Journal of Pathology
J Pathol January 2022; **256:** 83–92
Published online 29 October 2021 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/path.5811

ORIGINAL PAPER

Inflammatory capacity of exosomes released in the early stages of acute pancreatitis predicts the severity of the disease

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Abstract

As acute pancreatitis progresses to the severe form, a life-threatening systemic inflammation is triggered. Although the mechanisms involved in this process are not yet well understood, it has been proposed that circulating exosomes may be involved in the progression of inflammation from the pancreas to distant organs. Here, the inflammatory capacity and protein profile of plasma exosomes obtained during the first 24 h of hospitalization of patients diagnosed with acute pancreatitis were characterized and compared with the final severity of the disease. We found that the final severity of the disease strongly correlates with the inflammatory capacity of exosomes in the early stages of acute pancreatitis. Exosomes isolated from patients with mild pancreatitis had no effect on macrophages, while exosomes isolated from patients with severe pancreatitis triggered NFκB activation, TNFα and IL1β expression, and free radical generation. To delve deeper into the mechanism involved, we performed a proteomic analysis of the different exosomes that allowed us to identify different groups of proteins whose concentration was also correlated with the clinical classification of pancreatitis. In particular, an increase in the amount of S100A8 and S100A9 carried by exosomes of severe pancreatitis suggests that the mechanism of action of exosomes is mediated by the effect of these proteins on NADPH oxidase. This enzyme is activated by S100A8/S100A9, thus generating free radicals and promoting an inflammatory response. Along these lines, we observed that inhibition of this enzyme abolished all the pro-inflammatory effects of exosomes from severe pancreatitis. All this suggests that the systemic effects, and therefore the final severity of acute pancreatitis, are determined by the content of circulating exosomes generated in the early hours of the process.

Keywords: acute pancreatitis; inflammation; exosomes; S100A8; S100A9

Received 7 April 2021; Revised 14 September 2021; Accepted 29 September 2021

No conflicts of interest were declared.

Introduction

Acute pancreatitis (AP) is a sudden inflammatory process of the pancreas and one of the most common indications for inpatient hospital care in the gastrointestinal field [1,2]. Although in most cases AP is a self-limiting illness, in patients with moderately severe or severe AP, there may be organ failure, as well as local or systemic complications [3]. The Atlanta classification system, developed in 1992 and revised in 2012 [4,5], delineates three grades of severity in AP – mild, moderately severe, and severe.

In mild AP, there is no organ failure and no local or systemic complications. In moderately severe AP, there may be transient organ failure that resolves within 48 h, as well as local or systemic complications. Finally, severe AP progresses with persistent (lasting more than 48 h) organ failure and a high mortality rate [3]. The early stages of severe forms of AP are associated with a systemic inflammatory response syndrome that frequently evolves into acute lung injury. It has been reported that one third of the deaths associated with AP occur during the first week and 50% of those deaths are associated with severe lung injury [6].

Our understanding of the pathophysiology of AP has greatly improved in recent years, although the precise mechanism that links the local pancreatic damage with systemic inflammation, and in particular lung injury, remains elusive. A number of studies have revealed the involvement of cytokines, pancreatic enzymes, reactive oxygen species, and bioactive lipids [7–9], but, unfortunately, this knowledge has not resulted in significant advances in the treatment of severe AP. Recently it has been found that extracellular vesicles, in particular exosomes, may also be involved in this process [10,11]. In experimental studies using a model of taurocholate-induced AP in rats, we have demonstrated that at least two different populations of exosomes are released into the bloodstream in the early stages of AP. The first is released from the pancreas and is retained by the liver, while the second population appears to be released by the liver into the systemic circulation. These hepatic exosomes reach the alveolar space and could be taken up by alveolar macrophages, which in turn are activated, thus generating pro-inflammatory cytokines into the lung microenvironment [11,12].

Despite the obvious interest of these results, they have the usual limitations associated with experimental studies. Herein, we evaluated how the final severity of acute pancreatitis correlates with the characteristics of circulating exosomes obtained from the plasma of patients in the first 24 h after hospital admission. We found a clear correlation between the severity of AP and the pro-inflammatory activity of the exosomes. In addition, the proteomic study of exosomes allowed us to identify three proteins (C-reactive protein, S100A8, and S100A9) strongly correlated with the severity of the process. This has allowed us to suggest the mechanism involved in exosome-dependent macrophage activation during acute pancreatitis.

Materials and methods

Patients

This study was carried out in accordance with the standards of good clinical practice and the international ethical principles applicable to medical research in humans (Declaration of Helsinki) [13]. The study protocol was approved by the Institutional Review Boards of Hospital General Universitario de Alicante (Alicante, Spain), Hospital Universitario Ramón y Cajal (Madrid, Spain), Hospital Clínico Universitario de Valencia (Valencia, Spain), Hospital Costa del Sol (Marbella, Spain), and Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain). The collection of samples from patients with AP was prospective and required informed consent. Plasma samples were collected within a year, within 24 h of admission, from patients with a diagnosis of AP and stored at −70 °C until used for exosome isolation. Diagnosis was based on at least two of the following parameters: increase in serum amylase and/or lipase above three times the upper limit of normal, imaging compatible with AP, and/or typical abdominal pain appearance [5]. Healthy controls were obtained from hospital staff, and also required informed consent. The final severity of AP was categorized retrospectively according to the revised Atlanta classification system as mild, moderately severe, or severe [5]. Exclusion criteria included infections, pregnancy, and time between the onset of symptoms and blood collection greater than 48 h. The clinical characteristics of patients are presented in Table 1.

Exosome isolation

Exosomes were isolated as described previously [10], with some modifications. Plasma samples were centrifuged at 2000 × *g* and 10 000 × *g* for 10 and 30 min, respectively, at 4 °C. The 10 000 × *g* supernatant was recovered, resuspended in PBS, filtered through a 0.22 µm filter, and ultracentrifuged at 120 000 × *g* for 70 min in an ultracentrifuge (Beckman Coulter, Optima L-90K, Brea, CA, USA). Pellets were resuspended in PBS and the remaining soluble proteins were removed

Table 1. Clinical characteristics of patients from the different groups included in the study.

			Gender		Etiology		
	n	Age (range), years	Male	Female	Biliary	Alcohol	Other
Control	20	60 (51–68)	12	8	n/a	n/a	n/a
Mild	20	57 (26–86)	11	9	13	4	3 Idiopathic (2), hyper-TG (1)
Moderate	20	59 (37–89)	11	9	11	7	2 Idiopathic (1), post-ERCP (1)
Severe	20	64 (31–88)	14	6	8	4	8 Idiopathic (6), post-ERCP (1), pharmacological (1)

n/a, not applicable; hyper-TG, hypertriglyceridemia; post-ERCP, post-endoscopic retrograde cholangiopancreatography.

by a final filtration through a 30 kDa cutoff filter. Isolated exosomes were quantified by measuring their protein content. For the proteomic analysis, 10 μg of exosomes from five patients was pooled, thus generating four pools for each group. Each pool included samples of both genders and etiologies. It has been reported that although men had alcohol-induced AP more frequently than women, there was no significant association between gender and the severity of pancreatitis [14].

Additionally, exosome-depleted plasma was generated by mixing plasma, at a 5:1 ratio, with 50% polyethylene glycol 6000 (Merck, Darmstadt, Germany). After incubation at 4 °C for 12 h, samples were centrifuged at 1500 × *g* for 30 min and supernatants were collected and used as exosome-free plasma [15]. Complete plasma was processed in the same way, adding saline solution instead of PEG.

Nanoparticle tracking analysis

The size distribution and concentration of exosomes were measured using a NanoSight LM10 machine (NanoSight, Salisbury, UK). All the parameters of the analysis were set at the same values for all samples and three 1-min-long videos were recorded in all cases. Background was measured by testing filtered PBS, which revealed no signal.

SDS-PAGE and western blotting

Exosome proteins were extracted in RIPA buffer supplemented with protease inhibitors (PMSF, aprotinin, trypsin inhibitor). Cells were lysed using the Nuclear Extract Kit from Active Motif (Carlsbad, CA, USA) under conditions for preparation of nuclear and cytoplasmatic extracts. The concentration of the isolated proteins was determined using a Bradford assay. Proteins were separated on a 12% SDS-PAGE and electrophoretically transferred under wet conditions onto a PVDF membrane (Immun-Blot; Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h in 5% nonfat milk powder in PBS, followed by overnight incubation at 4 °C with antibodies against TSG101 (14497-1-AP) (1:500), ALIX (12422-1-AP) (1:500), calnexin (10427-2-AP) (1:1000), CD81 (10630D) (1:500) (ProteinTech, Rosemont, IL, USA), p65(c20) (1:500) (sc372; Santa Cruz Biotechnology, Dallas, TX, USA), and histone H3 (1:500) (PA5-16183; Thermo Fisher Scientific, Waltham, MA, USA). Blots were washed and incubated for 1 h 30 min at room temperature with the DyLight 800-conjugated secondary antibody. Immunoreactive bands were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) (supplementary material, Figure S1). Western blotting was performed in duplicate. Densitometry was performed using ImageJ/Fiji open source software version 1.51J (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij).

Cell lines and treatments

Human THP-1 cells (Merck) were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher, Waltham, MA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were differentiated into macrophages through a first incubation with 100 nM phorbol 12-myristate 13-acetate (PMA) (Merck) for 24 h. After that, the PMA-containing medium was discarded and replaced with fresh medium for a further 24 h. To evaluate the ability of exosomes to activate macrophages and trigger inflammation, cells

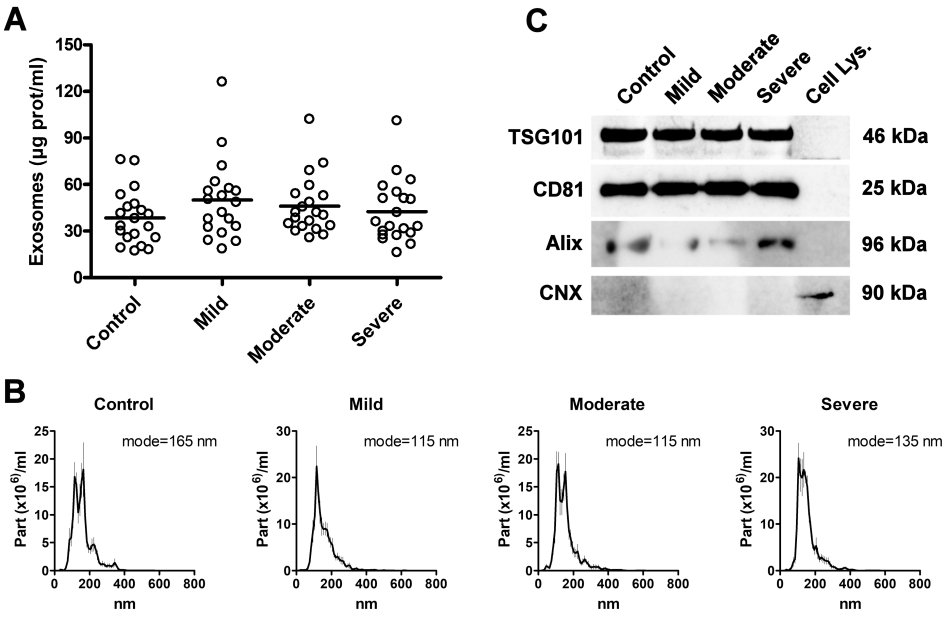

Figure 1. (A) Exosome levels, expressed as μg prot/ml, detected in the plasma of patients with different severities of AP. $n = 20$ per group. (B) Nanovesicle tracking assay confirms that the size of the extracellular vesicles obtained corresponds to exosomes. (C) Western blotting analyses confirm the presence of exosomal protein markers from the different groups. THP1 cell lysates and calnexin (CNX) were included as negative controls.

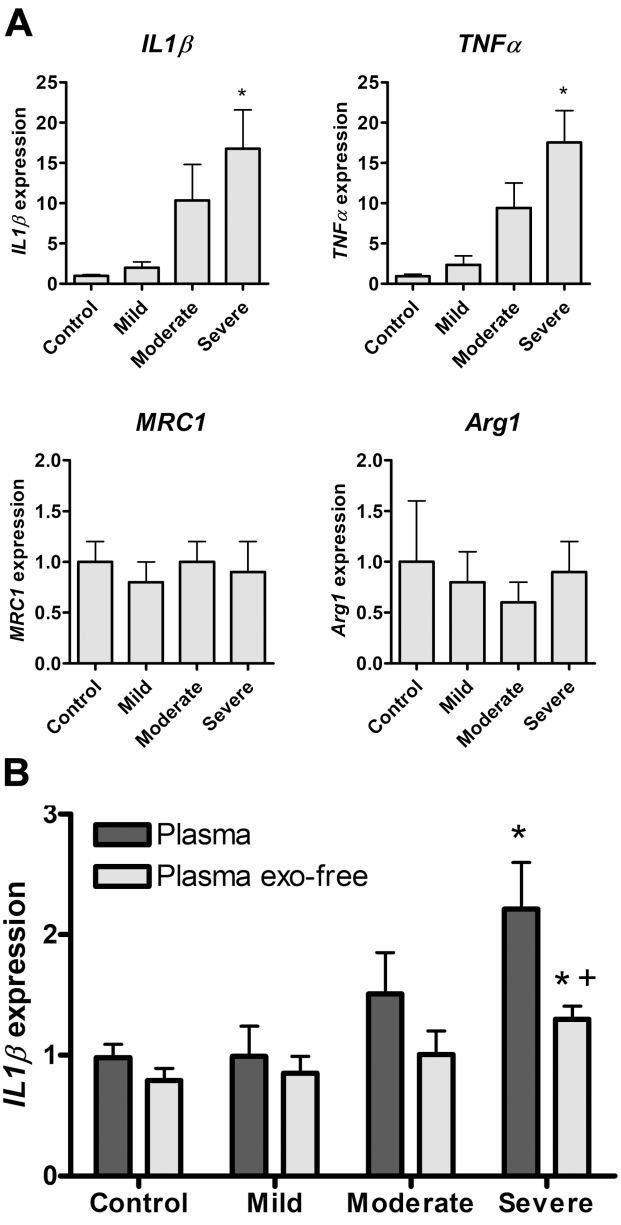

Figure 2. (A) Induction of *IL1β*, *TNFα*, *MRC1*, and *Arg1* mRNA expression in THP1 macrophages treated with 10 µg/ml exosomes for 3 h. (B) Expression of IL1β in THP1 macrophages treated with plasma (15 µl/ml cell culture medium) or with exosome-depleted plasma (exo-free) for 3 h. $n = 20$ per group; $^{*}p < 0.05$ versus control; $^{+}p < 0.05$ versus complete plasma.

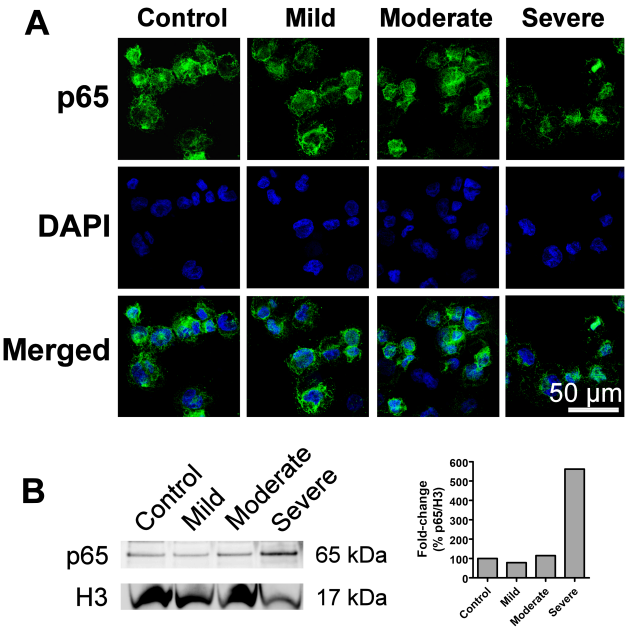

Figure 3. (A) The activation of NFκB was evaluated by immunofluorescence analysis of p65 nuclear translocation after treating THP1 macrophages with 10 µg/ml exosomes for 45 min. Nuclear translocation was observed only in macrophages treated with exosomes from severe pancreatitis. (B) Western blotting for p65 in nuclear extracts of THP1 cells and its densitometric analysis.

were incubated with 10 µg/ml exosomes [10] for 3 h and changes in the expression of inflammatory cytokine mRNAs were evaluated by RT-qPCR. In some experiments, the NADPH inhibitor DPI (10 µM) was added 1 h before the administration of exosomes.

RNA isolation and RT-qPCR

Total RNA from cells was extracted using TRizol reagent (Invitrogen, Waltham, MA, USA) as reported previously [12]. After extraction, cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and the relevant primers (supplementary material, Table S1). The expression of target genes relative to *GAPDH* was calculated by the ΔC(t) formula.

Digestion and TMT labeling

Protein extracts, prepared as described above, were digested with Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) using the FASP (filter aided sample preparation) digestion protocol [16]. Each tryptic peptide mixture was isotopically labeled with the corresponding TMT6plex reagent (Thermo Fisher) based on the standard procedure. TMT-labeled peptide mixtures were combined in a low-bind 1.5 ml Eppendorf tube, evaporated, and desalted using a C18 SPE cartridge (Agilent Technologies, Santa Clara, CA, USA). Quadruplicates for each condition were used. The set of all these samples was analyzed in three parallel TMT-label experiments.

Liquid chromatography–mass spectrometry

Peptides were analyzed using an Orbitrap Fusion Lumos Tribrid mass spectrometer coupled to a Thermo Scientific Dionex Ultimate 3000 ultrahigh-pressure chromatographic system (Thermo Fisher Scientific) as described previously [17].

ELISA for S100A8/S100A9 heterodimer

Since S100A8 and S100A9 are included in the proteins that increase their concentration in exosomes from severe AP and could form heterodimers with pro-inflammatory activity, we measured the presence of this

heterodimer in exosomes using a commercial ELISA (DS8900; R&D Systems, Minneapolis, MN, USA).

Subcellular location of NFκB

To monitor NFκB nuclear translocation, macrophage-differentiated THP1 cells were incubated with exosomes in a humidified atmosphere of 95% air, 5% CO_2. After treatments, cells were fixed with 3.5% formaldehyde for 5 min at room temperature. The cells were incubated with anti-p65 antibody [p65(c20) sc372; Santa Cruz Biotechnology] (1:400) and Alexa Fluor 488-conjugated anti-goat secondary antibody (A11008; Life Technologies, Carlsbad, CA, USA) (1:1000). Nuclear localization was examined by fluorescence microscopy.

Fluorescence microscopy

Cells were observed using an inverted Nikon Eclipse Ti2-E microscope (Nikon Instruments, Melville, NY, USA) attached to an Andor Dragonfly spinning disk unit. Samples were excited with 405 and 488 nm laser diodes. Cells were imaged using a high-resolution scientific complementary metal oxide semiconductor (sCMOS) camera (Zyla 4.2, 2.0 Andor; Oxford Instruments Company, Abingdon, UK). Image processing and analysis were performed using ImageJ/Fiji open source software version 1.51J.

Measurement of reactive oxygen species (ROS) generation

ROS production was measured with the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; ab113851; Abcam, Cambridge, UK). Macrophage-differentiated THP1 cells were plated in 96-well plates. After 24 h, cells were washed and incubated with 10 μM H_2DCFDA in D-PBS for 15 min at 37 °C. Cells were then treated with 10 μg/ml exosomes for 3 h and ROS production was measured using a fluorimeter unit SPECTRAmax GEMINI XS (Molecular Devices, San José, CA, USA) (excitation $\lambda = 484$ nm, emission $\lambda = 535$ nm).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software v. 4.02 (GraphPad Inc, San Diego, CA, USA). Data are presented as mean ± SEM. Data were analyzed using a two-tailed Student's *t*-test for comparison of two groups, and one-way analysis of variance (ANOVA) analysis followed by Tukey's *post hoc* test when three groups were compared. Differences were considered statistically significant when $p < 0.05$.

For mass spectrometry analysis, database searching was conducted using Proteome Discoverer v2.1 (Thermo Fisher Scientific) with a 1% false discovery rate (FDR) and the UniProt 2018-10 database restricted to human and contaminants. Search parameters were precursor and fragment tolerance, 20 ppm; enzyme, trypsin; missed cleavages, 1; fixed modifications, TMTsixplex (N-terminal, K), carbamidomethyl (C); and variable modifications, oxidation (M). DanteR was used for relative quantification from the spectrometric data. Only unique peptides were considered for the analysis. ANOVA was performed using a linear model and *P* values were adjusted by using the Benjamini–Hochberg FDR correction. Regulated proteins were determined using an adjusted *P* value cutoff of 0.05 and a fold-change lower than 0.67 (down) or higher than 1.5 (up). Quantified proteins were submitted to hierarchical clustering using the Python Seaborn package (https://seaborn.pydata.org/): Method: average; z_score:1; metric: Euclidean (Figure 4B). Proteins with a *P* value less than 0.05 were analyzed using VSClust [18]. Number of clusters = 3; minimum membership = 0.35 (Figure 4C, only those proteins with a membership equal to or higher than 0.5 are displayed in the graphic).

Results

Characterizing circulating exosomes in AP patients

The amount of circulating exosomes isolated from the blood of patients with AP, as well as from healthy

Table 2. Proteins from exosomes that showed increased levels (>2-fold) in severe pancreatitis compared with the control group.

	Mild		Moderate		Severe	
Protein	**FC**	***p***	**FC**	***p***	**FC**	***p***
C-reactive protein	2.98	1.0E-14	6.33	1.3E-32	8.43	3.0E-39
Protein S100A9	1.02	9.5E-01	2.24	1.1E-03	6.07	3.3E-07
Protein S100A8	1.34	1.3E-01	1.96	2.5E-04	4.11	9.6E-12
Alcohol dehydrogenase 1B	2.28	1.0E-01	2.32	9.1E-02	3.82	5.7E-03
Neprilysin	2.02	1.6E-08	2.28	1.4E-10	3.06	1.1E-14
CD9 antigen	2.28	1.7E-03	1.42	1.6E-01	2.74	1.4E-04
Elastase 2A	2.01	1.5E-02	3.28	4.7E-04	2.56	4.4E-03
BAI-associated protein 2	1.15	7.4E-01	1.15	7.4E-01	2.54	4.0E-02
Keratin	1.14	7.8E-01	2.34	4.2E-02	2.48	5.0E-02
Lipopolysaccharide-binding protein	1.98	2.0E-13	2.18	1.2E-16	2.47	2.9E-22
Integrin alpha IIb	2.50	4.3E-03	1.75	7.1E-02	2.43	5.3E-03
Pantetheinase	2.09	1.5E-01	2.46	8.9E-02	2.42	1.1E-01
von Willebrand factor 2	1.81	1.1E-19	2.77	9.9E-48	2.40	3.5E-39
Olfactomedin-4	0.94	8.8E-01	1.57	1.4E-01	2.33	9.3E-03

A complete list of identified proteins is presented in supplementary material, Table S2.
FC, fold-change.

donors, was determined by measuring the total protein content in the exosome extract (Figure 1A). No significant differences were detected between the different groups. The nanoparticle tracking assay also revealed a similar size of vesicles, which is also compatible with the expected size of exosomes (less than 200 nm) in all the groups (Figure 1B), and western blotting confirmed the presence of exosome markers Alix, CD81, and TSG101, as well as the absence of calnexin (Figure 1C). The clinical characteristics of patients from the different groups are summarized in Table 1.

Pro-inflammatory activity of circulating exosomes correlates with the severity of AP

To evaluate the effect on the activation of macrophages, differentiated THP-1 cells were incubated with 10 µg/ml exosomes for 3 h and the expression of mRNAs encoding the M1 markers IL-1β and TNFα and M2 markers MRC1 and Arg1 was evaluated by RT-qPCR. Although we measured RNA and not the final generation of proteins, this allowed us to evaluate the activation of pro- or anti-inflammatory pathways. The results indicated that exosomes obtained from the control and mild AP

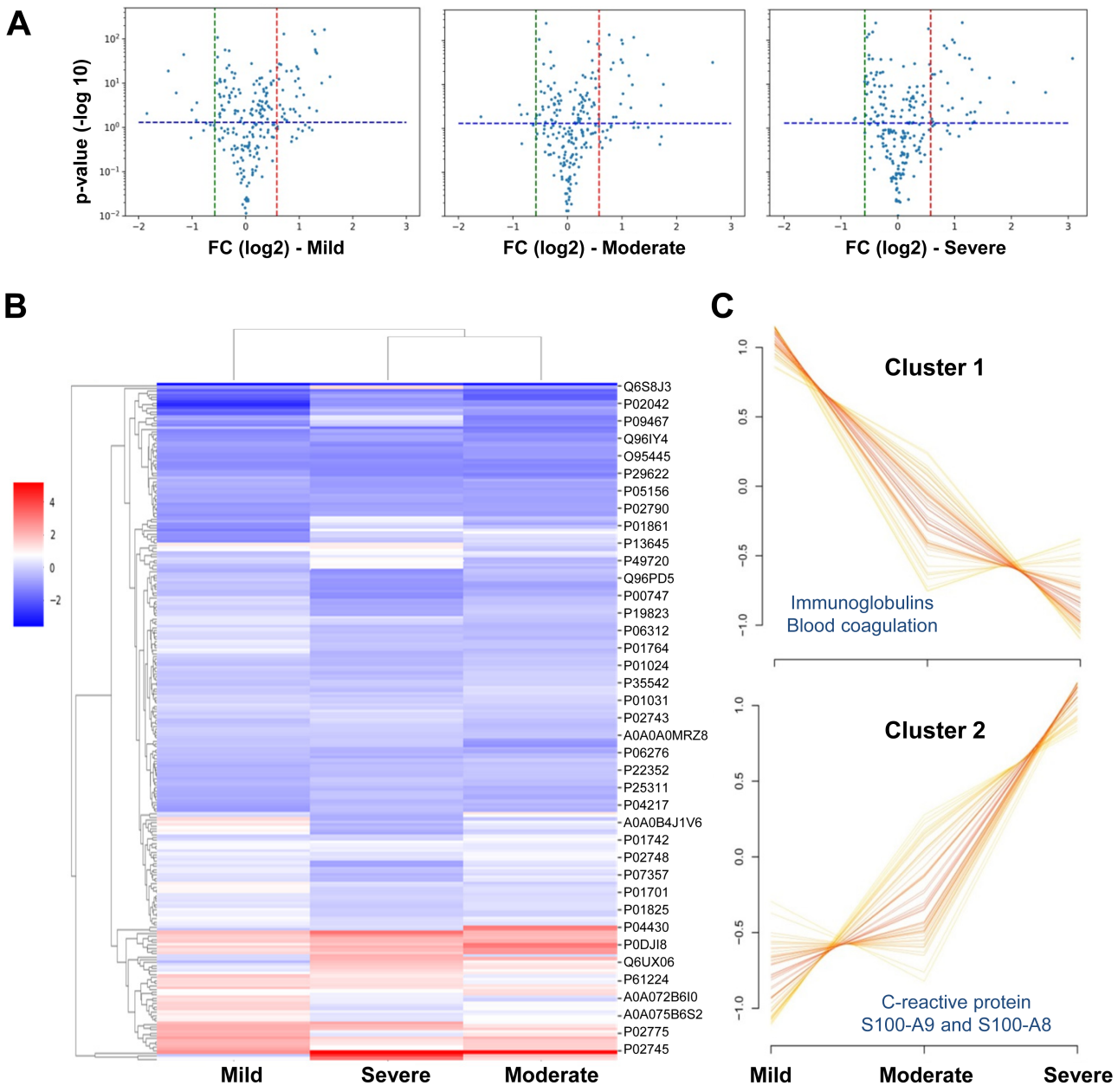

Figure 4. Analysis of the proteome in mild, moderate, and severe AP versus control. (A) Volcano plots showing proteins with decreased [<0.67 fold-change (FC), $p < 0.05$, top-left quadrant] or increased abundance (>1.5 FC, $p < 0.05$, top-right quadrant). (B) Hierarchical clustering and heatmap visualization of protein expression (z-score of $\log_2$ fold-change) calculated based on the proteins detected in each site. (C) Clustering of the proteins identified in the different conditions [18]. Proteins belonging to each cluster are listed in supplementary material, Table S3. Enriched KEGG pathways associated with proteins from each cluster are indicated. $n = 4$ pools of five samples per group.

groups had no effect on the expression of these cytokines. By contrast, when macrophages were incubated with exosomes from moderate and severe pancreatitis, the expression of mRNAs for IL-1β and TNFα was increased (Figure 2A). Interestingly, increases in the expression of these cytokine mRNAs were significantly higher in response to exosomes from severe AP patients, again suggesting that the intensity of the systemic inflammatory response during AP is linked to differences in the content of circulating exosomes.

Exosomes are not the only agents involved in the systemic inflammatory response during AP, and a number of soluble inflammatory mediators are released along the progression of the disease [7,8,19]. Therefore we evaluated the relative importance of exosomes in the induction of inflammatory response by incubating macrophages with exosome-depleted plasma from the different groups. We found that removal of exosomes resulted in a significant decrease of the induction of expression of *IL1β* mRNA in macrophages, although a certain level of activity remained in the severe pancreatitis group, which could be attributed to the presence of soluble mediators in the plasma (Figure 2B).

Final evaluation of the pro-inflammatory activity of exosomes from severe pancreatitis was carried out by measuring its effects on the activation of signal transduction pathways. Since the induction of inflammatory cytokines is mediated by the activation of NFκB, the effect of exosomes on the nuclear translocation of the p65 subunit of this nuclear factor was evaluated by immunohistochemistry. The results revealed nuclear translocation after macrophages were incubated with exosomes from severe AP. No effects were observed on the subcellular localization of p65 after incubation with control or mild AP exosomes (Figure 3A). This result was confirmed by western blotting for p65 in the nuclear fraction of the cells (Figure 3B).

Changes in the proteome of circulating exosomes in AP

Mass spectrometry-driven proteomics analysis allowed us to identify a total of 279 proteins with high confidence (supplementary material, Table S2). As reported previously in animal models, the presence of relevant proteins with alleged hepatic origin (apolipoproteins, C-reactive protein, retinol binding protein, alpha-2-macroglobulin) and the low abundance of proteins of pancreatic origin are worthy of note. Of all the proteins identified, 38 showed enrichment in severe AP (fold-change >1.5, supplementary material, Table S2). Higher enrichments in the severe AP group were observed for C-reactive protein (>8.4-fold) as well as in the two components of calprotectin (>6.1-fold for S100A8 and >4.1-fold for S100A9). These proteins were also significantly increased, although at lower levels, in exosomes from moderate AP (Table 2).

These differences allow the three clinical conditions to be distinguished based on the protein composition of circulating exosomes (see hierarchical clustering in Figure 4B) in plasma obtained at hospital admission. A large separation between the proteome of exosomes from mild patients versus moderate and severe was observed, the latter also being distinguishable. Clustering analysis revealed two different protein dynamics profiles. Proteins involved in blood coagulation and immunoglobulins decreased with the severity of the

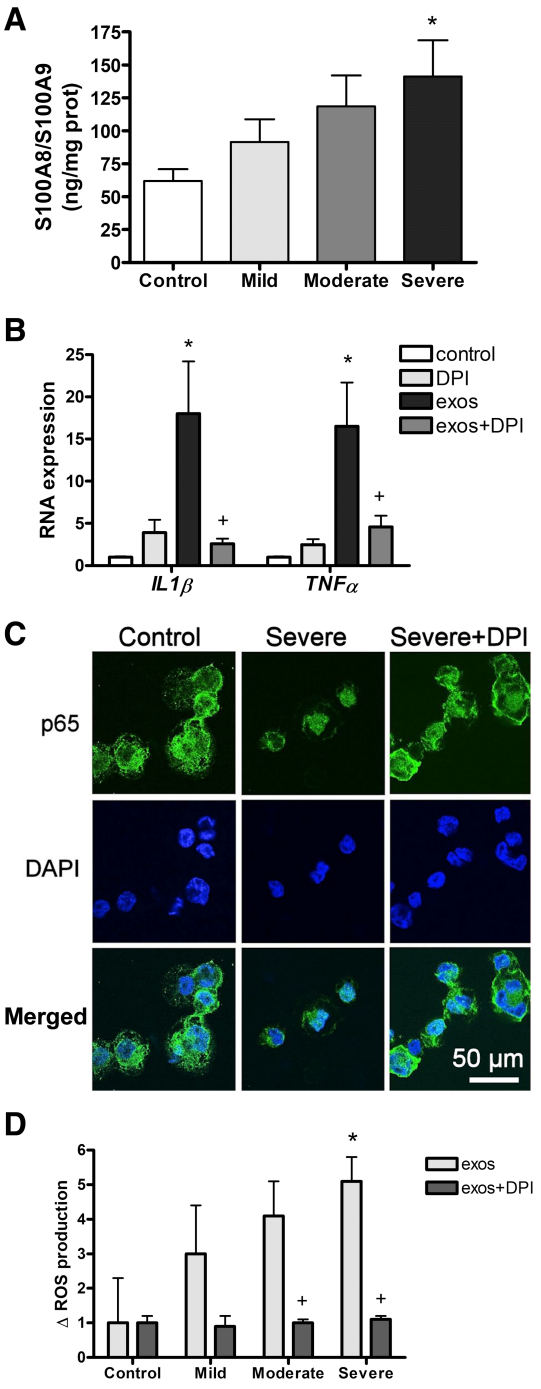

Figure 5. (A) Levels of S100A8/S100A9 heterodimer in exosomes, measured by ELISA. (B) The expression of *IL1β* and *TNFα* mRNAs induced in THP1 cells by exosomes from severe AP patients (10 μg/ml) was abolished by treatment with the inhibitor of NADPH oxidase DPI (10 μM). (C) DPI treatment also inhibited the nuclear translocation of the p65 subunit of NFκB induced by exosomes from severe AP patients. (D) ROS generation induced by exosomes. $n = 20$ per group; $^{*}p < 0.05$ versus control; $^{+}p < 0.05$ versus exos.

disease, whereas proteins related to inflammation and acute response increased with disease progression (Figure 4C).

The concomitant increase in S100A8 and S100A9 suggests the presence of heterodimer S100A8/S100A9 in exosomes from severe AP patients. This fact was verified by measuring its presence with a specific ELISA. The results confirmed that increases in the amount of these proteins parallel an increase in the heterodimer (Figure 5A).

Exosome-induced macrophage activation is dependent on NADPH oxidase activation

The increase detected in the amount of S100A8/S100A9 proteins, as well as in the heterodimer, suggests a potential mechanism involved in the pro-inflammatory activity of exosomes released during severe AP. Intracellular S100A8/S100A9 calcium-loaded complex could induce NFκB activation and cytokine generation through the activation of NADPH oxidase [20]. To test the potential involvement of NADPH oxidase in the increases in the expression of inflammatory mediators induced by exosomes from severe pancreatitis, we used the NADPH oxidase inhibitor diphenyleneiodonium (DPI), which selectively interferes with intracellular ROS production [21]. DPI pretreatment blocked the expression of *IL1β* and *TNFα* mRNAs induced by exosomes obtained from patients with severe AP (Figure 5B). In addition, immunofluorescence analysis revealed that NADPH oxidase inhibition with DPI also prevented the nuclear translocation of the p65 unit of NFκB in macrophages treated with these exosomes (Figure 5C).

Finally, the generation of ROS in macrophages after treatment with exosomes was evaluated using H_2DCFDA as a probe. Treatment of macrophages with mild or severe PA exosomes significantly increased ROS production, while NADPH oxidase inhibition with DPI completely abolished these increases (Figure 5D).

Discussion

Increasing evidence points to circulating exosomes being relevant mediators in the pathogenesis of AP. In particular, they appear to play a role in the progression from local pancreatic damage to systemic inflammation, and in particular to the development of acute lung injury in patients with the severe form of pancreatitis. Experimental studies in rats [10] and mice [11] revealed that circulating exosomes have the ability to reach the alveolar compartment and trigger the activation of NLRP3 inflammasome alveolar macrophages, initiating the local generation of inflammatory mediators and the inflammatory cascade in the lung. However, for human patients, the situation is more complex and AP has been divided into different levels of severity, depending on the degree of local and systemic effects. In this study, we found that the characteristics of exosomes isolated from the blood of patients with AP in the first 24 h of hospital admission strongly correlate with the final severity of the disease.

Our results indicate that exosomes from patients with severe AP have the ability to trigger the inflammatory activation of macrophages *in vitro*, while exosomes from mild AP patients have no significant effect on these cells (Figure 2A). The effect of exosomes from severe AP patients was closely similar to that observed in experimental studies using alveolar macrophages and exosomes obtained in a model of taurocholate-induced severe AP in rats [12]. Exosomes from moderately severe AP induced a moderate increase in the expression of inflammatory cytokines that did not achieve statistical significance. Since the expression of inflammatory cytokines is largely regulated by the activation of NFκB, we evaluated the activation of this nuclear factor by following the nuclear translocation of the p65 subunit (Figure 3). Nuclear localization of p65 was observed only after treating macrophages with exosomes from severe AP patients. Again, these results agree with the previous findings in experimental models [10,11] and confirm the potential role of exosomes in the progression of inflammation in severe AP. However, it should be noted that in addition to exosomes there is a wide range of soluble molecules with inflammatory potential circulating in the plasma of patients with severe pancreatitis. In all likelihood, acute phase proteins, soluble cytokines, and other mediators with different cellular and tissue origins that have been reported to increase during AP may also be involved in triggering systemic inflammation. In order to evaluate the specific relevance of exosomes, we evaluated the ability of plasma to activate macrophages after removing exosomes by precipitating them with PEG. The results indicated that exosome depletion significantly reduced the response of macrophages to plasma (Figure 2B). Nevertheless, this reduction was not complete and a level of activity remained which must be related to the presence of soluble mediators.

It should be noted that the experiments were performed using the same exosome concentration in the different groups. This was chosen because no significant changes were detected in the amount of circulating exosomes between the different groups of pancreatitis or with respect to the controls (Figure 1A). This suggests that the inflammatory effect of exosomes is related to changes in its content; so a proteomic analysis was performed and a number of proteins were identified that increased as the severity of pancreatitis increased (Figure 4).

The proteomic analysis of the exosome content revealed different sets of proteins whose concentration was either reduced or increased in patients relative to controls (Figure 4A). These changes strongly agree with the clinical classification of pancreatitis severity and allow us to differentiate between them in a cluster analysis where the moderate and severe states were classified in a branch different to that of the mild state (Figure 4B). Several groups of proteins are observed with different dynamics depending of the final degree of severity. Thus, immunoglobulins and proteins related to coagulation decrease with severity,

while a set of proteins increasing with severity is enriched in inflammation and acute response-related proteins (Figure 4C). It is interesting to note that despite some proteins diminishing with severity, volcano plots suggest that there is a slight general trend towards positive change ratios with disease severity (Figure 4A).

Of particular interest are the increased amounts of CRP, S100A8, and S100A9 detected in exosomes from moderate and severe AP patients. These proteins mediate a number of both extracellular and intracellular biological functions including the regulation of inflammatory response [22]. Circulating S100A8 and S100A9, which are mostly found as heterodimers, have the ability to bind the receptors TLR4 and RAGE, thus leading to the activation of different inflammatory mediators [23]. Although in this study we have focused on the role of mediators transported within exosomes, potential synergies with soluble mediators is a relevant topic that will require future studies. Inside the cells, S100A8/S100A9 (Figure 5A) act as regulators of NADPH oxidase, the activation of which leads to the oxidative burst and the activation of inflammatory pathways [20]. Since these proteins are transported by exosomes, their main effect can be expected to take place through intracellular mechanisms. Consequently, we evaluated the activation of NADPH oxidase in response to exosomes by treating macrophages with DPI, an NADPH oxidase inhibitor that selectively interferes with intracellular ROS production [21]. Our results show that DPI significantly impaired the increase in the levels of *IL1β* and *TNFα* mRNAs induced by exosomes from severe AP (Figure 5B). DPI also inhibited the activation of NFκB promoted by exosomes (Figure 5C). Finally, an increase in ROS generation was observed after treating macrophages with exosomes, and DPI also inhibited this increase (Figure 5D). It is important to note that although the increase observed is not enough to suggest a relevant level of direct oxidative damage, it does allow activation of signal transduction pathways to be triggered.

The role of ROS, and of NADP oxidase in particular, in the pathogenesis of AP has been previously suggested by experimental studies [24,25]. Although it was generally accepted that oxidative stress plays a role in the development of pancreatitis, the ineffectiveness of the antioxidant treatments tested led to a decrease in interest for this route. Our results indicate that the intracellular mechanism involved in the amplification of systemic inflammation induced by exosomes is mediated by an increase in the intracellular oxidative stress.

On the other hand, a different role appears to be played by CRP. It has been reported that this protein can bind to the surface of extracellular vesicles, undergoing structural changes that increase their pro-inflammatory activity [26]. This has been observed in different inflammatory situations and could explain the presence of CRP in exosomes from severe AP. In the experimental studies, the exosomes detected in plasma appear to be released by the liver. Since this organ is also the main source of CRP, it could be hypothesized that CRP becomes attached to the surface of exosomes in the hepatic microenvironment where both exosomes and CRP are released. This possibility remains to be explored.

Taken together, our results revealed that the final severity of AP is largely conditioned by the composition of circulating exosomes during the early stages of the process. In the case of severe pancreatitis, changes in the profile of proteins carried by these exosomes give them the ability to activate the inflammatory response in systemic inflammatory cells through an NADPH oxidase-dependent mechanism.

Acknowledgements

This work was supported by the projects PI16/00060 from Instituto de Salud Carlos III, 2019AEP057 CVSCI, and a grant 'Gonzalo Miño' from the Asociación Española de Gastroenterología. The Biologial and Environmental Proteomics group is a member of Proteored-PRB3 and is supported by Grant PT17/0019/0008 of the PE I+D+I 2013–2016, funded by ISCIII and FEDER.

Author contributions statement

DC and MC conceived the study. AA-B, DC and MC performed experiments. DC, MC and JA analyzed the data. Ede-M, KC-J, GG-R, RR, RMMM, IP-M and MG collected samples. All the authors critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Images of whole gel western blots and protein markers

Table S1. Primers used for qPCR amplification

Table S2. Mass spectrometry-driven proteomics analysis allowed the identification of a total of 279 proteins with high confidence

Table S3. Proteins included in clusters 1 and 2 in Figure 4C