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ORIGINAL ARTICLE

Diabetes increases pancreatitis induced systemic inflammation but has little effect on inflammation and cell death in the lung

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SUMMARY

Acute pancreatitis (AP) can lead to a systemic inflammatory response that often results in acute lung injury and single or multiple organ failure. In a previous study we demonstrated that diabetes aggravates the local pathophysiological process during AP. In this study we explore, if diabetes also increases pancreatitis induced systemic inflammation and causes lung injury. Acute pancreatitis was induced in untreated and streptozotocin-treated diabetic mice by injection of cerulein. Systemic inflammation was studied by IL-6 ELISA in blood plasma and white blood cell count. Lung inflammation and lung injury were quantified by chloroacetate esterase staining, evaluation of the alveolar cellularity index and cleaved caspase-3 immunohistochemistry. In normoglycaemic mice AP increased the IL-6 concentration in plasma and caused lymphocytopenia. Diabetes significantly increased the IL-6 concentration in plasma and further reduced the number of lymphocytes during AP, whereas diabetes had little effect on these parameters in the absence of pancreatitis. However, diabetes only marginally increased lung inflammation and did not lead to cell death of the lung epithelium during AP. We conclude that diabetes increases parameters of systemic inflammation during AP, but that this increase is insufficient to cause lung injury.

Keywords
acute lung injury, chloroacetate esterase staining, cleaved caspase-3, sepsis, systemic inflammatory response syndrome

About 15%–20% of patients with acute pancreatitis (AP) develop severe symptoms, such as pancreatic parenchymal necrosis, a systemic inflammatory response and concomitant single or multiple organ failure (Forsmark & Baillie 2007). The AP-induced inflammatory response often causes acute lung injury (ALI) with a mortality rate of up to 40% (Zhou *et al.* 2010). This is often associated with lymphopenia and an increase in the concentration of the pro-inflammatory cytokine IL-6 in the blood (Takeyama *et al.* 2000; Gregoric *et al.* 2010). IL-6 is not only of prognostic value, but has also been demonstrated to be an essential mediator of pancreatitis-associated lung injury (Zhang *et al.* 2013). The pro-inflammatory milieu during AP leads to activation and infiltration of neutrophil granulocytes in the lung and to an increase in the alveolar cellularity index (Guice *et al.* 1989; Frossard *et al.* 1999; Pastor *et al.* 2006; Tascilar *et al.* 2007). Pulmonary inflammation can cause lung injury, which is often characterized by apoptosis of lung epithelial cells (Yuan *et al.* 2000; Nakamura *et al.* 2003). Although several proteins, such as the cytokine induced neutrophil chemoattractant, the CD40 ligand or the toll-like receptor 4, have been implicated in the pathophysiological process of AP-induced ALI, underlying mechanisms and contributing parameters of this process are still not well understood (Bhatia *et al.* 2000; Frossard *et al.* 2001; Sharif *et al.* 2009; Zhou *et al.* 2010).

One parameter that has been described to aggravate AP is diabetes. Several correlative studies in patients have suggested that diabetes leads to a higher risk for pancreatitis (Seicean *et al.* 2006; Noel *et al.* 2009; Girman *et al.* 2010; Xue *et al.* 2012) and that hyperglycaemia may predispose patients with AP to systemic organ failure (Mentula *et al.* 2008). In addi-

tion, blood glucose level is an accurate predictor of outcome in gallstone pancreatitis and an important criterion for the Ranson score, which is used to assess the prognosis of AP (Ranson *et al.* 1974; Rajaratnam & Martin 2006).

We have previously demonstrated in experimental settings that diabetes indeed aggravates the local pathophysiological process during acute as well as chronic pancreatitis (Zechner *et al.* 2012, 2013, 2014). The purpose of this study was to explore if the aggravation of AP by diabetes leads to increased systemic inflammation, and if adequate alterations in the lung can be observed.

Methods

Animals

Eight to 12-week-old C57BL/6J mice were allowed access to water and standard laboratory chow *ad libitum*. The mice were treated as published previously (Zechner *et al.* 2012). Diabetes was caused by i.p. injection of 50 mg/kg streptozotocin (STZ; Sigma-Aldrich, Steinheim, Germany) on 5 consecutive days (day 1-5), whereas AP was induced on day 22 and day 23 by administration of eight i.p. injections per day of 50 μg/kg cerulein (Sigma-Aldrich) at a rate of one every hour. All control mice were Sham-treated appropriately (0.9% wt/vol. NaCl solution instead of cerulein, 50 mmol/l sodium citrate pH 4.5 instead of STZ). At 2 h before induction of pancreatitis, and up to the time point of tissue preservation, all mice received drinking water containing 800 mg/l metamizol (Ratiopharm, Ulm, Germany) and 1 g/l BrdU (Sigma-Aldrich Chemie GmbH). For sampling blood and tissue the animals were anaesthetized with 75 mg/kg ketamine (Bela-Pharm, Vechta, Germany) and 5 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany). All experiments were executed in accordance with German legislation, the local animal wellfare committee and the EU-directive 2010/63/EU.

Analysis of plasma and tissue

Blood samples were taken 2 hours after the last cerulein injection on day 23. A differential blood cell count was performed with an automated hematology analyzer Sysmex KX 21 (Sysmex Cooperation, Kobe, Japan) as previously published (Bobrowski *et al.* 2013). Concentrations of interleukin (IL)-6 were measured in blood plasma with commercially available enzyme-linked immunosorbent assay (ELISA) kits from Thermo Fisher Scientific (Rockford, IL, USA) following the manufacturer's instructions, and data were plotted as fold induction compared to the average of IL-6 in Sham-treated mice. Tissue samples were taken on day 23 (2 hours after the last cerulein injection) or on day 30 (7 days after the last cerulein injection). Naphthol AS-D chloroacetate esterase (CAE) staining, primarily staining neutrophil granulocytes, or hematoxylin/eosin staining was performed on paraffin-embedded tissue to evaluate lung inflammation and histology. To evaluate the alveolar cellularity index (nuclei/septum), the number of nuclei crossing three gridlines of the integrating eyepiece (using a 100× objective) was divided by the number of septa crossing these gridlines (Tascilar *et al.* 2007;). We analysed 10 randomly chosen microscopic fields from each lung. Cell death was analysed by immunohistochemistry using a rabbit-anti-mouse cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology Inc., Denver, USA, code 9661, dilution 1:500) and a HRP-conjugated goat-anti-rabbit secondary antibody (code P0448; Dako Deutschland GmbH, Hamburg, Germany). Cell proliferation was evaluated by immunohistochemistry using mouse anti-BrdU (clone: Bu20a, dilution: 1:50) and the Universal LSAB™+ Kit/HRP kit (Dako Deutschland GmbH). Quantification of inflammation, BrdU incorporation and cell death was performed on 10 random fields per mouse using a 40× objective.

Statistics

Data are given as means and standard deviation respectively. The significance of data was assessed by SigmaStat 3.5 software (SigmaStat; Jandel Corporation, San Rafael, CA, USA). In all cases where the assumption of normality or the homogeneity of variance across groups failed, the Mann–Whitney rank sum test was performed, including correction of the α-error according to the Bonferroni probabilities for repeated analysis. In other cases, the unpaired Student's *t* test including the correction of the α-error according to the Bonferroni was performed. The criterion for significance was $P < 0.05$ divided by the number of meaningful comparisons.

Results

Diabetes aggravates systemic inflammation during pancreatitis

To evaluate if STZ-induced diabetes has an influence on parameters of systemic inflammation during cerulein-induced AP, the IL-6 concentration in plasma was determined in cerulein-treated diabetic mice (STZ + Cer) and compared to healthy normoglycaemic mice (Sham), cerulein-treated normoglycaemic mice (Cer) and diabetic mice without pancreatitis (STZ). Administration of cerulein in normoglycaemic mice during the acute phase of pancreatitis (on day 23, 2 hours after the last cerulein administration) increased the IL-6 plasma concentrations when compared to healthy mice (Figure 1a). STZ plus cerulein treatment lead to an even more pronounced increase in IL-6 concentration when compared to Sham, cerulein or STZ-treated mice (Figure 1a). Evaluation of the blood cell count revealed that administration of cerulein only slightly reduced the number of leucocytes in normoglycaemic animals (Figure 1b). However, STZ plus cerulein treatment lead to a significant decrease in the number of leucocytes when compared to cerulein-treated mice (Figure 1b). A decrease in

the number of lymphocytes was observed after administration of cerulein in normoglycaemic mice compared to healthy mice (Figure 1c). STZ plus cerulein treatment lead to an even more pronounced decrease in the number of lymphocytes when compared to Sham, cerulein or STZ-treated mice (Figure 1c). The number of monocytes plus granulocytes was increased by cerulein administration, but was not significantly influenced by STZ (Figure 1d).

Diabetes only marginally aggravates lung inflammation during pancreatitis

To evaluate if the observed aggravation of systemic inflammation by diabetes has an influence on lung inflammation, infiltrating inflammatory cells were identified by CAE staining on lung sections on day 23 (Figure 2a–d). Administration of cerulein in normoglycaemic or diabetic mice significantly increased the number of CAE^+ cells in the lung tissue when compared to Sham or STZ-treated mice (Figure 2e). STZ plus cerulein treatment lead to a slight increase ($P = 0.023$) in the number of CAE^+ cells when compared to cerulein-treated mice (Figure 2e). The alveolar cellularity index was marginally increased by cerulein treatment ($P = 0.143$), but barely influenced by STZ treatment (Figure 2f).

Diabetes does not induce cell death in the lung epithelium

To evaluate if STZ-induced diabetes has an influence on cell death in the lung during the acute phase of pancreatitis (on day 23, 2 hours after the last cerulein administration), cleaved caspase-3^+ cells were identified by immunohistochemistry (Figure 3a). However, the administration of cerulein, STZ or STZ plus cerulein did not result in an increased number of cleaved caspase-3^+ cells in the lung when compared to Sham-treated animals (Figure 3b). To evaluate if this lack of cell death in the lung epithelium might be caused by inadequate severity of pancreatitis we evaluated the histology of the pancreas on day 23 and on day 30. Normal histology of the pancreas was observed in Sham and

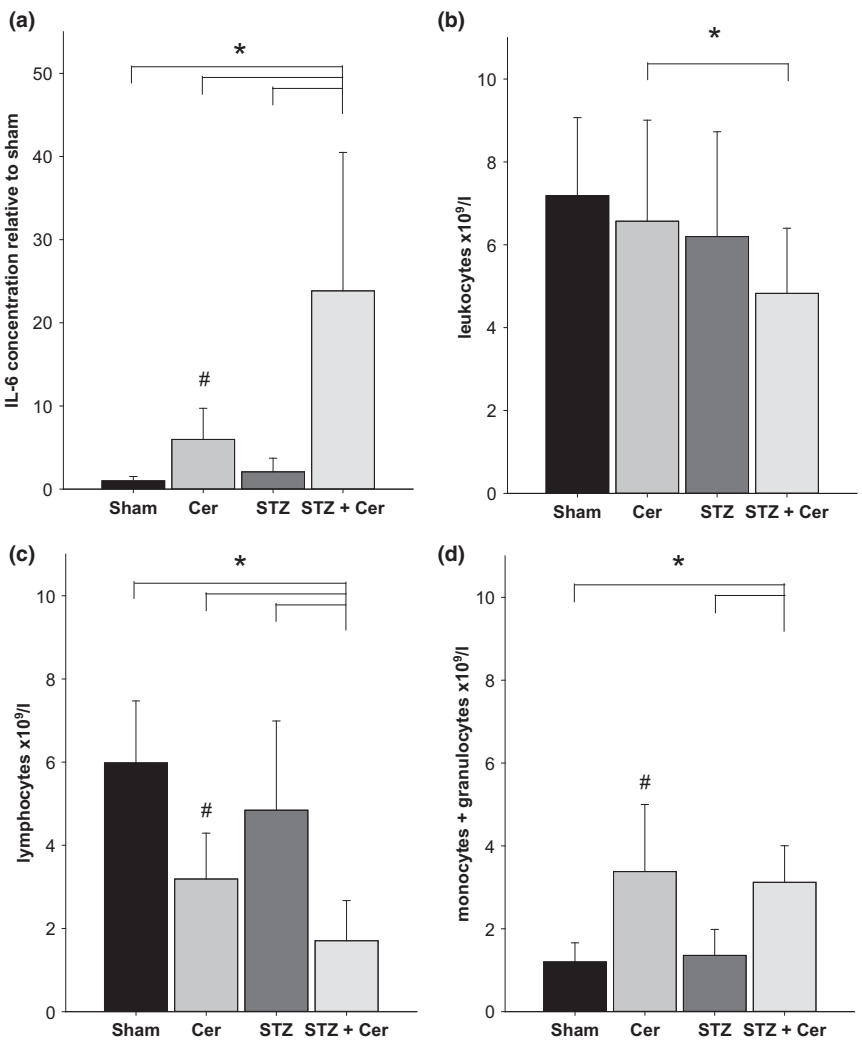
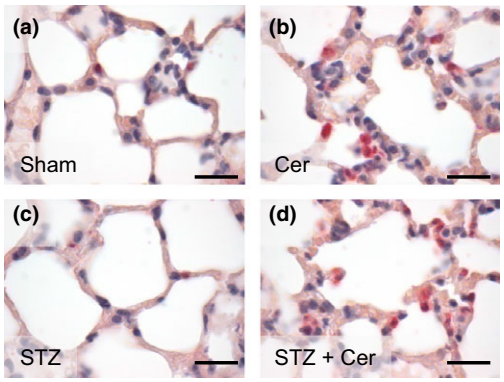
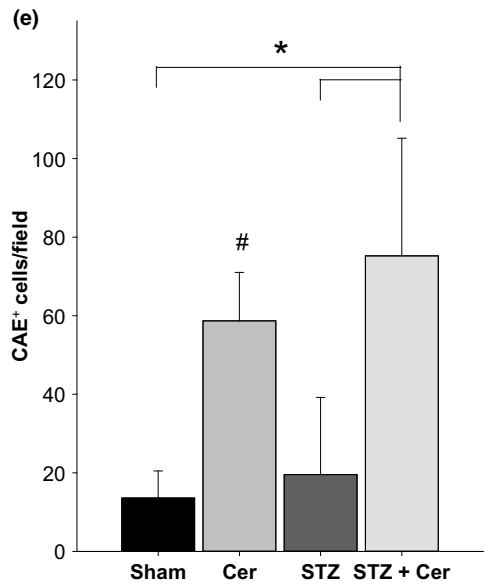


Figure 1 Diabetes aggravates pancreatitis induced systemic inflammation. The concentration of IL-6 in blood plasma (a), and the concentration of leucocytes (b), lymphocytes (c) or monocytes plus granulocytes (d) in the blood was determined on day 23 (2 hours after the last cerulein administration) in control mice (Sham), mice with AP (Cer), diabetic mice (STZ) and diabetic mice with AP (STZ + Cer). Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 8$ (Sham), $n = 19$ (Cer), $n = 11$ (STZ), $n = 21$ (STZ + Cer) in panel a and $n = 6$ (Sham), $n = 20$ (Cer), $n = 7$ (STZ), $n = 19$ (STZ + Cer) in panel b to d. Significant differences between the cohorts are indicated, Mann–Whitney rank sum test: $^*P \leq 0.001$ (a, c, d), $^*P = 0.008$ (b), $^{\#}P \leq 0.001$ compared to Sham-treated mice (a, c, d).





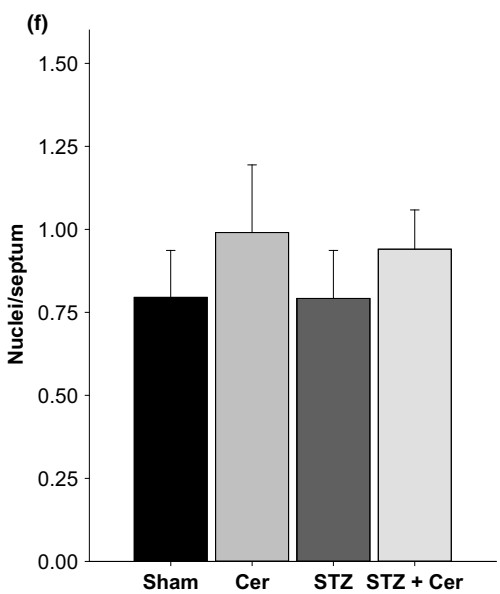
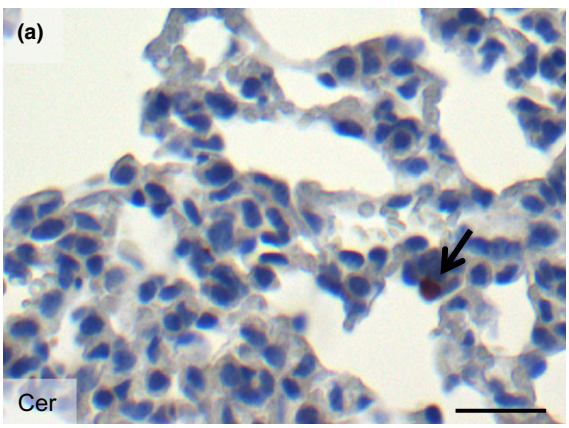


Figure 2 Diabetes and pancreatitis influence lung inflammation and alveolar cellularity on day 23. Representative images of CAE^+ inflammatory cells in the lung in control mice (a), mice with AP (b), diabetic mice (c) and diabetic mice with AP (d). The number of CAE^+ inflammatory cells per field (e) and the alveolar cellularity index (f) was quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 9$ (Sham), $n = 17$ (Cer), $n = 12$ (STZ), $n = 17$ (STZ + Cer) in panel e and $n = 4$ (Sham), $n = 5$ (Cer), $n = 4$ (STZ), $n = 6$ (STZ + Cer) in panel f. Significant differences between the cohorts are indicated, Mann–Whitney rank sum test: $^*P < 0.001$, $^\#P < 0.001$ compared to Sham-treated mice. Bar = 20 μm.

STZ-treated mice (data not shown). In cerulein-treated mice features of AP such as oedema, and tissue-infiltrating inflammatory cells were observed on day 23, but pancreatitis was reversible as judged by histology of the pancreas on day 30



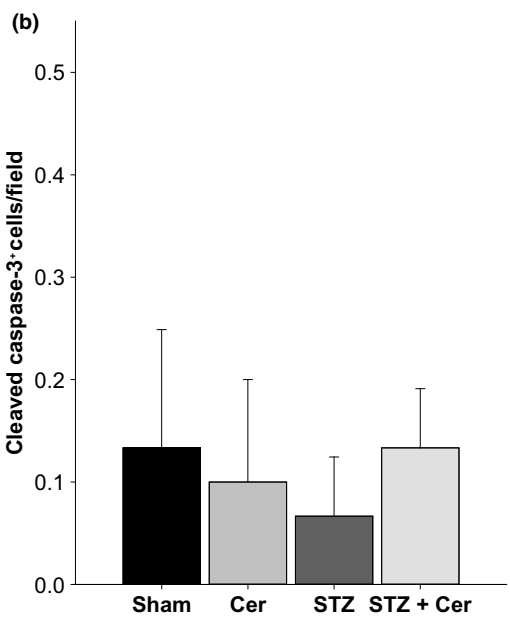


Figure 3 Diabetes does not enhance cell death in the lung epithelium on day 23. Representative image of cleaved caspase-3^+ cells in the lung of a cerulein-treated mouse (a). The number of cleaved caspase-3^+ cells in the lung epithelium per field (b) was quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 4$. No significance was observed by Mann–Whitney rank sum test followed by Bonferroni correction for repeated analysis. Bar = 50 μm.

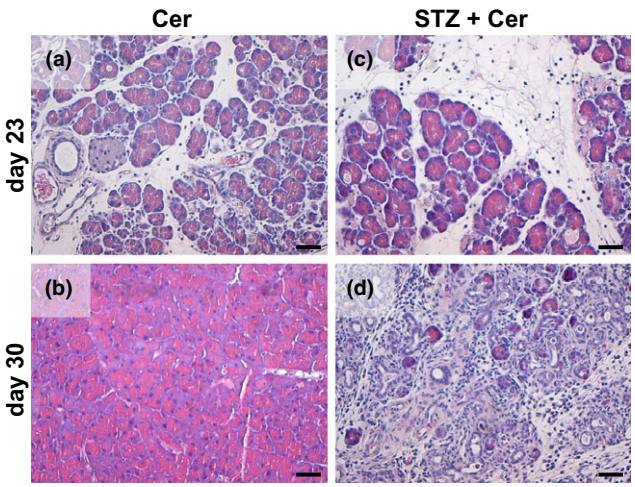


Figure 4 Histology of the pancreas after induction of acute pancreatitis. Representative images of hematoxylin/eosin-stained pancreas sections of cerulein (a, b) or STZ plus cerulein (c, d)-treated mice on day 23 (a, c) and day 30 (b, d). Bar = 50 μm.

(Figure 4a,b). In STZ plus cerulein-treated mice an even stronger induction of oedema and increased infiltration of inflammatory cells was observed in the pancreas on day 23 when compared to cerulein-treated mice (Figure 4a,c). In addition, AP continued until day 30 leading to a massive reduction of acinar cells (Figure 4d). These data suggest that cerulein induces a mild reversible form of pancreatitis, whereas STZ plus cerulein treatment leads to a more severe form of pancreatitis resulting in an impressive difference in the histology of the pancreas on day 30.

Analysis of inflammation and cell death on day 30

On day 30, STZ plus cerulein-treated mice have a marginally increased number of CAE$^+$ cells ($P = 0.114$) in the lung epithelium when compared to cerulein-treated mice (Figure 5a). In addition, the alveolar cellularity index was also slightly increased ($P = 0.114$) by STZ plus cerulein treatment when compared to cerulein-treated mice (Figure 5b). However, we observed no increase in the number of cleaved caspase-3$^+$ cells in STZ plus cerulein-treated mice when compared to cerulein-treated mice (Figure 5c). As lung injury can cause proliferation of lung epithelial cells, we also quantified BrdU incorporation into the nuclei of lung epithelial cells during 8 days of AP (day 22–30). We observed no increase in the number of BrdU$^+$ cells in STZ plus cerulein-treated mice when compared to cerulein-treated animals (Figure 5d).

Discussion

The presented data demonstrate that diabetes during AP (i) enhances IL-6 concentration in blood plasma while decreasing the number of lymphocytes in the blood, (ii) only marginally increases pancreatitis induced lung inflammation, but

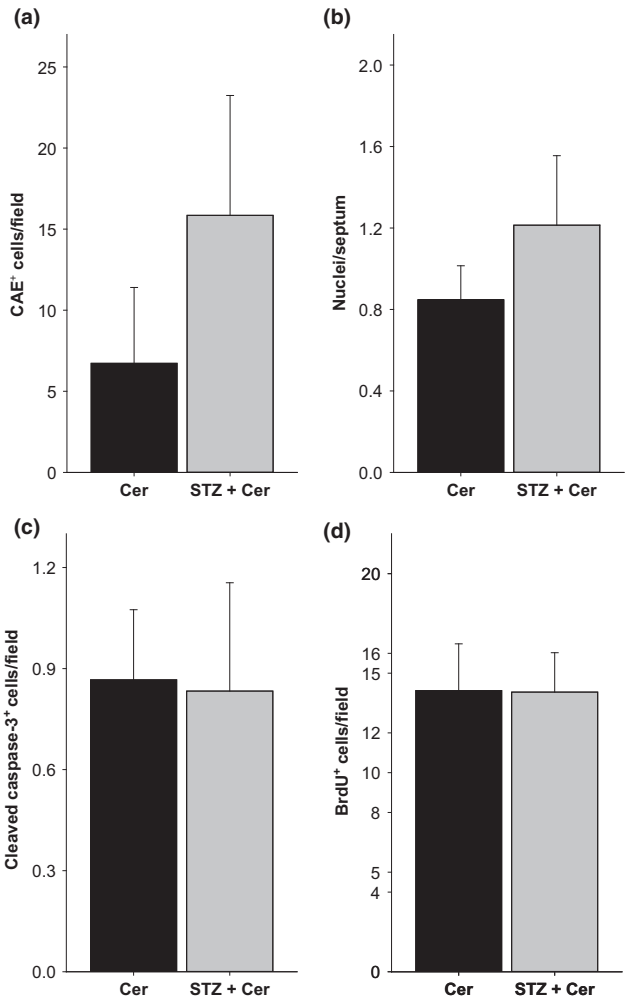


Figure 5 Evaluation of inflammation and cell death in the lung on day 30. The number of CAE$^+$ inflammatory cells per field (a), the alveolar cellularity index (b), the number of cleaved caspase-3$^+$ cells in the lung epithelium per field (c) and the number of BrdU$^+$ cells per field (d) were quantified. Bar charts indicate the average and standard deviation. The number of animals evaluated for each cohort was $n = 4$. No significance was observed by Mann–Whitney rank sum test followed by Bonferroni correction for repeated analysis.

(iii) does not lead to major cell death or proliferation in the lung epithelium. We conclude that diabetes has a fundamental influence on the progression of pancreatitis at a local level as published previously (Zechner *et al.* 2012, 2013, 2014) and can also increase systemic inflammatory parameters such as IL-6 concentration in blood plasma. However, these data also suggest that the observed strong aggravation of pancreatitis by diabetes leads neither to strong enhancement of lung inflammation nor to induction of cell death in the lung epithelium.

Redundant administration of cerulein causes an oedematous form of AP, which is associated with lung inflammation and a very mild form of lung injury (Elder *et al.*

2011). This animal model system should be ideal to test if additional parameters such as diabetes aggravate lung injury, but will not detect a possible inhibition of pancreatitis induced lung injury. The seemingly contradictory result, that diabetes worsens AP leading to a severe form of pancreatitis, but does not cause lung injury, could be explained by the following assumption. Possibly diabetes aggravates pancreatitis, but at the same time reduces the risk for lung injury. This conclusion that lung injury is not aggravated but rather reduced by diabetes is supported by clinical as well as experimental studies. For example, diabetes predicts mortality in critically ill patients, but is not associated with ALI (Koh *et al.* 2012). A meta-analysis also suggests that pre-existing diabetes leads to reduced rather than increased risk of lung injury in critically ill patients (Gu *et al.* 2014). Diabetes also does not increase, but reduces the risk for lung dysfunction in patients with sepsis (Esper *et al.* 2009; Yang *et al.* 2011). Experiments in rats demonstrate that sepsis-induced ALI is milder in diabetic rats than in normoglycaemic controls (Filgueiras *et al.* 2012). Although a few studies suggest that in specific model systems, pre-existing diabetes can also increase the risk of lung injury (Hagiwara *et al.* 2011; Xiong *et al.* 2013) a consensus seems to develop that diabetes is protective against lung injury (Honiden & Gong 2009). Our presented data and the above cited literature, therefore, suggest that it might be especially valuable to carefully adjust glucose concentration to avoid local complications during AP, but that hyperglycaemia might not increase the risk of lung injury during AP.

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Conflict of interests

The authors declare that there is no conflict of interest.

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