67 on subcutaneous bladder cancers treated with DMSO, Nec1s and Shikonin. Left panel 100x, right panel 400x magnification.

5. DISCUSSION

This study investigated the role of necroptosis in bladder cancer, by using two distinct murine bladder cancer models. The results obtained from the BBN model show that necroptotic cell death becomes a significant phenomenon only in later tumor stages i.e. in invasive tumors (Figure 5). Apoptotic cell death behaves in a similar fashion. These findings are most likely explained by the fact that tumors in advanced stages of development have a bigger immune infiltrate and therefore a more diverse and potent concentration of inflammatory mediators within the tumor microenvironment. This notion is supported by RNA-sequencing that shows how almost all molecules involved in necroptotic signaling are upregulated in advanced stages of tumor development (Figure 6). A significant increase is particularly seen in TNF-alpha, a major inducer of the necroptotic signaling pathway (Figure 6). The second important observation is that apoptotic and necroptotic hotspots present at separate locations within the same sample (Figure 5 A-D). In other words, areas with these respective hotspots do not seem to overlap. A possible explanation for this finding may be the way how the TNF-alpha induced signaling pathway works (Figure 2). Following this signaling pathway, a cell can enter only into either necroptosis or apoptosis. Finding areas of apoptosis and necroptosis in separate clusters (hotspots) lets one to hypothesize that the inflammatory environment in one part of the tissue section dictates which type of cell death is activated. Presumably, once one type of cell death is activated, the remaining cells within that cluster will undergo that same type of cell death. Finally, results showed a significant increase of *IL33* and *CXCL1* expression (Figure 5F, Figure 6). These molecules are found downstream of the necrosome. IL33 has been shown to induce T-regulatory cells (44,45). CXCL1 was described as the main mediator of an immunosuppressive tumor environment in pancreatic ductal adenocarcinoma (31). Further investigation is needed to examine whether these molecules are really under the control of the necrosome in this particular situation and what their influence on carcinogenesis is. The BBN bladder cancer model closely mimics the human counterpart, so the results that were obtained here can be extrapolated to human bladder cancer to a certain degree.

In the subcutaneous bladder cancer model, Necrostatin-1s treated mice showed a significantly higher tumor volume at day 21 of the experiment when compared to the control (Figure 7). However, this effect tapered off and was not observed at day 28 of the experiment. This shows that whatever effect Necrostatin-1s might have had on carcinogenesis in tumors of smaller volume at day 21, was lost in bigger tumors at day 28 of the experiment (Figure 7). This could explain why we could not detect any decrease in p-MLKL i.e. in necroptosis in the Nec1s treated group. On the contrary, our Western blot results, although not statistically significant,

indicate that the Nec1s treated group had even higher levels of necroptosis than the control group. This finding might be explained by the slightly larger tumor volume in the Nec1s group on day 28 of the experiment (Figure 7). Further, one could hypothesize that because Nec1s increased tumor volume at day 21, necroptosis actually protects against tumor progression in small tumors and that its induction rather than its inhibition could have therapeutic value, perhaps even by immune modulating mechanisms. This is under the presumption that necroptosis was indeed inhibited by Nec1s. To prove this hypothesis, the experiment would need to be repeated and ended at day 21. The specificity of Nec1s on necroptosis inhibition and potential effects on the immune system would need to be revaluated and confirmed. Additionally, it was interesting to observe that cells that stained positive for p-MLKL (necroptotic cells) were clustered around the capsule of the subcutaneous tumor. This is a region with high rates of cellular proliferation and therefore high metabolic activity, as shown immunohistochemistry sections stained against Ki67 (Figure 9C). This is indicating once more that necroptosis is an active process which needs an adequate metabolic environment and comes opposed to unregulated necrosis that is merely a passive phenomenon and results mostly from the lack of adequate blood supply (9).

Shikonin did not show any significant effect on tumor volume up to day 21 of the experiment. However, from day 21 onward, tumor growth slows down and at the end of the experiment differs significantly from the control (Figure 7). Shikonin significantly induces apoptosis as well as necroptosis in subcutaneous tumors. Although the results suggest that Shikonin preferentially induces apoptosis, its effects on tumor volume cannot be attributed to either one of these types of cell death with certainty (Figure 8). The reason for this non-specific induction of both types of cell death can likely be explained by its mechanism of action. Shikonin acts as an inhibitor of pyruvate kinase isozyme M2 (PKM2), an enzyme which is important for the shift in energy production from the Krebs cycle to glycolysis (38,39). Malignant cells, more so than normal cells, are dependent on glycolysis as their primary source of ATP. This is known as the Warburg effect (39). Therefore, Shikonin interferes with the energy metabolism of tumor cells and likely non-specifically induces cell death by depleting tumor cells of ATP (39). Which type of cell death is induced therefore varies with the cell type that is being investigated (39,40). In MB49 cells Shikonin seems to preferentially induce apoptosis (Figure 8). Noteworthy, cell division did not seem to be influenced by Shikonin, as shown with tissue sections of subcutaneous tumors stained against Ki67 using immunohistochemistry (Figure 9). To elucidate if the decrease in tumor volume is a result of

apoptosis or necroptosis, or perhaps some other mechanism, future experiments could block apoptotic cell death by co-administering a pan caspase inhibitor with Shikonin to exclude the effects of apoptosis. Also, administering Shikonin together with Nec1s could exclude the effects of necroptosis.

Furthermore, the results show that tumors of mice treated with Shikonin had an increased percentage of FoxP3 (T-regulatory cells) (Figure 9). Shikonin has already been described to induce T-regulatory cells (44). A possible explanation for this finding could be an indirect induction of T-regulatory cells by Shikonin via the necrosome. The necrosome has been shown to directly induce IL33 production (45). IL33, on the other hand, has been described as an inducer of T-regulatory cells (46,47). Based on these findings, one could theorize that Shikonin increases the amount of IL33 via the induction of necroptosis. This in turn increases the number of T-regulatory cells. If the induction of T-regulatory cells is indeed dependent on the necrosome could be tested by preventing the formation of the necrosome by administering Shikonin together with a necroptosis inhibitor such as Nec1s.

The fact that Shikonin increases the number of T-regulatory cells seems paradoxical when considered that at the same time it slows down tumor growth. This is because T-regulatory cells are usually regarded as immunosuppressive and tumor promoting (31). A possible explanation could be that Shikonin's interference with the energy metabolism of tumor cells and the subsequent induction of cell death simply overshadowed the immunosuppressive effects of T-regulatory cells. Notably, we did not observe any changes in T-cells (CD4, CD8) or immunosuppressive macrophages (CD163) in the Shikonin-treated group (data not shown).

In summary, it was shown that necroptotic cell death is a late finding during tumor development and that necroptosis and apoptosis occur in separate hotspots. Nec1s treated mice had significantly higher tumor volumes at day 21 of the experiment. This effect was not observed by the end of the experiment on day 28. Nec1s did not show the expected inhibitory effect on necroptosis on day 28 of the experiment. Therefore, further improvement needs to be made in the experimental outline when it comes to the Nec1s group. The experiment should be ended at day 21, because at his time point the effects of Nec1s seem to be most prominent. Then one could evaluate the specificity of Nec1s as a necroptosis inhibitor. One could also confirm or refute the effects on tumor growth and clarify if these effects are related to immune modulating mechanisms. Shikonin significantly slowed down tumor growth. This was likely caused by a dual induction of apoptosis and necroptosis. However, the effects on apoptosis and

necroptosis would need to be studied separately in order to clarify which one of these two types of cell deaths primarily influences tumor growth. Also, future experiments should clarify if additional mechanisms are at work. Shikonin also promotes a counterintuitive induction of T-regulatory cells, which warrants further investigation. Overall, Shikonin is a new compound with a significant inhibitory effect on tumor growth. This justifies the use of Shikonin in autochthonous induced bladder cancer models, such as in the BBN-induced bladder cancer model. Shikonin was already used in topical applications for inflammatory mouth lesions and showed good tolerance as well as clinical improvement (37). Were Shikonin to show the same antitumorigenic results in the BBN-induced bladder cancer model and other preclinical models, more clinical trials could be conducted with the purpose of gaining more data on this new compound and its possible clinical applications.

6. CONCLUSION

1. Necroptotic cell death is a late finding during tumor development in a BBN bladder cancer model.
2. Necroptosis and apoptosis occur in spatially separate hotspots in a BBN bladder cancer model.
3. Necrostatin-1s accelerates, while Shikonin slows down tumor growth in a subcutaneous tumor model with MB49 cells.
4. Shikonin induces both, necroptosis, and apoptosis in a subcutaneous tumor model with MB49 cells.
5. Shikonin increases the percentage of T-regulatory cells in a subcutaneous tumor model with MB49 cells.
6. Shikonin has therapeutic potential in human MIBC.

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8. SUMMARY

Objectives: Although great progress has been achieved in terms of bladder cancer management, survival rates for muscle invasive bladder cancer are still dismal, which warrants for the search of new therapeutic approaches. Necroptosis is a programmed type of cell death, whose role has not been fully elucidated in bladder cancer. The objective of this study is to assess the prevalence of necroptosis at different time points during tumor progression and to investigate whether induction or inhibition of necroptosis influences tumor growth.

Methods: This study used two distinct murine bladder cancer models. The first one was an autochthonous BBN-induced bladder cancer model. The second one was a subcutaneous tumor model, made using MB49 cells. Mice with subcutaneous tumors were treated with Necrostatin-1s and Shikonin, two compounds that should have the opposite effects of inhibition and induction of necroptosis, respectively. The tumors were analyzed using gene expression studies, immunohistochemistry and immunoblotting.

Results: It was shown that necroptotic cell death is a late finding during tumor development and that necroptosis and apoptosis occur in separate hotspots in BBN-induced bladder cancers. Necrostatin-1s significantly accelerated tumor growth. This effect on tumor growth tapered off and was not significantly present by the end of the experiment. Further, Necrostatin-1s did not show the expected inhibitory effect on necroptosis. Shikonin-treated mice had significantly smaller tumor volumes by the end of the experiment and it was shown that Shikonin induced both necroptosis and apoptosis. Additionally, it was observed that Shikonin increased the number of T-regulatory cells in subcutaneous tumors.

Conclusion: Necroptotic cell death is a late finding during tumor development, indicating that this type of cell death primarily occurs towards invasive tumor stages. Necroptosis and apoptosis occur in separate hotspots, presumably because the inflammatory environment in one part of the tissue dictates the type of cell death being activated. Necrostatin-1s accelerates tumor growth, which allows one to hypothesize that necroptosis is protective against tumor progression. Shikonin slows down tumor growth likely via a dual induction of necroptosis and apoptosis. This justifies the use of Shikonin in autochthonous induced bladder cancer models, such as in the BBN-induced bladder cancer model. Were Shikonin to show the same antitumorigenic results in the BBN-induced bladder cancer model and other preclinical models, more clinical trials could be conducted with the purpose of gaining more data on this new compound and its possible clinical applications.

9. CROATIAN SUMMARY

Naslov: Uloga nekroptoze u tumoru mokraćnog mjehura

Ciljevi: Iako je postignut veliki napredak u liječenju tumora mokraćnog mjehura, preživljenje kod osoba s mišićno invazivnim tumorom mokraćnog mjehura i dalje je nisko što opravdava potrebu za novim terapijskim pristupima. Nekroptoza je programirani tip stanične smrti, čija uloga nije do kraja razjašnjena u tumoru mokraćnog mjehura. Cilj ovog istraživanja je proučiti prisutnost nekroptoze u različitim vremenskim točkama tijekom progresije tumora i istražiti utječe li indukcija ili inhibicija nekroptoze na rast tumora.

Metode: Studija koristi dva različita mišja modela tumora mišjeg mjehura. Prvi je autohtoni model tumora mokraćnog mjehura induciran BBN-om. Drugi je model potkožni tumor iniciran pomoću tumorskih stanica MB49. Miševi s potkožnim tumorima tretirani su s Necrostatin-1s i Shikoninom, dva spoja koji bi trebali imati suprotne učinke - inhibicija i indukcija nektoptoze. Kod tumora je analizirana ekspresija gena pomoću metode Q-PCR, te ekspresija proteina pomoću imunohistokemije i imunoblotinga.

Rezultati: Pokazano je da se nekroptoza javlja u kasnim uznapredovalim fazama tumora mokraćnog mjehura izazvanog BBN-om te da se nekroptoza i apoptoza pojavljuju u prostorno odvojenim nakupinama (hotspots). Miševi tretirani s Necrostatin-1s imali su značajno veće volumene tumora u početnim fazama tretmana ali se je taj efekt izgubio do kraja eksperimenta. Na kraju eksperimenta Necrostatin-1s nije pokazao očekivani inhibitorni učinak na nekroptozu. Miševi tretirani sa Shikoninom imali su značajno manji volumen tumora na kraju eksperimenta, a pokazano je da Shikonin inducira nekroptozu i apoptozu. Uz to, uočeno je da Shikonin povećava broj T-regulatornih stanica u potkožnim tumorima.

Zaključak: Nekroptoza se ponajprije pojavljuje u uznapredovalim stadijima invazivnog tumora mokraćnog mjehura. Nekroptoza i apoptoza prisutna je u prostorno odvojenim nakupinama u tumoru, vjerojatno zbog različitog upalnog mikrookoliša u tumoru koji diktira koja vrsta stanične smrti će se aktivirati. Nekrostatin-1 ubrzava rast tumora, što upućuje zaštitnu ulogu nekroptoze kod progresije tumora. Shikonin usporava rast tumora pri čemu inducira nekroptozu i apoptozu. To opravdava upotrebu Shikonina u autohtonom modelu BBN-om induciranog tumora mokraćnog mjehura. Daljnja istraživanja antitumorskog učinka Shikonina na modelu tumora mokraćnog mjehura izazvanog BBN-om kao i na drugim pretkliničkim modelima bila bi dobra osnova za potencijalna buduća klinička ispitivanja i eventualnu kliničku primjenu Shikonina u liječenju tumora mokraćnog mjehura.

10. CURRICULUM VITAE

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Korac-Prlic J, Degoricija M, Vilović K, **Haupt B**, Ivanišević T, Franković L, Grivennikov S, Terzić, J. Targeting Stat3 signaling impairs the progression of bladder cancer in a mouse model. Cancer Lett. 2020.

Others

Poster prize from the European Association for Cancer Research (EACR)

Korac-Prlic J, Degoricija M, Vilović K, **Haupt B**, Ivanišević T, Franković L, Grivennikov S, Terzić, J. "STAT3 is essential for bladder cancer progression"

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