Intracapillary leucocyte accumulation as a novel antihaemorrhagic mechanism in acute pancreatitis in mice

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► Seven supplementary video files (1A,B; 2A,B; and 3A–C) are published online only at http://gut.bmj.com/content/vol58/issue11

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ABSTRACT

Background: Pancreatic infiltration by leucocytes represents a hallmark in acute pancreatitis. Although leucocytes play an active role in the pathophysiology of this disease, the relation between leucocyte activation, microvascular injury and haemorrhage has not been adequately addressed.

Methods: We investigated intrapancreatic leucocyte migration, leucocyte extravasation and pancreatic microperfusion in different models of oedematous and necrotising acute pancreatitis in lys-EGFP-ki mice using fluorescent imaging and time-lapse intravital microscopy.

Results: In contrast to the current paradigm of leucocyte recruitment, the initial event of leucocyte activation in acute pancreatitis was represented through a dose- and time-dependent occlusion of pancreatic capillaries by intraluminally migrating leucocytes. Intracapillary leucocyte accumulation (ILA) resulted in dense filling of almost all capillaries close to the area of inflammation and preceded transvenular leucocyte extravasation. ILA was also initiated by isolated exposure of the pancreas to interleukin 8 or fMLP, demonstrating the causal role of chemotactic stimuli in the induction of ILA. The onset of intracapillary leucocyte accumulation was strongly inhibited in LFA-1$^{-/-}$ and ICAM-1$^{-/-}$ mice, but not in Mac-1$^{-/-}$ mice. Moreover, prevention of intracapillary leucocyte accumulation led to the development of massive capillary haemorrhages and transformed mild pancreatitis into lethal haemorrhagic disease.

Conclusions: ILA represents a novel protective and potentially lifesaving mechanism of haemostasis in acute pancreatitis. This process depends on expression of LFA-1 and ICAM-1 and precedes the classical steps of the leucocyte recruitment cascade.

Circulating leucocytes are considered a constitutive part of blood with the potential to leave the vasculature and transmigrate into tissue to fulfil their role as immune cells. According to the current paradigm, leucocyte recruitment during inflammation follows a well-defined cascade of events,[1] starting with the capture of free-flowing leucocytes and leucocytes rolling along the endothelium. Triggered by chemotactic signals, leucocytes then arrest on the vascular endothelium and prepare for their migration into tissue.[1] Recently, a post-arrest leucocyte activation step was described as a complex and extremely dynamic process which includes spreading of attached leucocytes on the endothelium followed by intraluminal leucocyte migration (crawling).[2–5] Thereafter, leucocyte migration occurs via a paracellular or transcellular route[6–8] and migration through the basement membrane.[8] Although the diameter of neutrophils and monocytes exceeds the diameter of capillaries, their presence does not disturb blood flow through narrow blood vessels under healthy conditions. Nevertheless, leucocytes can substantially plug capillaries under pathological conditions including ischaemia/reperfusion, diabetes or shock[9 10] leading to disturbances in microvascular blood flow which induces a so-called "no-reflow" condition with subsequent tissue damage.[10 11] This phenomenon was previously identified as a secondary process following the injury of the capillary endothelium and described in different tissue types such as skeletal muscles,[12] myocardium,[13 14] lung,[15] brain[16] and retina.[17]

Pancreatic infiltration by inflammatory leucocytes represents one of the key features in acute pancreatitis which significantly contributes to the pathogenesis of this disease.[18–20] It has been shown that the recruitment of leucocytes into pancreatic tissue is caused by chemokine release from the pancreas.[19 21] Once recruited, activated leucocytes aggravate the local damage of the pancreatic tissue by release of inflammatory cytokines.[19 20] Although acute pancreatitis is frequently characterised by intrapancreatic haemorrhage, the relationship between activated leucocytes and haemorrhages has not been recognised.

In recent years, progress in digital technologies has led to a substantial improvement of standard methods for intravital imaging. We recently described a novel technology for the investigation of leucocyte locomotion by digital time-lapse intravital microscopy in vivo.[3] This technique includes a continuous video documentation of leucocyte movement with optional time compression. This method allows the monitoring of single adherent or migrating leucocytes and offers dramatic improvements in the study of leucocyte locomotion.

In the present study, leucocyte accumulation in the pancreatic microcirculation was investigated using conventional and digital time-lapse intravital microscopy. Leucocyte activation was analysed both after induction of acute pancreatitis or after direct intrapancreatic application of chemoattractants. The results show for the first time a crucial role of intracapillary leucocyte accumulation in preventing the development of haemorrhagic complications during acute pancreatitis.

MATERIALS AND METHODS

Animals

Lys-EGFP-ki mice were used which express the enhanced green fluorescent protein (EGFP) under control of the lysozyme M promoter leading to an excellent visualisation of neutrophils and monocytes by fluorescence microscopy.[22] To investigate the role of adhesion molecules, lys-EGFP-ki mice were mated with macrophage antigen (Mac)-$1^{-/-}$, lymphocyte function-associated antigen (LFA)-$1^{-/-}$[4] and intercellular adhesion molecule (ICAM)-$1^{-/-}$[23] mice. Male mice, aged 8–12 weeks, were used for experiments.

Induction of acute pancreatitis

Each animal was anaesthetised using an intraperitoneal injection of xylazin (10 mg/kg, Rompun; Bayer, Leverkusen, Germany) and ketamin (50 mg/kg, Ketanest; Parke Davis, Berlin, Germany). Three previously described models of acute pancreatitis were used: (1) five intraperitoneal injections of cerulein at intervals of 1 h (50 μg/kg, Takus; Pfizer Italia, Milan, Italy);[24] (2) intraductal infusion of 50 μl of 2% sodium taurocholate (Sigma Aldrich, Deisenhofen, Germany);[25 26] and (3) intraductal infusion of 50 μl (5 U/ml) of enterokinase solution (Sigma).[27 28] Saline injections under the same conditions served as controls. Leucocyte infiltration was assessed 0, 2, 6 and 24 h after infusion. For microscopic access, the pancreas was removed as a whole, placed onto the microscope slide, and covered with a cover slip. Five capillary fields (total surface 3.75 mm^2) expressing maximal accumulation of EGFP-labelled leucocytes and one venular field were identified using an upright fluorescence microscope (Leica DMRB; Leica, Wetzlar, Germany) and recorded using a colour video camera (CF 20/4DX; Kappa, Gleichen, Germany) and computer. Analysis of intra- and extravascular leucocyte accumulation (expressed as cells per mm^2) was performed using Capimage 8.02 software (Zeintl, Heidelberg, Germany). Spatial distribution of leucocytes in the pancreas was investigated using laser scanning confocal microscopy and three-dimensional reconstruction (TE2000E; Nikon, Düsseldorf, Germany). Pancreatic microvessels were additionally visualised using intravenous injection of tetrarhodamine isothiocyanate (TRITC)-labelled albumin (25 mg/kg BW, Sigma). The changes of pancreatic microperfusion (n = 3/group) were studied 2 h after induction of acute pancreatitis using intravital microscopy as described below.

In an additional series of experiments, the effect of neutrophil depletion on the progression of acute pancreatitis was studied. Neutrophils were depleted from systemic microcirculation by intravenous application of 1 mg/kg BW of anti-Gr1 monoclonal antibody (mAb) (clone RB6-8C5; BioLegend, San Diego, California, USA)[29] prior to the intraductal injection. The control group received isotype mAb (rat IgG2; BD Biosciences, Heidelberg, Germany). The efficacy of neutrophil depletion was controlled by leucocyte counting in blood smears.

One fragment of the pancreas was fixed in 4% buffered formalin. Sections of 5 μm thickeness were cut and stained with haematoxylin (Fluka, Steinheim, Germany) and eosin (Riedel-de-Haën, Seelze, Germany). Histomorphological changes such as oedema, necrosis and haemorrhages were assessed using a scoring system (0 = none; 1 = mild; 2 = moderate; 3 = severe changes) as described previously.[30] Another fragment of the pancreas was snap-frozen in liquid nitrogen for the measurement of trypsin activity. Trypsin activity was analysed using pancreatic tissue extract and the fluorochromic substrate Gln-Ala-Arg-AMC (where AMC is 7-amino-4-methylcoumarin) as previously described.[31 32] Serum amylase and lipase were measured using the ADVIA-2400 chemistry system (BayerHealthCare, Leverkusen, Germany).

Intrapancreatic injection of chemotactic substances

Local intrapancreatic release of chemotactic substances is a triggering mechanism of leucocyte recruitment in acute pancreatitis. In order to analyse the role of chemotactic stimulation, this mechanism was mimicked by the direct injection of chemoattractants into the pancreas of anaesthetised animals. For this purpose, the abdomen was opened by midline incision under aseptic conditions. Saline (10 μl) or one of the following chemotactic substances was injected into the pancreas using a 20 μl microsyringe (Hamilton, Reno, Nevada, USA): interleukin 8 (IL8, 20 μg/ml; R&D Systems, Minneapolis, Minnesota, USA), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (10^{-4} mol/l, Sigma) or phorbol 12-myristate 13-acetate (PMA) (10 μg/ml, Sigma). Leucocyte infiltration was assessed 2 h after intrapancreatic injection as described above. To identify the population of infiltrating leucocytes, the pancreas was removed 1 h after injection of IL8, minced through a 40 μm nylon mesh (BD Biosciences) and analysed using flow cytometry (Epics XL-MCL; Beckman Coulter, Fullerton, California, USA). To demonstrate the role of coagulation and thrombocytes in capillary occlusion, intrapancreatic injection of IL8 was accompanied by an intravenous injection of heparin (75 U; B. Braun, Melsungen, Germany), thrombocyte-depleting mAb MwReg30 (30 μg; BD Biosciences) or isotype mAb (rat immunoglobulin G (IgG)). To analyse the role of specific adhesion molecules, IL8 was injected into lys-EGFP-ki/Mac-$1^{-/-}$, lys-EGFP-ki/LFA-$1^{-/-}$ and lys-EGFP-ki/ICAM-$1^{-/-}$ mice.

The expression of ICAM-1 on endothelial cells was analysed in non-treated wild-type and in ICAM-$1^{-/-}$ animals using immunofluorescence on histological slides with Alexa488-labelled anti-ICAM-1 (clone YN1/1.7.4; eBioscience, San Diego, California, USA) and Alexa568-labelled anti-CD31 mAb (clone MEC13.3; BD Biosciences).

Table 1 Histomorphological changes and pancreatic enzymes 6 h after induction of acute pancreatitis: three models of acute pancreatitis (intraperitoneal application of cerulein, intraductal injection of taurocholate or enterokinase solution) were used

Parameter	Saline, ip	Cerulein, ip	Saline, id	Taurocholate, id	Enterokinase, id
Oedema	0	1.8 (0.4)*	0	1.2 (0.3)*	1.3 (0.2)*
Necrosis	0	0.4 (0.2)*	0	1.5 (0.3)*	1.3 (0.4)*
Infiltration	0	1.4 (0.4)*	0	0.8 (0.4)*	0.8 (0.3)*
Amylase (kU/l)	1.5 (0.1)	22.4 (6.9)*	26.4 (6.1)	28.0 (9.0)	29.1 (5.1)
Lipase (kU/l)	0.2 (0.1)	2.4 (0.9)*	3.3 (1.2)	5.1 (2.7)	3.7 (1.1)
Trypsin (RFU/mg protein)	<0.01	0.03 (0.01)*	0.03 (0.01)	0.30 (0.08)*	2.04 (0.10)*

Results are given as mean (SEM).
*Significant differences ($p<0.01$) compared to the respective control (saline ip or id) group.
id, intraductally; ip, intrapancreatically; RFU, relative fluorescence unit.

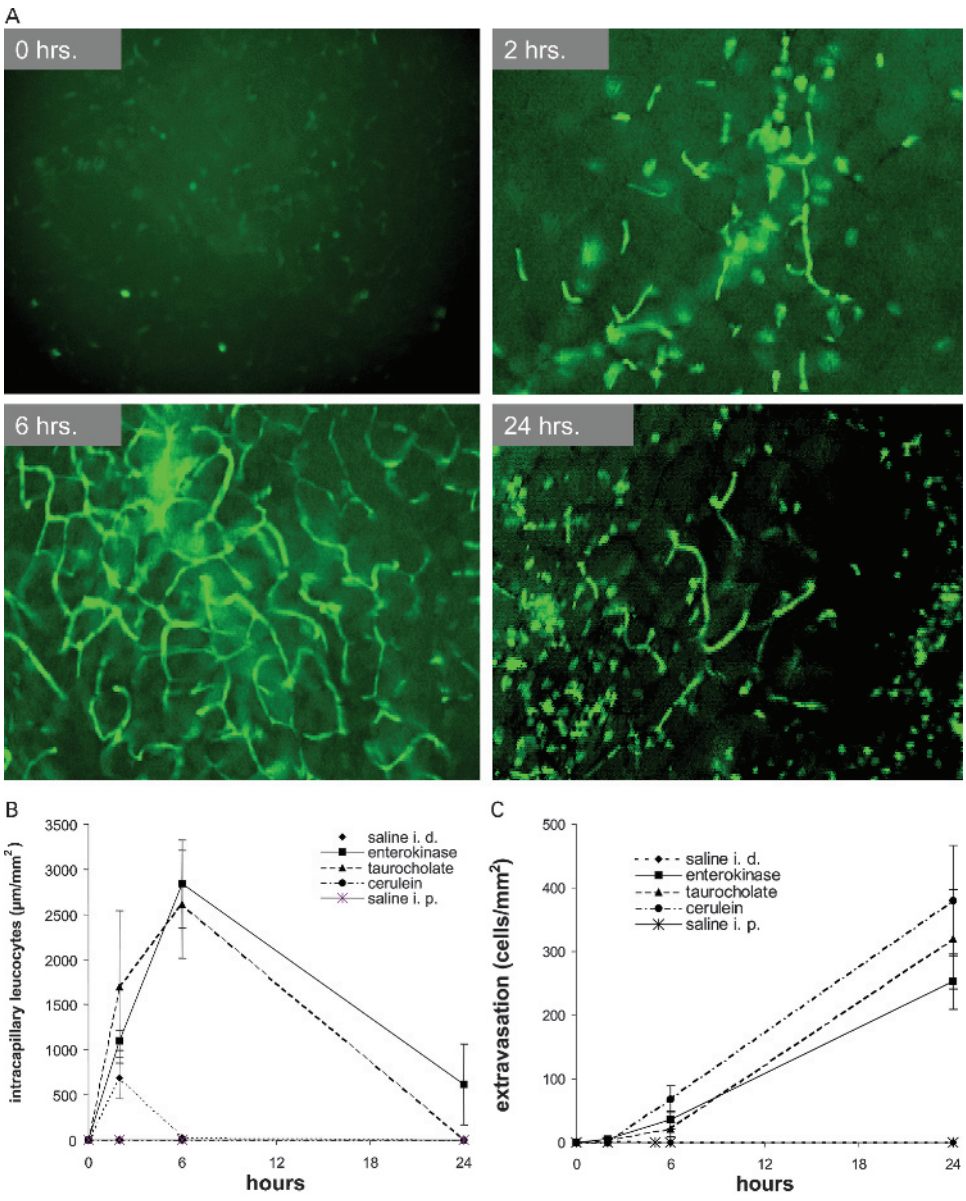

Figure 1 Progression of intracapillary leucocyte accumulation (ILA) during acute pancreatitis: Acute pancreatitis was induced by intraductal injection of enterokinase (5 U/ml; 50 µl). Intracapillary leucocyte accumulation and extravasation were assessed by fluorescence microscopy. (A) Representative images show typical distribution of EGFP fluorescent leucocytes in the pancreas: absent infiltration (baseline), moderately expressed intracapillary accumulation (2 h), strong capillary accumulation (6 h), stage of mixed intracapillary accumulation/extravasation (24 h). Intracapillary leucocyte accumulation preceded extravasation, reached a maximal level 6 h after induction of acute pancreatitis and decreased in reversed correlation with the progress of leucocyte extravasation. (B) Kinetics of ILA. (C) Kinetics of leucocyte extravasation. n = 6/group. EGFP, enhanced green fluorescent protein.

Digital time-lapse intravital microscopy

Animals were anaesthetised as described above. A polyethylene catheter (diameter 0.5 mm; RCT, Heidelberg, Germany) was inserted into the right internal jugular vein as described above. A second catheter was placed into the left carotid artery for monitoring of arterial blood pressure using a custom-made monitoring system. Attention was paid to preserve sterile conditions during the surgical preparation and during intravital microscopy. The mouse was placed on a special stage where temperature was automatically maintained at 37°C. A single pancreatic lobe was immobilised in a temperature-controlled (37°C) immersion chamber which contained sterile 80 ml buffered Ringer's solution.

To investigate the effect of fMLP, the animals were allocated to one of the following groups (n = 6/group): control, local application of 10^{-4} mol/l, 10^{-5} mol/l or 10^{-6} mol/l fMLP, which was dissolved in 100 µl DMSO and applied locally into the immersion chamber. The effect of IL8 (50 ng/ml) was investigated after local application of these proteins into the immersion chamber and compared with controls (n = 6/group).

Intravital microscopy was performed using a custom-made microscopy workstation (LaVision Biotec, Bielefeld, Germany). The perfusion of pancreatic capillaries was visualised by the injection of PKH-26-labelled rat erythrocytes (1 ml/100 g of body weight (BW), haematocrit 50). Labelling of erythrocytes with PKH-26 (Sigma) was performed according to the manufacturer's instructions. The imaging was started with the real-time recording of pancreatic perfusion. Briefly, two random microscopic fields containing microvessels filled with PKH-26-labelled erythrocytes were recorded for 10 s on video tape using an analogue video camera. These recorded images were used for further off-line analysis of erythrocyte velocity. For recording freeze images, five microscopic fields with accumulated leucocytes were selected and recorded at the following time points: 0, 60, 120 and 180 min. Time-lapse microscopy was performed at the following time periods: 0–30, 60–90 and 120–150 min. For the time-lapse mode, digital images were saved on a computer as a line of consecutive images (1 image/10 s, TIF format) using the Imspector software (LaVision Biotec). Exposure time was set to 800 ms for recording of PKH-26-labelled erythrocytes

which were tracing the capillary network within a single frame. The exposure time for leucocytes was adjusted to 100 ms. The images were superimposed and processed to video sequences in uncompressed AVI format. These sequences exhibited the leucocyte movement and capillary perfusion with optional acceleration.

The software Capimage was used for computer-assisted video analysis.[3] The erythrocyte velocity and the velocity of migrating leucocytes was measured by using "frame-to-frame" analysis. The capillary perfusion was measured by capillary length, which was indicated by fluorescent erythrocytes, calculated as a mean value of all five fields and expressed as its proportion to the baseline value.

Statistical analysis

Statistical analysis was performed using SPSS software (version 11.5.1). All data are given as mean with the SEM. The Wilcoxon or Mann–Whitney U-test was used if appropriate. The Kaplan–Meier estimation was used to analyse animal survival after induction of acute pancreatitis. The associations between control and mAb-treated animals were examined by the log-rank test. Statistical significance was assumed at $p \leqslant 0.05$.

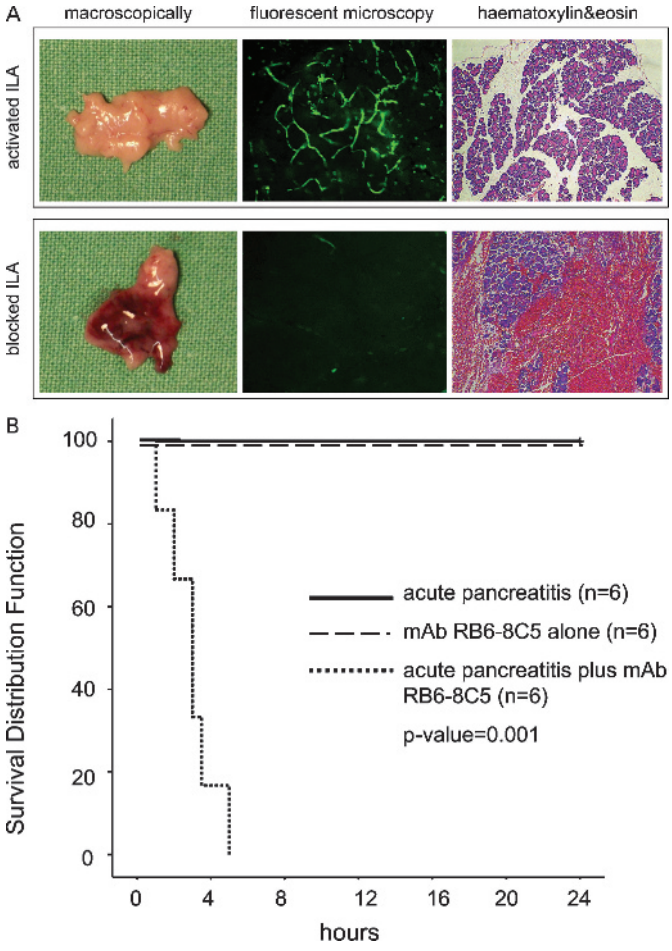

Figure 2 Effect of the prevention of intracapillary leucocyte accumulation (ILA) on the progression of acute pancreatitis (enterokinase model). (A) Representative macroscopic view, fluorescent image of intracapillary leucocytes and histological image of pancreatic tissue; upper panels: acute pancreatitis; lower panels: acute pancreatitis combined with ILA prevention. (B) Kaplan–Meier analysis of survival time. Prevention of ILA by RB6-8C5 monoclonal antibody resulted in the development of massive intrapancreatic haemorrhages (A) and in 100% lethality during 1–5 h after induction of acute pancreatitis (B).

RESULTS

Acute pancreatitis

Histomorphological changes and enzymes after induction of acute pancreatitis

Histomorphological and biochemical parameters of three models of acute pancreatitis are summarised in table 1. Cerulein-induced pancreatitis was characterised by minimal trypsin activity and by lowest necrosis. In contrast, intraductal applications of taurocholate or enterokinase resulted in a strong increase of trypsin activity and were accompanied by formation of extended necrotic areas. Intraductal injection of saline induced a short-time increase of amylase and lipase without histological changes.

Intracapillary leucocyte accumulation precedes leucocyte extravasation during the initial phase of acute pancreatitis

We investigated the time course of intra- and extravascular leucocyte accumulation during progression of acute pancreatitis. Fluorescence microscopy of pancreatic tissue showed that the baseline leucocyte count was near zero in healthy pancreatic tissue. Following induction of acute pancreatitis, ILA occurred. Surprisingly, accumulating leucocytes were not located in the extravascular space, but rather formed dense structures within pancreatic capillaries and obstructed the capillary lumen (fig 1A). Because the margins of single leucocytes within leucocyte accumulation could not be distinguished, the density of ILA was measured as the total length and expressed per mm^2. ILA showed the highest expression 6 h after induction of acute pancreatitis which resulted in dense filling and occlusion of almost all visible capillaries in some areas of the pancreas (fig 1B,C). ILA was most evident after taurocholate or enterokinase injection, but was absent after application of cerulein (fig 1A,B). ILA was reversed 24 h after induction of acute pancreatitis and was accompanied by a concomitant increase of leucocyte extravasation (fig 1A,C). Interestingly, ILA was identifiable only in the whole-mount pancreatic tissue (fig 1A; supplementary videos 1A,B), but not in thin histological slides.

Intravital microscopy of pancreatic tissue demonstrated that intracapillary erythrocyte velocity strongly increased from 0.57 (SEM 0.08) mm/s in the control to 1.44 (SEM 0.07) mm/s in cerulein pancreatitis ($p<0.05$). Intracapillary erythrocyte velocity decreased in enterokinase pancreatitis (0.33 (SEM 0.05) mm/s).

Prevention of intracapillary leucocyte accumulation transforms non-lethal acute pancreatitis into lethal haemorrhagic disease

To address the importance of intracapillary leucocyte accumulation on disease progression in acute pancreatitis, we temporarily depleted neutrophils from the peripheral circulation by systemic injection of anti-Gr1 mAb RB6-8C5. The number of blood neutrophils decreased from 25 (SEM 7)% at baseline to zero within 1–5 min and remained at zero level up to 60 min after mAb injection. Neutrophil depletion resulted in an effective blockade of ILA as demonstrated by fluorescence microscopy (fig 2A). Interestingly, prevention of ILA led to the development of severe acute pancreatitis and death of the animal within 1–5 h after induction of acute pancreatitis (fig 2A). Histologically, neutropenic mice developed fulminant intrapancreatic haemorrhage (fig 2B) while control mice

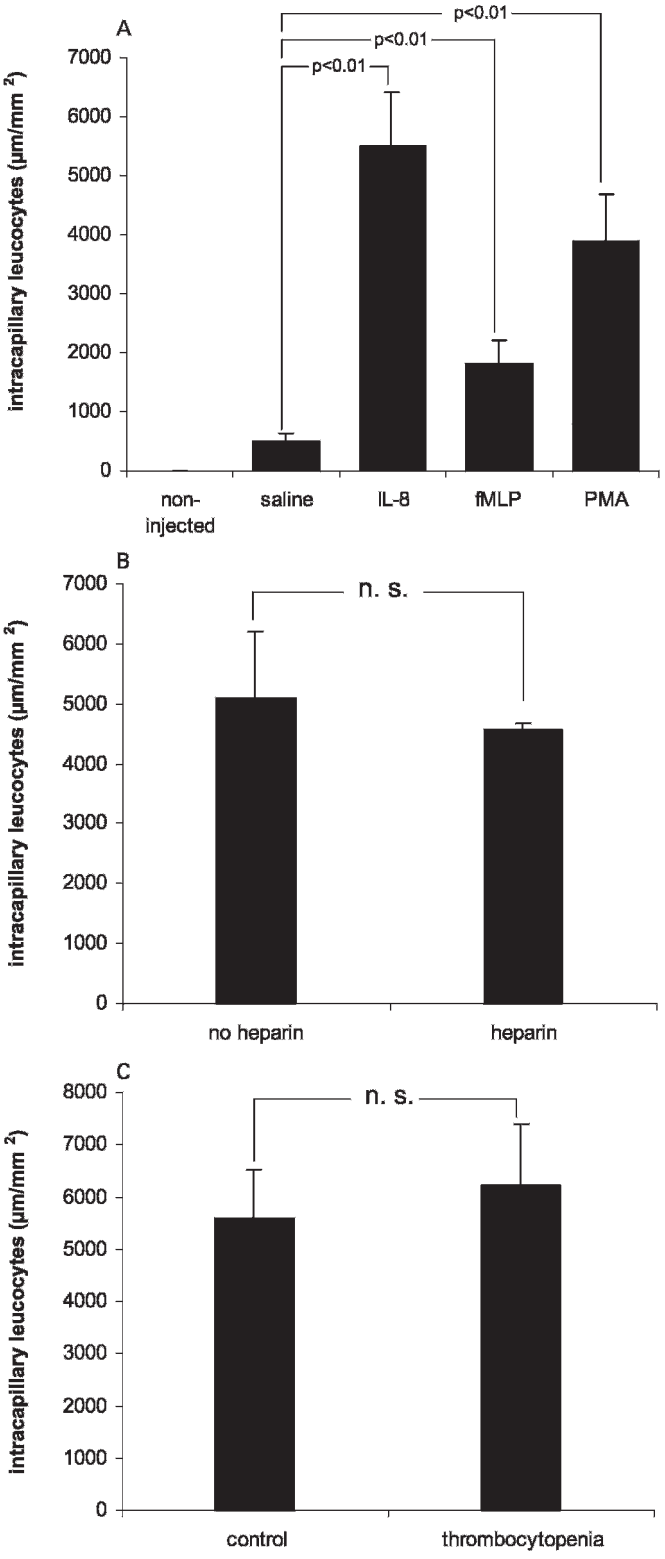

Figure 3 (A) Induction of intracapillary leucocyte accumulation (ILA) by different chemotactic substances. Ten microlitres of chemoattractant solution (IL8, fMLP or PMA) or saline were injected into the pancreas. ILA was analysed 2 h after injection. All chemoattractants induced strong ILA in the pancreas. (B) Effect of anticoagulation or (C) thrombocytopenia on ILA. Heparin, monoclonal antibody MwReg30 or rat immunoglobulin G were injected intravenously. ILA was measured 2 h after intrapancreatic injection of IL8. No significant differences between treatment and control groups were found. fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IL, interleukin; PMA, phorbol 12-myristate 13-acetate.

survived acute pancreatitis without any haemorrhagic changes (fig 2).

Intrapancreatic injection of chemotactic substances

Intracapillary leucocyte accumulation is a result of chemotactic signalling

In order to study the relationship between chemotactic signalling and leucocyte recruitment without induction of complex acute pancreatitis, chemotactic substances including IL8, fMLP or PMA were directly injected into the pancreas. All chemoattractants induced ILA (fig 3) which was morphologically identical to ILA induced in the pancreatitis model. Histological analysis of pancreatic tissue showed no necroses although almost all capillaries were obstructed by leucocytes in the areas where chemoattractants had been applied.

To identify leucocyte populations participating in ILA, we injected IL8 intrapancreatically and removed the pancreas 1 h thereafter. Prior to removal of the pancreas, the development of ILA and the absence of leucocyte extravasation were confirmed by fluorescence microscopy. Flow cytometric analysis of leucocytes isolated from removed pancreatic tissue showed that ILA was merely caused by neutrophils (86 (SEM 3)%) and to a minor degree by monocytes (14 (SEM 3)%).

Intracapillary leucocyte accumulation is not affected by anticoagulation or thromocytopenia

To exclude a contribution of the coagulation system or platelets in ILA, we performed additional experiments where we injected heparin or the anti-gpIIb mAb MwReg30 into mice before injection of IL8. The platelet count decreased to 29 (SEM 4)% of baseline value 2 h after injection of anti-gpIIb mAb MwReg30. Both anticoagulation and thrombocytopenia did not cause significant changes of ILA ($p>0.05$; fig 3B,C) rendering a role of the coagulation system or platelets in mediating ILA in vivo unlikely.

Intracapillary leucocyte accumulation is attenuated in ICAM-$1^{-/-}$ and LFA-$1^{-/-}$, but not in Mac-$1^{-/-}$ mice

Because IL8 induced the strongest induction of ILA when compared to fMLP and PMA, IL8 was selected for further experiments, which aimed to identify the molecular mechanisms of intracapillary accumulation in the pancreas. IL8 was injected intrapancreatically into lys-EGFP-ki/ICAM-$1^{-/-}$, lys-EGFP-ki/LFA-$1^{-/-}$, and lys-EGFP-ki/Mac-$1^{-/-}$ mice. As shown in the fig 4A, ILA was strongly attenuated in ICAM-$1^{-/-}$ and LFA-$1^{-/-}$ mice compared to control lys-EGFP-ki mice ($p<0.01$) although some ILA could still be found in the absence of ICAM-1 and LFA-1. In contrast, there was no significant difference in the occurrence of ILA between Mac-$1^{-/-}$ and control mice. Of note, transvenular leucocyte extravasation was strongly attenuated in lys-EGFP-ki/ICAM-$1^{-/-}$, lys-EGFP-ki/LFA-$1^{-/-}$, and lys-EGFP-ki/Mac-$1^{-/-}$ mice compared to control lys-EGFP-ki mice (fig 4B).

Immunofluorescence staining of histological slides showed that almost all pancreatic microvessels express ICAM-1 (fig 4C). No binding of YN1/1.7.4 mAb was found in ICAM-$1^{-/-}$ mice.

Digital time-lapse intravital microscopy

Intracapillary leucocyte accumulation in resting and stimulated pancreas

ILA was investigated in fMLP- or IL8-treated pancreatic tissue using digital time-lapse intravital microscopy. Throughout the microscopic observation period, arterial blood pressure was

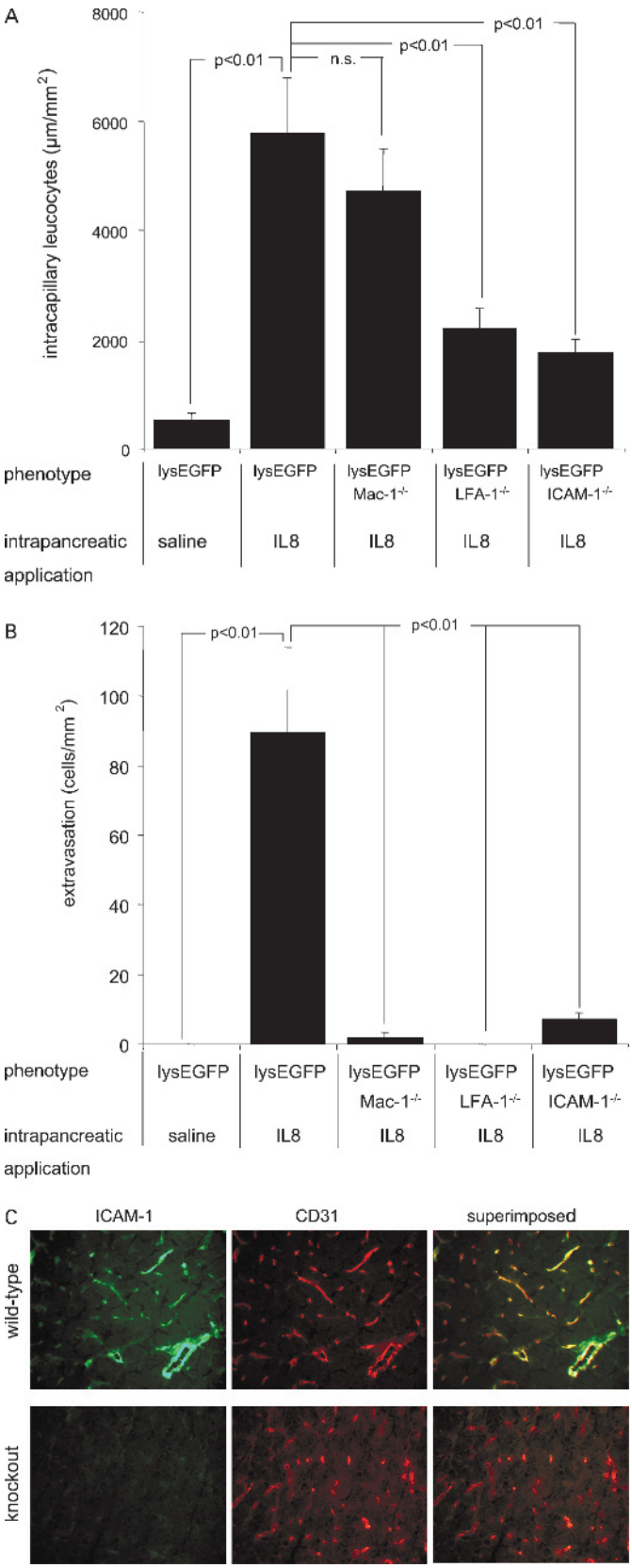

Figure 4 Role of adhesion molecules in the induction of intracapillary leucocyte accumulation (ILA). Ten microlitres of IL8 was injected into the pancreas of control lys-EGFP-ki mice and of the following knockout mice: lys-EGFP-ki/ICAM-1$^{-/-}$, lys-EGFP-ki/LFA-1$^{-/-}$ and lys-EGFP-ki/Mac-1$^{-/-}$ mice. ILA (A) and transvenular leucocyte extravasation (B) were analysed 2 h after injection of IL8. ILA was strongly attenuated in ICAM-1$^{-/-}$ and LFA-1$^{-/-}$ mice compared with control animals. There was no significant difference of ILA between Mac-1$^{-/-}$ and control mice. Leucocyte extravasation was strongly attenuated in all three knockout strains compared to the control. (C) Representative fluorescent images of ICAM-1 expression in the pancreas. To label ICAM-1-expressing microvessels, histological slides were stained with Alexa488-labelled anti-ICAM-1 mAb (green) and Alexa568-labelled anti-CD31 mAb (red). All pancreatic capillaries and venules of wild-type animals expressed ICAM-1. Blood vessels of ICAM-1$^{-/-}$ mice did not bind anti-ICAM-1 mAb. EGFP, enhanced green fluorescent protein; ICAM, intercellular adhesion molecule; IL, interleukin; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; Mac, macrophage antigen.

stable. Capillary perfusion and mean erythrocyte velocities did not significantly change in control mice during 3 h of observation (fig 5B; supplementary video 2A). In unstimulated pancreatic tissue, only single leucocytes were found to occasionally plug capillaries and did that only for a short time (several seconds) before re-entering the circulation (fig 5A). In contrast to controls, the local application of both fMLP and IL8 caused a rapid ILA (fig 5A,E; supplementary video 2B). Time-lapse video sequences showed that the first plugged leucocytes appeared in superficial layers of the pancreas within 2–4 min after application of chemotactic agents (supplementary video 3A). Once plugged, leucocytes occluded the capillary lumen and migrated actively within the capillary network. Migrating leucocytes accumulated in the capillary network of outer pancreatic layers causing stable occlusion of the capillary cross section in those capillaries (fig 6; supplementary videos 3A–C). The number of accumulating leucocytes (fig 5A), the aggravation of capillary perfusion (fig 5B) and the reduction in erythrocyte velocity (fig 5C) were strongly dependent on the concentration of the chemotactic substance. If the fMLP- or IL8-containing immersion solution was exchanged by Ringer's solution, ILA stopped, plugging leucocytes slowly lost their polarised shape, became round, detached from endothelium and re-entered the peripheral circulation.

Intracapillary leucocytes crawl actively but do not extravasate

Digital time-lapse microscopy showed that the migration velocity of intracapillary leucocytes was highest at the beginning of the observation period and decreased continuously thereafter (fig 5D). Surprisingly, leucocytes migrating in the capillary lumen never entered the extravascular space (fig 6/frame A). Using time-lapse microscopy, we found that leucocyte extravasation occurred only via postcapillary and collecting venules of pancreatic tissue and peripancreatic fat tissue, but not via capillaries (fig 6/frame B; supplementary video 3A–C). Without chemoattractants, leucocytes did not firmly adhere to the venular endothelium. However, application of fMLP or IL8 to the immersion chamber induced firm leucocyte adhesion to the venular endothelium without extravasation during early time points. Leucocyte extravasation was observed only in those experiments where 10^{-4} mol/l fMLP was applied for more than 60 min or 10^{-5} to 10^{-6} mol/l fMLP was given for more than 120 min.

DISCUSSION

In the present study we demonstrate, for the first time, that intracapillary leucocyte accumulation represents an initial step of leucocyte activation during acute pancreatitis consisting in the rapid promotion of leucocyte adhesion to capillary endothelium and intraluminal intracapillary crawling of leucocytes and leading to sustained capillary occlusion. The vast majority of intracapillary accumulating leucocytes is

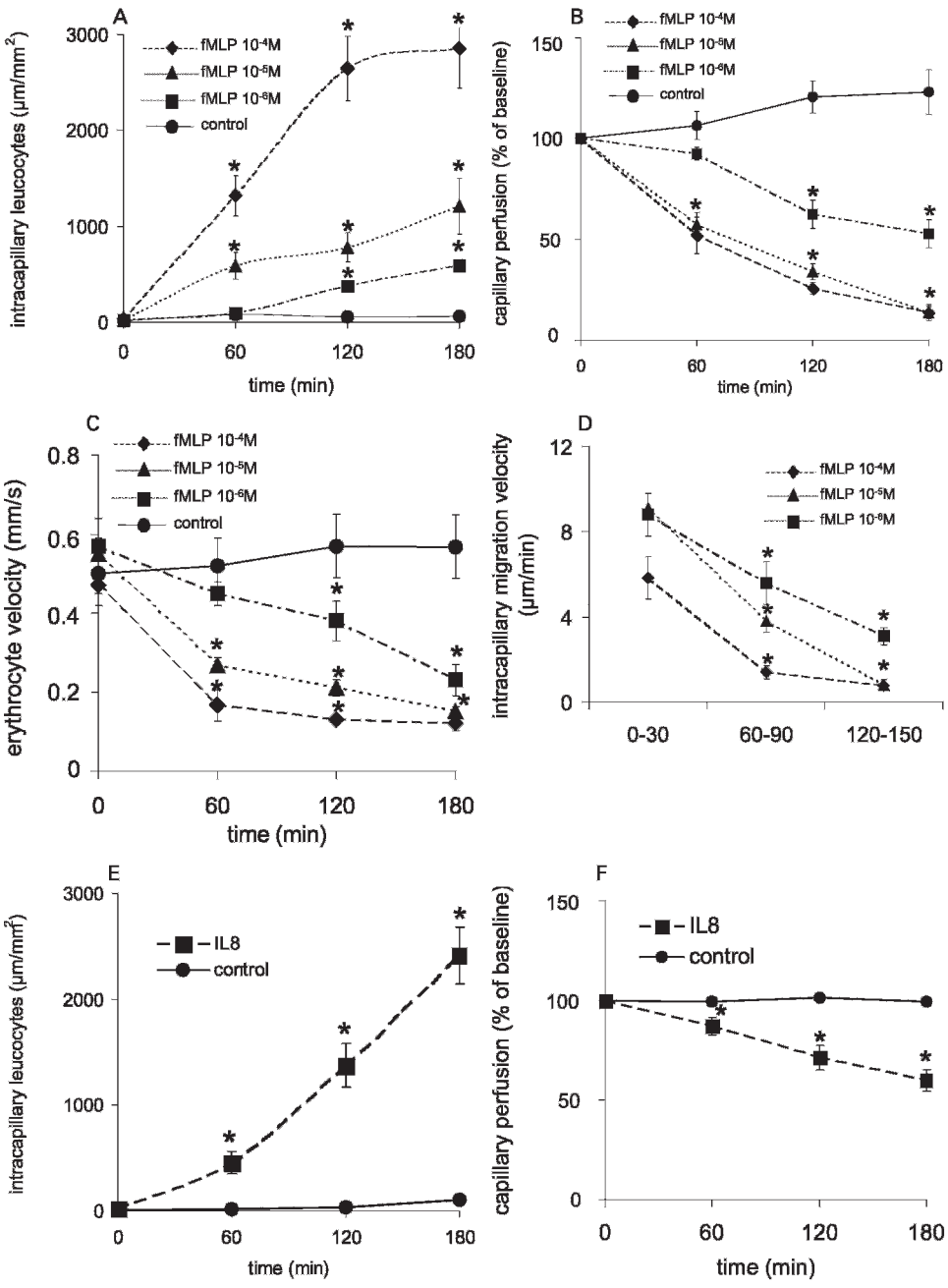

Figure 5 Analysis of intracapillary leucocyte accumulation (ILA) using digital time-lapse microscopy, which was used to study the accumulation of leucocytes in pancreatic capillaries. (A–D) Effect of fMLP. Dose- and time-dependent progression of ILA (A), progressed aggravation of capillary perfusion (B), erythrocyte velocity in perfused capillaries (C), and the migration velocity of intracapillary leucocytes (D). Groups: control (circles, solid line), 10^{-6} mol/l (squares; dash–dot line), 10^{-5} mol/l (triangles; dotted line), 10^{-4} mol/l (diamonds; long-dash line). (E,F): Effect of IL8. Immersion of the pancreas with IL8 induced ILA (E) and a significant reduction of microperfusion (F). *Significant differences compared to controls. fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IL, interleukin.

represented by neutrophils and restricted to the area where the stimulus had been applied. ILA was only identifiable in the whole-mount tissue, not in conventional histological slides, demonstrating the three-dimensional nature of this phenomenon.

Activation of trypsinogen to trypsin is the key mechanism of the enzyme activation cascade during acute pancreatitis.[33 34] In the present study, high trypsin activity and extended necrosis were found in taurocholate- or enterokinase-induced pancreatitis, but not in cerulein-induced pancreatitis. Furthermore, non-haemorrhagic necrotising pancreatitis was immediately transformed into lethal haemorrhagic disease if the intracapillary leucocyte accumulation was prevented. Probably, activated pancreatic enzymes injured exocrine tissue as well as pancreatic capillaries which became permeable for erythrocytes. Interestingly, the capillary occlusion through the rapid induction of ILA completely prevented capillary haemorrhage and was lifesaving. Thus, ILA represents a newly discovered haemostatic mechanism which has a protective function and aims to prevent capillary haemorrhage by closing injured capillaries. This finding is closely related to the clinical situation since intrapancreatic haemorrhage frequently accompanies pancreatic necrosis in humans.[35] We propose that intrapancreatic haemorrhage can only be prevented if the speed of ILA balances the speed of progression of capillary injury. Haemorrhage appears if capillary injury exceeds ILA progression. Our experiments strengthen this hypothesis and show that the intraductal injection of high enterokinase concentration (15 U/ml) causes rapid development of haemorrhagic pancreatitis in spite of ILA induction.

Acute pancreatitis in humans and in animal models is frequently accompanied by haemostatic abnormalities.[36 37] Both ILA and thrombosis can occur in acute pancreatitis.

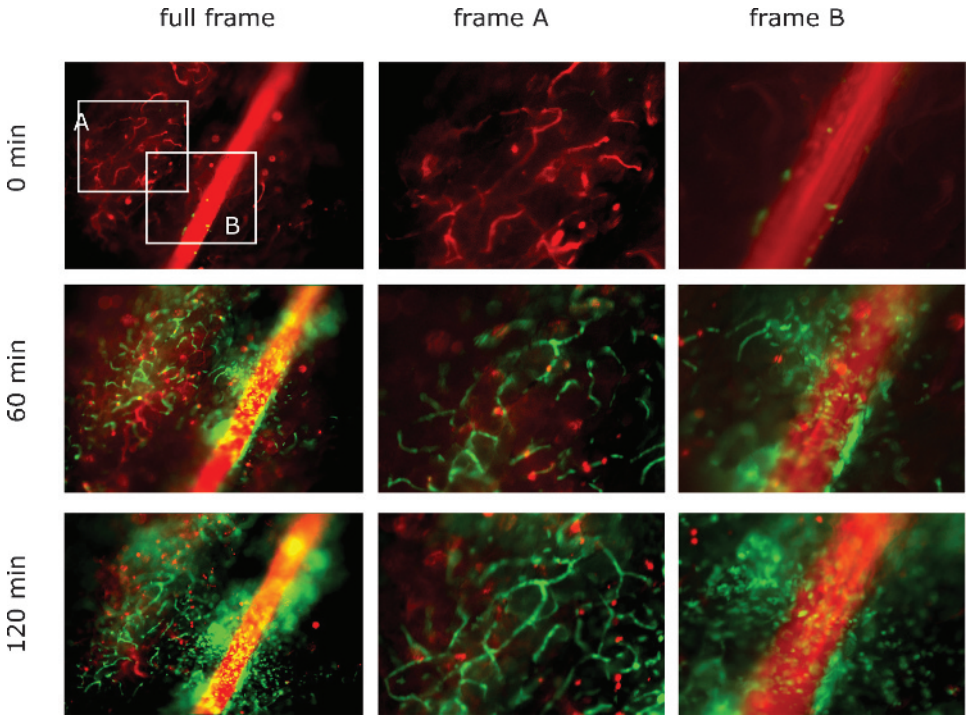

Figure 6 Representative images of the progressing capillary occlusion by active leucocytes and transvenular leucocyte extravasation after local application of fMLP. EGFP+ leucocytes are shown as green cells. Perfused capillaries are shown in red. Occluding leucocytes were absent at baseline time (0 min). Intracapillary leucocyte accumulation was initiated 3 min after application of fMLP and was continuously increased during the observation period. The number of perfused capillaries decreased continuously over time in accordance with the progressing occlusion of capillaries by leucocytes. Frame A depicts intracapillary accumulating leucocytes. Frame B shows transvenular leucocyte extravasation. The time after fMLP application is indicated. EGFP, enhanced green fluorescent protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

Although ILA and thrombosis have a haemostatic function, they show several fundamental differences. In contrast to thrombosis, which can occur in all part of the vascular system, ILA can be found only in capillaries. ILA is fast-resolving; it reached the maximum at 6 h and disappeared 24 h after induction of acute pancreatitis. Furthermore, ILA is independent of coagulation and platelets since we have shown that inhibition of coagulation or thrombocytopenia did not influence ILA.

In contrast to enterokinase pancreatitis, only cerulein pancreatitis was accompanied by a strong increase in erythrocyte velocity in pancreatic capillaries. This phenomenon of cerulein-induced hyperperfusion has been previously described in the rat pancreas.[38 39] We believe that the increase in shear stress may represent the factor that is responsible for the absent ILA in cerulein-induced pancreatitis. The absent ILA did not result in haemorrhage because cerulein pancreatitis was accompanied by very low trypsin activity and tissue damage. It is also possible that active proteases contribute to ILA through activation of adhesion molecules.[40 41]

The protective function of leucocytes in acute pancreatitis is novel since leucocytes, especially infiltrating neutrophils, were believed to contribute to disease progression. Previous experimental studies showed that infiltrating neutrophils are strongly involved in the damage of pancreatic and lung tissue.[42] In addition, it has been shown that inhibition of leucocyte extravasation or the depletion of neutrophils reduces the severity of pancreatitis and lung injury.[43 44] These results are not contradictory to the findings of the present study. ILA and leucocyte extravasation/infiltration may be considered to represent two distinct processes with different biological functions: ILA occurs early during leucocyte activation and exhibits a protective effect whereas subsequent leucocyte extravasation leads to leucocyte infiltration which can cause significant tissue damage. Since leucocyte extravasation but no ILA occurs in a cerulein model of acute pancreatitis, neutrophil depletion in this model may be accompanied by beneficial effects which have been described by other authors.[45 46]

Furthermore, extravasation is not a primary function of ILA. If intracapillary leucocytes emigrate from capillaries, they would not be able to maintain the stable occlusion of injured capillaries and therefore prevent haemorrhage as shown in our experiments. Thus, leucocytes adhere and migrate both in venules and in capillaries, but their functions are different: intravenular adhesion is a preceding step of leucocyte extravasation whereas intracapillary adhesion represents a protective anti-haemorrhagic mechanism. We believe that the different presentation of adhesion molecule or chemokines on venular and capillary endothelium may be responsible for the absent extravasation from capillaries.

Interestingly, the pancreatic tissue itself can resist ILA-related hypoperfusion since chemotattractant-induced ILA led to extensive capillary occlusion, without inducing hypoperfusion-related oedema and necrosis. This finding contrasts with previous studies which attributed capillary occlusion by plugged leucocytes as the only factor inducing ischaemic tissue injury.[9 10] However, ILA progressed only in the initial stage after induction of acute pancreatitis, while it decreased and disappeared in the later stage of the disease. Persistent capillary occlusion would likely cause tissue damage and necrosis.

IL8 and fMLP activate Mac-1 and LFA-1[47 48] which classically interact with endothelial ICAM-1 and promote leucocyte adhesion to venular endothelium.[1] In the current study, all applied chemoattractants showed strong activation of ILA. Interestingly, IL8 of human origin acts as a powerful chemotactic substance in mice, although mice do not produce IL8.[49]

In the current study, we analysed transvenular leucocyte extravasation and showed that it was strongly reduced in ICAM-1$^{-/-}$, LFA-1$^{-/-}$ and Mac-1$^{-/-}$ mice. Interestingly, the molecular mechanisms of ILA differ from those seen during leucocyte extravasation, since ILA was attenuated in LFA-1$^{-/-}$ and ICAM-1$^{-/-}$, but not in Mac-1$^{-/-}$ mice. The important role

of ICAM-1 as well as functional differences between LFA-1 and Mac-1 in the leucocyte adhesion cascade have previously been shown by several investigators.[50 51]

Another important question arises from the present study: is ILA a pancreas-specific phenomenon or not? Preliminary results of our group show that ILA is not an organ-specific process. Dense leucocyte accumulation was also induced by local chemotactic stimulation in the liver sinusoids. Additional investigations will be required to clarify the role of intracapillary leucocyte accumulation in other organs.

Taken together, we provide substantial evidence that the occlusion of pancreatic capillaries by actively migrating leucocytes can be considered a preceding event of the classical leucocyte adhesion cascade. This process is LFA-1- and ICAM-1-dependent and plays a protective and potentially lifesaving role in the progression of acute pancreatitis.

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Intracapillary leucocyte accumulation as a novel antihaemorrhagic mechanism in acute pancreatitis in mice

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