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Loss of TLR3 and its downstream signaling accelerates acinar cell damage in the acute phase of pancreatitis

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ABSTRACT

Background: Acute pancreatitis is accompanied by acinar cell damage releasing potential toll-like receptor 3 (TLR3) ligands. So far, TLR3 is known as a pattern recognition receptor in the immune signaling cascade triggering a type I interferon response. In addition, TLR3 signaling contributes to programmed cell death through the activation of caspase 8. However, the functional role of TLR3 and its downstream toll-like receptor adaptor molecule 1 (TICAM1) in the inflamed pancreas is unknown.

Methods: To uncover the role of TLR3 signaling in acute pancreatitis, we induced a cerulein-mediated pancreatitis in *Tlr3* and *Ticam1* knockout (KO) mice and in wildtype animals. The exocrine damage was determined by blood serum analysis and histological examination. Immunohistochemistry, gene expression and immunoblot analysis were conducted to study TLR3 function.

Results: After the induction of an acute pancreatitis, wildtype mice showed a high endosomal TLR3 expression in acinar cells. In comparison to wildtype and *Ticam1* KO mice, *Tlr3* KO mice exhibited the highest severity of pancreatitis with an increased NF-κB activation and elevated expression of the pro-inflammatory cytokines *Il6* and *Tnf*, although the amount of infiltrating immune cells was unaffected. Additionally, we detected a strong elevation of acinar cell necrosis and reduced levels of cleaved caspase 8 in *Tlr3* and *Ticam1* KO mice.

Conclusions: TLR3 and its downstream adaptor TICAM1 are important mediators of acinar cell damage in acute pancreatitis. They possess a critical role in programmed cell death and our data suggest that TLR3 signaling controls the onset and severity of acute pancreatitis.

Introduction

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) and primarily described as mediators of the innate immune response. Besides their function in regulating host defense after microbial invasion and the recognition of conserved pathogen-associated molecular patterns (PAMPs), TLRs detect damage-associated molecular patterns (DAMPs). DAMPs are released during tissue stress and injury, for instance during pancreatitis [1]. Until now, ten human and twelve mouse TLRs have been identified, unique through their binding of different ligands and the activation of either an inflammatory response or controlled cell death [2]. TLR3 recognizes double-stranded RNAs and TLR4 bound bacteria cell wall components [3]. Notably, TLR3 shows an endosomal localization and induces a type 1 interferon response, whereas TLR4 is localized in the cell membrane mediating NF-κB (nuclear factor of kappa light polypeptide gene enhancer) signaling and a pro-inflammatory reaction through the expression of the cytokine IL6 (interleukin 6) and TNF (tumor necrosis factor) [3]. After ligand

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binding TLR4 utilizes either the adapter protein MYD88 (myeloid differentiation primary response 88) or TICAM1 (toll-like receptor adapter molecule 1, hereinafter referred to as TRIF) as a direct downstream signaling factor, whereas TLR3 signaling is processed only by TRIF [4]. There are increasing evidences that the presence of TLRs is not restricted to immune cells [5]. Breast cancer tumor cells express functional TLR3 to trigger caspase 8-dependent extrinsic apoptosis via the adaptor protein TRIF [6]. Studies in melanoma cells demonstrated that the TLR3/TRIF/caspase 8 axis is controlled by the inhibitory apoptosis proteins XIAP (X-linked inhibitor of apoptosis) and FLIP (CFLAR, Casp8 and Fadd like apoptosis regulator) [7]. Remarkably, the inhibition of caspase-related apoptosis results in the activation of programmed cell necrosis, which is a major complication in acute pancreatitis (AP) [8,9]. The extent of necrosis directly correlates with the severity of the disease, thus, the proportion of apoptotic to necrotic cell death is an important factor in AP [10].

AP has an annual incidence of 13–45/100,000 persons and is often related to excessive alcohol consumption or gallstone obstruction [11]. Severe forms of the disease occur in around 15–25% of the cases. Here, an uncontrolled systemic inflammatory response can lead to multiple organ failure, which is associated with a mortality risk between 10 and 24% [12]. The pathophysiological mechanisms of AP comprise an intracellular activation of trypsinogen and other digestive enzymes in the pancreatic parenchyma, entailing autodigestive injury of the organ. The severe acinar cell damage is followed by a strong inflammatory response characterized by the infiltration of various immune cells and the secretion of pro-inflammatory cytokines, such as TNF and IL6 [13]. Potential TLR ligands such as DNA and RNA complexes are released from apoptotic and necrotic cells during tissue inflammation, but the functional relevance of the intrinsic TLR3/TRIF-dependent signaling cascade in remnant acinar cells remains elusive. In this study, we investigated the onset and severity of pancreatitis in the acute phase of inflammation in two different transgenic mouse models, lacking the expression of TLR3 and TRIF.

Material and methods

Mouse lines. The transgenic *Tlr3* knockout mouse line B6; 129S1-Tlr3^{tm1Flv}/J (*Tlr3* KO) was obtained from Jackson Laboratory (stock: 005217; Bar Harbor, USA) and described previously [14]. Mutant, inactive Trif$^{Lps2/Lps2}$ (*Trif* KO) mice were kindly provided by Bernhard Holzmann (Munich, Germany) [15]. Both mouse models were housed under pathogen-free conditions on a pure C57BL/6 background at the Klinikum rechts der Isar, Technical University Munich, Germany. C57BL/6J mice served as wildtype control animals and were obtained from Charles River Laboratories (Sulzfeld, Germany). All mouse experiments and procedures were in accordance with the German Federal Animal Protection Laws and approved by the government of Upper Bavaria for Laboratory Animal Welfare with the reference number 55.2-1-54-2531-147-09.

Acute pancreatitis. Pancreatitis was induced in around eight-week-old wildtype, *Tlr3* KO and *Trif* KO mice. In total, ten intraperitoneal injections of 50 μg/kg body weight cerulein (Sigma Aldrich, Steinheim, Germany) in a volume of 100 μl 0.9% physiological saline were administered, with one injection per hour. Wildtype, *Tlr3* KO and *Trif* KO control mice received similar injections of physiological saline. Animals were sacrificed one hour after the final cerulein injection. Blood and pancreata were harvested for further analysis.

Blood serum analysis. Serum Amylase, Lipase and LDH levels were measured (Cobas 8000) in the Department of Clinical Chemistry at the Technical University Munich, Germany.

Histology and Immunohistochemistry. For histological analysis, paraffin-embedded tissue sections (3 μm thick) were stained with hematoxylin and eosin (H&E) according to standard conditions. Immunohistochemistry staining was performed using antibodies against TLR3 (Novus Biologicals, Southpark, CO, USA), CD45 (BD Pharmingen, San Diego, CA, USA) and p65 (Lab Vision/NeoMarkers, Fremont, CA, USA) followed by color reaction with the DAB + Substrate Chromogen System (Dako, Hamburg, Germany) or the AEC + High Sensitivity Substrate Chromogen (Dako), respectively. To detect apoptotic and necrotic cells, TUNEL assay was performed with the Apo-BrdU-IHC™ In Situ DNA Fragmentation Kit from BioVision (Milpitas, CA, USA) according to manufacturer's protocol, however half of the antibody concentration was used. Light microscopic pictures were taken with the Axioskop Zeiss microscope (Zeiss, Jena, Germany). Whole slide scans were generated with a Pannoramic MIDI II slide scanner (Sysmex Deutschland GmbH, Norderstedt, Germany). Staining intensity of TLR3 was quantified by manually scoring (0, 1, 2, 3) the whole tissue slide. Quantification of p65 antibody staining was performed by examining five high-power fields (HPFs) per pancreas and manual counting of positive and negative cells. CD45 staining was quantified from the whole slide scan using QuPath (version 0.1.2, open source software). Areas of acinar cell necrosis were assessed with TUNEL staining and quantified on five HPFs per pancreas as percentage of the whole tissue area with ImageJ (NIH, Bethesda, MD, USA). Histological changes, such as edema, immune cell infiltration and quantification of apoptotic cells and necrosis were scored (0, 1, 2, 3) on H&E stains by two experienced pathologists (KS, AMS) in a blinded manner.

RNA isolation and cDNA synthesis. Total RNA was extracted immediately after pancreas dissection. Tissue was homogenized in Tri-Reagent (Sigma Aldrich, Steinheim, Germany) with a tissue disrupter (TissueLyser LT, Qiagen, Hilden, Germany), depleted from DNA and purified using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reverse transcription of total RNA was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit from Thermo Fisher Scientific (St. Leon-Rot, Germany).

Quantitative real-time PCR (qRT-PCR). PCR amplification of the respective genes was carried out with 40 ng of cDNA, 500 nM forward and reverse primer and SYBR Green I Mastermix (Roche Diagnostics, Penzberg, Germany) in a final volume of 20 μl. PCR reactions were run in doublets for 40 cycles consisting of 15 s denaturation at 95 °C, primer annealing for 15 s at 55 °C, and extension for 15 s at 72 °C. We used the following primer pairs (5′-3′ orientation): *Ifna* fw-CCTGACCCAGGAAGATGCCCTGCTGG, rev-AGCACATTGGCAGAGGAAGACAGGGCT; *Ifnb* fw-AGCTACGCCTGGA TGGTGGTCCGA, rev-GCCTGCAACCACCACTCATTCTGAGGC; *Il6* fw-TCCTCTCTGCAAGAGACTTCCATCC, rev-CCTCTGTGAAGTCTCCTCTCC GG; *Tnf* fw-TCGGGGTGATCGGTCCCCAA, rev-TGGTTTGCTACGACGT GGGCT and *Ppib* (housekeeping gene) fw-GGAGCGCAATATGAAG GTGC, rev- CTTATCGTTGGCCACGGAGG. Quantitative RT-PCR was performed on a LightCycler® 480 System (Roche). Relative quantification of gene expression and normalization to wildtype controls was calculated with the ΔΔCt method as described earlier [16].

Immunoblot analysis

Mouse pancreatic tissue was homogenized and lysed in tissue lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% SDS, 1% sodium deoxycholate, 30 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM $NaVO_3$, 1 mM DTT). 30 μg of protein extracts were subjected to immunoblot analysis with the following primary antibodies: cleaved caspase 8 (Asp387) (D5B2) (Cell signaling, Massachusetts, USA) and GAPDH (Meridian Life Science, Memphis, USA).

Statistics. Data are presented as mean ± SEM. Statistical significance was determined by non-parametric Mann-Whitney test, non-parametric Kruskal-Wallis test and One-way ANOVA with a Tukey's multiple comparison as indicated in the figure legends using GraphPad Prism 8 software (GraphPad Software Inc.). *P* values less than 0.05 were considered as significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Results

Elevated expression of TLR3 in acute pancreatitis

To assess the expression and localization of TLR3 in normal and inflamed pancreatic tissue of wildtype mice, we performed TLR3 immunohistochemistry staining. Healthy wildtype control animals at eight weeks of age exhibited merely a weak expression of TLR3 in the exocrine compartment of the pancreas, whereas some cells of the endocrine glands, likely glucagon-secreting alpha cells, demonstrated an intense TLR3 staining (Fig. 1A). Acute pancreatitis was induced in eight-week-old wildtype mice by ten intraperitoneal injections of the cholecystokinin analogue cerulein administered hourly. Pancreatic tissue was harvested one hour after the final cerulein-injection. Compared to healthy controls, significantly increased TLR3 expression was detected in acinar cells (Fig. 1A and B), with a clear endosomal localization of TLR3 (Fig. 1A, high magnification image). Additionally, a few of the infiltrated immune cells were positive for TLR3 expression (Fig. 1A). In summary, TLR3 is highly activated in the acute phase of pancreatitis and TLR3 expression is massively apparent in epithelial acinar cells showing endosomal localization.

Loss of TLR3 and TRIF accelerates acute pancreatitis

Next, we investigated the impact of activated TLR3 signaling in acute pancreatitis. Thus, we characterized two different transgenic mouse models with either a global gene knockout (KO) of *Tlr3* or its downstream signaling adaptor *Trif*. Under physiological conditions, the histological examination revealed that the *Tlr3* KO and *Trif* KO mice displayed no pathological alterations in the pancreas at eight weeks of age. In addition, the distribution of pancreatic acinar cells, ducts, blood vessels and endocrine islets was comparable to wildtype mice (Fig. 2A, control). These data suggest that the loss of TLR3/TRIF signaling during embryogenesis has no influence on pancreatic organ development. Further, *Tlr3* KO, *Trif* KO and wildtype mice received consecutive injections of cerulein to induce an acute pancreatitis. The pancreata were harvested one hour after the final injection. The initial histological assessment of H&E stains of the inflamed pancreatic tissue revealed accelerated acinar cell damage in *Tlr3* KO and *Trif* KO mice compared to wildtype mice (Fig. 2A, AP 1h). We scored the grade of edema, necrosis and immune cell infiltration, which are specific characteristics of acute pancreatitis (Fig. 2B) [17]. *Tlr3* KO showed a significant increase in edema compared to wildtype animals and *Trif* KO mice. Notably, *Tlr3* KO mice revealed a significantly elevated level of necrosis, whereas no difference in infiltrating immune cells was detectable (Fig. 2B). Furthermore, we analyzed the blood serum levels of Amylase, Lipase and LDH (lactate dehydrogenase) under normal and inflamed conditions determining the severity of acute pancreatitis. Under physiological conditions, we observed once more that wildtype, *Tlr3* KO and *Trif* KO control mice displayed no phenotypical differences indicated by similar Amylase, Lipase and LDH blood serum levels (Fig. 2C). After the induction of an acute pancreatitis, Amylase serum levels were highly increased, with no significant difference between wildtype, *Tlr3* KO and *Trif* KO mice (Fig. 2C). On the contrary, the pancreatitis-mediated release of Lipase into the blood was significantly elevated in *Tlr3* KO and *Trif* KO mice compared to wildtype mice (Fig. 2C). Similarly, LDH levels were strongly increased and drastically enriched in the blood serum of *Tlr3* KO and *Trif* KO mice, showing the highest level in *Tlr3* KO mice (Fig. 2C). According to the histological results, wildtype and *Trif* KO mice demonstrated a mild acute pancreatitis, whereas *Tlr3* KO mice exhibited a moderate acute pancreatitis. Although the blood serum analysis did not fully corroborate the gradual difference between *Tlr3* KO and *Trif* KO mice, we assume that TLR3 deficiency worsens the outcome of acute pancreatitis.

Absence of TLR3 and TRIF does not influence immune cell recruitment in acute pancreatitis

It has been described that TLR3 signaling is essential for immune cells to initiate the innate and adaptive immune response [5]. Thus, we wanted to analyze in more detail whether the loss of TLR3 or TRIF has an influence on the immune cell infiltration in the healthy pancreas and in acute pancreatitis. By performing immunohistochemistry (IHC) staining against CD45, a common marker for immune cells, we first quantified the cells in 0.9% NaCl-treated wildtype, *Tlr3* KO and *Trif* KO control mice (IHC staining pictures not shown). *Tlr3* KO and *Trif* KO mice exhibited a significantly higher proportion of resident immune cells in the healthy pancreas compared to wildtype mice (Fig. 3A). Next, we determined the recruitment of inflammatory cells after the induction of an acute pancreatitis. Unexpectedly, wildtype, *Tlr3* KO and *Trif* KO mice exhibited comparable levels of infiltrated immune cells (Fig. 3B and C). In summary, the global deficiency for TLR3 or TRIF changed the amount of resident immune cells in the healthy organ, whereas the quantity of infiltrating immune cells in the acute phase of

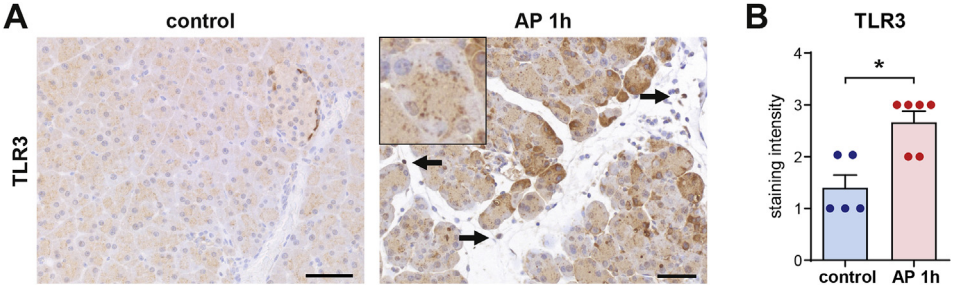

Fig. 1. Elevated expression of TLR3 in acute pancreatitis. (A) Representative pictures of immunohistochemistry staining of TLR3 of healthy eight-week-old wildtype control animals and wildtype mice with an acute pancreatitis receiving 10 hourly intraperitoneal injections of 50 μg/kg cerulein. Tissue was harvested one hour after the final cerulein administration (AP 1h). High power picture demonstrates endosomal TLR3 localization. Arrows mark TLR3-positive immune cells. Magnification 400x, scale bar 50 μm. (B) Quantification of TLR3 staining intensity of pancreata from control and acute pancreatitis (AP 1h) wildtype mice. All data are assessed from 5 to 6 animals per group and presented as mean ± SEM; p-values were calculated with non-parametric Mann-Whitney test; $^{*}p < 0.05$.

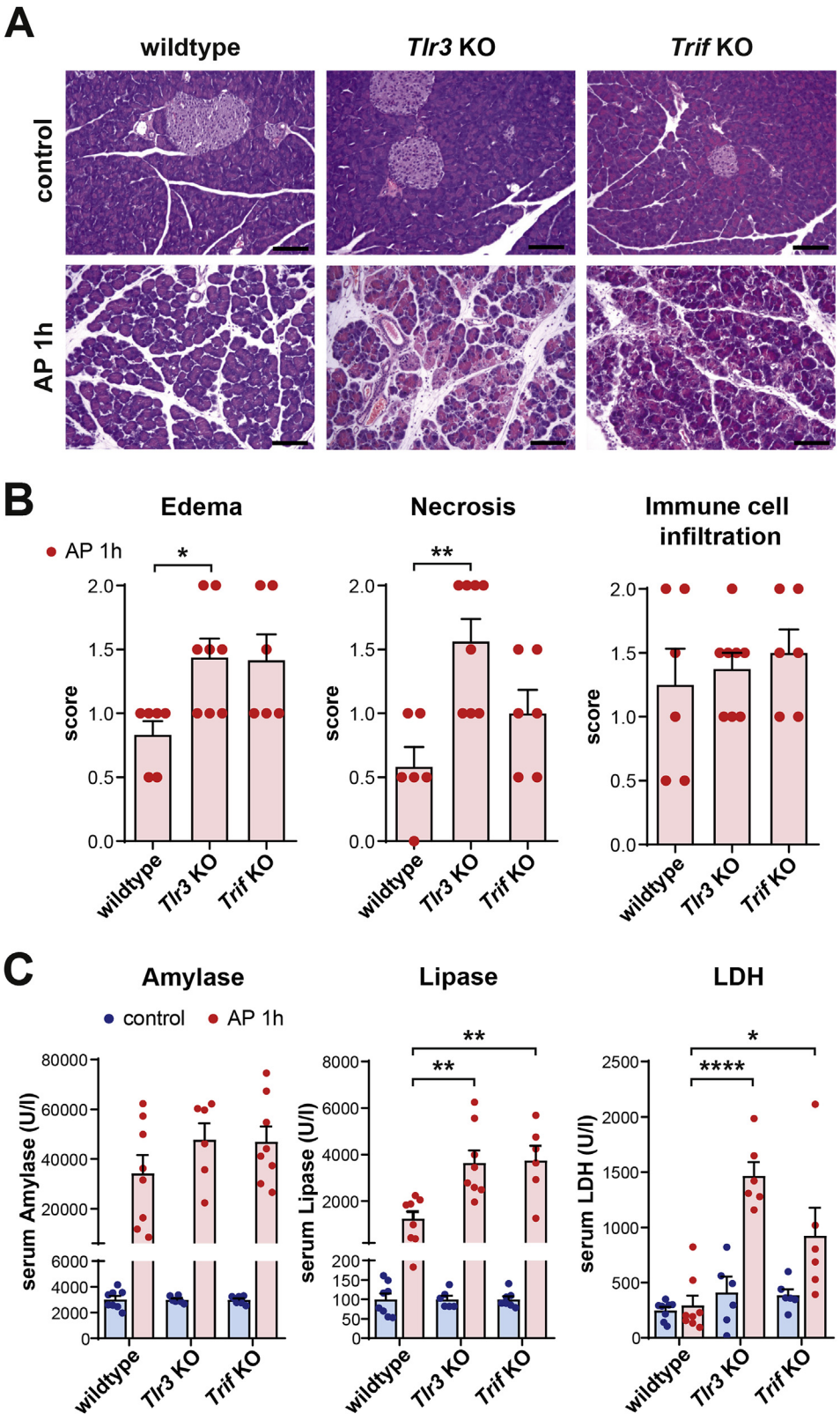

Fig. 2. Loss of TLR3 and TRIF accelerates acute pancreatitis. (A) Representative H&E pictures of healthy eight-week-old wildtype, *Tlr3* KO and *Trif* KO control mice and mice one hour after receiving 10 hourly intraperitoneal injections of 50 μg/kg cerulein for the induction of an acute pancreatitis (AP 1h). Magnification 100x, scale bar 100 μm. (B) Histological scoring for edema, necrosis and immune cell infiltration of wildtype, *Tlr3* KO and *Trif* KO mice after receiving cerulein (AP 1h). (C) Measured blood serum levels of Amylase, Lipase and LDH in U/l from wildtype, *Tlr3* KO and *Trif* KO control animals and mice with an acute pancreatitis (AP 1h). All data are assessed from 6 to 8 animals per group and presented as mean ± SEM; p-values were calculated with a non-parametric Kruskal-Wallis test (B) or with a One-way ANOVA (C); *$p \leq 0.05$, **$p < 0.01$, ****$p < 0.0001$.

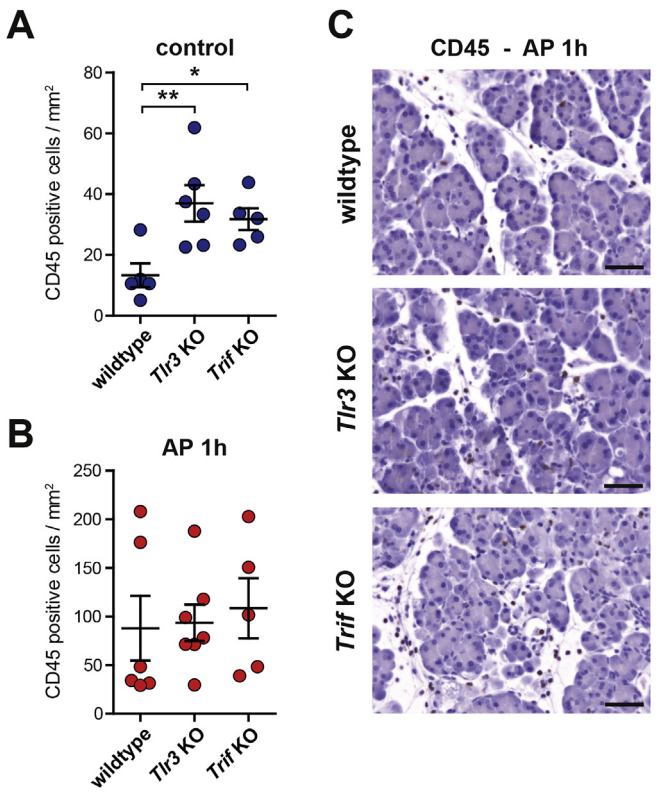

Fig. 3. Absence of TLR3 and TRIF does not influence immune cell recruitment. (A) Quantification of CD45-positive cells in whole tissue slide scan of control wildtype, *Tlr3* KO and *Trif* KO animals. (B) Quantification of CD45-positive cells in whole tissue slide scan of wildtype, *Tlr3* KO and *Trif* KO mice with an acute pancreatitis (AP 1h). (C) Representative pictures of immunohistochemistry staining of the immune cell marker CD45 in wildtype, *Tlr3* KO and *Trif* KO mice one hour after receiving 10 hourly intraperitoneal injections of 50 μg/kg cerulein (AP 1h). Magnification 200x, scale bar 50 μm. All data are assessed from 5 to 7 animals per group and presented as mean ± SEM; p-values were calculated with One-way ANOVA; $^{*}p \leq 0.05$, $^{**}p < 0.01$.

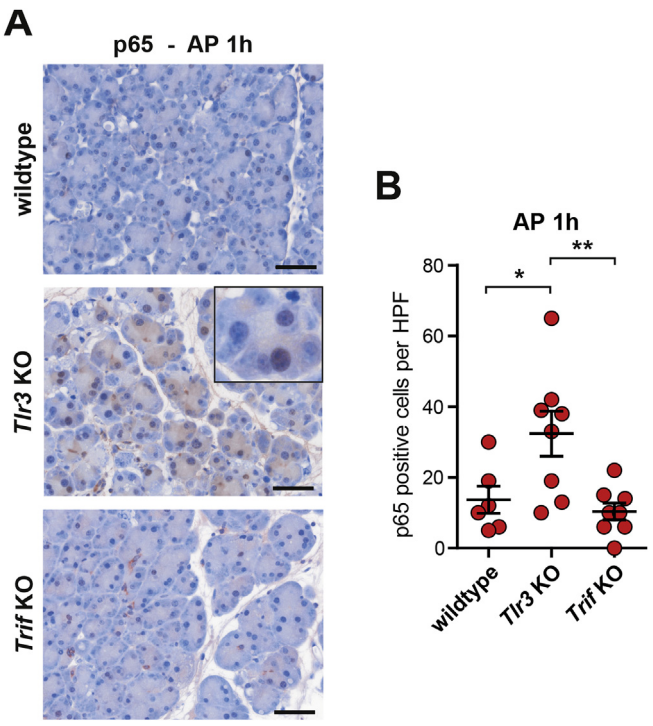

Fig. 4. High levels of activated NF-κB are restricted to *Tlr3* KO mice. (A) Representative pictures of immunohistochemistry staining of NF-κB/p65 in wildtype, *Tlr3* KO and *Trif* KO mice, one hour after receiving 10 hourly intraperitoneal injections of 50 μg/kg cerulein (AP 1h). High magnification picture shows p65-positive and -negative nuclei. Magnification 400x, scale bar 50 μm. (B) Quantification of p65-positive cells in wildtype, *Tlr3* KO and *Trif* KO with an acute pancreatitis (AP 1h). All data are assessed from 6 to 8 animals per group and presented as mean ± SEM; p-values were calculated with a One-way ANOVA; $^{*}p \leq 0.05$, $^{**}p < 0.01$.

pancreatitis did not differ in comparison to wildtype mice. Thus, the data indicate that the loss of TLR3 and TRIF did not affect the rate of immune cell infiltration in the early phase of acute pancreatitis.

High levels of activated NF-κB are present in Tlr3 KO mice

The inflammatory response in acute pancreatitis promotes the activation of the nuclear factor kappa B (NF-κB). Existing data claim that high levels of NF-κB favor pro-inflammatory mechanisms, which result in acinar cell necrosis, whereas basal NF-κB levels support protective and pro-survival effects of the acinar cells [18,19]. The recruitment of the adaptor protein TRIF to either TLR3 or TLR4 effects NF-κB signaling. Consequently, we examined the NF-κB activation in our mouse models after cerulein-induced pancreatitis. The predominant heterodimer of NF-κB consists of the p65 and p50 subunits, which translocate into the nucleus and functions as a transcriptional activator of specific NF-κB target genes [20]. Using immunohistochemistry, we stained p65 in healthy and inflamed pancreata of wildtype, *Tlr3* KO and *Trif* KO mice (Fig. 4A). We could detect no basal NF-κB activity in the exocrine compartment of the healthy pancreata, all three control-treated mouse models showed a negative staining for p65 (data not shown). However, after the induction of an acute pancreatitis, NF-κB was activated and the p65 staining was localized in the nucleus of the acinar cells. A high magnification picture showed p65-positive and -negative nuclei in an acinar cell complex (Fig. 4A). The quantification of positive acinar cells demonstrated the strongest activation of NF-κB in *Tlr3* KO mice, whereas *Trif* KO and wildtype mice exhibited only a few positive nuclei for p65 (Fig. 4B). Altogether, the elevated expression of p65 and the increased histopathological score in *Tlr3* KO mice suggest that highly activated NF-κB signaling favors acinar cell damage and exacerbates the grade of pancreatitis in *Tlr3* KO mice.

Increase of the pro-inflammatory cytokines IL6 and TNF in Tlr3 KO mice

Next, we determined the expression of the type I interferon response genes *Ifna* and *Ifnb* (Interferon alpha and beta), which are mainly regulated by TLR3/TRIF signaling as well as the pro-inflammatory cytokines *Il6* and *Tnf*, controlled by TLR4/MYD88-dependent NF-κB signaling. RNA was extracted from bulk pancreatic tissue of healthy control animals and mice with an acute pancreatitis. The gene expression of *Ifna*, *Ifnb*, *Il6* and *Tnf* did not differ between the 0.9% NaCl-treated wildtype, *Tlr3* KO and *Trif* KO control mice (Fig. 5A and B). After the induction of an acute pancreatitis, wildtype mice demonstrated a strong increase of *Infa* and *Infb* expression, whereas *Tlr3* KO and *Trif* KO mice revealed nearly no elevated expression of the type I interferon response genes (Fig. 5A). Furthermore, we analyzed the expression of NF-κB target genes *Il6* and *Tnf*, which were highly activated in all three mouse models after the induction of pancreatitis (Fig. 5B). Particularly, *Tlr3* KO mice exhibited a significant upregulation of *Il6* in comparison to wildtype mice (Fig. 5B). Notably, the gene expression data confirmed a strong activation of NF-κB and its downstream

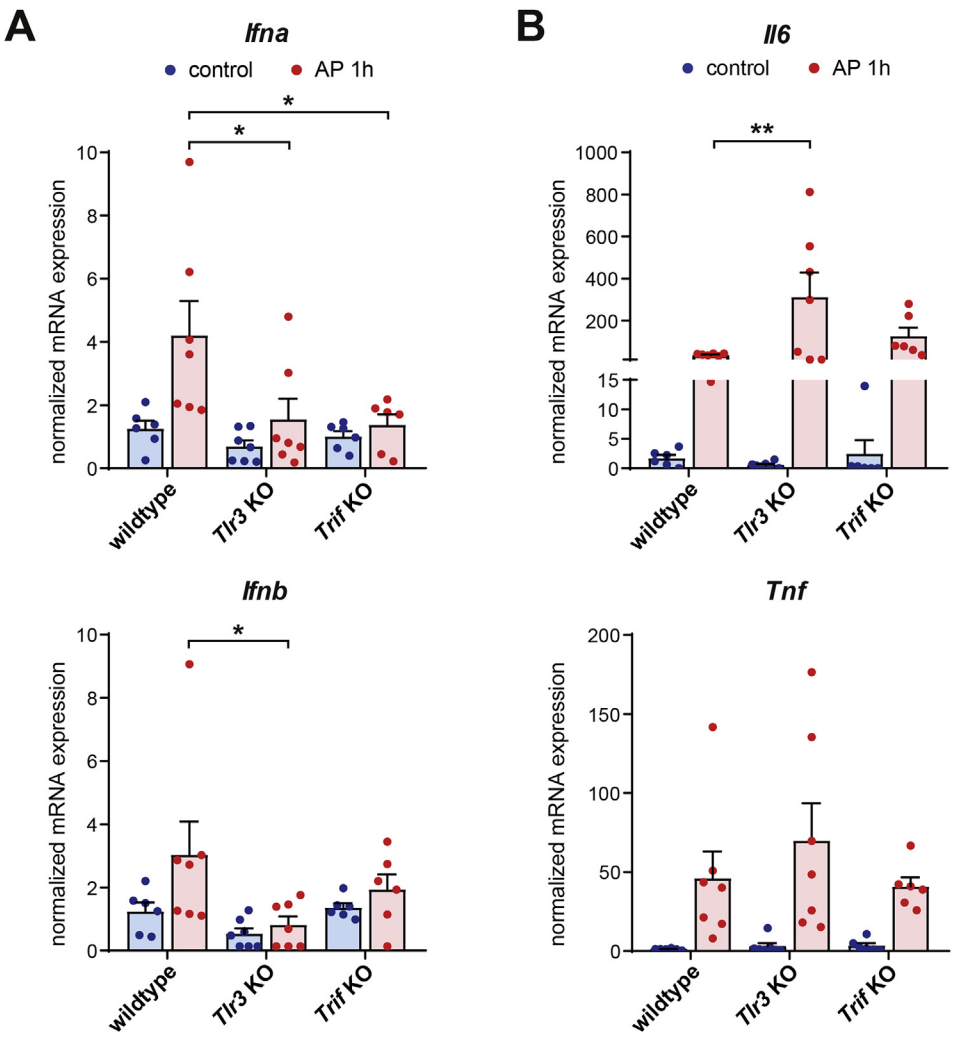

Fig. 5. Increase of the pro-inflammatory cytokines IL6 and TNF in *Tlr3* KO mice. Cytokine expression profile of healthy wildtype, *Tlr3* KO and *Trif* KO control animals and corresponding treatment group receiving 10 hourly intraperitoneal injections of 50 μg/kg cerulein (AP 1h). (A) Gene expression level of type 1 interferon response genes Interferon alpha (*Ifna*) and Interferon beta (*Ifnb*). (B) Gene expression level of NF-κB-regulated genes Interleukin-6 (*Il6*) and tumor necrosis factor alpha (*Tnf*). All data are assessed from 6 to 7 animals per group, normalized to wildtype controls and presented as mean ± SEM; p-values were calculated with One-way ANOVA; *p ≤ 0.05, **p < 0.01, ***p < 0.001.

targets in cerulein-treated *Tlr3* KO mice.

Increased necrosis in Tlr3 KO mice

TLR3 can act as a death receptor and promotes programmed cell apoptosis through the activation of caspase 8 [21]. To investigate the rate of cell death in the phase of acute pancreatitis, we performed a TUNEL assay, demonstrating the highest level of cell death in *Tlr3* KO mice compared to *Trif* KO and wildtype mice (Fig. 6A and B). Since, the assay fails to discriminate between apoptosis and necrosis we carefully examined H&E stains (shown in Fig. 2) and manually quantified areas of necrosis as well as the amount of apoptotic cells. In comparison to wildtype mice, the apoptosis/necrosis ratio was significantly reduced in *Tlr3* KO and *Trif* KO mice, with the lowest ratio in *Tlr3* KO mice, indicating that TLR3 and TRIF depletion favors acinar cell necrosis to apoptosis (Fig. 6C). To determine whether the suppression of apoptosis was regulated by the two apoptotic inhibitors FLIP (CFLAR, Casp8 and Fadd like apoptosis regulator) and XIAP (X-linked inhibitor of apoptosis), we analyzed their gene expression in the phase of acute pancreatitis. *Tlr3* KO mice demonstrated significantly elevated *Xiap* expression, whereas *Flip* was only slightly increased in *Tlr3* KO and *Trif* KO mice, not showing significant changes (Fig. 6D). Highly diminished levels of cleaved caspase 8 were detected in *Tlr3* KO and *Trif* KO mice, implying reduced apoptotic activity and a switch towards necrotic cell death in cerulein-mediated pancreatitis (Fig. 6E). In summary, our data indicate that TLR3/TRIF signaling is an important mediator in controlling acinar cell apoptosis and necrosis, determining the severity of acinar cell damage in the onset of acute pancreatitis.

Discussion

Acute pancreatitis is characterized by an intracellular activation of digestive enzymes triggering an autodigestive injury of the exocrine compartment [22]. Concomitant pancreatic acinar cell damage provokes the release of endogenous molecules, which act as DAMPs and are detected by PRRs, such as TLR3 [23]. So far, TLR3 signaling has been described as a mechanism of the innate immune response against viral infections. The role of the signaling pathway in pancreatitis in the absence of pathogens, so called sterile inflammation, is largely unknown [24]. Our data demonstrated that in the healthy pancreas the TLR3 expression was very weak, whereas a strong enrichment and a clear endosomal localization of TLR3 was demonstrated in the exocrine compartment after the

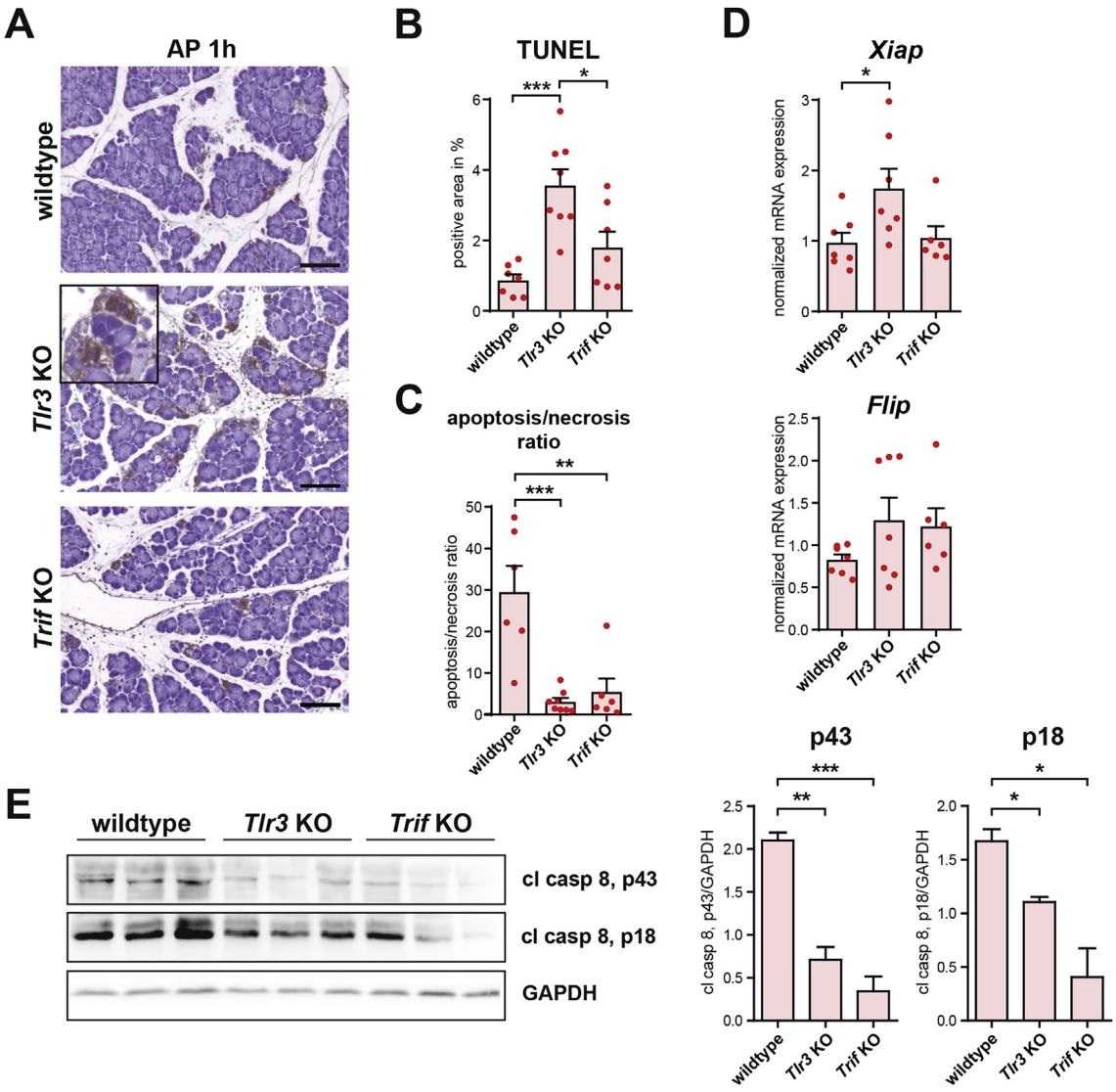

Fig. 6. Elevated necrosis in *Tlr3* KO mice. (A) Representative pictures of TUNEL assay staining in wildtype, *Tlr3* KO and *Trif* KO mice, one hour after receiving 10 hourly intraperitoneal injections of 50 µg/kg cerulein (AP 1h). Magnification 100x, scale bar 100 µm. High magnification picture shows positive staining of necrotic cells. (B) Quantification of positive areas in percent to whole tissue, calculated from five HPFs of wildtype, *Tlr3* KO and *Trif* KO mice with an acute pancreatitis. (C) Apoptosis/necrosis ratio is given as number of apoptotic cells divided by area of necrosis in percent of whole tissue from five HPFs of wildtype, *Tlr3* KO and *Trif* KO mice with an acute pancreatitis. (D) Normalized mRNA expression of *Xiap* and *Flip* in wildtype, *Tlr3* KO and *Trif* KO mice with an acute pancreatitis. (E) Representative picture of immunoblot analysis with indicated antibodies of wildtype, *Tlr3* KO and *Trif* KO mice ($n = 3$) with an acute pancreatitis. Subunits of cleaved caspase 8 levels were quantified according GAPDH levels. Unless otherwise stated, all data are assessed from 6 to 8 animals per group and presented as mean ± SEM; p-values were calculated with One-way ANOVA; $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

induction of an acute pancreatitis. As expected, a few immune cells showed also TLR3 positivity. TLR9, another endosomal receptor, was also not detectable in pancreatic acinar cells under physiological conditions. Here, only bone marrow-derived CD45-positive cells demonstrated TLR9 positivity [25]. Another study, analyzing autoimmune pancreatitis, depicted various TLRs with the highest enrichment of TLR7-, TLR8-and TLR10-positive immune cells in the inflamed pancreata [26]. Our data demonstrated that the genetic depletion of *Tlr3* and *Trif* resulted in an increase of endogenous CD45-positive immune cells in the pancreata of untreated animals compared to wildtype mice; however, the effect was no longer observable after cerulein administration. Given that TLR3 expression was highly elevated in acinar cells and that the number of infiltrating immune cells was not affected in the inflamed tissue, we supposed, that TLR3 signaling modulates the acinar cell response during pancreatitis.

Thus, we assessed the function of TLR3 signaling and its downstream signaling molecule TRIF after cerulein-triggered acute pancreatitis in global *Tlr3* and *Trif* KO mice. Our results of the histological scoring, the LDH serum levels and the quantification of fragmented DNA from apoptotic and necrotic cells, examined with a TUNEL assay, clearly demonstrated a significant increase in acinar cell damage in *Tlr3* KO mice. In contrast to our data, Hoque et al. did not detect alterations in edema or acinar cell apoptosis in $Tlr3^{-/-}$ mice after the induction of an acute pancreatitis [25]. Since the mouse model was not clearly defined, we can just speculate that the genetic background of the mice might differ or that less cerulein injections did not recapitulate the strong phenotype of acinar cell necrosis as we observed. Further characterization of our *Tlr3* KO mouse model revealed an increase in NF-κB signaling, which was determined by nuclear p65 immunohistochemistry staining and a significant upregulation of the pro-inflammatory cytokine *Il6* in

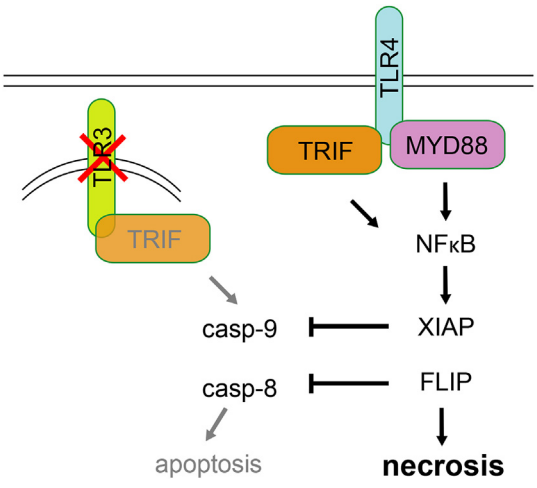

Fig. 7. Our proposed model of acinar cell apoptosis versus necrosis suggests that TLR3/TRIF signaling initiates cell apoptosis via caspase 8 cleavage. In the absence of TLR3, the downstream adaptor TRIF triggers NF-κB-mediated acinar cell necrosis, which worsens the onset and severity of the acute pancreatitis.

comparison to *Trif* KO and wildtype mice. We assume that the TLR3 depletion leads to an enhanced activity of the TLR4/TRIF-signaling axis, triggering NF-κB activation (Fig. 7). In contrast, the loss of TRIF prevents TLR3 signaling and causes merely a weak basal TLR4/MYD88-mediated NF-κB response. Studies exist, describing that NF-κB signaling can either aggravate or ameliorate pancreatitis [27]. Thus, recent data showed that highly activated NF-κB levels provoke a pro-inflammatory phenotype with induced acinar cell necrosis [18], whereas low basal levels of NF-κB attenuate the induction of severe pancreatitis [19]. In our study, increased NF-κB activation was accompanied by highly accelerated acinar cell necrosis in *Tlr3* KO mice. Notably, the apoptosis/necrosis ratio shifted significantly towards acinar cell necrosis in *Tlr3* KO and *Trif* KO mice and our results furthermore depicted a concomitant reduction of cleaved caspase 8 in the two mouse models. Due to the genetic depletion of *Trif* and *Tlr3*, it is most likely that the TLR3/TRIF-dependent activation of caspase 8 was absent and prevented TLR3-mediated cell apoptosis in the *Tlr3* KO and *Trif* KO mice (depicted in Fig. 7) [7,21]. The additional induction of XIAP, an inhibitor of apoptosis, in *Tlr3* KO mice supported our findings of an apoptosis to necrosis switch in the mouse model. The group of Gukovsky and Gukovskaya described in detail that caspase inhibition and stable XIAP expression promotes necrotic events, whereas XIAP degradation or inhibition triggers caspase-mediated apoptosis in acute pancreatitis [9]. Acinar cell necrosis is a severe complication in acute pancreatitis and from our results, we can conclude that in the onset of acute pancreatitis TLR3 functions as a mediator of programmed cell death, preventing unrestricted cell necrosis and severe organ damage.

Disclosures

No conflicts of interest to disclose.

Author contributions

IR, SRa, JK, CWM designed the study and wrote the manuscript. IR, SRa, SB, CM, SRi, GL performed experiments and collected data. IR, SRa, JM, BK, JK, CWM analyzed and interpreted the data. KS, AMS, IE and IR evaluated tissue histology. IE, JM, BK, JK, CWM gave administrative, technical, or material support. All authors revised the manuscript critically for important intellectual content and approved the final version to be published.

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