**ORIGINAL RESEARCH**

**Contribution of RIP3 and MLKL to immunogenic cell death signaling in cancer chemotherapy**

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**ABSTRACT**

Chemotherapy can reinstate anticancer immunosurveillance through inducing tumor immunogenic cell death (ICD). Here, we show that anthracyclines and oxaliplatin can trigger necroptosis in murine cancer cell lines expressing receptor-interacting serine-threonine kinase 3 (RIP3) and mixed lineage kinase domain-like (MLKL). Necroptotic cells featured biochemical hallmarks of ICD and stimulated anticancer immune responses \textit{in vivo}. Chemotherapy normally killed \textit{Rip3}\textsuperscript{-/-} and \textit{Mlkl}\textsuperscript{-/-} tumor cells and normally induced caspase-3 activation in such cells, yet was unable to reduce their growth \textit{in vivo}. RIP3 or MLKL deficiency abolished the capacity of dying cancer cells to elicit an immune response. This could be attributed to reduced release of ATP and high mobility group box 1 (HMGB1) by RIP3 and MLKL-deficient cells. Measures designed to compensate for deficient ATP and HMGB1 signaling restored the chemotherapeutic response of \textit{Rip3}\textsuperscript{-/-} and \textit{Mlkl}\textsuperscript{-/-} cancers. Altogether, these results suggest that RIP3 and MLKL can contribute to ICD signaling and tumor immunogenicity.

**Introduction**

Neoplasia preferentially develops and progresses in the context of insufficient immunosurveillance, \textit{i.e.} when the immune system fails to recognize tumor-associated antigens and to specifically eliminate malignant cells. This concept, which was initially developed in mice,\textsuperscript{1} has been validated in humans.\textsuperscript{2} In addition to an ever-expanding arsenal of immunotherapies,\textsuperscript{3} conventional anticancer chemotherapies constitute a strategy for reinstating anticancer immunosurveillance.\textsuperscript{4} Accordingly, there is ample evidence that the long-term fate of breast cancer patients treated with anthracyclines or that of colorectal cancer patients treated with oxaliplatin (OXA) is largely determined by the density of the immune infiltrate (in particular memory effector T cells) at diagnosis,\textsuperscript{5-7} as well as by dynamic changes in the ratio of cytotoxic T lymphocytes (CTL) versus regulatory T cells occurring shortly after chemotherapy.\textsuperscript{8} Loss-of-function alleles of toll-like receptor 4 (TLR4) and formyl peptide receptor 1 (FPR1) also have a negative impact on the therapeutic response of mammary and colorectal carcinoma patients to adjuvant chemotherapies.\textsuperscript{9-11} Further supporting the notion that the immune system dictates (at least part of) the therapeutic response.

Anthracyclines and OXA fall into the particular category of anticancer agents that are capable of triggering ICD, meaning that cancer cells killed by these compounds stimulate a protective anticancer immune response upon their subcutaneous injection even in the absence of any adjuvant.\textsuperscript{12-14} ICD has been initially studied in two model cell lines, namely CT26 colon cancers and MCA205 fibrosarcomas.\textsuperscript{12,13} In these cell lines, anthracyclines and OXA induce caspase-dependent apoptosis. Although caspase inhibition fails to prevent chemotherapy-induced cell death (which then occurs in a non-apoptotic fashion), it does prevent ICD due to the suppression of calreticulin (CRT) exposure (which is an “eat-me” signal facilitating the transfer of tumor antigens into immature dendritic cells (DC))\textsuperscript{15} and the reduction of ATP release (which serves as a chemotactic signal for the attraction of immune cells into the

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tumor bed).16,17 CT26 and MCA205 cells that have been lysed by freeze-thawing fail to immunize mice against cancer.12 These two cell lines, when killed by chemotherapy in the context of caspase inhibition, undergo necrotic cell death, which is non-immunogenic as well.13,15 Based on these results, we concluded that necrotic cell death is less immunogenic than caspase-dependent ICD.18

One particular form of necrosis is necroptosis (programmed necrosis), which can be elicited by the ligation of surface receptors (such as the tumor necrosis factor receptor, TNFR), in particular when caspases are inhibited.19-22 Necroptosis involves a series of essential signaling molecules, in particular receptor-interacting serine/threonine-protein kinase 1 and 3 (RIP1, RIP3) and MLKL.22-28 In a typical necrototic signaling sequence, the TNFR-associated death domain (TRADD) protein signals to RIP1, which recruits RIP3 to form the so-called necosome. RIP3 then phosphorylates MLKL, causing its oligomerization, insertion into cellular membranes and fatal permeabilization of the plasma membrane.23-25,29 Necroptotic cell death is accompanied by the release of danger-associated molecular patterns (such as ATP and high-mobility group protein B1, HMGB1),30 which are involved in ICD.18,31 While ATP is known to act on purinergic receptors to mediate immunostimulatory signals,16,25,32 HMGB1 interacts with TLR4 expressed in DC to stimulate tumor antigen presentation.9

Driven by these considerations, we investigated the potential role of necroptosis in ICD. We found that, in contrast to CT26 and MCA205 cells, which lack the expression of RIP3, other murine cancer cell lines that can undergo ICD, such as the TC-1 lung carcinoma,33 as well as the EL4 thymoma,9 express such molecules. To our surprise, necrototic cancer cells exhibit all biochemical hallmarks of ICD (CRT exposure, ATP and HMGB1 release) and are able to induce a protective anticancer immune response. Moreover, the knockout of RIP3 or MLKL reduced ICD-associated signals in TC-1 and EL4 cells responding to anthracyclines or OXA. Thus, TC-1 and EL4 tumors lacking RIP3 or MLKL became chemoresistant in vivo because they failed to stimulate an antitumor immune response upon chemotherapy. Altogether, these results indicate that the necrototic signaling molecules RIP3 and MLKL play a facultative role in ICD signaling.

Results

Immunogenicity of necrototic cancer cells

The combination of recombinant tumor necrosis factor-α, a synthetic second mitochondria derived activator of caspase (SMAC) mimetic, and the caspase inhibitor z-VAD-FMK (TSZ)20 can induce cell death in TC-1 lung cancer cells, as well as in EL4 thymoma cells, causing the cells to sustain positively with phycoerythrin-labeled recombinant Annexin V protein (which detects phosphatidylserine on the plasma membrane surface of intact cells or within dead cells), and to permeabilize their plasma membrane to the vital DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 1A, B). Importantly, CT26 and MCA205 cells failed to undergo necroptosis in response to TSZ (Fig. 1A, B). Massive death of TC-1 and EL4 cells was only detectable when all three reagents (TSZ) were applied together and was partially inhibited by addition of necrostatin-1 (Nec-1), a specific RIP1 inhibitor19, supporting the contention that this cell death is bona fide necroptotic (Fig. 1C, D; S1A, B). TC-1 and EL4 cells expressed the entire set of necroptosis-relevant signaling molecules (RIP1, RIP3 and MLKL), while CT26 and MCA205 cells lacked detectable RIP3 expression (Fig. 1E), a finding that might explain the relative TSZ resistance of the latter two cell lines.

Knockout of RIP3 or Mlkl by CRISPR/Cas9 technology using two distinct guide RNA (gRNA) constructs (g1, g2) for each gene (Fig. 2A) yielded TC-1 cells that became resistant to TSZ (Fig. 2B). Similarly, EL4 cells subjected to the knockout of RIP3 or Mlkl (with g1) became resistant to TSZ-induced necroptosis (Fig. S2A, B). Moreover, in response to TSZ, only wild type (WT), neither Rip3-/- nor Mlkl-/-, TC-1 or EL4 cells exhibited the common hallmarks of ICD including release of ATP (Fig. 2C, S2C), exposure of CRT on the surface of the plasma membrane of still viable cells (Fig. 2D, E), and release of HMGB1 (Fig. 2F, S2D). The clonogenic potential of TC-1 cells was significantly reduced upon prolonged exposure to TSZ, while 2 h of drug exposure to anthracycline mitoxantrone (MTX) was sufficient to abolish their colony-forming activities (Fig. 2G, H). Accordingly, TSZ-treated TC-1 cells injected subcutaneously into the right flank of immunocompetent C57BL/6 mice were able to elicit an immune response that protected the animals against rechallenge with live tumor cells injected into the opposite flank one week later. This effect was comparable to the positive control (i.e., TC-1 cells cultured with the anthracycline MTX (Fig. 2I, J). In contrast, freeze-thawed (F/T) TC-1 cells failed to immunize mice in a comparable setting, though necessary measures have been taken to optimize the preservation of short-lived danger signals (Fig. 2J). Altogether, these results suggest that necroptotic cells may display features of ICD, while necrotic cells generated by F/T cycles lack the capacity to induce ICD, in accord with our prior observations.12

Necroptotic signaling molecules contribute to chemotherapy-induced ICD

Based on the finding that necroptotic signalings may lead to ICD, we wondered whether this phenomenon might be involved in chemotherapy-elicited ICD. Chemotherapy with MTX caused MLKL phosphorylation, which can be augmented in the presence of a pan-caspase inhibitor z-VAD-FMK (Fig. 3A). Pre-treatment with lambda phosphatase significantly reduced the level of phosphorylated MLKL (pMLKL) in cell lysates, while phosphatase inhibitor cocktails could prevent pMLKL dephosphorylation (Fig. 3A). MTX was able to induce cell death events that resembled those triggered by TSZ, as determined by transmission electron microscopy (Fig. 3B). It is noteworthy that TC-1 cells engineered to stably express a RIP3-green fluorescent protein (GFP) fusion protein34 in an MLKL-/- background (to avoid lethal effects of RIP3-GFP over-expression in WT TC-1) manifested redistribution of the green fluorescent signal from a diffuse pattern to discrete cytoplasmic speckles in response to MTX. This phenomenon was largely suppressed by Nec-1 (Fig. 3C, D), suggesting that it reflects the
formation of necrosomes and hence induction of necroptosis. In conclusion, MTX can induce cell death that is accompanied by the features of necroptosis.

In response to chemotherapy with MTX, Rip3-/- and Mlkl-/- TC-1 and EL4 cells showed similar sensitivity to MTX-induced cell death compared to WT TC-1, as measured by assessing plasma membrane permeabilization and Annexin V staining, although cell death occurred with a transient delay in TC-1 cells (Fig. 3E), but not EL4 cells (Fig. S3A). Quantifying the frequency of cells with activated caspase-3 (Casp3a, which defines apoptosis at the biochemical level) using the specific antibody or fluorogenic substrate (Fig. 3F, G, S3B), or condensed nuclei (which defines apoptosis at the morphological level) with Hoechst staining (Fig. S3C, D) demonstrated that WT, Rip3-/- and Mlkl-/- TC-1 cells underwent a similar level of apoptosis after chemotherapy with MTX, both in vivo, in established tumors (Fig. 3F, G) and in vitro, in cultured cells (Fig. S3B, C, D). WT, Rip3-/- and Mlkl-/- cells similarly lost their clonogenic potential upon short-term exposure to MTX (Fig. S3E). However, necrosis induction was reduced in Rip3-/- and Mlkl-/- cells in vivo. In established tumors, a fraction of WT TC-1 tumor cells treated with MTX exhibited a loss of HMGB1 staining in the nuclei without any sign of concomitant chromatin condensation, indicating that they underwent necrosis. Again, this sign of MTX-induced necrosis induction was strongly reduced in Rip3-/- and Mlkl-/- tumors, as revealed in (D). Altogether, we conclude that MTX-induced cell death does not require RIP3 and MLKL presumably because Rip3-/- and Mlkl-/- cells normally activate the apoptotic pathway. However, MTX-induced HMGB1 release (as a sign of necrosis) largely depended on RIP3 and MLKL.

Although Rip3-/- and Mlkl-/- cells normally died in response to MTX in vitro, they were totally unable to elicit protective anticancer immune responses in vaccination assays consisting in the inoculation of dead/dying tumor cells followed by live tumor cell rechallenge (Fig. 4A, B). More importantly, only WT TC-1 and EL4 tumors reduced their growth in vivo in response to chemotherapy with MTX or OXA, respectively, while Rip3-/- and Mlkl-/- tumors failed to do so (Fig. 4C–E). This experiment was done in conditions, in which the growth retardation by chemotherapy was entirely dependent on a cellular immune response, because TC-1 cells (Fig. S4) and EL4 cells implanted in athymic nu/nu mice failed to respond to chemotherapy, while they did so when developed on mouse.
immunocompetent mice. Hence, Rip3−/− and Mlkl−/− cancers are chemoresistant in vivo, and this chemoresistance could be linked to their incapacity to induce an anticancer immune response responsible for tumor growth reduction.

**Defective ICD in RIP3 or MLKL-deficient TC-1 and EL4 cells**

To understand the incapacity of Rip3−/− and Mlkl−/− cells to undergo ICD, as well as their associated chemoresistance, we characterized all known biochemical hallmarks of ICD in such cells.
cells, comparing them to their WT parental control. In response to MTX or OXA, Rip3−/− and Mlkl−/− cells exhibited reduced ATP release (Fig. 5A, S5A), normal CRT exposure (Fig. 5B, S5B), as well as strongly reduced HMGB1 release (Fig. 5C, S5C). ATP release, occurring in the context of caspase-dependent ICD, has been linked to premortem autophagy.17,36 However, Rip3−/− and Mlkl−/− exhibited a normal autophagic response to MTX in vitro (Fig. S5D), suggesting that differences in autophagy cannot explain the reduced ATP release. The absence of RIP3 (but less so that of MLKL) also reduced the expression of several genes linked to the type 1 interferon (IFN) response after stimulation with MTX,37 such as the chemokines Cxcl9 and Cxcl10, the master transcription factor Irf7 and others including the pathogen resistance genes.
Mx1 and Mx2 (Fig. 5D, E, S5G, H), yet had no major detrimental effects on the induction of such genes by recombinant interferon-α (Fig. S5I–K). This finding suggests that RIP3 may play a specific role in signal transduction pathways that link lethal signaling to the induction of the type 1 IFN response.

To further explore the chemoresistance of Rip3−/− and Mlkl−/− tumors, we characterized the immune infiltrate in untreated tumors as well as post-chemotherapy. No differences in the frequency of CD11c+CD86+ cells (which are bona fide activated DC) and CD3+CD8+ cells (which are bona fide CTL) could be found between WT and Rip3−/− and Mlkl−/− tumors, suggesting that baseline immunosurveillance was not influenced by necroptotic signaling. However, 48 h post-chemotherapy only WT (not Rip3−/− or Mlkl−/−) tumors exhibited a
A major increase in the frequency of infiltrating CD11c<sup>+</sup>CD86<sup>+</sup> cells. Similarly, only WT (but not Rip3<sup>−/−</sup> or Mlkl<sup>−/−</sup>) cancers exhibited an augmented accumulation of CD3<sup>+</sup>CD8<sup>+</sup> cells 7 d post-chemotherapy (Fig. 6A–D).

The aforementioned findings suggest that chemotherapy-elicited RIP3- and MLKL-dependent necroptotic signals may contribute to ICD in a decisive fashion. Since the release of ATP and HMGB1 is compromised in Rip3<sup>−/−</sup> and Mlkl<sup>−/−</sup> cells responding to chemotherapy (Fig. 5A, C, S5A, C), we wondered whether compensation of these ICD parameters would restore the efficacy of chemotherapy against Rip3<sup>−/−</sup> and Mlkl<sup>−/−</sup> tumors. For this, we combined systemic MTX administration with the injection of the synthetic TLR4 ligand dendrophilin A (DENA) (which can replace HMGB1 to ligate TLR4)<sup>38</sup> and the apyrase inhibitor ARL67156 (ARL, which causes an elevation of extracellular ATP within the tumor).<sup>36</sup> DENA plus ARL failed to affect tumor growth when given without chemotherapy. However, DENA plus ARL had a strong tumor growth-reducing effect when

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**Figure 5.** Requirement of Rip3 and Mlkl for the manifestation of some hallmarks of immunogenic cell death. (A) Comparison of ATP release from WT, Rip3<sup>−/−</sup> or Mlkl<sup>−/−</sup> TC-1 treated with MTX at indicated time points. (B) Cytoplasmic CRT exposure by WT, Rip3<sup>−/−</sup> or Mlkl<sup>−/−</sup> TC-1 in response to MTX, as determined by immunofluorescence staining and cytofluorometry. (C) HMGB1 release from WT, Rip3<sup>−/−</sup> or Mlkl<sup>−/−</sup> TC-1 treated with MTX. (D, E) Induction of type 1 IFN response genes in WT, Rip3<sup>−/−</sup> or Mlkl<sup>−/−</sup> TC-1 treated with MTX, as determined by quantitative RT-PCR reaction. Results are shown as fold increase compared to the untreated control WT cells. Histograms indicate means ± SD of triplicates from one representative experiment out of two–six repeats. p values were calculated by means of the unpaired Student’s t test. *p < 0.05, **p < 0.01. All date shown in this figure were obtained with the knockout clones generated with gRNA1 for Rip3 and Mlkl. See also data obtained with another series of knockout clones in Fig. S5.
combined with MTX for the treatment of Rip3−/− and Mlk1−/− cancers (Fig. 7A–C). Altogether, these results indicate that TLR4 ligation and ATPase inhibition can compensate for deficient ICD signaling in Rip3−/− and Mlk1−/− tumors, thereby improving the efficacy of chemotherapy.

**Discussion**

Our data provide strong evidence indicating that a necroptotic cell death pathway involving RIP3 and MLKL can contribute to the induction of chemotherapy-relevant ICD. The strongest proof in favor of this interpretation is the observation that systemic administration of chemotherapy normally induces apoptosis (i.e. cell death with chromatin condensation and caspase-3 activation), yet is unable to induce necroptosis in Rip3−/− and Mlk1−/− cancer cells *in vivo* (i.e., cell death culminating in the release of HMGB1 from the nuclei of cancer cells without that such nuclei would exhibit the chromatin condensation typically found in apoptosis). This failure to induce necroptosis is coupled to a lack of immune infiltration of the tumors post-chemotherapy, as well as to the failure of tumor growth reduction in response to treatment with anthracyclines or OXA. *In vitro* experiments confirmed the conclusion that RIP3 and MLKL are required for optimal release of both HMGB1 and ATP from tumor cells treated with chemotherapeutics, although such cells showed a normal rate of apoptosis induction and only a minor if any reduction in the rate of cell death. In

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**Figure 6. Requirement of Rip3 and Mlk1 for the MTX-elicited immune infiltration in vivo.** (A–D) Mice bearing established TC-1 tumors (WT, Rip3−/− or Mlk1−/−) with the indicated genotypes were treated with vehicle or MTX. Tumors were recovered at day 2 after MTX treatment and stained for the detection of CD11c+CD86+ dendritic cells (A). Alternatively, tumors were excised at day 7 post-chemotherapy for the immunofluorescence detection of CD3+CD8+ cytotoxic T lymphocytes (B). Representative images of MTX-treated tumor are exemplified in (A, B) and quantitative results are shown in (C, D). Results are shown as Box-and-Whisker plots merged with dot plots showing individual values. *p* values were calculated by means of the unpaired Student’s *t* test. *p* < 0.01, **p** < 0.001.
response to anthracyclines, necroptosis-deficient cells also manifested reduced expression of genes linked to type 1 IFN response. Hence, the necroptotic component of lethal signaling can contribute to ICD. Accordingly, the deficient immune-related chemotherapeutic response of both Rip3−/− and Mlkl−/− tumors could be fully restored by the administration of a synthetic TLR4 ligand DENA plus an apyrase inhibitor ARL, without the need of type 1 IFN supplementation. TLR4 ligation is well known to induce type 1 IFN responses, which might explain the capacity of the treatment to restore the chemotherapeutic response in both Rip3−/− and Mlkl−/− cancers.

It should be noted that the present study concentrated on the contribution of RIP3 and MLKL to immune activation within cancer cells, and hence did not characterize the possible role of the necroptotic signaling pathway in immune cells.

Figure 7. Restoration of deficient chemotherapeutic responses of Rip3−/− or Mlkl−/− cancers. (A–C) Mice bearing established WT, Rip3−/− or Mlkl−/− TC-1 cancers received intraperitoneal injections of mitoxantrone (MTX) alone or together with dendrophilin A (DENA, injected intravenously) and ARL67156 (ARL, injected locally into the tumor) on the same day, and cancer growth was monitored as indicated in (A). Complete tumor growth curves are shown in (B). For quantitative comparisons, the final tumor sizes were indicated for each individual mouse (C). Dot plots indicate means ± SEM. p values were calculated by means of Mann–Whitney U-test. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.
Myeloid cells can undergo necroptosis in response to a variety of pathogenic signals. Hence, future studies must address the possible contribution of chemotherapy-induced necroptosis in inflammatory and immune cells in anticancer immunosurveillance. Irrespective of this limitation, the present data support the notion that RIP3 and MLKL may participate to chemotherapy-induced ICD. In accord with this conclusion, artificial activation of a RIP3 construct that has been rendered susceptible to dimerization by chemical agents can stimulate ICD. In that paper, RIP3 activation by dimerization was shown to lead to the activation of RIP1, which in turn stimulated an NF-κB-mediated transcriptional program that contributed to the immunogenicity of dying cells. Anthracyclines potently induce the NF-κB pathway in a RIP1-dependent fashion, suggesting that this pathway may contribute to chemotherapy-elicited ICD as well.

Cancers may escape from immunosurveillance by means of three major strategies, namely by (i) producing immunosuppressive factors that condition the local microenvironment or induce systemic immunosuppression; (ii) immunoeediting to avoid the expression of highly immunogenic tumor-associated antigens; and (iii) the loss of adjuvant signals, including those involved in ICD. There is indeed evidence that human cancers can lose CRT expression, which is associated with reduced T cell infiltration and poor prognosis. Similarly, loss of HMGB1 expression is associated with cancer progression and constitutes a negative prognostic marker in breast cancer treated with adjuvant chemotherapy. Deficient autophagy suggested by low expression of Beclin-1 protein is also a negative prognostic marker in breast cancer and in colorectal cancer. Accordingly, the absence of cytoplasmic LC3B puncta (which suggests a defect in autophagy) indicates poor prognosis in breast cancer. It is tempting to speculate that the reduction of RIP3 expression, that is often found in cancer due to DNA methylation, may also perturb ICD signaling in response to therapeutic maneuvers including chemotherapy and radiotherapy, thus contributing to the resistance of cancers to the immune-related beneficial effects of conventional treatments.

ICD can be induced in specific tumor cell types (such as CT26 and MCA205 cells) that lack RIP3 expression in a fully caspase-dependent fashion, yet can also be induced in cells that express RIP3 by activating necroptosis in the presence of a caspase inhibitor. At least in some RIP3-expressing cells, chemotherapy-induced ICD (but not cell death as such) is suppressed by deleting the genes coding for RIP3 or its downstream effector MLKL. Together these findings point to a facultative, cell type- and context-dependent contribution of necrotic signaling to ICD induction. While the pathways leading to ICD are apparently heterogeneous (as exemplified by caspase-dependent ICD in CT26 and MCA205 cells as opposed to RIP3/MLKL-dependent ICD in TC-1 and EL4 cells), the hallmarks of ICD apparently are similar as they include ATP release, CRT exposure, the exposure of HMGB1 from the nucleus, as well as the expression of type 1 IFN-related genes. The exposure of phosphatidylserine and CRT usually require caspase activation, while they could also happen in a caspase-independent manner. Our findings confirm and extend the idea that ICD can be stimulated by distinct pathways. Thus, a caspase-8-dependent type 1 ICD induced by anthracyclines has been distinguished from a caspase-8-independent type 2 ICD induced by photodynamic stress. A recent study suggested that B16F10 melanoma cells can be driven into ICD in the presence of the pan-caspase inhibitor z-VAD-FMK. Hence, the current results altogether suggest that there is not a single ICD pathway but multiple distinct signaling cascades that can culminate in ICD.

Necroptosis has been strongly linked to the innate immune response against viral infection. Thus, viruses can trigger necroptosis, and RIP3-deficient mice are more susceptible to infection by a number of pathogenic viruses including herpes simplex virus 1 and murine cytomegalovirus, supporting the idea that necroptosis can restrain viral infection. In accord with this speculation, there is ample evidence that pathogenic viruses can manipulate RIP3-dependent necroptosis, perhaps as a strategy for immune escape. In this regard, it should be noted that viruses also subvert pathways linked to ICD including caspase-8 activation, endoplasmic reticulum stress, autophagy, and type-1 IFN responses. Successful anticancer chemotherapeutic agents induce all these hallmarks of ICD, suggesting that they induce a sort of ‘viral mimicry’. From this point of view, the capacity of ICD inducers to trigger necroptosis-associated ICD would not consider an exception. By stimulating necroptosis, some widely used anticancer chemotherapeutics may restore anticancer immunosurveillance.

Materials and methods

Mice, cell lines and plasmids

Naive female C57BL/6 mice aged between 6 and 8 weeks were purchased from Harlan and used for experiment one week after delivery. Female athymic nude mice (Nu/Nu) aged between 8 and 12 weeks were bred and ordered from the animal facility of Gustave Roussy Cancer Campus. Murine CT26 colorectal carcinoma (H2b), MCA205 fibrosarcoma (H2b), TC-1 lung carcinoma (H2b) and EL4 thymoma (H2b) were cultured in Dulbecco’s Modified Eagle Medium containing L-glutamine (DMEM), high glucose supplemented with 10% FBS, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES and 100 U/mL penicillin and streptomycin. All culture reagents were purchased from Life Technologies (Saint Aubin, France). TC-1 and EL4 cells were used to generate stable Rip3−/− and Mlkl−/− subclones using the Clustered Regularly Interspaced Short Palindromic Repeats (Crispr)/Cas9 technology. Cripsr/Cas9 tool plasmid pX330-U6-Chimeric_BB-CBh2-hSpCas9 (Plasmid #42230) containing human codon-optimized Cas9 (hCas9) and guide RNA expressing expression cassettes, mRIP3 GFP wt plasmid (#41382, based on pEGFP-N1 vector backbone) were obtained from Addgene.

Transmission electron microscopy

TC-1 cells were seeded in culture dishes with a diameter of 10 cm at the density of 2 × 106 cells/10 mL medium per dish. After overnight recovery, pre-warmed fresh medium, or the medium containing either TSZ or MTX were added into each dish. Forty hours later, all cells in suspension were collected by centrifugation, combined with attached cells collected by
scraping. The pellets were resuspended gently and fixed in 2% glutaraldehyde, centrifuged and post-fixed in 2% osmium tetroxide. Following ethanol dehydration, cell pellets were embedded in Epon™ 812. Ultrathin sections were stained and observed with a Tecnai 12 electron microscope (FEI, Eindhoven, Netherlands).

**Clonogenic assay**

TC-1 cells were seeded at 10^5/200 μL/well in 96-well plates, treated with TSZ as indicated above or 1 μM MTX. At different time points, cells were trypsinized, collected and resuspended in fresh medium. Then 100 cells/well were seeded in six-well plates and clone formation was tested one week later by 0.5% crystal violet solution staining. This experiment were triplicated and repeated twice.

**Statistical analyses**

All results were expressed as the means ± SEM (for animal study or pooled experiments) or SD (data from a representative experiment) when appropriate. Each independent experiment was based on at least three parallel assessments. All experiments were repeated at least two–three times (refer to detailed explanations above). The statistical significance was determined using unpaired, two-tailed Student’s t test. Tumor growth kinetics was compared using the Mann–Whitney U-test. The log-rank test was used to analyze Kaplan–Meier survival curves. Data visualization and statistical analyses were performed using R and GraphPad Prism 5 software (San Diego, CA, USA).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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