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The neuropeptide receptor subunit RAMP1 constrains the innate immune response during acute pancreatitis in mice

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ABSTRACT

Objectives: The importance of the Calcitonin-gene-related-peptide-pathway (CGRP) as neuronal modulator of innate immune responses in mice has been previously demonstrated. The CGRP-receptor is composed of two subunits: the receptor-activity-modifying-protein-1 (RAMP1) and the calcitonin-receptor-like-receptor (CLR). CGRP can influence immune cells and their capacity of producing inflammatory cytokines. Using a RAMP1 knockout-mouse (RAMP1$^{-/-}$) we examined the role of the CGRP-receptor in the acute-phase of cerulein-induced pancreatitis.

Methods: Hourly cerulein-injections for a period of 8 h in RAMP1$^{-/-}$ and wild-type mice were performed. To compare severity and extent of inflammation in RAMP1$^{-/-}$ and wild-type mice, histological analyses were done and cytokine levels were assessed using qRT-PCR 8 h, 24 h, 2 days, and 7 days post-cerulein-treatment. Furthermore, serum activities of LDH and lipase were determined.

Results: After 8 h RAMP1$^{-/-}$ mice showed a higher pancreas-to-body-weight-ratio, increased tissue edema and immune cell infiltration with higher amount of F4/80-positive cells as compared to wild-type mice. Overall infiltration of immune cells at 24 h was increased in RAMP1$^{-/-}$ mice and composed predominantly of MPO-positive neutrophils. In addition, after 24 h RAMP1$^{-/-}$ mice presented a higher pancreas-to-body-weight-ratio, higher expression of *Ccl3*, *Il6*, and *Il1b* and increased number of cleaved caspase 3 positive cells. Serum lipase correlated with the extent of tissue damage in RAMP1$^{-/-}$ compared to wild-type mice 24 h post-cerulein treatment.

Conclusion: Mice lacking RAMP1 showed increased inflammation, tissue edema, and pancreas injury particularly in the early phase of acute pancreatitis. This study highlights the essential role of CGRP for dampening the innate immune response in acute pancreatitis.

Introduction

Acute pancreatitis is one of the most common diseases in the field of gastroenterology leading to high morbidity, mortality and hospitalization with increase of indirect treatment costs [1–3]. Nevertheless, a specific therapy is still not available for these patients. Therefore, there is a need to better understand underlying mechanisms that regulate the inflammatory reaction in patients with acute pancreatitis.

Growing evidence supports the concept that immune responses can be modified and influenced by the nervous system [4–7]. In the

Abbreviations: ADM, Acinar-to-ductal metaplasia; CD, Cluster of differentiation; CGRP, Calcitonin-gene-related-peptide; CLR, Calcitonin-receptor-like-receptor; cCasp3, cleaved caspase 3; HPF, High-power-field; HE, Hematoxylin and eosin; ICER, Inducible-cAMP-early-repressor; IL, Interleukin; LDH, Lactate-dehydrogenase; MPO, Myeloperoxidase; qRT-PCR, Quantitative real-time polymerase chain reaction; RAMP1, Receptor-activity-modifying-protein 1; RNA, Ribonucleic acid; SEM, Standard error of the mean; STAT3, Signal transducer and activator of transcription 3; Wnt, Wingless/Integrated.

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peripheral nervous system, this activity may be mediated by the neuropeptide calcitonin gene related peptide (CGRP). CGRP is a 37 amino-acid peptide neurotransmitter that plays an important role in the central and peripheral nervous systems [8,9]. CGRP is able to activate the CGRP-receptor consisting of two subunits: the receptor-activity-modifying-protein 1 (RAMP1) and the calcitonin-receptor-like-receptor (CLR) [10–12]. Activation of the receptors leads to the induction of a diverse range of downstream signaling pathways where the precise pathway is likely to be cell-specific [13].

In immune cells, the activation of the CGRP-receptor leads to the stimulation of $G\alpha_s$ proteins with consecutive elevation of cAMP-levels and activation of protein kinase A [13]. Thereby, CGRP can directly influence immune cells and their capacity of producing inflammatory cytokines. Previous studies have shown a reduction of inflammatory cytokines, such as TNFα, IL-12, IL-1β, and CCL4 and an increased production of the anti-inflammatory cytokine IL-10 after treating dendritic cells or macrophages with CGRP [7,14–16]. The reduced expression of inflammatory genes may also involve an upregulation of the inducible-cAMP-early-repressor [7,17], that inhibits the expression of inflammatory genes by competing with the activating transcription factor ATF-2 for binding to cytokine promoters [7,18].

Although the CGRP-RAMP pathway has been extensively studied in inflammatory conditions like septic peritonitis, where the deficiency of the RAMP1 subunit of the CGRP-receptor led to an impaired anti-bacterial defence [19] to date little is known about its function in affecting the inflammatory reaction of acute pancreatitis. In previous studies, CGRP immunoreactivity has been detected in nerve fibres innervating the pancreas [20]. In addition, Schneider et al. [21] were able to show an improved pancreatic microcirculation and reduced inflammation in experimental acute pancreatitis after treating rats with capsaicin, a substance of red hot chili peppers inducing a release of endogenous CGRP.

By using a RAMP1 knockout model ($RAMP1^{-/-}$), we were able to show an important role and function of the CGRP-pathway during the early phase of cerulein-induced acute pancreatitis. This conclusion is supported by a more severe acute pancreatitis in RAMP1-deficient mice, exhibiting more tissue edema and damage, immune cell infiltration, apoptosis, a higher expression of pro-inflammatory cytokines, and reduced acinar cell proliferation.

Methods

Mouse strains

All experiments were performed according to the German Federal Animal Protection Laws and were approved by the Institutional Animal Care and Use Committees of the government of Bavaria and the Technical University of Munich. In the experimental setup, $RAMP1^{-/-}$ females were used as experimental group, and C57BL/6 N wild-type mice (Charles River) as controls. $RAMP1^{-/-}$ mice were obtained from Dr. Tsujikawa [22] and were backcrossed for 10 generations to the C57BL/6 N background.

Cerulein treatment

For the induction of acute pancreatitis, cerulein diluted in phosphate buffered saline (0.2 μg/injection) (Bachem) was administered by hourly intraperitoneal injections for a period of 8 h one day only (modified according to Jensen et al. [23]), to 8- to 10-week-old mice (mean body weight: RAMP1−/− mice 19.8 g, C57BL/6 N mice 19.5 g). Mice were sacrificed at 8 h, 24 h, 2d and 7d after the first cerulein injection for analysis. Performing the analysis at 8 h and 24 h permits evaluation of the acute pancreatic damage. 2d after the pancreatitis induction, regeneration period begins with acinar to ductal metaplasia. To further evaluate the regeneration capacity, we analysed day 7. At this time point wild type mice pancreata are fully recovered [24,25]. As an internal control, $RAMP1^{-/-}$ and C57BL/6 N mice received intraperitoneal injections with phosphate buffered saline (PBS) and were sacrificed 7 days after the first injection.

Blood sample extraction and pancreatic weight analysis

Mice were sacrificed with an overdose of isoflurane and exsanguination by punction of the inferior caval vein. Next the whole pancreas was carefully isolated without any adjacent tissue and immediately weighed on a precision scale.

Microscopy and immunohistochemistry

Pancreatic sections were stained with hematoxylin and eosin. Edema, inflammation, and necrosis (0 = condition was absent, to 4 = maximum) were scored separately and used to calculate an acute pancreatitis score for 7 to 9 mice per group and timepoint ($n \geq 7$) (modified according to Folias et al., and Rongione et al.[26,27]). For the evaluation of edema and inflammation, the whole slide was scored. For necrosis ten high power fields were analysed using Aperio ImageScope (Leica Biosystems) and scored as shown in Supplementary Table 1. Furthermore, ADM Area was measured 2 days post-cerulein treatment for 5 mice per group ($n = 5$).

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: mouse *anti*-Ki67 (BD Pharmigen, Dilution 1:400), rabbit anti-Cleaved caspase 3 (Cell Signaling, Dilution 1:200), rabbit *anti*-CK19 (Abcam, Dilution 1:1000), goat *anti*-CPA1 (RD Systems, Dilution 1:300), rabbit *anti*-F4/80 (Abcam, Dilution 1:300), rat anti-CD4 (Dianova, Dilution 1:100), rat anti-CD8 (Dianova, Dilution 1:100), mouse *anti*-MPO (Santa Cruz Biotechnology, Dilution 1:350), rabbit *anti*-NFkappaB p65 (Santa Cruz, Dilution 1:500). Biotin-conjugated secondary antibodies were incubated for 1 h at room temperature, following development with ABC and DAB kits (both Vector Laboratories). Nuclear counterstaining was done using hematoxylin.

qRT-PCR

After the isolation, a small piece of the pancreas was homogenised with RNAlater stabilization reagent (Qiagen, Hilden, Germany). Then, total RNA was extracted using Maxwell®16 Total RNA Purification Kit. The extracted RNA (2 μg) was then reversely-transcribed with SuperScript™ II Reverse Transcriptase (ThermoFisher). Afterwards, the transcripts were amplified using Fast SYBR Green Master Mix (Roche, Unterhaching, Germany) and a LightCycler 480 PCR machine (Roche Applied Science). The Primers used are listed in Supplementary Table 2. The expression levels of each transcript were normalised to the housekeeping gene XS13 using the ΔΔCt method. All RT-PCR experiments were performed in duplicates using 8 to 10 individual biological samples per group ($n \geq 8$).

Lipase and LDH levels

In order to analyse pancreatic injury, serum activity of LDH and lipase were measured using photometry (Roche, Cobas) at the Institute of Clinical Chemistry and Pathobiochemistry of the Technical University of Munich for 6 to 10 biological samples per group ($n \geq 6$).

Statistical analysis

Quantification of immunohistochemistry stainings ($n \geq 5$, 5 to 9 biological samples per group) was done using Aperio ImageScope (Leica Biosystems). Ten random high-power fields from each mouse pancreas were chosen for quantification (cCasp3/Ki67: 862 Pixel; MPO/F4/80/CD4/CD8: 1190 Pixel), for NFkappaB five to ten fields were taken and quantified using Aperio ImageScope. The Wilcoxon-Mann-Whitney-Test was used for the comparison between groups (Prism V6.0, GraphPad Software Inc.). All data are

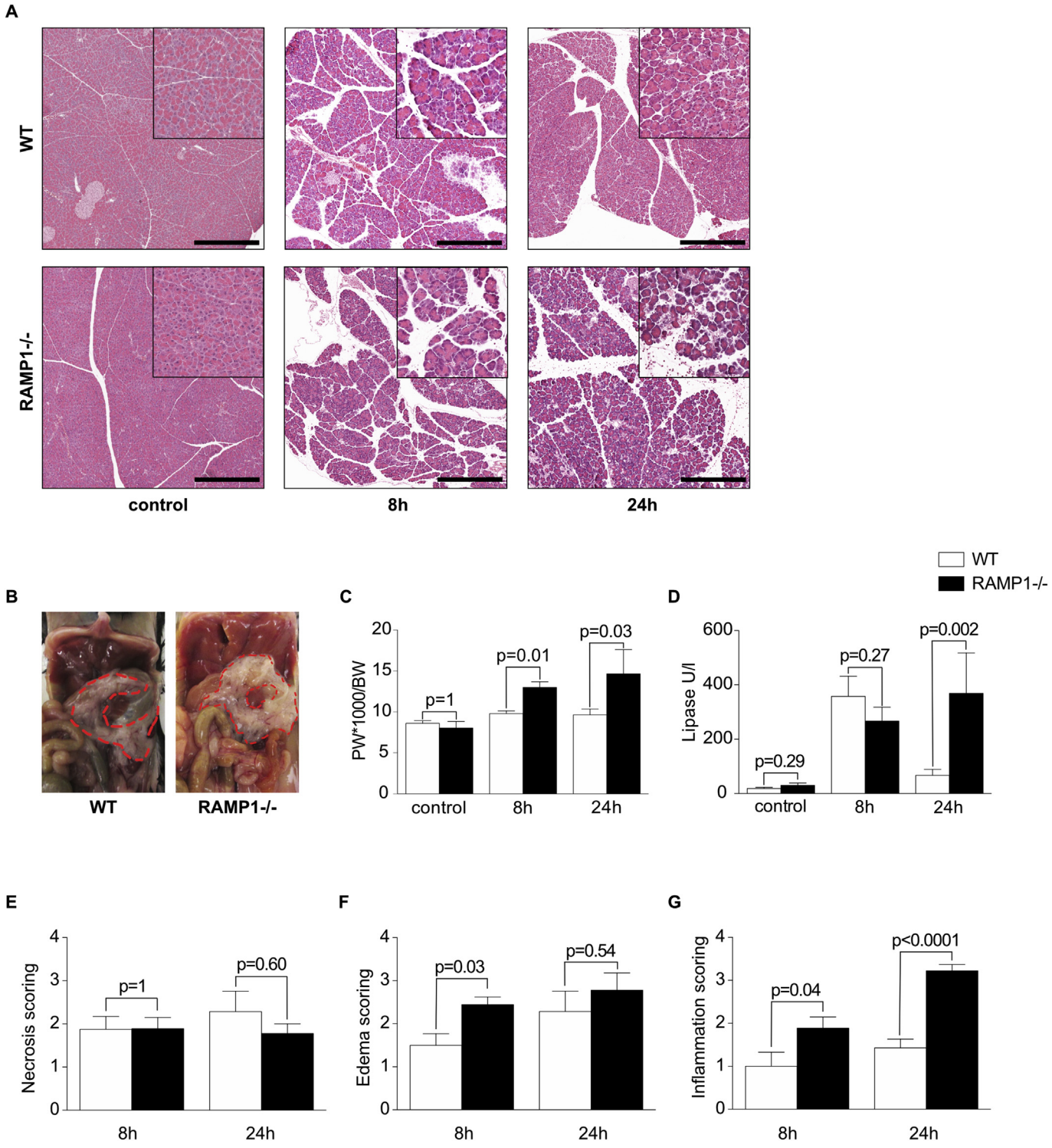

Fig. 1. Increased pancreatic damage after acute pancreatitis in RAMP1$^{-/-}$ mice. (A): Representative H&E staining of random pancreatic sections of the indicated genotypes: in untreated mice and 8 h/24 h post-pancreatitis induction. (B) Macroscopic images of representative pancreata of the indicated genotypes 24 h post pancreatitis induction. (C) Pancreas-to-body-weight-ratio and (D) lipase activity of the indicated genotypes in control mice and 8 h/24 h post-cerulein injections. (E–G) Histopathological semi-quantitative acute pancreatitis severity score of the indicated genotypes concerning: (E) necrosis, (F) edema and (G) inflammation (0 = condition was absent, 4 = maximum) 8 h and 24 h post-cerulein injections. Data are presented as mean ± SEM. Scale bar for H&E staining represents 500 μm.

presented as mean values ± SEM in the figures. Differences between experimental groups were considered significant for p-values less than 0.05.

Results

RAMP1 deficiency increases pancreatic damage after acute pancreatitis in mice

Pancreata from untreated RAMP1$^{-/-}$ and wild-type mice did not reveal any architectural differences, as shown by HE, CPA1, and CK19 staining. Furthermore, comparable pancreas-to-body-weight ratios and lipase values (Suppl. Fig. 1A–C) indicate that RAMP1-deficiency does not appear to affect pancreas organogenesis.

Upon induction of acute pancreatitis, RAMP1$^{-/-}$ mice showed a higher pancreas-to-body-weight ratio at 8 h and 24 h post-cerulein injection (8 h $p = 0.008$; 24 h $p = 0.032$) (Fig. 1B and C). Accordingly, the pancreatitis-score of RAMP1$^{-/-}$ mice exhibited more severe tissue edema at 8 h ($p = 0.03$) and increased infiltration of inflammatory cells at 8 h ($p = 0.04$) and 24 h ($p < 0.0001$) post-cerulein induction compared to wild-type mice (Fig. 1A,F,G). Moreover, the serum lipase activity correlated with the extent of tissue damage showing significantly elevated lipase activities in RAMP1$^{-/-}$ mice ($p = 0.002$) compared to the wild-type group at 24 h (Fig. 1D). In addition, RAMP1−/− mice exhibited a trend towards higher NFkappaB-activity 8 h post-cerulein treatment ($p = 0.093$, Suppl. Fig. 2). The LDH-activity did not show significant differences between the two groups at any time point. Despite developing more severe pancreatitis, RAMP1$^{-/-}$ mice showed a comparable ADM amount as wild-type mice two days after the cerulein injection (Suppl. Fig. 3) and a normal regeneration seven days post-cerulein treatment with complete recovery of pancreatic tissue (Suppl. Fig. 4).

RAMP1 loss reduces proliferation and promotes apoptosis upon pancreatic damage

In accordance with previously published data, indicating that CGRP stimulates proliferation in a variety of tissues including endothelial cells, osteoblasts or alveolar epithelial cells [28–32], RAMP1$^{-/-}$ mice exhibited a significantly reduced number of Ki67-positive cells ($p = 0.004$) compared to wild-type mice 8 h post-cerulein treatment (Fig. 2A and B). Furthermore, an increased number of cleaved caspase 3-positive cells was observed 24 h ($p = 0.002$) after pancreatitis induction in RAMP1$^{-/-}$ mice (Fig. 2C and D).

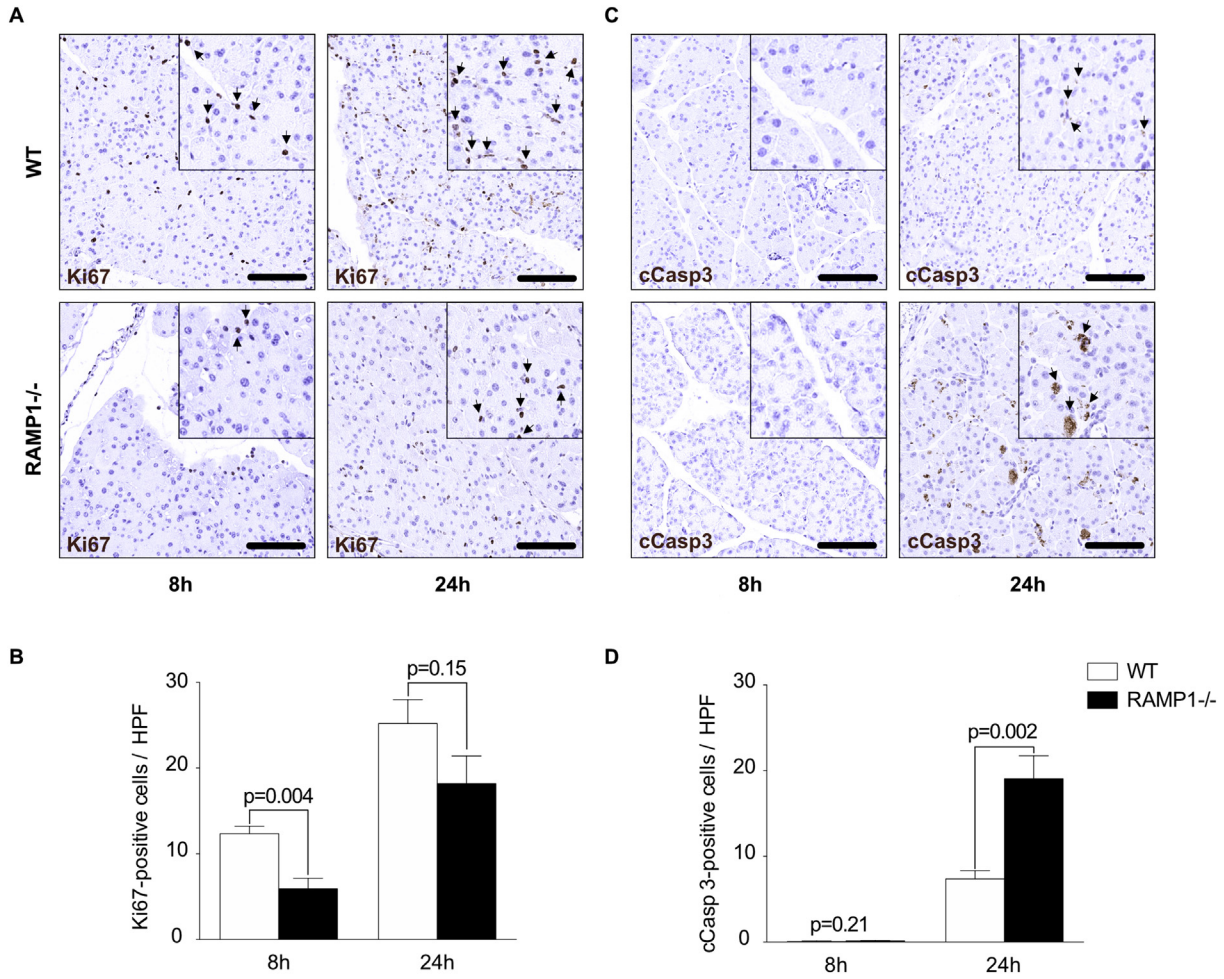

Fig. 2. RAMP1 loss reduces proliferation and promotes apoptosis upon pancreatic damage. (A) Representative immunohistochemical staining for Ki67 8 h and 24 h after pancreatitis induction; black arrows show Ki67-positive cells. (B) Quantification of Ki67-positive cells per HPF. (C) Representative immunohistochemical staining for cleaved caspase 3 (= cCasp3) 8 h and 24 h after pancreatitis induction; black arrows show cleaved caspase 3 positive cells. (D) Quantification of cleaved caspase 3-positive cells per HPF. Data are presented as mean ± SEM. Scale bar represents 100 μm.

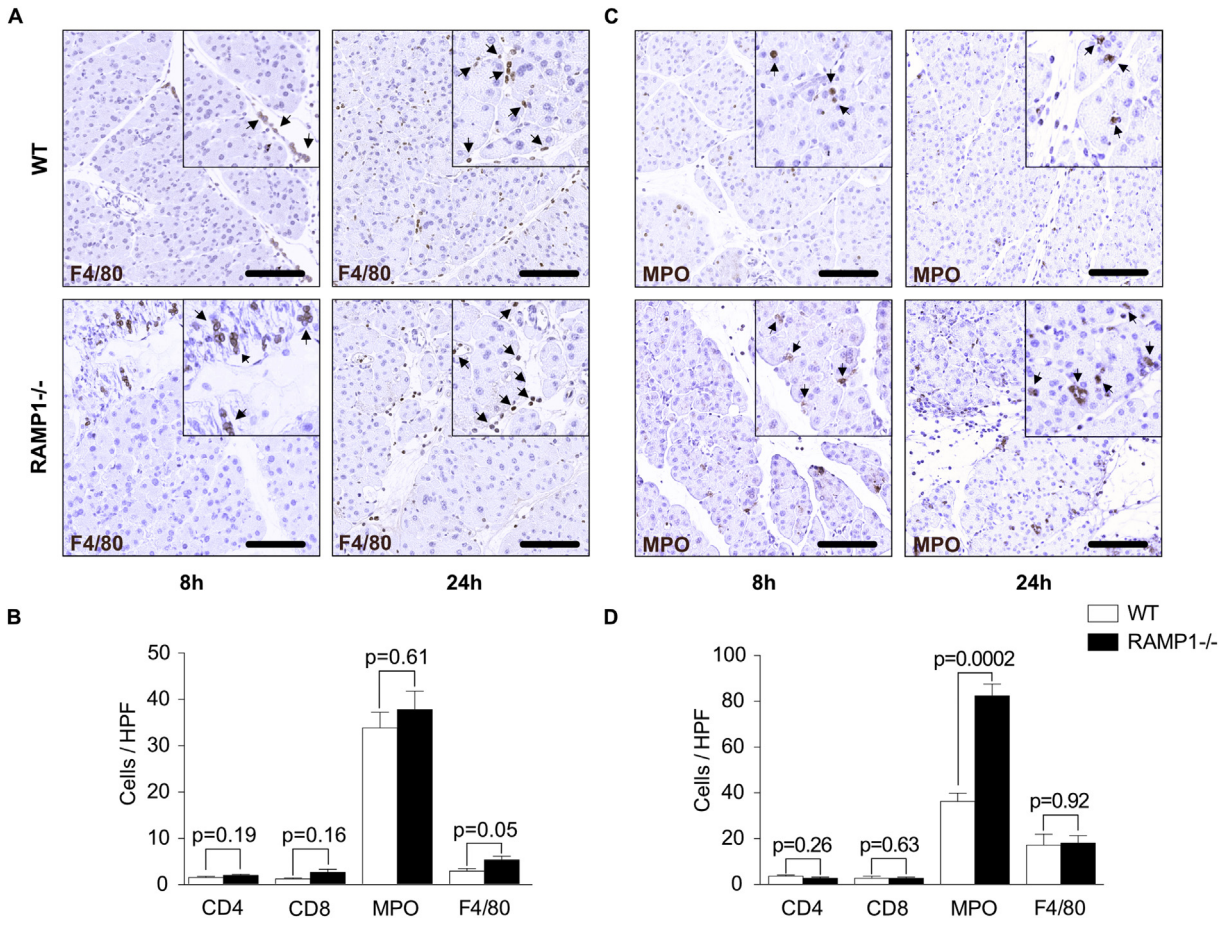

Fig. 3. Characterisation of the immune cell infiltrate. (A) Immunohistochemical staining for F4/80 8 h and 24 h post-cerulein injections; black arrows show F4/80- positive cells. (B) Quantification of $CD4^-$, $CD8^-$, MPO- and F4/80-positive cells per HPF 8 h after pancreatitis induction. (C) Immunohistochemical staining for MPO 8 h and 24 h post-cerulein injections; black arrows show MPO-positive cells. (D) Quantification of $CD4^-$, $CD8^-$, MPO-, and F4/80-positive cells per HPF 24 h after pancreatitis induction. Data are presented as mean ± SEM. Scale bar represents 100 μm.

RAMP1 deficiency enhances infiltration of macrophages and neutrophils in pancreatitis

To further characterise the immune infiltrate, immunohistochemistry was performed. $RAMP1^{-/-}$ mice showed a significantly higher amount of F4/80-positive macrophages 8 h post-cerulein treatment ($p = 0.046$) compared to the control group (Fig. 3A and B). Additionally, after 24 h, significantly higher amounts of MPO-positive neutrophils in $RAMP1^{-/-}$ mice ($p = 0.0002$) without any differences in the number of $CD4^-$and $CD8^-$positive lymphocytes was observed (Fig. 3C and D). These findings suggest an enhanced recruitment and activation of macrophages and neutrophils in RAMP1-deficient mice during the early phase of acute pancreatitis.

Cytokine expression in response to acute pancreatitis

To elucidate potential cytokine cues that direct the attraction of immune cells, cytokine expression in response to acute pancreatitis was measured. RAMP1-deficient mice displayed significantly elevated levels of the pro-inflammatory cytokines CCL3 ($p = 0.008$), IL-6 ($p = 0.003$), and IL-1β ($p = 0.01$) 24 h post-cerulein injection (Fig. 4A–C). Furthermore, RAMP1-deficient mice exhibited a trend towards higher expression of TNFα ($p = 0.083$) compared to wild-type mice, even though, this was not statistically significant (Fig. 4F). Previous studies have indicated that CGRP might promote the production of the anti-inflammatory cytokine IL-10 during acute inflammatory conditions [33]. However, $RAMP1^{-/-}$ mice exhibited a significantly increased IL-10 ($p = 0.028$) expression 24 h after pancreatitis induction compared to wild-type mice (Fig. 4D).

Discussion

Using a murine model deficient for the CGRP-receptor component RAMP1, this study underlines the essential role of the CGRP-pathway as a central modulator of the innate immune response during acute pancreatitis. RAMP1-deficient mice exhibited a severely increased production of cytokines and recruitment of inflammatory cells during the early phase of acute pancreatitis. Accordingly, pancreatic damage was increased in $RAMP1^{-/-}$ mice. These findings are in line with previous *in vitro* experiments that have shown an inhibition of innate and adaptive immune cells by CGRP [7,14,33–35]. Furthermore, other studies have shown *in vivo*, that CGRP has a potent immunosuppressive activity in experimental colitis and endotoxaemia [7,15,36–38].

In comparison to wild-type mice, RAMP1-deficient mice exhibited a significantly reduced proliferation 8 h post-cerulein injections and an increased apoptosis 24 h after the cerulein treatment. These findings are in accordance with previously published data underlining the impact of CGRP on proliferation and apoptosis [28,29,31,32]. For example, Mrak et al. [29] could show an anabolic effect of CGRP via the stimulation of canonical Wnt-signaling pathway and apoptosis-inhibition in human osteoblasts.

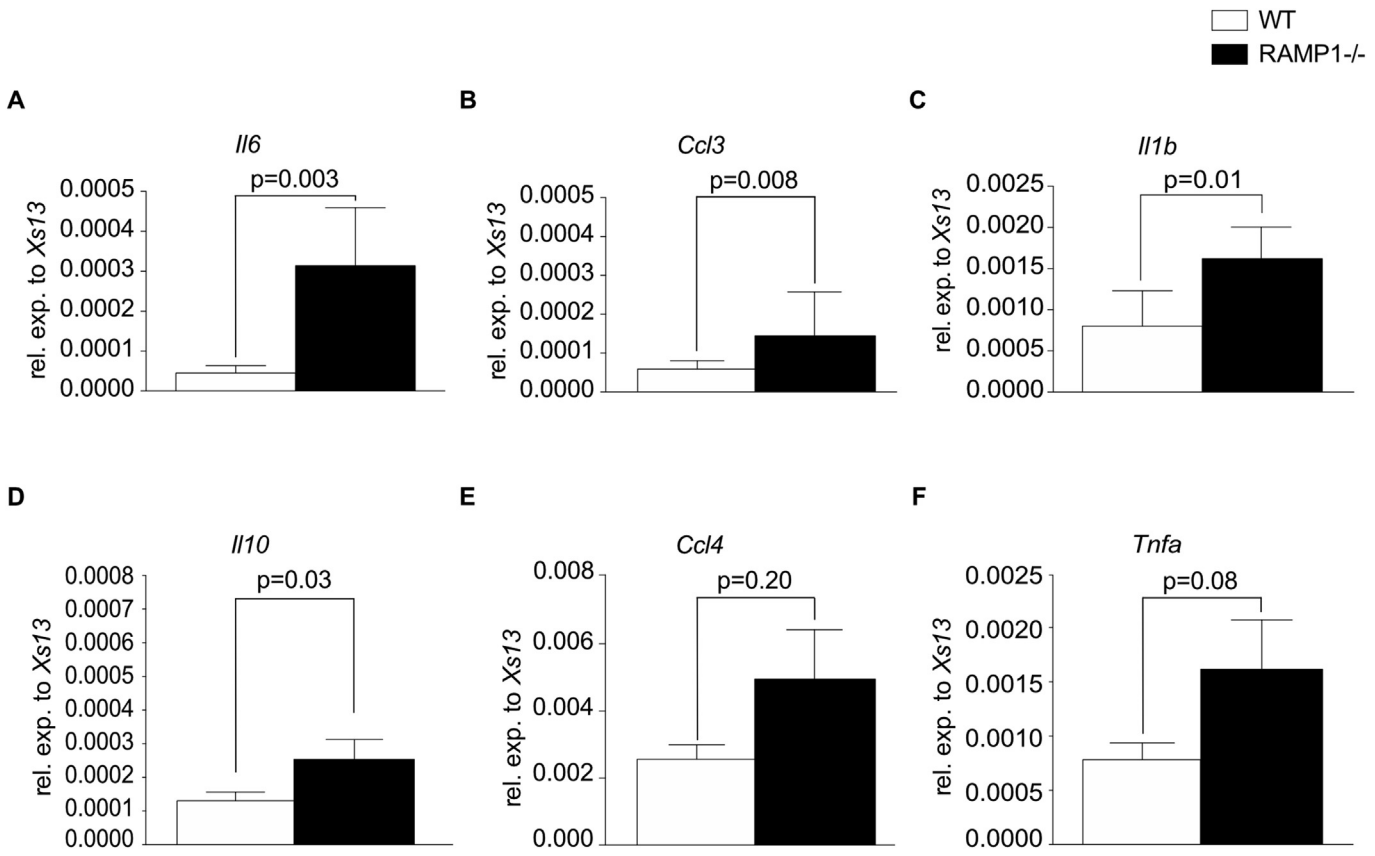

Fig. 4. Cytokine expression in response to acute pancreatitis. RNA expression of (A) *Il6*, (B) *Ccl3*, (C) *Il1b*, (D) *Il10*, (E) *Ccl4*, and (F) *Tnfa* 24 h post-cerulein injections of the indicated genotypes normalised to the housekeeping Gene XS13. Data are presented as mean ± SEM.

However, these early alterations of cell proliferation and death did not appear to influence long-term organ regeneration in our mouse model, since seven days after pancreatitis induction the pancreatic tissue was completely recovered in both RAMP1$^{-/-}$ and wild-type mice (Supp. Fig. 2A).

Further analysis of the immune cell infiltrate showed that the enhanced inflammatory environment in RAMP1$^{-/-}$ mice was mostly composed of macrophages and MPO-positive neutrophils. On the RNA-level, we measured a significantly enhanced expression of the chemokine CCL3 (MIP-1α) and a slightly higher expression of CCL4 (MIP-1β) in RAMP1-deficient mice (Fig. 4B, E). Both act as chemoattractants for polymorphonuclear leucocytes, natural killer cells, and other immune cells [39]. These results suggest that the up-regulated expression of chemokines may be responsible for the excessive recruitment of macrophages and neutrophils into the pancreas in RAMP1$^{-/-}$ mice. In contrast, we could not find any difference in the number of CD4^{-}and CD8-positive lymphocytes in the pancreas following acute pancreatitis.

In accordance with the anti-inflammatory activity of CGRP, a significantly increased expression of *Il1b* and *Il6* in RAMP1$^{-/-}$ mice compared to wild type mice 24 h post cerulein-treatment was observed (Fig. 4A, C). In human patients, the IL-6 level in serum appears to correlate with the severity of pancreatic inflammation [40,41]. Furthermore, IL-1β is one of the key effector cytokines in the innate immune response to sterile injury and there is evidence suggesting a role of IL-1β in the initiation of a sterile inflammatory response following pancreatic injury in humans [42]. In addition, studies have shown a pivotal role of IL-1β in encouraging organ failure during the course of severe acute pancreatitis [43].

A possible mechanism for anti-inflammatory effects of the CGRP-pathway in the peripheral nervous system is related to increased transcription of the anti-inflammatory cytokine IL-10. Nonetheless, in the present study no reduction in IL-10 expression of RAMP1$^{-/-}$ mice could be observed. However, there are numerous transcription factors known besides the cAMP response element binding protein to be essential or critical factors in IL-10 regulation [44]. For example, IL-6 can induce IL-10 transcription via the activation of STAT3 [45,46]. Hence, in this study, the increased IL-10 levels in RAMP1$^{-/-}$ mice may be due to compensatory mechanisms in response to a more severe inflammation.

In conclusion, this study shows that RAMP1-deficiency results in a more severe acute pancreatitis with significantly increased tissue edema, organ injury, apoptosis, and immune-cell infiltration composed of macrophages and neutrophils. In addition, RAMP1$^{-/-}$ mice exhibited higher levels of the pro-inflammatory cytokines IL-6, IL-1β and CCL3 and a reduced pancreatic cell proliferation. In the context of previous studies, these findings underline the importance of the CGRP-pathway in maintaining the balance between pro- and anti-inflammatory components during acute pancreatitis. With the recent release of a fully human monoclonal antibody against the canonical CGRP-receptor for the treatment of migraine [47], it is important to consider the evidence gathered from mouse models including the one presented here with regards to potential pro-inflammatory side effects when interfering with this pathway.

Conflicts of interest

The authors declare to have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.05.455.

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