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Allergy and inflammation

Research Article

Toll-like receptor 3 expression in myeloid cells is essential for efficient regeneration after acute pancreatitis in mice

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Stringent regulation of the inflammatory response is crucial for normal tissue regeneration. Here, we analyzed the role of Toll-like receptor 3 (TLR3) in pancreatic regeneration after acute pancreatitis (AP). AP was induced by caerulein treatment in mice with global TLR3 deficiency ($TLR3^{OFF}$) or in mice re-expressing TLR3 exclusively in the myeloid cell lineage ($TLR3^{Mye}$). Compared to WT mice, $TLR3^{OFF}$ mice had a markedly increased formation of acinar-to-ductal metaplasia (ADM) that persisted until day 7 after initiation of AP. Pancreatic tissue of WT mice was completely regenerated after 5 days with no detectable ADM structures. The enhancing effect of TLR3-deficiency on ADM formation was closely linked with an increased and prolonged accumulation of macrophages in pancreata of $TLR3^{OFF}$ mice. Importantly, the phenotype of $TLR3^{OFF}$ mice was rescued in $TLR3^{Mye}$ mice, demonstrating the causative role of myeloid cell selective TLR3 signaling. Moreover, in vitro stimulation of macrophages through TLR3 initiated cell death by a caspase-8-associated mechanism. Therefore, these findings provide evidence that TLR3 signaling in myeloid cells is sufficient to limit inflammation and ADM formation and to promote regeneration after AP. Notably, resolution of inflammation after AP was associated with macrophage sensitivity to TLR3-mediated cell death.

Keywords: acute pancreatitis · ADM · macrophages · myeloid cells · TLR3

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas, caused by premature zymogene-activation resulting in pancreatic autodigestion [1, 2]. Within gastrointestinal diseases, AP is the most common reason for hospitalization with an increasing global incidence rate of currently 34 cases per 100 000 individuals [3–5]. The main causative factors include gallstones and alcohol abuse [6, 7].

In AP, pancreatic autodigestion evokes severe tissue necrosis, resulting in the release of damage-associated molecular patterns (DAMPs) like nucleic acids from dead cells [1]. The activation of DAMP receptors recruits immune cells to the pancreas, thereby initiating and orchestrating the inflammatory response [8]. As part of the subsequent regeneration process, acinar cells reversibly transdifferentiate to a ductal-like progenitor cell state [9–11]. This transient so-called acinar-to-ductal metaplasia (ADM) enables the recovery of the exocrine pancreas [11]. In contrast, in the context of oncogenic signaling, ADM persists and represents the initial step of pancreatic intraepithelial neoplasia development and pancreatic ductal adenocarcinoma (PDAC) carcinogenesis [12–15]. Of note, chronic pancreatitis represents a risk factor for pancreatic cancer, demonstrating that pancreatic inflammation has an important role in PDAC development [16, 17].

Immune cells have a bipartite function in AP [6, 18–22]. Neutrophils and macrophages support tissue repair by the tightly regulated secretion of pro- and anti-inflammatory cytokines [6]. Macrophage-secreted cytokines potentiate immune cell infiltration in the pancreas and therefore enhance the inflammatory response [23]. Accordingly, macrophage activation directly correlates with the severity of AP, while depletion of macrophages reduces AP severity [24, 25]. Furthermore, macrophages directly promote acinar transdifferentiation by the secretion of tumor necrosis factor (TNF) and chemokine (C-C motif) ligand 5 (CCL5) [18]. However, dysbalanced or sustained cytokine secretion counteracts tissue regeneration and leads to severe inflammation and chronic diseases [26, 27]. Therefore, the balanced activation and termination of the immune response is essential for restoring the tissue function. Recently, the DAMP receptor Toll-like receptor 3 (TLR3) was described as a new modulator of various tissue regenerative processes [28–31].

TLR3 is an endosomal receptor for double-stranded RNA (dsRNA) [32]. Originally discovered as receptor of the innate immune response for sensing viral infections, it is now evident that TLR3 is also activated by endogenous dsRNA liberated by necrotic cells [33, 34]. TLR3 is expressed by immune cells, like macrophages or DCs, but also in various other cell types including pancreatic epithelial cells [30, 35, 36]. While TLR3 is primarily localized in endosomes, it was also shown to be present on the surface of human epithelial cells and fibroblasts [37]. The horseshoe-shaped ectodomain of TLR3 consists of 23 leucine rich repeats, which are essential for ligand binding [38], leading to the recruitment of Toll/IL-1 receptor-domain adapter-inducing IFN-β (TRIF) to the cytosolic Toll/IL-1 receptor (TIR) homology domain of TLR3 [32, 39]. TRIF subsequently activates the transcription factors IRF3, AP-1, and NF-κB, which potentiates the secretion of proinflammatory cytokines, like TNF and IL-6, and type 1 IFNs [32, 40, 41]. Additionally, TLR3 induces the formation of cleaved caspase-8 via TRIF and thereby initiates cell death [42–47].

In this study, we aimed to analyze the cell specific function of TLR3 in acute pancreatitis, induced by caerulein treatment. In the early phase of AP, LDH and amylase serum levels were increased in $TLR3^{OFF}$- or $TLR3^{Mye}$-deficient mice, while morphologically no differences in necrosis, edema, or immune cell filtration were detected. In late stages of AP, we observed enhanced and persistent ADM, accompanied by an increased and sustaining high immune cell infiltrate of predominantly macrophages. We were able to trace back these findings to TLR3 exclusively expressed in myeloid cells. We showed that TLR3 induces cell death of murine macrophages in vitro, which could provide a new mechanism for the termination of immune responses. Thus, TLR3 dysfunction may cause the continuous inflammatory response observed in TLR3-deficient mice after AP.

Results

Influence of TLR3 deficiency on the early phase of acute pancreatitis

The present study investigates the role of TLR3 in acute pancreatitis using mouse strains with global TLR3-deficiency ($TLR3^{OFF}$), generated by the insertion of a floxed transcriptional termination element in the gene locus of *Tlr3*, or selective TLR3 expression in myeloid cells ($TLR3^{Mye}$) by lysozyme M Cre-mediated deletion of the transcriptional termination element. TLR3 deficiency or its exclusive expression in myeloid cells did not affect pancreatic tissue architecture as shown by H&E staining (Fig. 1A). Acinar and duct cells were morphologically normal as shown by CPA1 and Krt19 staining (Supporting Information Fig. S1). In order to analyze the role of TLR3 during the early phase of acute pancreatitis, we examined the effect of TLR3 deficiency ($TLR3^{OFF}$) or re-expression in the myeloid lineage ($TLR3^{Mye}$) 8 h and 24 h after caerulein challenge. In untreated mice, serum concentrations of amylase and LDH were slightly higher in $TLR3^{Mye}$ mice as compared with WT controls (Supporting Information Fig. S2). In both, $TLR3^{OFF}$ and $TLR3^{Mye}$ mice, the serum concentrations of amylase and LDH were increased 8 h after AP induction compared to WT mice, while after 24 h the concentrations decreased to basal values (Supporting Information Fig. S2). There was no significant difference in the pancreas-to-body weight ratios in untreated controls and after 24 h AP between the various mouse strains (Fig. 1B). In $TLR3^{Mye}$ mice, the pancreas-to-body weight ratio was elevated after 8 h AP. Neither the histological examination of the tissue nor the Spormann index (combined score for necrosis, edema, leukocytes, and hemorrhage) revealed a major influence of global TLR3 deficiency or myeloid cell-specific re-expression of TLR3 in the early phase of AP (Fig. 1A and C, Supporting Information Table S1).

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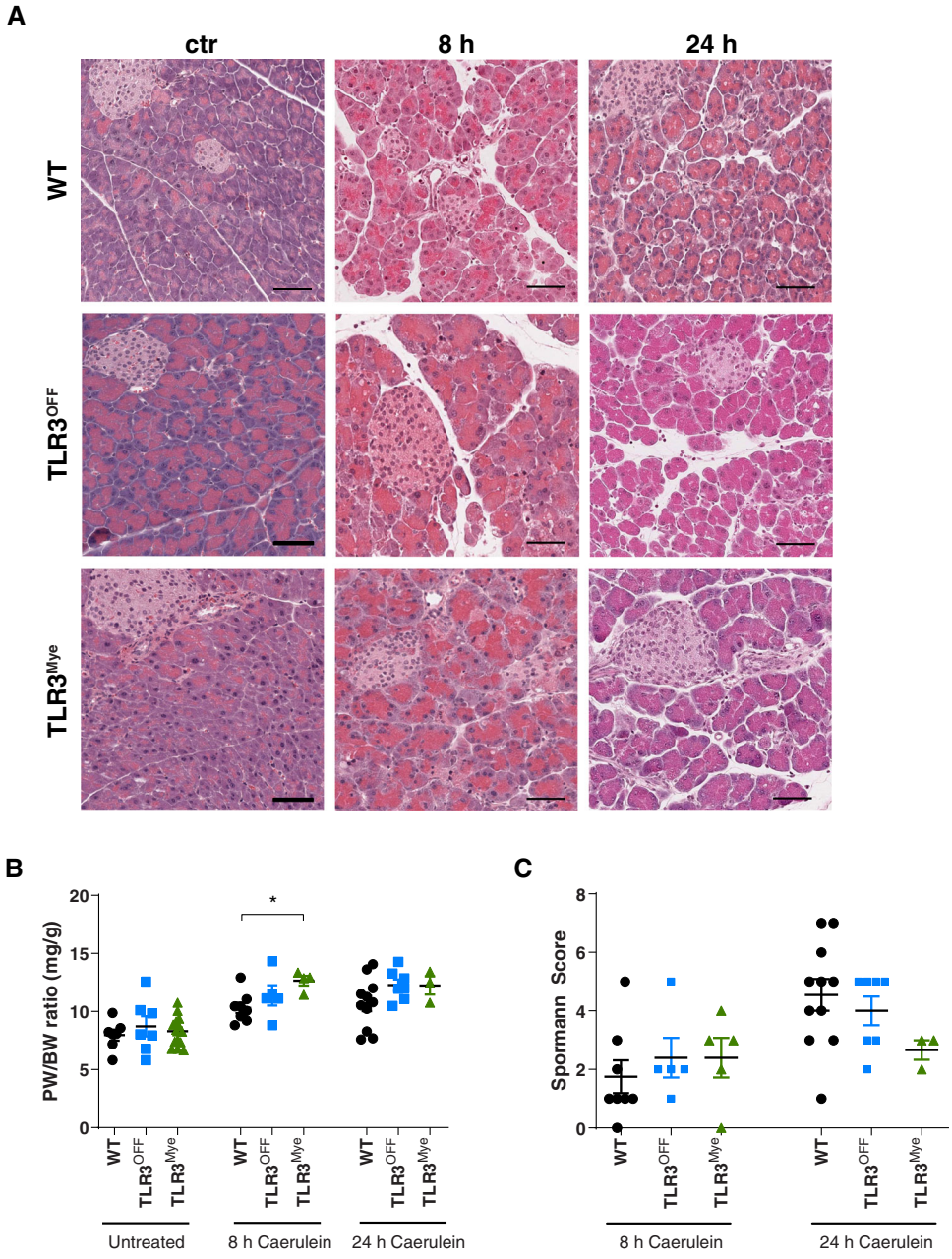

Figure 1. Influence of TLR3 deficiency on the early phase of acute pancreatitis. (A) Representative H&E pictures of pancreatic sections from WT, TLR3^{OFF}, and TLR3^{Mye} mice at 8 h and 24 h after repetitive caerulein injections compared to untreated controls. Scale bars indicate 100 μm. (B) Pancreas to body weight ratio of TLR3^{OFF} and TLR3^{Mye} mice compared to WT controls at the specified time points. (C) Spormann scoring of WT, TLR3^{OFF}, and TLR3^{Mye} mice at the specified time points. (B and C) For each time point and group, samples from N ≥ 3 mice were analyzed representing pooled data from at least three independent experiments and were analyzed using the Mann–Whitney test. Data are presented as mean ± SEM. $^{*}p < 0.05$.

TLR3-deficient mice show a defective regenerative response

Next, we investigated the role of TLR3 at later time points after AP induction. At 2 days after AP induction, H&E staining of the pancreas from WT mice showed apparent ADM, characterized by acinar cells with ductal-like cell morphology (Fig. 2A). After 5 and 7 days, pancreatic tissue of WT mice was completely regenerated with no histologically detectable ADM structures (Fig. 2A and C). In contrast, ADM formation was significantly increased in TLR3^{OFF} mice 2 days after AP (Fig. 2A and C). Strikingly, these ADM structures persisted and were still elevated even 7 days after AP induction, indicating a crucial role of TLR3 for pancreatic regeneration in the late phase of AP.

Exclusive expression of TLR3 in myeloid cells partially rescued the regenerative defects observed for global TLR3 deficiency on

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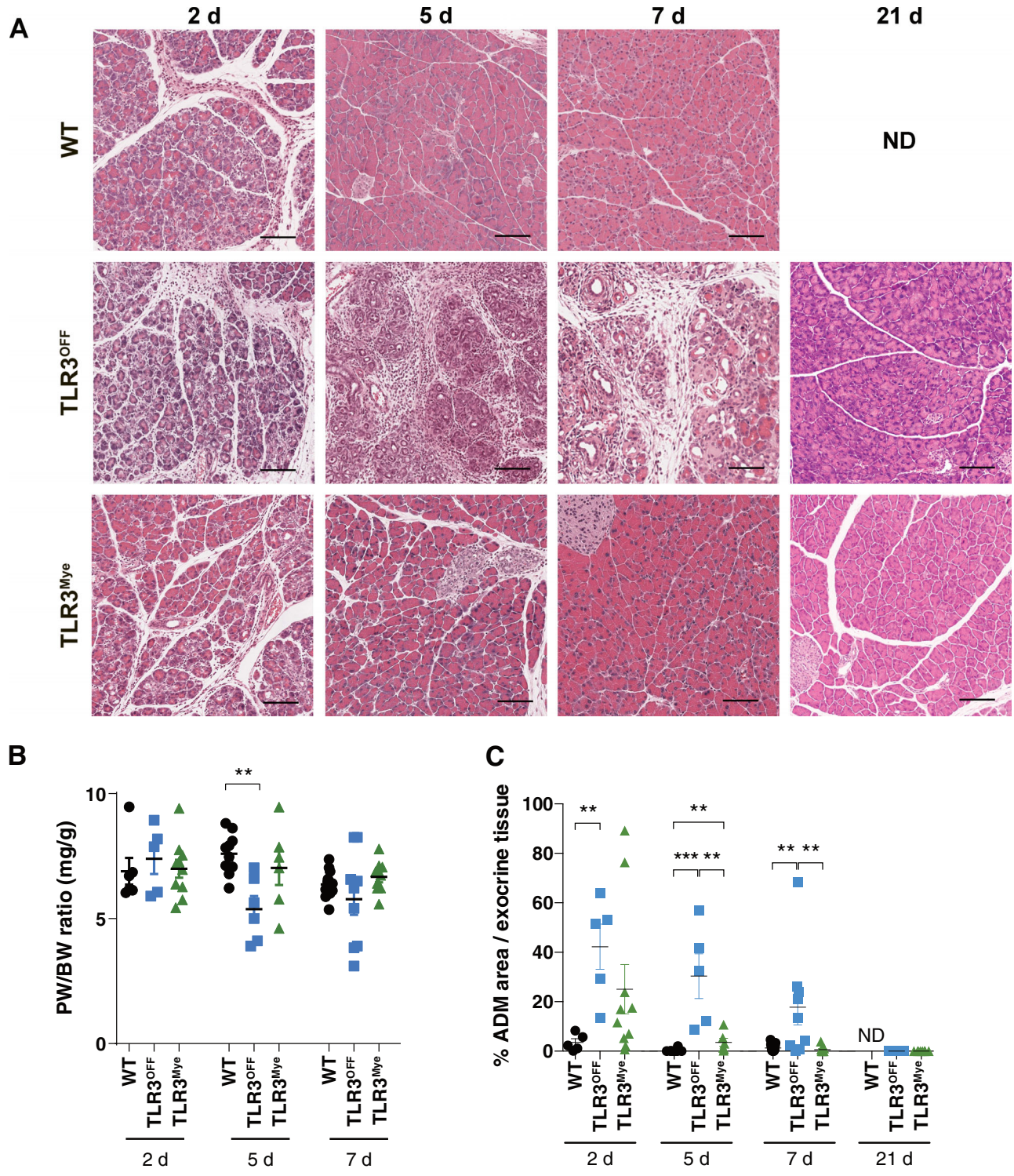

Figure 2. TLR3-deficient mice show a defective pancreatic regeneration after AP. (A) Representative H&E pictures of pancreatic sections from TLR3^{OFF} and TLR3^{Mye} mice compared to WT controls at 2, 5, 7, and 21 days after induction of acute pancreatitis. Scale bars indicate 200 μm. (B) Pancreas-to-body weight ratio of WT, TLR3^{OFF}, and TLR3^{Mye} mice at the specified time points. (C) Quantification of ADM area in the exocrine pancreas from TLR3^{OFF} and TLR3^{Mye} mice compared to WT controls at 2, 5, 7, or 21 days after caerulein injection. Because the pancreas from WT mice was completely regenerated after 7 days, ADM area after 21 days was not further analyzed. (B and C) For each time point and group, samples from N ≥ 5 mice were analyzed representing pooled data from at least five independent experiments and were analyzed using the Mann–Whitney test. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ND = not determined.

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days 2 and 5 after AP induction, while a full rescue was observed on day 7. There were significantly less ADM areas compared to TLR3^{OFF} mice after 5 days and the pancreatic tissue was completely regenerated after 7 days, comparable to the WT controls (Fig. 2A and C). These results demonstrate that pancreatic regeneration following AP is dependent on the expression of TLR3 in myeloid cells.

At 21 days after AP induction, the pancreata of all mouse strains were fully recovered (Fig. 2A). Only one animal from the TLR3^{OFF} cohort developed an area of less than 5 % of the tissue with signs of chronic pancreatitis. There was no difference in pancreas-to-body weight ratio between the mouse strains, except for a decreased ratio in TLR3^{OFF} mice 5 days after AP, which is in line with the observed delay in regeneration (Fig. 2B).

Therefore, global deletion of TLR3 resulted in a markedly increased formation of ADM and a delayed regeneration of the pancreas after AP. This defect was rescued by myeloid cell selective TLR3 expression, demonstrating the importance of TLR3 expression in myeloid cells for pancreatic regeneration after AP.

Increased ADM marker expression after AP caused by systemic TLR3 deficiency

To further corroborate the role of myeloid cell TLR3 in acinar regeneration, pancreatic differentiation markers were investigated at protein and RNA levels in pancreata of mice at 2 and 5 days after AP induction.

At 2 days after AP induction, expression of *CPA1* in combination with high numbers of Krt19 and SOX9 positive metaplastic cells was detected, confirming ADM on a molecular basis (Fig. 3A and B). In agreement with the increased formation of ADM observed in TLR3^{OFF} mice, global TLR3 deficiency lead to increased mRNA expression of the ductal markers *Krt19* and *SOX9*, as well as to an increased number of nuclear SOX9 positive metaplastic cells, compared to WT and TLR3^{Mye} mice (Fig. 3A and B).

After 5 days, acinar cells were fully restored in WT mice as shown by the normal *CPA1* expression and the restriction of Krt19 and nuclear SOX9 to ductal and centroacinar cells (Fig. 4A and B). These results confirm the complete tissue regeneration in WT mice 5 days after AP induction. In contrast, TLR3^{OFF} mice presented ADM areas with a significantly reduced number of CPA1 positive acinar cells and a significantly higher number of Krt19 positive cells with nuclear SOX9 compared to WT mice, demonstrating ongoing tissue regeneration and the persistence of ADM (Fig. 4A and B). In TLR3^{Mye} mice, mRNA expression of *CPA1*, *Krt19*, and *SOX9* was similar to WT mice, while the number of Krt19 positive cells was increased but lower than in TLR3^{OFF} mice, confirming the partial rescue of the regenerative defect by myeloid cell specific TLR3.

Examination of the proliferation marker Ki67 and the apoptosis marker cleaved caspase-3 (CC3) in the pancreatic tissue showed no significant differences between the different mouse strains at 2 and 5 days after AP induction (Supporting Information Fig. S3A and B).

Taken together, these results demonstrate the pro-regenerative function of myeloid cell specific TLR3 in AP.

TLR3 expression in myeloid cells is required for immune cell clearance after AP

Immune cells play a pivotal role in pancreatic regeneration [6]. To further corroborate the importance of TLR3 expression in myeloid cells for pancreatic regeneration, the AP-induced immune cell infiltration was characterized immunohistochemically.

In the untreated state and 8 h after AP induction, low numbers of pancreatic immune cells were detected and the numbers did not significantly differ between the various mouse strains (Fig. 5A). Notably, TLR3^{OFF} mice had significantly increased amounts of F4/80, CD3, and MPO positive cells compared to WT mice on day 2 (Fig. 5A and B). Accordingly, mRNA levels of *IL-6* and *TNF* were elevated in TLR3^{OFF} as compared with TLR3^{Mye} and WT mice (Supporting Information Fig. S4). Significantly increased accumulation of F4/80 and CD3 positive cells in TLR3^{OFF} mice persisted until day 5 after AP (Fig. 5A and C). In contrast, the numbers of infiltrating immune cells in TLR3^{Mye} mice were similar to those of WT mice both 2 and 5 days after AP induction. Accordingly, immune cell infiltration was significantly reduced in TLR3^{Mye} as compared to TLR3^{OFF} mice. Interestingly, quantification of pancreatic immune cells revealed macrophages to be the predominant cell type (Fig. 5).

These results show that TLR3 signaling exclusively in myeloid cells is sufficient to resolve the inflammatory immune cell infiltrate after AP and may therefore play a central role in the termination of the immune response after acute pancreatitis, which, in turn, may limit ADM formation and reduce ADM persistence.

TLR3 signaling induces cell death of murine macrophages

Since macrophages represent the main cell population in the pancreas after AP (Fig. 5) and due to their central importance for the regulation of the immune response, we further investigated the effect of TLR3 on macrophages. To characterize the effect of TLR3 in macrophages, conditionally immortalized murine BM-derived progenitor cells (BMDCs) from WT mice were in vitro differentiated to macrophages. The differentiation status was verified via flow cytometry (Supporting Information Fig. S5A). To determine the effect of TLR3 signaling on murine macrophages, the cells were stimulated with different concentrations of the TLR3 agonist polyinosinic:polycytidylic acid (poly(I:C)). After 48 h of stimulation, WT macrophages showed a dosage-dependent reduction of cell viability as detected by MTT assay (Fig. 6A). Notably, even low concentrations of poly(I:C) were sufficient to cause a reduction of cell viability.

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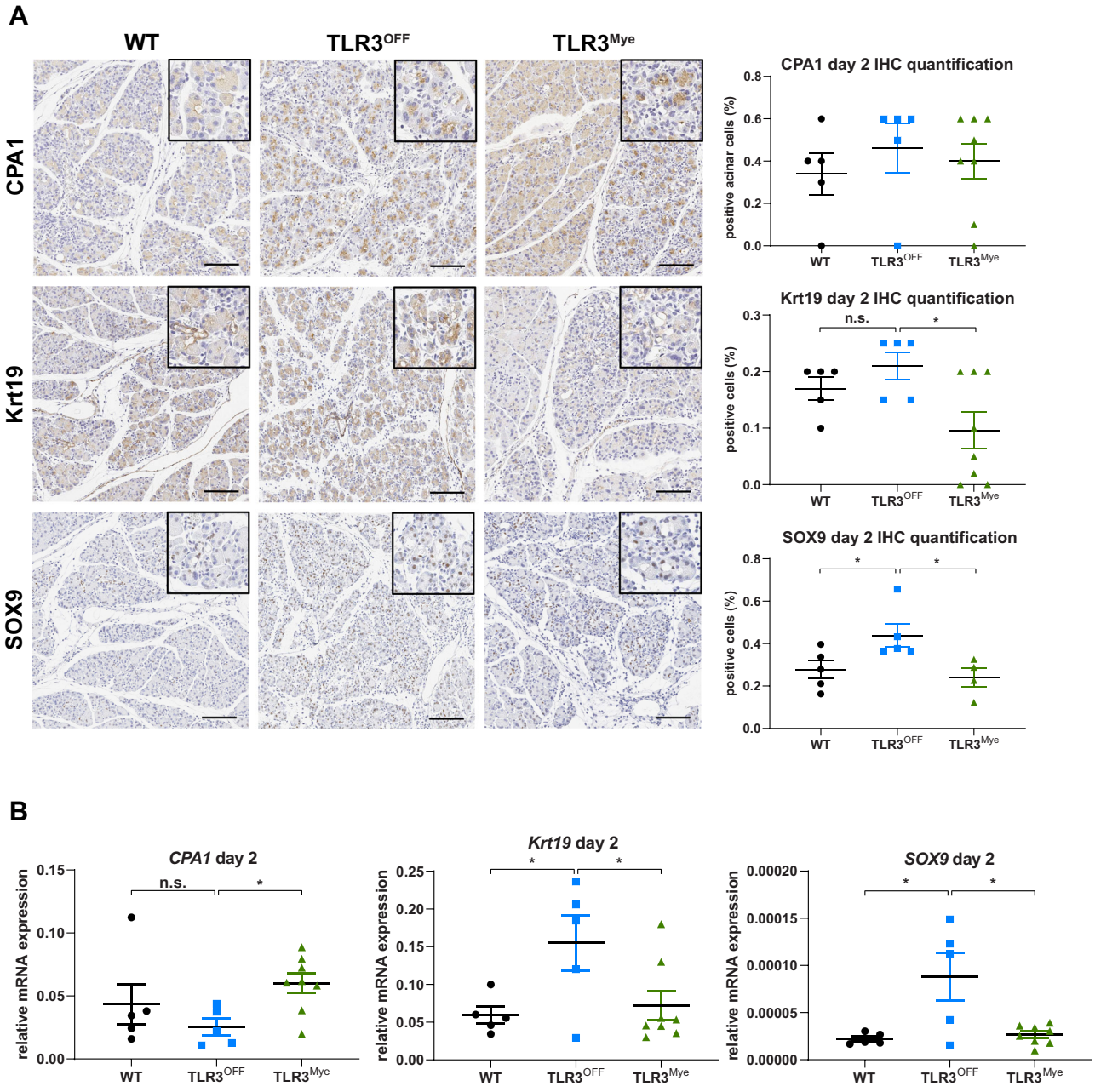

Figure 3. Immunohistochemical analysis of cell identity markers 2 days after AP induction. (A) Representative pictures and quantification of the immunohistochemical staining of CPA1, Krt19, and SOX9 from TLR3^{OFF} and TLR3^{Mye} mice compared to WT controls at 2 days after AP induction. Scale bars indicate 200 μm. (B) Pancreatic mRNA expression levels of *CPA1*, *Krt19*, and *SOX9* normalized to *Rps13* at 2 days after AP induction analyzed by qPCR. For each group, samples from $N \geq 4$ mice were analyzed representing pooled data from at least four independent experiments and were analyzed using the t-test. Data are presented as mean ± SEM. $^{*}p < 0.05$. n.s. = not significant.

To exclude effects of other dsRNA receptors, conditionally immortalized BMDCs from TLR3^{OFF} and TLR3^{Mye} mice were generated and in vitro differentiated to macrophages. There were no differences in the differentiation status of WT as compared with TLR3^{OFF} and TLR3^{Mye} progenitor cell-derived macrophages as shown by FACS analysis of macrophage-specific differentiation markers (Supporting Information Fig. S5A). *TLR3* expression in TLR3^{Mye} and WT macrophages was comparable, while it was absent in TLR3^{OFF} macrophages (Supporting Information Fig. S5C). Consistent with the lack of TLR3, macrophages from TLR3^{OFF} mice did not produce cytokines in response to poly(I:C) (Supporting Information Fig. S5B). While stimulation of WT and TLR3^{Mye} macrophages with 1 μg/mL poly(I:C) led to a substantial reduction in cell viability, viability of TLR3^{OFF} macrophages was not significantly reduced (Fig. 6B). Immunoblot analysis showed an induced formation of cleaved caspase-8 at

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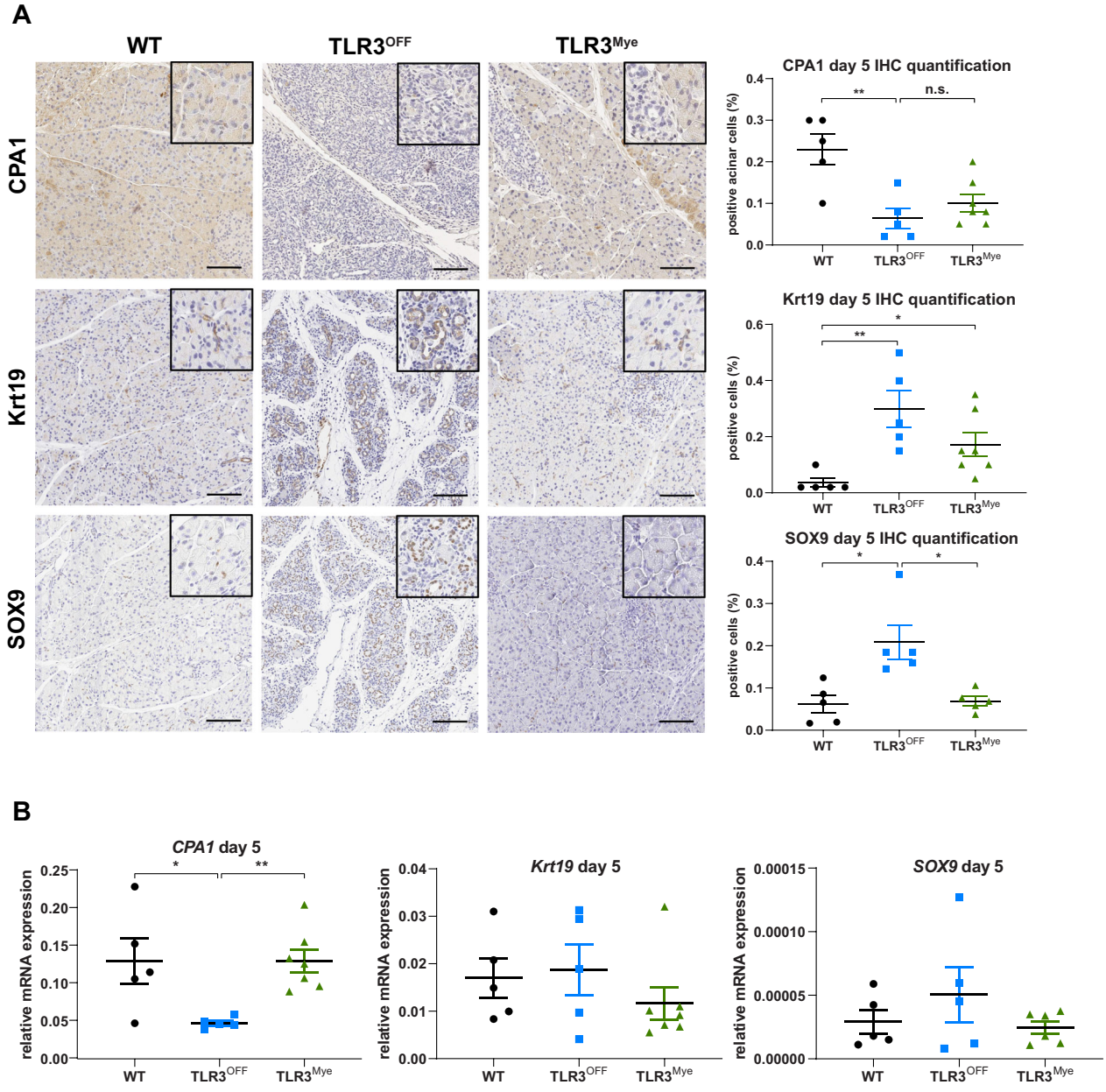

Figure 4. Immunohistochemical analysis of cell identity markers 5 days after AP induction. (A) Representative pictures and quantification of the immunohistochemical staining of CPA1, Krt19, and SOX9 from TLR3^{OFF} and TLR3^{Mye} mice compared to WT controls at 5 days after AP induction. Scale bars indicate 200 μm. (B) Pancreatic mRNA expression levels of *CPA1*, *Krt19*, and *SOX9* normalized to *Rps13* at 5 days after AP induction analyzed by qPCR. For each group, samples from $N \geq 5$ mice were analyzed representing pooled data from at least five independent experiments and were analyzed using the *t*-test. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. n.s. = not significant

48 h after stimulation of WT macrophages with poly(I:C), indicating a TLR3-mediated induction of caspase-8 dependent cell death by poly(I:C) (Fig. 6C; Supporting Information Fig. S6).

Collectively, stimulation of murine macrophages with poly(I:C) led to a TLR3-dependent reduction of cell viability by induction of cleaved caspase-8-mediated cell death. Because macrophages are of central importance for the recruitment of other immune cells and the resolution of the immune response [23, 27, 48], TLR3-dependent resolution of the immune reaction in AP appears to be crucial for terminating pancreatic regeneration. Interestingly, this effect seems to be associated with the sensitivity of macrophages to TLR3-induced cell death.

Discussion

Acute pancreatitis is associated with tissue necrosis resulting in the liberation of DAMPs, like nucleic acids [1]. Various studies have shown that TLR3 signaling is not only induced by viral dsRNA but also by RNA released from damaged tissue [28, 29, 33,

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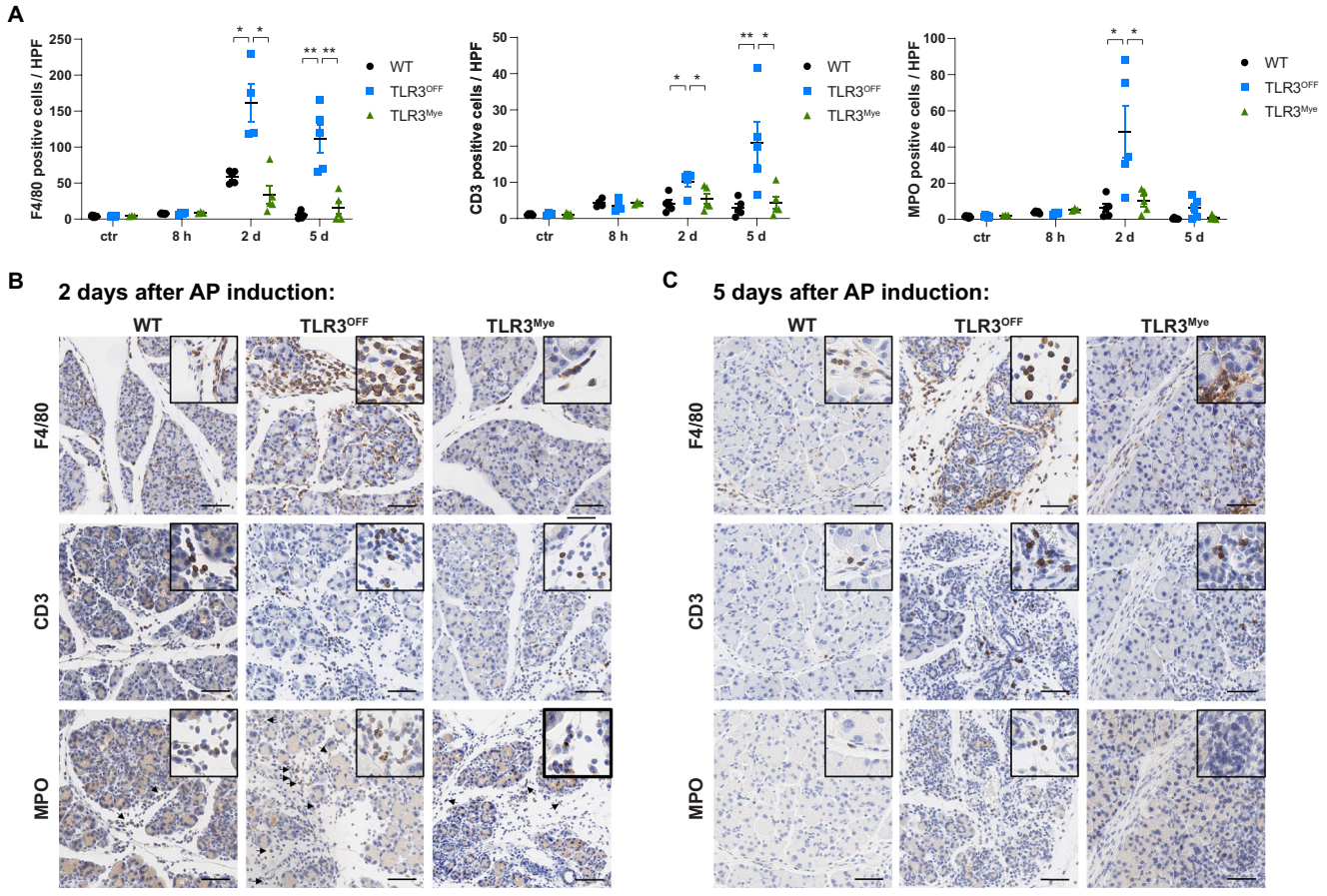

Figure 5. Characterization of the immune cell infiltrate 2 days and 5 days after AP. (A) IHC-based quantification of F4/80-, CD3-, and MPO-positive cells in the pancreas of WT, TLR3^{OFF}, and TLR3^{Mye} mice, at 8 h, 2 days and 5 days after AP induction, compared to untreated controls. (B) Representative pictures of the immunohistochemical staining of CD3, MPO, and F4/80 in WT, TLR3^{OFF}, and TLR3^{Mye} mice at 2 days after AP induction. Scale bars indicate 100 μm. (C) Representative pictures of the immunohistochemical staining of CD3, MPO, and F4/80 in WT, TLR3^{OFF}, and TLR3^{Mye} mice at 5 days after AP induction. Scale bars indicate 100 μm. (A–C) For each time point and group, samples from $N \geq 3$ independent mice were analyzed. Data were pooled from at least three independent experiments and were analyzed using the Mann–Whitney test. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

34]. Thereby, TLR3 fulfills versatile physiological functions including the regulation of tissue regeneration after myocardial infarction or skin neogenesis after injury [28, 29]. This pro-regenerative role of TLR3 is in agreement with the results in the present study, where the myeloid cell specific importance of TLR3 for pancreatic regeneration in the late phase of acute pancreatitis has been shown. Global TLR3 deficiency in mice led to increased formation of ADM, which persisted for up to 7 days after AP. This effect was partially rescued by myeloid cell selective expression of TLR3. These findings therefore highlight the functional importance of TLR3 in myeloid cells for pancreatic regeneration.

Induction of AP in TLR3^{OFF} mice was accompanied by a significantly increased infiltration of macrophages and, at lower numbers, also T cells and neutrophils in the pancreas. This effect was completely rescued by the expression of TLR3 in myeloid cells, showing that TLR3 signaling in myeloid cells limits the number of pancreatic immune cells. Furthermore, we demonstrated the induction of cleaved caspase-8-associated cell death by TLR3 in murine BM-derived macrophages. Thus, TLR3-mediated cell death provides a potential mechanism for the clearance of macrophages from the pancreas and the termination of the immune response after AP.

Macrophages fulfill a central role for the recruitment of immune cells, for example, CD4^{+} T cells, and thereby regulate inflammatory processes [23]. The degree of macrophage activation plays a determining role for the severity of AP [24, 25]. Folias et al. demonstrated that macrophage depletion reduced formation and persistence of acinar dedifferentiation [21]. It was further shown that macrophages promote ADM formation by the secretion of TNF and CCL5 [18]. Both studies are in accordance with our data, showing that highly increased ADM formation was associated with increased macrophage infiltration in TLR3^{OFF} mice.

Furthermore, we demonstrated that TLR3 directly induces the cell death of macrophages in vitro and that this effect is associated with the activation of caspase-8. The induction of cleaved caspase-8 by TLR3 is well documented in literature [42–45, 47]. Activated

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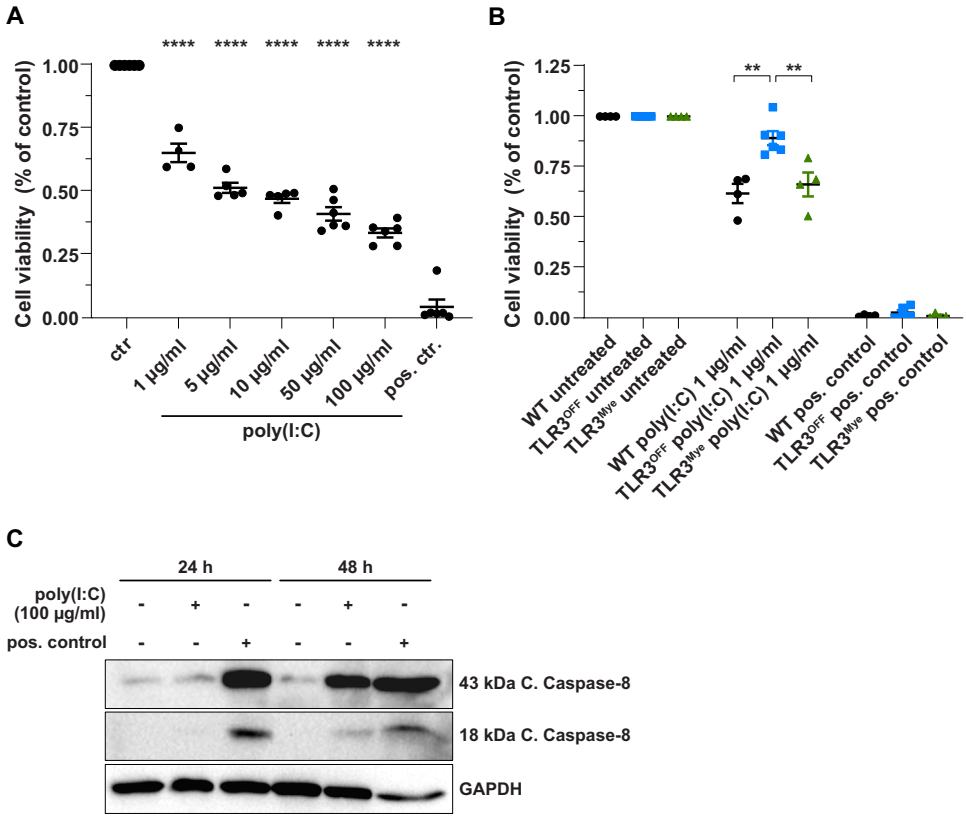

Figure 6. TLR3 signaling induces cell death of murine macrophages in vitro. (A) Cell viability of murine WT macrophages stimulated with different concentrations of poly(I:C) for 48 h. (B) Cell viability of macrophages from WT, TLR3^{OFF}, and TLR3^{Mye} mice stimulated with 1 μg/mL poly(I:C) for 48 h. (C) Representative immunoblot showing the formation of cleaved caspase-8 after 48 h stimulation of murine macrophages with 100 μg/mL poly(I:C) or brefeldin A, as positive control. GAPDH was used as loading control. (A and B) As positive control, apoptosis was induced by brefeldin A. Data were pooled from $N \geq 4$ independent experiments and were analyzed using the *t*-test. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

TLR3 binds TRIF, which subsequently may recruit the ripoptosome complex, formed by caspase-8, RIP1, FADD, and cFLIP isoforms [47]. Depending on the molecular composition of the ripoptosome, it then induces apoptosis or necroptosis, the later mediated by RIP3 [49]. Thus, the increased cell death of macrophages, observed in the present study occurs likely via the described formation of the ripoptosome complex.

While the initiation of ADM has been extensively studied, comparatively little is known about the termination of ADM and acinar re-differentiation. Macrophages not only secrete proinflammatory cytokines that promote tissue infiltration by immune cells to initiate inflammation, but they are also crucial for the resolution of inflammation. Thus, macrophages are involved in the phagocytic clearance of cell debris and apoptotic immune cells by efferocytosis and are subsequently cleared from the tissue by lymphatic drainage or apoptosis [48, 50]. Both, a dysregulated efferocytosis by macrophages, as well as impaired tissue clearance of macrophages may lead to persistent inflammatory diseases and autoimmunity [23]. The in vivo depletion of macrophages consequently was shown to reduce ADM [21]. In the present report, we provide evidence for a previously unrecognized mechanism for the termination of inflammation in the late phase of AP that is based on the activity of TLR3 in myeloid cells. The results suggest that the termination of inflammation requires clearance of macrophages by TLR3-induced cell death, which allows for the resolution of ADM and successful pancreatic regeneration. Hence, these findings are of potential relevance for the design of pro-resolution therapies that could be applied to directly initiate the termination of the immune response, especially for chronic inflammatory diseases like chronic pancreatitis, with decisive benefits compared to anti-inflammatory treatments [48].

An incomplete resolution of inflammation not only may cause chronic disease [27], but, in the case of chronic pancreatitis, it can also contribute to pancreatic carcinogenesis [17]. In the context of oncogenic signaling, ADM is assumed to be the initial step in pancreatic intraepithelial neoplasia formation and accordingly, in PDAC carcinogenesis [12, 14, 15]. Therefore, the molecular pathways involved in ADM also provide detailed insight into the carcinogenesis of PDAC and potentially offer new therapeutic targets. Because systemic TLR3 deficiency leads to pronounced and persistent ADM, TLR3 may also influence PDAC initiation. The role of TLR3 on cancer development and progression is controversially discussed in literature [44, 51–55]. It is conceivable, however, that diverse cell type specific functions of TLR3 underlay these

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divergent results. Thus, cell-specific analysis of TLR3 may also provide decisive insights in its role in pancreatic carcinogenesis.

A recent study suggests that TLR3 deficiency may also enforce AP severity in the early phase of AP by a NF-κB-regulated induction of acinar cell necrosis, but did not address the role of TLR3 in pancreatic regeneration at later time points [30]. In contrast, the present report focuses on the role of TLR3 in pancreatic regeneration and reveals a novel and myeloid cell specific function of TLR3 for downregulating ADM formation. Moreover, in our experimental set up, we could not detect any dominant TLR3-dependent effects, both 8 and 24 h after AP induction. The deviating results may be explained by the different protocols used for AP induction, varying mouse models and the divergent time points of investigation after AP induction. It should be noted, however, that our results are in agreement with those from Hoque et al., who also found no significant alteration of AP severity caused by TLR3 deficiency [56].

In summary, our results demonstrate the key role of TLR3 in myeloid cells for the termination of ADM after AP and pancreatic regeneration. TLR3 reduces the infiltration of immune cells and terminates the inflammatory response possibly via the induction of cell death in macrophages. Thus, TLR3 represents a central regulatory element for pancreatic regeneration and for the restoration of physiological tissue function.

Materials and methods

Mouse strains

$TLR3^{OFF}$ mice were generated by insertion of a floxed transcriptional termination element between exons 3 and 4 of the *Tlr3* gene resulting in global TLR3 deficiency [57]. $TLR3^{Mye}$ mice were generated by crossing LysMcre mice [58] into the $TLR3^{OFF}$ strain. Due to the cell-specific expression of the Cre recombinase, $TLR3^{Mye}$ mice show a selective expression of TLR3 in cells of the myeloid lineage. WT C57BL/6N mice were used as controls. All mouse models were housed under specified pathogen-free conditions and maintained on a pure C57BL/6N background either at the Klinikum rechts der Isar, Technical University of Munich (Germany) or at the Charles Rivers facility in Calco, Italy. All animal experiments were institutionally approved by the government of Upper Bavaria (license 55.2-1-54-2532-130-2016) and were performed in accordance with the guidelines and regulations.

Caerulein injection

Induction of pancreatitis was carried out in 8-week-old female mice who were fasting overnight prior to the caerulein (Bachem) treatment with 8 hourly intraperitoneal injections of 2 μg/injection, using a previously described protocol [11] modified to one day. This protocol induces a mild form of acute pancreatitis with less exocrine cell necrosis compared to protocols for severe pancreatitis [59, 60]. Mice were sacrificed 8 h, 24 h, 2 days, 5 days, 7 days, and 21 days after the first caerulein injection for analysis.

Body and pancreatic weigh analysis

Mice were sacrificed and immediately weighted on a high precision scale to obtain the body weight. Directly afterward, the whole pancreas was carefully excised without any adjacent tissue and straightaway weighed in a precision scale.

Serum analysis

Mice were sacrificed and exsanguinated by vena cava. Blood was transferred to a tube (Microvette, Sarstedt) and centrifuged at 10 000 rpm for 5 min. Serum was collected and stored at −80°C until assayed. Serum levels of Amylase and LDH were measured using a cobas 8000 modular analyzer (Roche, Germany) at the Institute of Clinical Chemistry and Pathobiochemistry of the Technical University of Munich, Germany.

Immunohistochemical staining

For immunohistochemical staining, sections of paraffin-embedded pancreata were rehydrated and antigens were retrieved using Antigen Unmasking Solution (Vector Laboratories). Overnight incubation with the following primary antibodies was performed: anti-CPA1 (1:300; RD Systems AF2765), anti-Krt19 (1:1000; Abcam ab133496), anti-Clusterin (1:200; Santa Cruz sc-6419), anti-SOX9 (1:1500; Millipore AB5535), anti-Ki67 (1:400; BD Pharmingen 550609), anti-cleaved caspase-3 (1:200; Cell Signalling 9661), anti-CD3 (1:200; BD Pharmingen 145-2C11), anti-MPO (1:350; Santa Cruz Biotechnology sc-390109), anti-F4/80 (1:300; Abcam ab16911). Biotin-conjugated secondary antibodies were incubated for 1 h at room temperature, following development with ABC and DAB kits (both Vector Laboratories). SOX9 and Krt19 were stained using the envision kit from Dako. Nuclear counterstaining was done using hematoxylin. CD3, MPO, and F4/80 were stained using a Leica bond XR^m staining robot.

Morphometric quantification

For the evaluation of ADM area, pancreatic sections were stained with H&E and then analyzed using Aperio ImageScope (Leica Biosystems). All slides were quantified blindly. The histological evaluation of pancreatitis severity was based on the scoring system of Spormann by generating a combined score for necrosis, edema, leukocyte infiltration, and hemorrhage as described [61]. For quantification of Ki67 positive cells and nuclear SOX9 positive cells, five randomly distributed high-power fields (HPF) across the tissue (250 μm × 250 μm, 20× magnification) were counted.

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For cleaved caspase-3, 10 HPF were counted. For CD3, MPO, and F4/80, 10 HPF (500 μm × 500 μm, 40× magnification) were counted. For CPA1 and Krt19, the whole slide was quantified.

Reverse transcription quantitative PCR

Pancreas sections were immediately stored at -80°C in RNAlater (Qiagen) until use. RNA was isolated using the Maxwell RSC simplyRNA Tissue Kit (Promega) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). For the quantitative real-time PCR, the Universal Probe library (Roche Diagnostics) was used with the following primers (specified in 5′ to 3′ orientation): mCCL5-fwd: tgcagaggactctgagacagc, mCCL5-rev: gagtggtgtccgagccat, mTNF-fwd: tgcctatgtctcagcctcttc, mTNF-rev: gaggccatttgggaacttct, mTLR3-fwd: gatacagggattgcacccata, mTLR3-rev: tcccccaaaggagtacattaga, mIL-6-fwd: gctaccaaactggatataatcagga, mIL-6-rev: ccaggtagctatggtactccagaa, mCPA1-fwd: tcccatcaatgtgctgaagt, mCPA1-rev: ggatgccagtgtcaatcca. The SYBR green mastermix (Roche Diagnostics) was used for the following primers: mKrt19-fwd: accctcccgagattacaacc; mKrt19-rev: caaggcgtgttctgtctcaa, mSOX9-fwd: ccacgtgtggatgtcgaag, mSOX9-rev: ctcagctgctccgtcttgat.

Cell lines

Murine BM-derived progenitor cells were isolated from the femur of WT, TLR3^{OFF}, and TLR3^{Mye} mice and conditionally immortalized by retroviral transduction with a plasmid encoding for estrogen receptor-coupled Hoxb8 according to Wang et al. [62]. Differentiation to macrophages was induced by M-CSF stimulation and simultaneous estradiol withdrawal for 4 days. Successful differentiation was verified using flow cytometry analysis using directly fluorescence-labeled antibodies for CD11b (Invitrogen RM2805), CD115 (eBioscience AFS98), and F4/80 (Invitrogen MF48004). The protein amount of cleaved caspase-8 (Cell Signaling D5B2) and GAPDH (Cell Signaling 14C10) was determined by immunoblot analysis using the antibodies at 1:1000 in 5% milk in PBS with 0.1% Tween-20. As positive control for apoptosis induction, cells were stimulated with 4.5 μg/mL brefeldin A for the indicated time points.

Cell viability assay

Cells were seeded in 96-well plates and stimulated with different concentrations of poly(I:C). After 48 h, MTT solution was added for 4 h with subsequent cell lysis and solubilization of the formazan salt complex in 0.1 M HCl with 10% SDS over night at 37°C. The amount of complex formed was determined by measuring the absorbance at 580 nm.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism6 (GraphPad Software Inc). Unless otherwise stated, the Mann–Whitney test for nonnormal distributed unpaired data was used for intergroup comparison. Differences with a p-value lower than 0.05 were considered significant, and the following scale was applied: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are presented as mean value ± SEM.

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Abbreviations: **ADM**: acinar-to ductal metaplasia · **AP**: acute pancreatitis · **BMDCs**: BM-derived progenitor cells · **DAMP**: Damage-associated molecular pattern · **PanIN**: pancreatic intraepithelial neoplasia · **PDAC**: pancreatic ductal adenocarcinoma · **poly(I:C)**: polyinosinic:polycytidylic acid

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