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REVIEW ARTICLE

MLKL in cancer: more than a necroptosis regulator

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Abstract

Mixed lineage kinase domain-like protein (MLKL) emerged as executioner of necroptosis, a RIPK3-dependent form of regulated necrosis. Cell death evasion is one of the hallmarks of cancer. Besides apoptosis, some cancers suppress necroptosis-associated mechanisms by for example epigenetic silencing of RIPK3 expression. Conversely, necroptosis-elicited inflammation by cancer cells can fuel tumor growth. Recently, necroptosis-independent functions of MLKL were unraveled in receptor internalization, ligand-receptor degradation, endosomal trafficking, extracellular vesicle formation, autophagy, nuclear functions, axon repair, neutrophil extracellular trap (NET) formation, and inflammasome regulation. Little is known about the precise role of MLKL in cancer and whether some of these functions are involved in cancer development and metastasis. Here, we discuss current knowledge and controversies on MLKL, its structure, necroptosis-independent functions, expression, mutations, and its potential role as a pro- or anti-cancerous factor. Analysis of MLKL expression patterns reveals that MLKL is upregulated by type I/II interferon, conditions of inflammation, and tissue injury. Overall, MLKL may affect cancer development and metastasis through necroptosis-dependent and -independent functions.

Facts

- MLKL is the executioner of necroptosis, a RIPK3-dependent process. Necroptosis may function as a tumor-suppressing mechanism and as a back-up cell death modality in case of apoptosis resistance.
- Necroptosis provokes inflammation that can fuel tumor growth.
- Some cancers suppress necroptosis by epigenetic silencing of RIPK3. MLKL expression is variable in cancer, but no epigenetic silencing mechanisms have been reported for MLKL to date.
- Little is known about the regulation and exact role of MLKL in cancer development and metastasis. Subcellular functions of MLKL such as receptor/ligand degradation and endosomal trafficking may operate independently of RIPK3.

Open questions

- Do necroptosis-independent functions of MLKL involve RIPK3-mediated phosphorylation? Which other kinases or mechanisms may activate MLKL?
- How is MLKL expression regulated in cancer?
- Does the role of MLKL in cancer involve its cell death-dependent or -independent mechanisms?
- Is there any evidence of functional MLKL mutations in cancer?

Introduction

Necroptosis, a form of regulated necrosis, is initiated by the kinase activity of the receptor-interacting protein kinase 3 (RIPK3) in conjunction with one of the other RHIM-containing proteins such as RIPK1, TRIF, or ZBP1, pending on the stimulus during infection and inflammation (reviewed in [1]) (Fig. 1). The mixed lineage kinase domain-like (MLKL) pseudokinase was identified as a downstream target of RIPK3 and final effector of

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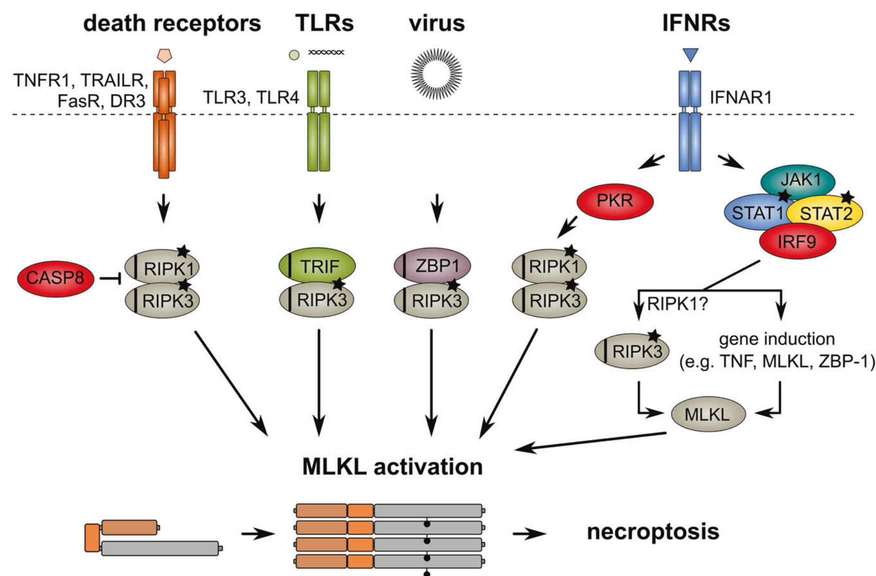


Fig. 1 Multiple stimuli lead to necroptosis. RIPK3-dependent necroptosis can be initiated by engagement of RIPK1, TRIF, or ZBP-1 through their respective RHIM domains (indicated as black stripe) upon activation by phosphorylation (asterisks). Various stimuli induce necroptosis by different pathways that all converge to the executioner of necroptosis, the pseudokinase MLKL. RIPK3-dependent phosphorylation (indicated as black dot on MLKL molecule) induces a conformational switch in MLKL and oligomerization that finally results in necroptosis. Necroptosis occurs especially under conditions of caspase-8 inhibition or ablation. See the legend to Fig. 2 for MLKL molecular domains.

necroptosis execution [2, 3]. Which RHIM-domain-containing protein will recruit and activate RIPK3, depends on the type of signaling receptor that is triggered. The canonical necroptosis pathway is initiated by TNF receptor family ligands (e.g. TNF, TRAIL, TL1A) and requires RIPK1 kinase activity, followed by recruitment and activation of RIPK3, which in its turn activates MLKL by serine-dependent phosphorylation. Various stimuli such as death receptor ligands (e.g. TNF), pathogen-associated molecular patterns (PAMPs) (e.g. LPS) and interferon (IFN) induce necroptosis by different pathways involving distinct RHIM-domain-containing proteins such as RIPK1, TRIF, and ZBP1, that all converge on RIPK3-mediated activation of the single known executioner of necroptosis, viz. MLKL (Fig. 1). RIPK3-dependent phosphorylation of MLKL results in a conformational switch and translocation of MLKL from the cytosol to diverse cellular membranes, including the plasma membrane where it causes loss of membrane integrity and eventually necrotic death. RIPK3 and MLKL constitute the core necroptosis machinery, whereas RIPK1 possesses a dual function as an essential survival factor through its scaffold function and as being a mediator of necroptosis (as well as certain types of apoptosis) through its kinase activity [4, 5]. Whether RIPK1 kinase activity mediates apoptosis or necroptosis depends on the inactivation of caspase-8 activity, a negative regulator of necroptosis [6, 7]. Necroptosis is implicated in a plethora of diseases ranging from degenerative diseases to inflammatory diseases [1] due to its lytic nature of cell death that provokes inflammation [8]. Its possible involvement in cancer is a topic of intense research and discussion [9–11]. Necroptosis in cancer can be regarded as a double-edged sword. Discovery of necroptosis as a back-up cell death mechanism gave rise to the concept to therapeutically target necroptosis in cancer cells that acquired apoptosis resistance [9]. Necroptosis, on the other hand, as an inflammatory/immunogenic mode of cell death can potentially fuel cancer growth and metastasis [12] or enhance antitumor immunity [13–15]. RIPK3 as the upstream kinase of MLKL has been studied extensively, but little is known about the exact role of MLKL and its regulation in cancer development and metastasis. Few existing literatures on the role of MLKL in cancer are fragmentary and controversial. MLKL and its role in cancer has been exclusively discussed in the context of necroptosis because of the known link between cell death and cancer. However, more recently other functions of MLKL beyond necroptosis are being reported such as receptor/ligand internalization, endosomal trafficking, EV, and exosome formation [16]. Therefore in this review we focus on these cell death-independent functions of MLKL to gain insight into the pleiotropic nature of this protein. In addition, it is known that cancer cells can suppress necroptosis by gene silencing and loss-of-function mutations of essential necroptotic factors. *RIPK3* expression is often silenced by promoter methylation in various cancer cells [17]. *MLKL* expression is variable in cancer, but there

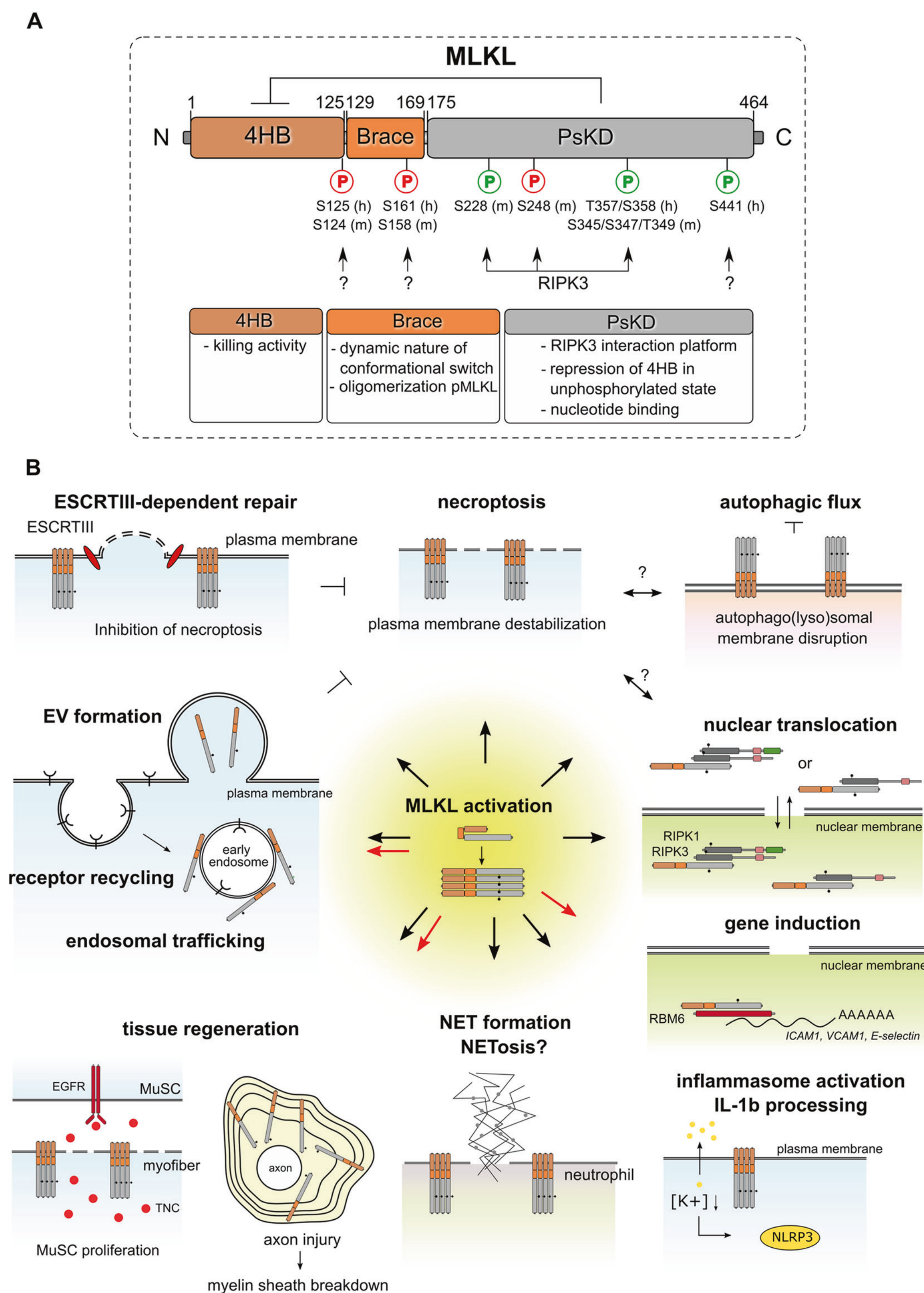
is no literature about epigenetic silencing mechanisms for MLKL. Although expression of both RIPK3 and MLKL is postulated to be a prerequisite to necroptosis susceptibility, little is known about the expression regulation of *MLKL*. Therefore we performed a global gene expression analysis of *MLKL* using a curated gene expression database to complement literature. Mutations in the *MLKL* gene in cancer are also reported but functional consequences are vastly unknown [18]. Finally, in the light of these diverse functions and expression regulation of *MLKL*, we discuss current knowledge and controversies surrounding MLKL and its possible role in cancer development and metastasis.

Molecular structure and function of MLKL

The MLKL protein has two functional domains: an N-terminal four-helix bundle (4HB) and a C-terminal pseudokinase domain (PsKD), connected by a brace domain consisting of two alpha helices [19] (Fig. 2). The 4HB domain is responsible for the killing activity of MLKL [20–24]. A PsKD lacks kinase activity but plays an important role in cellular signaling acting as a dynamic scaffold and modulator of protein-protein interaction [25]. The PsKD domain of MLKL acts as interface with the kinase domain of RIPK3 in a 1:1 constellation [19, 26]. Once RIPK3 is activated, it phosphorylates the PsKD of MLKL (S345, S347, and T349 in mMLKL and T357/S358 in hMLKL), leading to a conformational change in the PsKD that unlocks the 4HB domain followed by a release of MLKL from the RIPK3 activation platform [20, 27, 28] (Fig. 2). Unlike phosphomimetic mMLKL (S345D) in mouse cells, human phosphomimetic MLKL (T357E/S358D) was reported not to induce cell death in U937 and HT29 cells, suggesting that hMLKL may require additional activation steps than mMLKL beyond the phosphorylation of residues in the PsKD by RIPK3 [29]. However, others have reported that expression of the same human phosphomimetic can induce cell death upon stimulation in human cell lines such as RIPK3-HeLa and HT-29 [2, 24, 30]. The reasons for these discrepancies are currently unclear. Therefore, the question whether there are species differences between mMLKL and hMLKL with regard to its full activation remains unanswered. In addition, RIPK3-dependent activation of MLKL displays remarkable species selectivity as mRIPK3 cannot bind and activate hMLKL [2, 31]. A structural and reconstitution study revealed that distinct PsKD confirmations may account for such selectivity [31]. In the mouse context, a 'kiss and run' mechanism of RIPK3-dependent MLKL activation has been proposed where a transient interaction with RIPK3 is sufficient [32], whereas in human cellular systems more stable RIPK3-MLKL preformed complexes between RIPK3 KD and MLKL PsKD have been shown [2, 29, 33]. X-ray and NMR co-structures using a covalently bound inhibitor confirmed the interaction site between the auto-inhibitory brace region and the 4HB domain [34]. Other phosphorylation sites have been identified that fine-tune MLKL killing activity (both enhancing and inhibiting), including T376, S228, S158, S124, and S248 [35, 36] (Fig. 2). Not only phosphorylation, but also other structural changes alter MLKL's killing activity. For example, mutations in the brace region (D139V) and ATP-binding pocket (K219M, Q343A) are described that result in constitutive mMLKL killing activity [32, 37]. Activated MLKL oligomerizes [20, 24, 29, 38] and interacts via a patch of positively charged residues in the 4HB with negatively charged phosphatidylinositol phosphates (PIPs) in the plasma membrane or with cardiolipin [23, 39]. Besides the crucial role of PIPs in the recruitment to the plasma membrane, inositolphosphate (IP6) binding promotes the active conformation of MLKL by displacing the auto-inhibitory brace region [40]. Additionally, it was shown that phosphatidylinositol transfer protein alpha, a transporter of phosphatidylinositol, facilitates necroptosis by binding the N-terminal domain of MLKL and contributing to oligomerization in cisplatin-triggered cell death in human A549 lung cancer cells [41]. Whether this could be a general mechanism of action needs further investigation. Beside the membrane inserting structures described above, MLKL can also form large disulfide bond-dependent amyloid-like fibers consisting of α-helical structures similar to that of mitochondrial antiviral signaling protein and apoptosis-associated spec-like protein (ASC) [42] unlike RIPK1-RIPK3 amyloid-like fibers that mainly contain β sheet conformations [43].

Endosomal trafficking and ESCRT-III: detoxifying mechanisms of MLKL

MLKL, beyond its role in necroptosis execution, can serve a paradoxical role by counteracting its own cytotoxicity. This depends on the capacity of MLKL to contribute to proper endosomal trafficking from early to late endosomes and intracellular degradation of ligand/receptor complexes such as TNF/TNFR1 and EGF/EGFR, extracellular vesicle (EV) formation, and endosomal sorting complexes required for transport III (ESCRT-III)-dependent removal of damaged membrane during necroptosis. The endosomal trafficking processes involving MLKL do not require RIPK3 kinase activity and can operate during conditions of cellular homeostasis independent of cell death [30, 44]. RIPK3-dependent phosphorylation further enhances MLKL's ability to generate and release phospho-MLKL (p-MLKL)-containing EVs, thereby protecting against plasma membrane permeabilization and cell death. Moreover, blocking exosome release by silencing Rab27, increases the intracellular



p-MLKL levels and sensitizes cells to necroptotic death [30]. Finally, ESCRT-III, in conjunction with MLKL/p-MLKL, is implicated in the detoxifying mechanism of p-MLKL [44, 45]. Generation of p-MLKL causes a quick Ca^{2+} influx, followed by local exposure of phosphatidylserine (PS) to the cell surface and ESCRT-III-dependent release of 'bubbles' containing leaky or damaged plasma membrane at the site of MLKL/p-MLKL cluster formation. MLKL was not detected in those bubbles by time-lapse confocal microscopy. This process is thus likely to contribute to restore plasma membrane integrity and delays the onset of cell death [44]. This delay would create an additional time window for dying cells or cells bound to die to produce relatively more inflammatory cytokines and chemokines

◀ **Fig. 2 Structural domains of MLKL and their functions. A** The functions of each structural domain of MLKL are indicated. The N-terminal 4-helical bundle domain (4HB) shows homology with the N-terminal HeLo-like domain (HELL) of the fungal protein HELLP according to Hidden Markov Models [128]. The PsKD resembles a bi-lobal protein kinase domain, which binds ATP without hydrolyzing it, thus rendering the pseudokinase domain catalytically inactive [91]. Upon RIPK3-dependent phosphorylation (S345) of MLKL, the self-inhibitory pseudokinase domain (PsKD) of MLKL is released from the 4HB, thereby activating MLKL (pMLKL^{S345}) to induce necroptosis. The brace region contributes to the dynamic nature of the 4HB domain, the conformational change induced after activating phosphorylation of the PsKD and the oligomerization of activated MLKL. Phosphorylations that tune cell death activity of MLKL include inhibitory phosphorylation and activating phosphorylation (indicated in red and green respectively). Impact of the phosphorylation of MLKL on its cell death-independent functions is not known yet. h human site, m murine site. **B** Next to necroptosis execution, MLKL is also involved in ESCRT (Endosomal Sorting Complexes Required for Transport)-dependent repair of the plasma membrane (which restricts necroptotic cell death), release of extracellular vesicles, endosomal trafficking and receptor recycling, myelin sheath membrane breakdown and axon regeneration after injury, muscle stem cell (MuSC) proliferation after muscle injury, NET formation, inflammasome activation, possible nuclear functions including regulation of endothelial cell adhesion molecules such as ICAM1, VCAM1 and E-selectin through interaction with RNA-binding motif protein 6 (RBM6) and stabilization of mRNA, and inhibition of autophagic flux. TNC: tenascin-C. Black dot: phosphorylation of MLKL. Black arrows: processes that require RIPK3-dependent phosphorylation of MLKL, while red arrows: processes that require RIPK3-independent phosphorylation of MLKL.

(CXCL1, CXCL10), an important phenomenon to alert host immune system [44, 46]. These chemokines play an important role in cancer progression and metastasis by affecting various immune cells such as CD8^{+} T cells and myeloid cells [47, 48]. CXCL1 secreted from cancer cells attracts CD11b^{+}Gr1^{+} myeloid cells that in turn produce S1008/9 proteins, factors that promote cancer cell survival in metastatic sites [49]. Although the CXCL10/CXCR3 axis has an established role in tumor suppression, emerging evidence suggests its role in metastasis [50]. CXCL10 produced by brain astrocytes facilitates brain metastasis of melanoma [51]. Targeting of the CXCL10 receptor CXCR3 in either metastatic breast cancer cells or host compartment decreased metastasis of breast cancer cells [52]. Thus modulation of cell death by ESCRT-III dependent-repair mechanism may not only directly impacts the fate of primary tumor cells but also its complex interaction with the host immunity through paracrine and autocrine mechanisms that ultimately determine cell fate and tumor environment responses.

MLKL inhibits autophagic flux during necroptosis

MLKL can also target intracellular membranes in addition to the plasma membrane. The translocation of RIPK3-dependent activated MLKL (pMLKL^{S345}) to the autophagosomal and/or autophagolysosomal membranes results in disruption of membrane integrity, lysosomal dysfunctioning, and inhibition of autophagic flux in mouse dermal fibroblasts [53]. Increased MLKL expression and activation in the liver of high-fat diet-treated mice contributed to liver injury by inhibiting autophagy [54]. Autophagy plays a complex role in cancer [55–57], raising an intriguing possibility that MLKL might act via this pathway. Through lysosomal dysfunction, the necroptotic proteins RIPK1, RIPK3, and MLKL accumulated in neurons after spinal cord injury and potentiated necroptosis [58]. However, in fibroblasts and colorectal cancer cells, the canonical autophagic pathway apparently does not affect necroptotic cell death [53]. Therefore, the causal relationship between autophagy, MLKL and necroptosis may depend on the cell type and still needs further investigation.

MLKL in regeneration

MLKL can also contribute to tissue regeneration after injury. Although RIPK3 has been linked to tissue repair through kinase- and necroptosis-independent mechanisms [59], the mechanisms seem different. MLKL is induced in Schwann cells after axon injury and activated through Ser441 phosphorylation by a yet unidentified kinase different from RIPK3. Activated MLKL then translocates and binds to the sphingolipid sulfatide in the myelin sheath membrane in order to destroy this membrane structure to allow regeneration [16] (Fig. 2). MLKL also contributes significantly to muscle stem cell regeneration after muscle injury through necroptosis induction. Tenascin-C expression is induced in necroptotic myofibers and released in the extracellular stem cell microenvironment upon membrane rupture. This factor activates the EGFR signaling pathway in muscle stem cells, which in its turn facilitates muscle generation [60].

Nuclear functions of MLKL

Recently, a part of the MLKL pool has been observed to translocate from the cytosol to the nucleus following necroptotic stimuli [61]. This translocation occurs in association with RIPK1 and RIPK3 before onset of plasma membrane rupture but is apparently independent of cell death, as necrosulfonamide (an inhibitor of hMLKL [2]) treatment or N-terminal tagging of MLKL, disabling the killer domain of MLKL, has no impact on nuclear translocation. Phosphorylation of MLKL is required to expose the nuclear localization signal (NLS, AA 224–256) in the C-terminal domain. Mutations in the NLS sequence modestly decreased cytotoxicity of MLKL, suggesting nuclear

translocation may only partly contribute to cell death. Although this report illustrates that p-MLKL drives translocation of RIPK1 and RIPK3 as a complex to the nucleus, another publication indicated constitutive presence of RIPK1 in both nucleus and cytoplasm, implying only shuttling of RIPK3/MLKL between both compartments. Inhibition of nuclear export by Leptomycin B resulted in RIPK3/MLKL nuclear retention and delayed cell death [62]. Although these papers describe different mechanisms of the nuclear localization of RIPK1 and RIPK3 kinases and MLKL, both papers identified its nuclear shuttling as process partially contributing to necroptosis. The mechanism of how nuclear shuttling of MLKL contributes to necroptosis remains enigmatic. Recent papers describe MLKL as regulator of endothelial cell adhesion molecule expression [63, 64]. MLKL can promote vascular inflammation by directly regulating endothelial cell adhesion molecule expression such as ICAM1, VCAM1, and E-selectin, a process that seems to be independent from RIPK3 though. By directly interacting with RNA-binding motif protein 6 (RBM6), it stabilizes mRNA and enhances the expression of these adhesion molecules, which is necessary for the interaction of the endothelial cell with leukocytes [64]. This process is intimately linked to extravasation of tumor cells and contributes to metastasis [65].

MLKL in immune cells: NLRP3 inflammasome activation and NET formation

MLKL activation in monocytes and bone marrow-derived macrophages (BMDMs) also triggers NLRP3 inflammasome activation and caspase-1-dependent but gasdermin-D-independent IL-1β processing and release besides necroptosis [66, 67]. This process is dependent of RIPK3. In neutrophils MLKL contributes to multiple processes. Necroptosis stimulus-induced neutrophil extracellular traps (NETs), a network of extracellular DNA and microbiocidal proteins, entraps and destroys invading pathogens. This process is dependent on RIPK1, RIPK3, and MLKL, linking necroptosis players with NETosis and bactericidal activity [68]. Accordingly, MLKL deficient mice have more neutrophils in the peripheral blood after *Staphylococcus aureus* infection and are more susceptible to the infection [68]. Moreover, NETs act as a physical shield to protect tumor cells from T cell or NK cell-mediated cytotoxicity [69].

Analysis of a global gene expression database for *MLKL*

Expression of both RIPK3 and MLKL are required to induce necroptosis. As available literature on gene expression of MLKL is limited, we examined expression of MLKL in silico using curated gene/protein expression databases comprising thousands of experimental data. Data generated by the human protein atlas project (HPA) reveal highly differential expression of *MLKL* mRNA in human tissues, whereas the expression levels of *RIPK3* mRNA were more comparable (Fig. 3). *RIPK3* mRNA levels are the highest in the gastrointestinal tract and the skin, while *MLKL* mRNA levels are the highest in bone marrow and immune system (Fig. 3). *MLKL* mRNA is not only differentially expressed in tissues, but also strongly responsive to various stimuli comprising cellular environmental cues. Analysis of publically available transcriptomics data curated by Genevestigator illustrates that several perturbations, both in human and mouse, strongly impacts *MLKL* mRNA expression level, while this is often not the case for *RIPK3* mRNA. In mouse models of infection, inflammation, tissue injury, and a type of cancer driven by inflammation, *Mlkl* expression is highly inducible (Fig. 4). This induction might be crucial for proper functioning of MLKL.

Additionally, both type I interferon (IFN-α/β) and type II IFN (IFN-γ) seem to be a major regulator of *Mlkl* mRNA during infection, inflammation and cancer (Fig. 4). *Trypanosoma cruzi* infection, that activates expression of IFN-stimulated genes through type I IFN receptor (IFNAR1) signaling upregulates *Mlkl* mRNA [70]. IFNγ-treated BMDMs have increased *Mlkl* levels that are even further augmented when treated in combination with LPS, indicating that IFN and TLR signaling work in synergy to regulate MLKL expression. IFN-regulated transcription factors such as signal transducer and activator of transcription 1 (STAT1), STAT2 and IFN-regulatory factor 9 (IRF9) mediate the induction of *Mlkl* mRNA by IFNα, as respective gene ablation result in reduced MLKL levels. Also, tissue injury and inflammation-driven cancer upregulate *Mlkl* mRNA expression. Glycine N-methyltransferase (GNMT) gene deficient mice that develop nonalcoholic steatohepatitis (NASH), have a higher risk to develop hepatocellular carcinoma (HCC). NASH upregulate *Mlkl* mRNA expression in the liver, that is further augmented in HCC (Fig. 4). On the other hand, immune cells in melanoma tumor tissue have decreased *Mlkl* mRNA levels compared to splenocytes. Under these conditions that highly regulate *Mlkl* mRNA expression, *Ripk3* mRNA expression is rarely altered. Only upon certain infections (*Clostridium difficile* and *Mycobacterium tuberculosis*), inflammation and tissue injury, *Ripk3* mRNA levels are moderately increased (Fig. 4). Altogether, these data suggest a highly inducible *Mlkl* gene versus a *Ripk3* gene expression that remains rather unaffected. The highly responsive expression pattern of *Mlkl* gene may underlie experimental variability found in animal models and account for conflicting reports [71]. *RIPK3* gene expression seems to be dominantly regulated by promoter

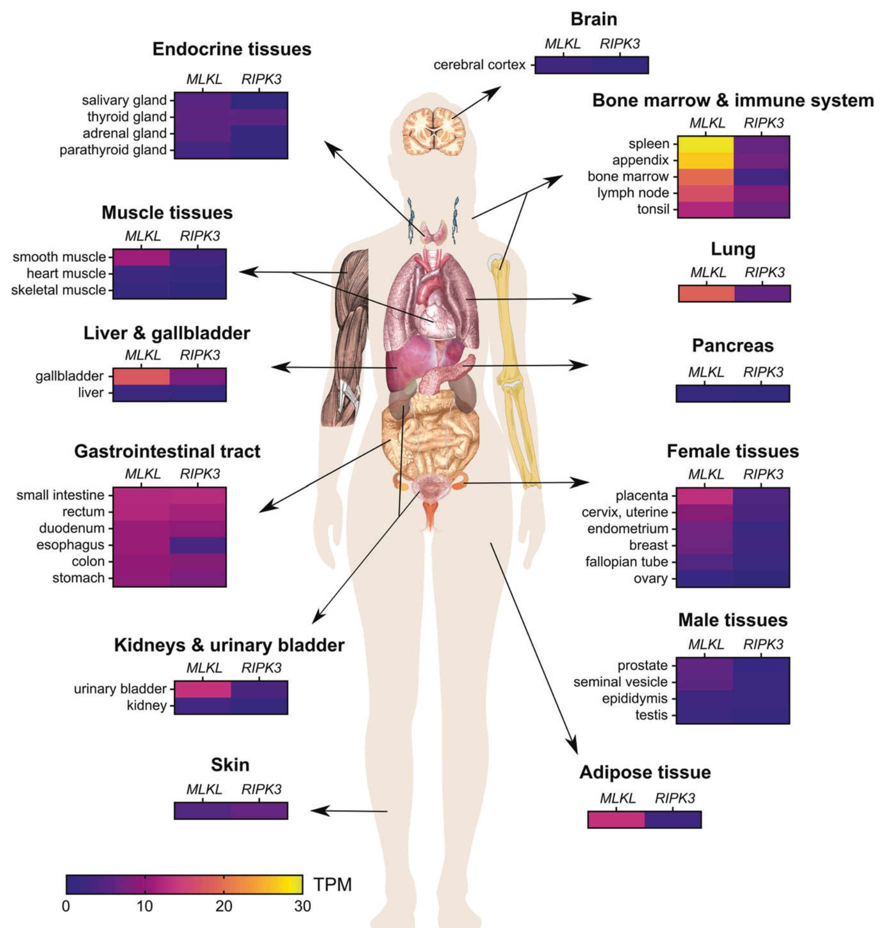


Fig. 3 ***RIPK3*** **and** ***MLKL*** **genes are differentially expressed in tissues during homeostasis.** *RIPK3* and *MLKL* mRNA expression data in human tissues, generated by the human protein atlas project (HPA) (www.proteinatlas.org). Bone marrow and immune system, lung, adipose tissue and gallbladder exhibit the highest *MLKL* gene expression, while the pancreas, brain, skin, and some muscle tissues reveal low *MLKL* mRNA levels. Overall, *RIPK3* mRNA expression levels are lower than *MLKL*. Both *RIPK3* and *MLKL* have low RNA expression in brain and pancreas. Additionally, *RIPK3* mRNA levels are the highest in the gastrointestinal tract and the skin (5–10 TPM), while *MLKL* mRNA levels are highest in bone marrow and immune system (20–30 TPM). TPM transcripts per million.

methylation as reported in many cancer cells [17]. This differential regulation of *Mlkl* and *Ripk3* mRNA may reflect their distinct cellular functions independent of necroptosis. Moreover, it is also conceivable that beside the regulation at the gene level, other kinases than RIPK3 may further regulate MLKL at the protein level in various cellular functions, as has been reported in case of striatal injury [16].

MLKL as an interferon-stimulated gene (ISG)

A link between IFN-signaling and necroptosis execution has been established in literature using macrophages and mouse embryonic fibroblast (MEF) cells [72–75] (Fig. 5). Many stimuli including TLR ligands, IFN-α/β and IFN-α/β-inducing factors result in activation of the IFN-stimulated gene factor 3 complex, consisting of STAT1, STAT2, and IRF9, followed by sustained formation of the necrosome and necroptosis execution. Type I interferon-signaling is important for necroptosis, as *Ifnar1* deficiency results in resistance to necroptosis in macrophages stimulated with LPS, poly-I:C, TNF or IFN-β in combination with caspase inhibitors [72, 73]. *Irf3/7* KO, *Ifnar1* KO, or *Stat2* KO BMDMs survive low-dose LPS treatment and have reduced MLKL expression [74, 76]. Pathogens such as *S. enterica* and *S. typhimurium* can take advantage of this by eliminating macrophages via IFN-α/β-induced RIPK3-dependent necroptosis [73]. Type I IFNs are also reported to augment the expression of other cell death regulating proteins by an epigenetic mechanism involving acetyltransferases *Kat2b* and *P300* [76] (Fig. 5). Although type I IFN-induced expression of MLKL is required for necroptosis, reconstitution with MLKL in *Ifnar1* KO BMDMs could not restore cell death sensitivity, indicating the involvement of other unknown ISGs [18, 77]. Steady-state levels of IFN-β determine expression of ISGs including STAT1/2 and MLKL in macrophages [77]. This steady-state expression of MLKL is important for its oligomerization and necroptosis. On the other hand, *Ifn-β*-deficient mice display higher p-MLKL levels in the bronchoalveolar lavage fluid during viral asthma exacerbation model [78]. It is likely that the impact of IFN-β may be dependent on cell type.

Not only type I IFNs but also type II IFNs induce MLKL (but not RIPK3) expression, resulting in necroptosis [79], consistent with the data from Genevestigator in the previous section. Upregulation of MLKL protein has been detected in liver tissues of patients with autoimmune hepatitis and of

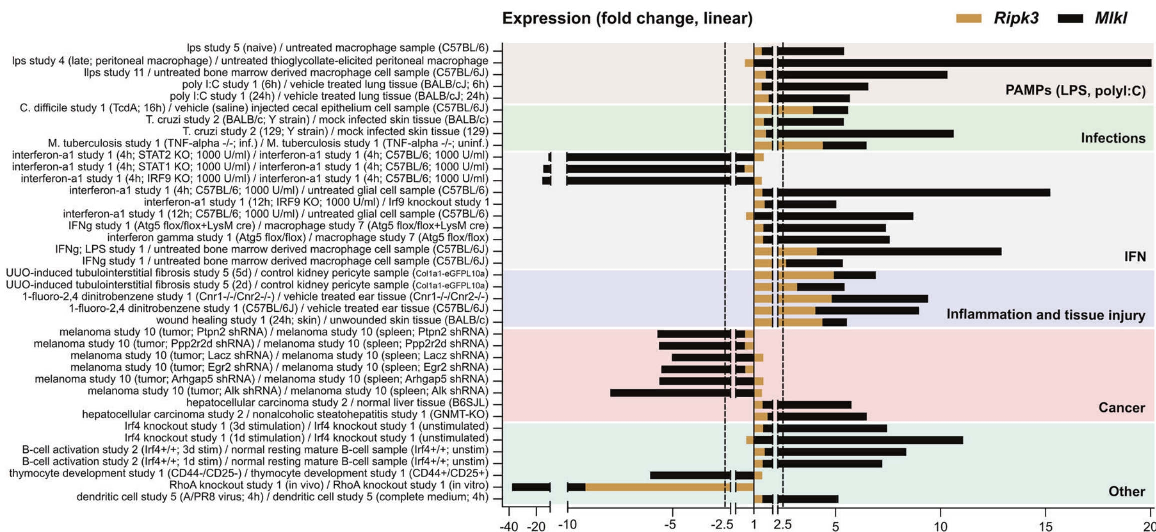


Fig. 4 ***Ripk3* and *Mlkl* are differentially expressed in tissues during homeostasis and during perturbations.** Overview of all perturbations tested in mouse that result in at least a 5-fold change in *mMlkl* mRNA levels both up- or downregulation. Data were collected from Genevestigator based on the 'affymetrix mouse genome 430 2.0 array' platform. The 2.5-fold change, a fold change considered to be low, is indicated by dotted line. *Mlkl* mRNA expression is highly inducible in mouse models of infection, inflammation, tissue injury, and cancer. Signals that activate innate immune response including pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS, a component of Gram-negative bacteria that activates both TLR4 and inflammasome), infections with parasites, viruses, and bacteria, wound healing as well as chemical-induced inflammation strongly induce *Mlkl* mRNA expression in various cell types and tissues. Also, IFN signaling induces *Mlkl* mRNA expression. *T. cruzi* infection, that activates expression of IFN-stimulated genes through type I IFN receptor (IFNAR1) signaling upregulates *Mlkl* mRNA [70]. Secondly, IFN-γ-treated BMDMs have increased *Mlkl* mRNA levels that are even further augmented when treated in combination with LPS, indicating that interferon and TLR/inflammasome signaling operate in synergy to regulate *Mlkl* mRNA expression. Finally, IFN-regulated transcription factors such as signal transducer and activator of transcription 1 (STAT1), STAT2, and IFN-regulatory factor 9 (IRF9) seem to mediate the induction of *Mlkl* by IFNα, as respective KOs result in reduced *Mlkl* mRNA levels. Mice that develop non-alcoholic steatohepatitis (NASH; *Gnmt* KO mice), have a higher risk to develop hepatocellular carcinoma (HCC). Not only does NASH upregulate *Mlkl* mRNA expression in the liver, but also HCC further increases its level (up to 7-fold change).

mice with ConA-induced hepatitis through IFN-γ-dependent activation of STAT1 that directly binds to the MLKL promoter and induces its transcription [80] (Fig. 5).

In contrast to MEF cells and macrophages, IFN-γ downregulates MLKL expression in splenocytes and protects against TNF/zVAD-fmk-induced necroptosis [81]. Also *Ifng* KO mice with collagen-induced arthritis have increased MLKL protein expression in the synovium [81]. Overall, IFN-γ may have a different impact on MLKL expression levels depending on cell type and disease context. Several cancer cell lines can be sensitized to necroptosis through IFN-γ- and STAT1/IRF1-dependent MLKL upregulation [82, 83]. Intercellular cues from the tumor microenvironment might therefore influence susceptibility of tumor cells to death-inducing stimuli. IFN-γ is secreted by tumor-infiltrating leukocytes such as CD8+ T cells and NK cells and alters tumor microenvironment [84, 85]. The regulation of MLKL by IFN signaling may be highly relevant in cancer biology and treatment options.

Finally, other transcriptional activators of *MLKL* gene expression have been described, such as the bromodomain protein BRD4. Together with acetylated IRF1, positive transcription elongation factor b (P-TEFb) and RNA polymerase II, BRD4 forms a transcriptional complex that binds to the MLKL promoter region [86]. Bromodomain and extra-terminal domain inhibitors (such as JQ-1) protect against necroptosis by downregulation of MLKL and moderately ameliorate TNF-induced shock in vivo [86]. The neurotoxicant non-dioxin-like polychlorinated biphenyl (PCB)-95 increases *Ripk1/3* and *Mlkl* gene expression and induces necroptosis in cortical neurons [87] (Fig. 5). This induced gene expression depends on both activation of RE1-silencing transcription factor (REST) and downregulation of cAMP responsive element binding protein (CREB).

MLKL expression and mutations in cancer

Deregulation of necroptosis signaling has been observed in many cancer types. In this context, RIPK1 upregulation, RIPK3 and CYLD downregulation, CYLD mutations resulting in truncated forms lacking its functional domain, *RIPK1/RIPK3* SNPs and *RIPK3 V458M* mutations have been described in different cancer types a.o. including non-Hodgkin lymphoma, glioblastoma, melanoma, pancreatic

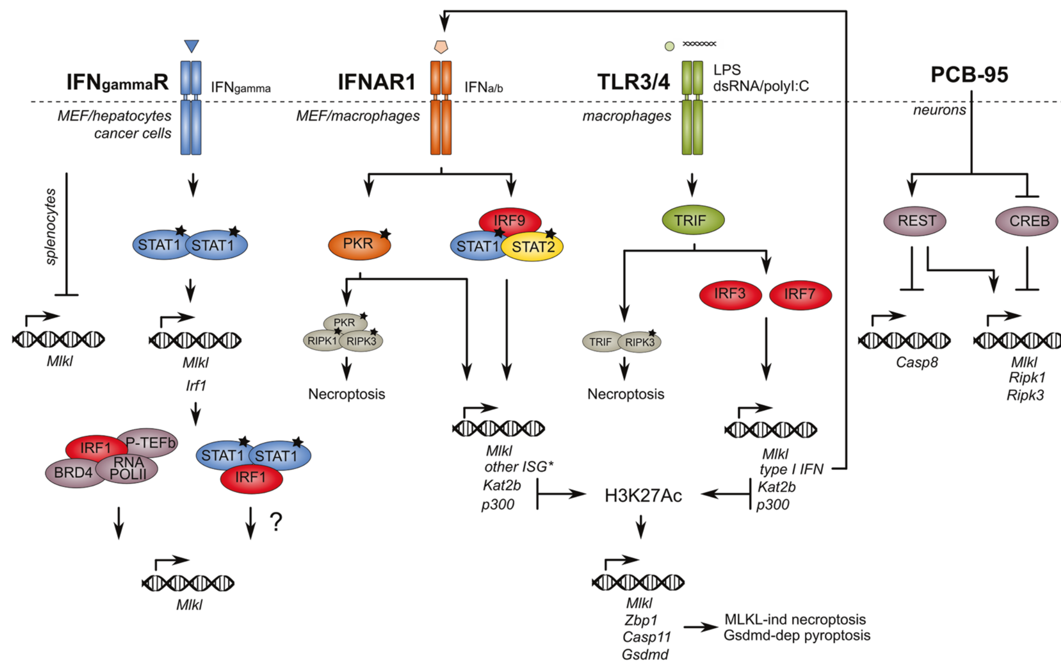


Fig. 5 Transcriptional regulation of *MLKL/Mlkl*. IFN signaling induces transcription of *MLKL* by different mechanisms, including direct binding of STAT1 to the promoter of *MLKL* and IRF-regulated expression of acetyltransferases that can activate transcription of MLKL by acetylation of its promoter. Also CREB can directly bind to the *MLKL* promoter, thereby repressing *MLKL* expression. Finally, REST can (in)directly activate *MLKL* transcription. * = another interferon-stimulated gene that might be involved in necroptosis signaling. *Irf1/3/7/9* interferon regulatory factor 1/3/7/9, *Gsdmd* gasdermin D, *Casp* 8/11 caspase-8/11, *Zbp1* Z-DNA binding protein 1, STAT1/2 signal transducer and activator of transcription 1/2, PKR protein kinase R, ISG interferon-stimulated gene, H3K27Ac histon 3 lysin 27 acetylation, *p300* histone acetyltransferase p300, *Kat2b* lysine acetyltransferase 2b, TRIF TIR-domain-containing adaptor-protein inducing IFN-β, PCB-95 polychlorinated biphenyl-95, LPS lipopolysaccharide, IFNgammaR interferon gamma receptor, IFNAR1 interferon alpha receptor, TLR3/4 toll-like receptor 3/4, REST RE1-silencing transcription factor, CREB cAMP responsive element binding protein, BRD4 bromodomain 4 protein, RNA-POLII RNA polymerase II, P-TEFb positive transcription elongation factor.

cancer, and hematopoietic cancers [18, 88]. *MLKL* mutation (L291P, a potential loss of function mutation) or deregulation is observed in many human cancer types, but needs further functional characterization [18]. Depending on the type of cancer, *MLKL* mRNA levels may differ largely (Fig. 6). A limited selection of human cancers from Genevestigator illustrates highly different transcript levels of *MLKL* (Fig. 6A). Cancers displaying high levels of *MLKL* mRNA include colon adenoma and hematopoietic cancers, while brain tumors display very low *MLKL* mRNA levels. This expression pattern may be reflecting different *MLKL mRNA* expression in these tissues (Fig. 3). Although *MLKL* mRNA level is low in skin tissue, different types of skin cancer have variable *MLKL* mRNA levels ranging from medium to high (Fig. 6A). Similar results are obtained with the few mouse cancer models analyzed to date (Fig. 6B). In COSMIC (catalogue of somatic mutations in cancer) opposing outcomes are described: both up- and down-regulation of *MLKL* mRNA expression, as well as copy number variation gain or loss, are observed over a wide range of cancer types. To date, three MLKL mutations have been investigated in human cancers. Two mutations, found in human gastric cancer, are located at highly conserved residues in the PsKD of human MLKL (F398I and L291P, corresponding with residue F385 and L280 in mMLKL) [32, 89, 90]. The corresponding F385I mMLKL mutant can apparently still induce necroptosis [18, 32], but the impact of this mutation on cell death-independent functions of MLKL, such as endosomal trafficking, EV formation and nuclear localization, have not been reported. On the other hand, the L291P MLKL mutation is likely a loss of function mutation since the corresponding mouse mutant (L280P) cannot induce necroptosis after stimulation [32]. Finally, the MLKL E351K mutation in the non-conventional GFE motif (instead of the DFG motif) at the activation loop of the PsKD has been detected in human lung carcinoma [18, 91]. Although the functional outcome and consequence for disease prognosis is not known, this mutation may alter the affinity for ATP binding [91].

MLKL as anti-cancerous factor

Low MLKL protein expression is associated with decreased overall survival in patients with resected pancreatic adenocarcinoma, independent from adjuvant chemotherapy

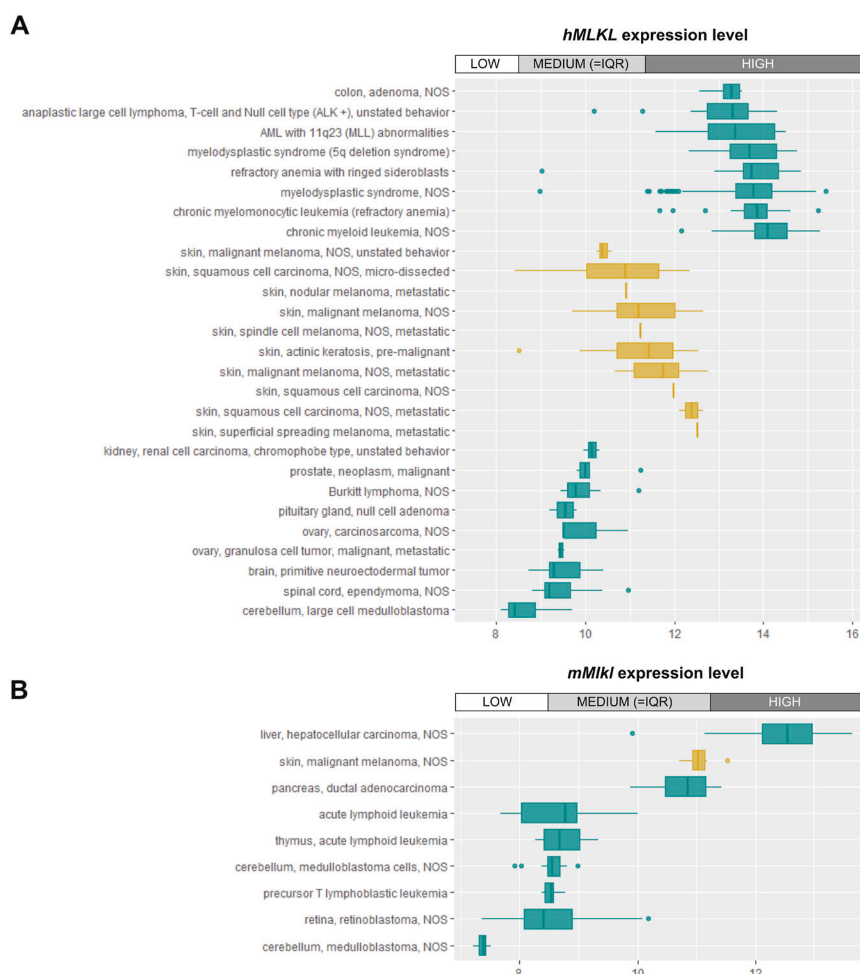


Fig. 6 High variation in *MLKL/Mlkl* expression between different types of cancer. *MLKL/Mlkl* mRNA expression data of a small selection of human (**A**) and mouse (**B**) cancer types. Cancers displaying high levels of *MLKL/Mlkl* mRNA include colon adenoma and hematopoietic cancers, while brain tumors display very low levels. This expression pattern may be reflecting different *MLKL/Mlkl* mRNA expression in these tissues (Fig. 2). Although *MLKL/Mlkl* mRNA level is low in skin tissue, different types of skin cancer have variable *MLKL/Mlkl* mRNA levels ranging from medium to high (orange). Data were collected from Genevestigator using 'affymetrix human genome U133 Plus 2.0 Array' (**A**) and 'affymetrix mouse genome 430 2.0 Array' (**B**) respectively as platform. Expression levels are indicated according to 'LOW', 'MEDIUM', and 'HIGH', referring to the expression value range determined by looking at all expression values of all genes over all samples for the platform used. LOW = first quartile range, MEDIUM = interquartile range (IQR), and HIGH = fourth quartile.

[92, 93]. Also in resected colon cancer, gastric cancer, cervical cancer and ovarian cancer similar correlations were observed [94–98]. Additionally, breast cancer and several subtypes of acute myeloid leukemia (AML) have reduced *MLKL* mRNA expression [18, 99–101]. The down-regulation of RIPK3 and MLKL in AML suggests the existence of selective forces that propagate AML progression by inducing necroptosis-resistance [100, 102]. A meta-analysis including 613 cancer patients indicated that low MLKL levels are indeed associated with advanced tumor stage and higher lymph node metastasis [103]. Down-regulation, loss-of-function mutations or SNPs are found in necroptosis-inducing genes in different types of cancer, suggesting that these factors can limit cancer development and metastasis [18]. Therefore, necroptosis-inducing therapy became an attractive alternative to kill chemotherapy-induced apoptosis-resistant tumors and to elicit immunogenic cell death [14, 104, 105]. Recent strategies include in vivo targeting of tumor cells with *Mlkl* mRNA through electroporation [105], immune-oncolytic therapy boosting antitumor immunity after tumor-cell specific introduction of *Mlkl* mRNA [106] or in vivo targeting of tumor cells with liposomes containing *Mlkl* pDNA together with SMAC mimetic BV6 and zVAD-fmk. These liposomes can reduce substantially the growth of subcutaneous CT26 tumors in syngeneic BALB-c mice [107].

The role of necroptosis mediators goes beyond the control of cancer cells and extends to host immune cells such as

tumor associated macrophages (TAM) that influence the tumor growth. *MLKL* mRNA and protein levels were upregulated in whole blood and peripheral blood mononuclear cells of cervical cancer patients and high MLKL level was associated with better overall survival [96], suggesting prognostic value of MLKL expression levels. Co-culturing U937-derived macrophages with cervical cancer cells results in a decrease in MLKL expression/phosphorylation upon LPS/zVAD-fmk stimulation, decreased necroptotic response and decreased M1 polarization, suggesting that cervical cancer cells apparently affect MLKL expression levels in TAMs to drive a more immunosuppressive environment [96]. Both RIPK3 and MLKL have been implicated in anti-cancer immune responses in vaccination assays in vivo [13, 14, 104, 105, 108]. Lung carcinoma deficient in MLKL or RIPK3 exhibited reduced response to cytostatic chemotherapy by mitoxantrone (MTX) or oxaliplatin (OXA) and revealed reduced anti-cancer immune response upon re-challenge [108]. Additionally, exogenous *Mlkl* mRNA induces antitumor immunity in melanoma and lymphoma [105]. All these findings are highly suggestive that induction of necroptosis may contribute to vaccination efficiency in these tumor cells. In human breast cancer tissue *MLKL* mRNA expression levels are positively correlated with immune cell infiltration and local antitumor immune responses by CD8$^+$ T cells and NK cells [101].

MLKL as pro-cancerous factor

In contrast to the concept of necroptosis as backup cell death mechanism to be exploited in anti-cancer therapy, recent evidence suggests that the necroptosis pathway may also contribute to cancer progression in certain conditions. Necroptosis of cancer cells ignites inflammation in the tumor microenvironment that can feed tumor growth. RIPK1/RIPK3-dependent necroptotic signaling induces CXCL1 and SAP130 expression in pancreatic ductal adenocarcinoma in mice, recruiting inhibitory TAMs and tumor-infiltrating myeloid cells respectively, creating an immunosuppressive tumor microenvironment with low T-cell infiltration and consequent tumor progression [109, 110]. Besides RIPK1/3 also MLKL protein expression is increased in patients [109], with increased MLKL levels found at the invasive front of human pancreatic cancer tissue [111]. *RIPK1/3* or *MLKL* gene ablation in the breast cancer cell line MDA-MB-231 resulted in sensitization to radiation, reduced growth in soft agar and delayed tumor growth when injected subcutaneously into nude mice [112]. Decreased overall survival is associated with increased p-MLKL (S358) levels in tumor tissue of esophagus, head and neck squamous cell carcinoma and colon cancer patients and with high MLKL expression in low grade glioma and glioblastoma [112–114]. How to explain this paradoxical finding? One may think of the function of MLKL in receptor turnover and endosomal trafficking [30] resulting in increased cancer cell survival or in detoxification of p-MLKL at the level of cancer cells [44, 45] which may contribute to cancer-promoting conditions.

A panel of AML cell lines were reported to express p-RIPK1/3 and p-MLKL (S358) constitutively in contrast to healthy hematopoietic stem/progenitor cells [115]. In these basal conditions, p-MLKL was found in the nucleus and on the centrosomes/spindle of dividing cells, suggesting that at least in some AMLs p-MLKL may execute cell death-independent functions contributing to cancer cell survival and proliferation.

Opposing roles of MLKL in metastasis

The role of MLKL in metastasis is also controversial, as opposing results have been described. Co-culturing human umbilical vein endothelial cells with MDA-MB-231 breast cancer cells induced p-MLKL (S358) activation, while knockdown of MLKL blocked tumor cell-induced endothelial cell necroptosis involving amyloid precursor protein binding to its cognate death receptor 6 [116]. Endothelial cell-specific gene ablation of *Ripk3* or *Mlkl* reduced the number of tumor nodules in the lung, indicating that tumor cell-induced necroptosis of the endothelial cells would promote metastasis by facilitating endothelial fenestration and extravasation of the tumor cells [117]. However, whether necroptosis in endothelial cells is really involved, was questioned in an independent report using similar tumor models. Although the resistance to tumor-induced killing of endothelial cells was reproduced in *Ripk3* KO mice, no prominent difference in lung colonization of B16F10 or LLC1 tumor cells was observed in *Mlkl* KO mice or *Ripk3* kinase-dead knockin mice, excluding necroptosis-dependent mechanisms [117]. Later, two other reports also specifically questioned the involvement of endothelial cell necroptosis in this model [118, 119]. On the other hand, MLKL-deficiency in cancer cells resulted in reduced metastasis in an orthotopic breast cancer model [118] and in nasopharyngeal carcinoma lung colonization model [120]. It is suggested that an inflammatory environment due to necroptotic cancer cell death in the primary tumor can drive metastasis [118]. Another report suggests that CXCL5 released by necroptotic cancer cells themselves can trigger neighboring cancer cells to migrate [111]. Chemokines attract distinct sets of immune cells to the tumor microenvironment, which promote metastasis by enhancing cancer cell migration and survival [47]. Release of cytokines by necroptotic cancer cells are important mechanisms to

govern tumor-host immunity, and can be modulated by ESCRT-III-dependent repair mechanism [44]. Alternatively, MLKL is suggested to directly contribute to cell migration by activating cell-surface proteases of the disintegrin and metalloprotease (ADAM) family [121] and to a shift in gene expression profile favoring epithelial-to-mesenchymal transition in nasopharyngeal carcinoma [120]. Overall, absence of MLKL in the tumor cells itself or in the tumor microenvironment will influence metastasis differently.

Conclusion and perspectives

MLKL does not only execute necroptosis, but is also involved in receptor turnover, endosomal trafficking, cytokine secretion, EV formation, NET formation, autophagic flux, tissue injury or regeneration and is also found in the nucleus for yet to be identified mechanisms. Several of these necroptosis-independent functions have been linked to cancer progression or metastasis [55–57] [122–124] [125–127]. Whether or not MLKL is involved in these processes needs further investigation. The impact of lack of MLKL protein expression in cancer might be more complex and extend beyond necroptosis resistance. Overall, the pro- or anti-cancerous role of MLKL in carcinogenesis and disease progression, including its regulation by extracellular factors (such as type I and type II IFNs), may depend on the cancer type, timing, and its cellular microenvironment (such as the type of infiltrating immune cells). For example, overexpression of exogenous *Mlkl* mRNA can result in antitumor immunity in melanoma and lymphoma [105], especially in combination with immune checkpoint blockade, but may also create immune suppressive tumor microenvironments in other tumors as was shown in pancreas cancer [109]. *MLKL* gene expression levels are very variable depending on the type of cancer (Fig. 6) in contrast to *RIPK3* gene that is primarily regulated by epigenetic silencing. MLKL protein deficiency in cancer cells may result in anti- or pro-cancerous conditions, depending on the balance between cancer cell elimination and nature of immune activation by the lytic mode of cell death (inflammatory, immunogenic, immune suppressive depending on the cancer cell types and infiltrate composition). Furthermore, cell death-independent functions of MLKL such as detoxification mechanisms involving inhibition of autophagic flux, modulate cancer cell survival. Distinct expression profiles of *RIPK3* and *MLKL* genes are highly suggestive of such MLKL functions playing a role in cancer in a highly context-dependent manner. Which exact function of MLKL plays a dominant role in a given cancer cell type might determine the outcome. Likewise, MLKL protein deficiency in the cells in the tumor stroma such as TAMs and endothelial cells determine the tumor microenvironment that modulates cancer initiation, growth, and metastasis through multiple processes such as modulation of antitumor immunity and tumor cell extravasation. Here again necroptosis-independent functions of MLKL might play a prominent role. An example of cell death modulation by ESCRT-III-dependent repair mechanism [44] not only directly affects the fate of the primary cancer cells, but also impact the intercellular communication between the cancer cells and the tumor microenvironment. Thus necroptosis and necoptosis-independent functions of MLKL form an intricate network that determines cancer cell survival, growth and metastasis. Better understanding of the complex and opposing functions of MLKL in cancer is needed in order to exploit or target MLKL in anti-cancer therapies. To this end, robust in vivo models such as tissue-specific KOs with well-controlled experimental settings could help to resolve the current controversies in future.

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Compliance with ethical standards

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