SUPPLEMENTARY INFORMATION

Necroptosis does not contribute to inflammation-associated or sporadic colon cancer development in mice

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Supplementary Methods

Supplementary Table 1. Table of key reagents used.

Supplementary References

Supplementary Figure Legends

Supplementary Figure 1, relates to Figure 1

Supplementary Figure 2, relates to Figure 1 and Figure 2

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Supplementary Figure 5, relates to Figure 3 and Figure 4

Supplementary Figure 6, relates to Figure 4

Supplementary Methods

Supplementary Table 1 **Table of key reagents used.**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotinylated Rat anti-MLKL (clone 3H1)	In-house[1]	Cat #MABC604 Merck-Millipore
Biotinylated Rat anti-mouse RIPK3 (clone 8G7)	In-house[2]	
Mouse anti-GAPDH (clone 6C5)	Merck-Millipore	Cat #MAB374
IRDye 800CW-conjugated streptavidin	Licor	Cat #926-32230
IRDye 800CW-conjugated donkey anti-mouse IgG	Licor	Cat #926-32212
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides and Recombinant Proteins		
Azoxymethane	Sigma-Aldrich	Cat#A5486
cOmplete Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#11697498001
Dextran Sodium Sulfate	MP Biomedicals	Cat#0216110
Critical Commercial Assays		
Bicinchoninic acid (BCA) Protein Assay Kit	ThermoFisher	Cat#23225
4-12% Bis-Tris NuPAGE gel system	ThermoFisher	Cat#NP0321BOX
Mouse IL-6 ELISA	Invitrogen	Cat#88-7064-88
Deposited Data		
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Apc^{Min} mice	Ref.[3]	
Mlkl$^{-/-}$ mice	Ref.[1]	
Ripk3$^{-/-}$ mice	Ref.[4]	
Recombinant DNA		
Software and Algorithms		
GraphPad Prism	GraphPad Prism	www.graphpad.com
Other		
Nikon Eclipse Ti-U microscope	Nikon	
Nikon DS-Ri2 camera	Nikon Instruments Inc.	Cat# MQA17000

Supplementary references

1. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S*, et al.* The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* 2013, **39**(3)**:** 443-453.

2. Petrie EJ, Sandow JJ, Lehmann WIL, Liang LY, Coursier D, Young SN*, et al.* Viral MLKL Homologs Subvert Necroptotic Cell Death by Sequestering Cellular RIPK3. *Cell reports* 2019, **28**(13)**:** 3309-3319 e3305.

3. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990, **247**(4940)**:** 322-324.

4. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 2004, **24**(4)**:** 1464-1469.

Supplementary Figure Legends

Supplementary Figure 1. ***Variability in experimental results in a model of colitis***

(**A, D**) Daily weight loss, presented as a percentage of the original weight, for mice of the indicated genotype. N > 5 mice per genotype. Data are presented as ± SEM. * P <0.05, Students unpaired T-test. Two independent experiments are shown, non-littermates (**A**) and littermates (**D**). All mice are female. Power for test in **(A)** is 0.999, power for test in **(D)** is 0.952.

(**B, E**) Colon length for mice of the indicated genotypes. N = 8 mice per genotype. Data are presented as ± SEM. Not significant, Students unpaired T-test. Two independent experiments are shown, non-littermates (**B**) and littermates (**E**). All mice are female. Power for test in **(B)** is 0.204, power of test in **(E)** is 0.424.

(**C, F**) Histological scoring of epithelial damage, mucosal inflammation and submucosal inflammation from mice of the indicated genotypes. N = 8 mice per genotype. Data are presented as ± SEM. Not significant, Students unpaired T-test. Two independent experiments are shown, non-littermates (**C**) and littermates (**F**). All mice are female. In **(C)** the power of epithelial damage PC, MC and DC tests are 0.05, 0.074, and 0.05 respectively. The power of mucosal inflammation PC, MC and DC tests are 0.354, 0.087 and 0.122 respectively. The power of submucosal inflammation PC, MC and DC tests are 0.069, 0.053 and 0.141 respectively. In **(F)** the power of epithelial damage MC and DC tests are 0.192 and 0.05 respectively. The power of the mucosal inflammation PC, MC and DC tests are 0.05, 0.104 and 0.05 respectively. The power of the submucosal inflammation PC, MC and DC tests are 0.1004, 0.231 and 0.05 respectively.

Supplementary Figure 2 ***Serum IL-6 expression levels in MLKL and RIPK3 mice in the colitis-associated cancer model***

Serum IL-6 levels for mice of the indicated genotype. N = 8 mice per genotype. Data are presented as ± SEM. Male and female mice are indicated. There are significant differences between female wt vs male wt mice, *p=0.02 (T-test), and between male wt vs male *Mlkl*$^{-/-}$ mice, *P=0.03 (T-test).

Supplementary Figure 3 ***MLKL and RIPK3 are expressed in tumors from the colitis-associated cancer model***

(**A**) Daily weight loss, presented as a percentage of the original weight, for mice of the indicated genotype. N > 9 mice per genotype. Data are presented as ± SEM.

(**B**) Colon length for mice of the indicated genotypes. N > 9 mice per genotype. Data are presented as ± SEM. *P <0.05, Students unpaired T-test. Male and Female mice are indicated. There are significant differences within the male mice, wt vs *Ripk3*$^{-/-}$ *P=0.02 (T-test) and *Mlkl*$^{-/-}$ vs *Ripk3*$^{-/-}$ *p=0.03 (T-test); and *Mlkl*$^{-/-}$ vs *Ripk3*$^{-/-}$ *p=0.04 for all mice (T-test).

(**C**) Representative immunoblot analysis of the non-tumor (N) and tumor (T) tissues from mice of the indicated genotype following the colitis-associated cancer model.

Supplementary Figure 4 ***MLKL and RIPK3 loss do not alter tumor burden in the colitis-associated cancer model***

(**A**) Tumor number at autopsy for mice of the indicated genotype. Littermate wild-type mice (*Mlkl*$^{+/+}$) were used as controls. N > 8 mice per genotype. Data are presented as mean ± SEM. Male and female mice are indicated, with no significant differences within the sexes recorded.

(**B**) Daily weight-loss, presented as a percentage of the original weight, for mice of the indicated genotype. N > 8 mice per genotype. Data are presented as ± SEM.

(**C**) Colon length for mice of the indicated genotypes. N > 8 mice per genotype. Data are presented as ± SEM. *P <0.05, Students unpaired T-test. Male and female mice are indicated, with no significant differences within the sexes recorded.

Supplementary Figure 5 ***IL-6 expression in the tumor tissue from MLKL and RIPK3 mice***

(**A**) IL-6 ELISA on extracts from the non-tumor (N) and tumor (T) tissues from N=3 mice of the indicated sex and genotype following the inflammation-associated cancer model. Values presented are relative to total protein content in the tissue lysates

(**B**) IL-6 ELISA on extracts from the non-tumor (N) and tumor (T) tissues from N=3 mice of the indicated sex and genotype following the sporadic cancer model. Values presented are relative to total protein content in the tissue lysates.

Supplementary Figure 6 **MLKL and RIPK3 are expressed in tumors from the sporadic cancer model**

Representative immunoblot analysis of extracts from the non-tumor (N) and tumor (T) tissues from mice of the indicated genotype following the sporadic cancer model.