**Lipids generated during acute pancreatitis increase inflammatory status of macrophages
by interfering with their M2 polarization**

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Abstract

Background: Necrosis of adipose tissue is a common complication of acute pancreatitis. The areas of steatonecrosis become a source of inflammatory mediators, including chemically modified fatty acids which could influence the progression of the systemic inflammation. In an experimental model of acute pancreatitis we analyzed the effects of lipids generated by two representative areas of adipose tissue on the switch to the M1 phenotype in macrophages.

Methods: Pancreatitis was induced in rats by intraductal administration of 5% taurocholate and after 6 h, lipids from retroperitoneal, mesenteric or epididymal adipose tissues were collected. Lipid uptake, phenotype polarization and the activation of PPAR γ and NF κ B were evaluated in macrophages treated with these lipids.

Results: After induction of pancreatitis, lipids from visceral adipose tissue promote the switch to an increased pro-inflammatory phenotype in macrophages. This effect is not related with a higher activation of NF κ B but with an interfering effect on the activation of M2 phenotype.

Conclusions: During acute pancreatitis, lipids generated by some areas of adipose tissue interfere on the M2 polarization of macrophages, thus resulting in a more intense pro-inflammatory M1 response.

Abbreviations: AP: Acute Pancreatitis; EWAT: Epididymal White adipose tissue; RWAT: Retroperitoneal White adipose tissue; MWAT: Mesenteric adipose tissue; MPO: Myeloperoxidase.

INTRODUCTION

Macrophages are involved in the innate immune response and play an important role in many host reactions and inflammatory processes. They show a remarkable capacity to produce and release a variety of reactive oxygen species, hydrolytic enzymes and both pro- and anti-inflammatory cytokines that modulate the progression and the resolution of the inflammatory process. These cells also exhibit a well-known capability to modify their phenotype adapting its functional properties in response to changes in local microenvironment [1].

In the early stages of inflammation, macrophages alter their gene expression profile to an M1 phenotype, or classical activation, characterized by the generation of pro-inflammatory mediators as IL-1 β , TNF α or iNOS. By contrast, the resolution of the inflammatory process is associated with the alternative or M2 phenotype of macrophages. These alternative activated macrophages inhibit the immune response, limit inflammation and promote the production of extracellular matrix and tissue remodeling [2]. The M2 phenotype has been also divided in regulatory, inhibitory or wound healing, according to the different genes expressed in each situation, but although this could be a useful classification, it only represents some points in the broad spectrum of phenotypes that can be acquired by macrophages.

Mediators present in the macrophage microenvironment are determinant in the acquisition of different phenotypes. Inflammatory cytokines and bacterial products strongly induce an M1 phenotype, while IL-4, IL-13 or IL-10 switch the phenotype to different alternative profiles. There are data indicating that phenotype could be also modulated by lipids released from adipose tissue. This fact could play a role in some pathological situations as the pro-inflammatory status associated to obesity or the progression of systemic inflammation in acute pancreatitis [3–5].

Macrophages are pivotal players in the pathogenesis of acute pancreatitis [6], an inflammatory disease of the pancreas that, in the severe forms, progresses to a systemic inflammation and multiple organ failure. Along this process, different populations of resident macrophages, including peritoneal [7], alveolar [8] and hepatic Kupffer cells [9], are reported to be activated to

an M1 phenotype. Although macrophage activation is triggered by cytokines, hydrolytic enzymes and DAMPs released from pancreatic tissue, there is increasing evidence suggesting a role for the white adipose tissue in the progression of inflammation during acute pancreatitis [4,10,11].

Necrosis of adipose tissue is a common complication of acute pancreatitis as a consequence of lipase released to bloodstream by damaged pancreas. The areas of steatonecrosis are surrounded by a marked inflammatory infiltrate and become sources of a number of inflammatory mediators, including cytokines and chemically modified fatty acids [11,12]. These lipids are involved in the hyperlipemic status observed in acute pancreatitis, but could also be involved in the activation of macrophages, thus influencing the progression of the systemic inflammation. Interestingly, there are remarkable differences between different areas of adipose tissue regarding their role on the inflammatory response [13]. In particular, visceral fat seems to be more involved in inflammatory processes than other regions of fat, not only in pancreatitis but also for diseases as metabolic syndrome or atherosclerosis [14–16].

In this study we confirm the differences between visceral and retroperitoneal adipose tissue. In addition, since macrophages acquire an M1 phenotype during severe acute pancreatitis, we analyzed the effects of lipids generated by adipose tissue on the switch to the M1 phenotype in macrophages.

METHODS

Animals

Male Wistar rats (250-300 g b.w.) were used in all experiments (Charles River, France). Animals were housed in a controlled environment, fed with standard laboratory pelleted formula (A04, Panlab, Barcelona, Spain) and tap water *ad libitum*. This study conformed to European Community for the use of experimental animals and the institutional committee of animal care and research approved it.

Animal model of acute pancreatitis

Animals (n=6 each group) were anesthetized with an i.p. administration of sodium pentobarbital (50 mg/kg). The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Pancreatitis was induced by retrograde perfusion of 5% sodium taurocholate (Sigma Aldrich, St Louis, MO) in a volume of 0.1 ml/100 g b.w. using a perfusion pump (Harvard Instruments, Edenbridge, UK). Although this model is very aggressive for the pancreas it provides a good induction of systemic effects, including the activation of distant inflammatory response and, in particular, the necrosis of adipose tissue [17]. In control animals, the same volume of saline solution was administered instead of taurocholate. Six hours after induction, samples of plasma, pancreas as well as retroperitoneal, mesenteric and epididymal adipose tissue were obtained, immediately frozen and stored at -80°C until analyzed. This time period is enough to observe the appearance of areas of necrosis, mainly in epididymal adipose tissue. Tissue samples of adipose tissues were also obtained for histological study and, in some animals, peritoneal and bronchoalveolar lavages were carried out to obtain peritoneal and alveolar macrophages.

Lipid extraction

Samples of adipose tissue were homogenized in PBS, and lipids were extracted by the method of Bligh and Dyer [18]. The organic solvent (methanol-chloroform mixture) was removed under a stream of nitrogen and lipids were reconstituted in HBBS followed by sonication to form lipid vesicles prior to their use.

Fatty acid levels

Total free fatty acid levels were measured by using the commercial colorimetric Free fatty acid Quantification kit (Biovision, Milpitas, CA), according to the supplier's specifications.

Lipase

Plasma lipase was determined by using the commercial turbidimetric assay kit from Randox (Antrim, U.K.), according to the supplier's specifications.

Myeloperoxidase

Neutrophilic infiltration was assessed by measuring myeloperoxidase (MPO) activity. MPO was measured photometrically with 3,3',5,5'-tetramethylbenzidine as a substrate. Tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer at pH 6.0. Homogenates were disrupted for 30 s using a Labsonic sonicator (Braun Biotech, Inc., Allentown, PA) at 20% power and submitted to three cycles of snap freezing in dry ice and thawing before a final 30 s sonication. Samples were incubated at 60 °C for 2 h and then spun down at 4000 xg for 12 min. Supernatants were collected for MPO assay. Enzyme activity was assessed photometrically at 630 nm.

Histological study

For histological studies, tissue samples were fixed in 10% neutral buffered formalin, paraffin-embedded, cut into 5 µm thick sections and stained with hematoxylin–eosin according to standard procedures. Sections were evaluated by light microscopy.

Cells

Human THP-1 cells were cultured in suspension in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95 % air, 5 % CO₂ at 37 °C. Cells were differentiated to macrophages by a first incubation with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St Louis, MO) for 48 h at 37 °C in 24-well plastic Petri dishes (Nunc; Nunc Inc., Naperville, Ill). PMA was removed before the experiments.

To induce the M1 phenotype, macrophage-differentiated THP-1 cells were incubated with a mixture of LPS + IFN γ at different doses (0.2, 1, 25 and 125 ng/ml). M0 macrophages were maintained in culture medium without the addition of cytokines.

To evaluate the effect of lipids on the induction of M1 phenotype in macrophages, lipid extracts (500 µg/ml) were added to THP-1 differentiated cells 2 h before the treatment with LPS + IFN γ . This concentration was selected since in toxicity analysis it was found that higher concentrations already impaired cell viability (data not shown). Lipid extracts were not removed from the culture media during all the experiment.

After treatment, cells were collected for the subsequent analysis of gene and protein expression by quantitative RT-PCR and Western Blot, respectively.

RNA isolation and qPCR.

Total RNA from cells was extracted using the TRizol® reagent (Invitrogen, Carlsbad, CA). The RNA was quantified by measurement of absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), and reverse transcription was then performed on 1 µg RNA sample by adding iScript reagents. The reaction was incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min, and then stored at -80 °C.

Subsequent PCR amplification was performed in a DNA Engine, Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) using IQTM SYBR Green Super mix (Bio-Rad Laboratories, Hercules, CA) and the correspondent primers: IL-1 β forward: 5'-TCGTTATCCCATGTGTCGAA-3' and reverse: 5'-GGACAAGCTGAGGAAGATGC-3'; MRC1 forward: 5'-TCTGGTAGGAAACGCTGGTC-3' and reverse: 5'-GGATGGATGGCTCTGGTG-3'; PPAR γ forward: 5'-TTGCAGTGGGGATGTCTCAT-3' and reverse: 5'-TTTCCTGTCAAGATCGCCCT; CD36 forward: 5'-AGATGCAGCCTCATTTCAC-3' and reverse: 5'-GCCTTGGATGGAAGAACAAA-3'; TGF β : forward: 5'-GTGGAAACCCACAACGAAAT-3' and reverse: 5'-CACGTGCTGTCTCACTTTTA-3'; TNF α forward: 5'-AGCCCATGTTGTAGCAAACC-3' and reverse: 5'-GGCACCACCAACTGGTTATC-5'; GAPDH forward: 5'-TGTGGTCATGAGTCCTTCCA-3' and reverse: 5'-GATCATCAGCAATGCCTCCT-3'. Reactions were performed in duplicate and threshold cycle values were normalized to GAPDH

gene expression. The specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to GAPDH was calculated by using the $\Delta C(t)$ formula.

SDS-PAGE and Western blot

Cells were lysed using the Nuclear extract kit from Active Motif (Carlsbad, CA) under conditions for preparation of whole cell or nuclear and cytoplasmatic extracts. SDS-PAGE was performed on 12% acrylamide gels calibrated using Precision Plus Protein Kaleidoscope Standards (Bio-Rad Laboratories, Hercules, CA) with markers covering a 10-250 kDa range. Proteins were electrotransferred onto nitrocellulose membranes using a semi-dry transfer apparatus TE 77&EPS 600 Power Supply (Pharmacia Biotech, Piscataway, NJ). Membranes were blocked with 5% non-fat milk in TBS-T for 1 h and probed with primary antibodies anti-I κ B α at 1/200; anti-PPAR γ at 1/400 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-tubulin at 1/2000 (Sigma Aldrich, St Louis, MO). After washing, the membranes were incubated with Dylight 800-labeled IgG secondary antibody at 1/10000 (Thermo Scientific, Waltham, MA) for 1 h and visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Immunofluorescence

To monitor NF κ B translocation, THP-1 cells were differentiated to macrophages on coverslips and treated with lipid extracts (500 μ g/ml) for 2 h before their polarization to an M1 phenotype with IFN γ (125 ng/ml) + LPS (125 ng/ml) for 30 min. Following treatment, cells were fixed with 4 % formaldehyde, permeabilized with 0,1 % triton X-100 and blocked with serum. The staining was performed by incubating with anti-p65 antibody at 1/400 (Santa Cruz Biotechnology, Santa Cruz, CA) and Chromeo 488-conjugated secondary antibody at 1/1000 (Acris Antibodies, Herford, Germany). Nuclear or cytoplasmic localization of p65 was examined by fluorescence microscopy.

Oil Red O Staining

The macrophage-differentiated THP-1 cells were carefully washed twice with PBS and then fixed and dried with 4% formaldehyde for 10 min. After removal of formaldehyde, 60%

isopropanol was added to each well for 3 min to remove any aqueous solution and avoid Oil Red O precipitation. Cells were then incubated with the prefiltered Oil red O working solution (0,5% in isopropanol mixed 3:2 with distilled water) for 10 min and washed three times with distilled water. Cells were also counterstained with Harris Hematoxylin for 30 s. The staining of lipid droplets and cell morphology was ascertained microscopically using a Nikon Eclipse E1000 microscope. Quantification was performed using the Adobe Photoshop program (Adobe Systems), measuring the percentage of stained pixels versus cell area pixels.

Alveolar and peritoneal macrophages were also stained in order to evaluate the changes in lipid uptake after induction of pancreatitis.

Statistical analysis

Data have been expressed as mean $\pm$ SEM. Means of different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed for evaluation of significant differences between groups.

RESULTS

Pancreatitis resulted in significant increases in lipase activity in plasma (Figure 1 A) as well as in myeloperoxidase activity in pancreas, mesenteric and epididymal adipose tissue (Figure 1 B). By contrast, no changes in myeloperoxidase were observed in retroperitoneal adipose tissue. This was confirmed by the histological analysis of adipose tissue samples. Pancreatitis resulted in a relevant increase in inflammatory infiltrate in epididymal (EWAT) and mesenteric (MWAT) adipose tissue, while no relevant changes were observed in the retroperitoneal areas (RWAT) (Figure 1 C).

Lipid uptake by macrophages

Macrophages incubated with the different lipid extracts clearly increased their lipid content as the Oil Red O staining revealed (Figure 2 A). Quantification of lipid stored in macrophages showed that treating macrophages with lipid extracts obtained after induction of pancreatitis resulted in a higher lipid storage. These increases correlated with the amount of free fatty acids present in the lipid mixture (Figure 2 B). *In vivo*, increased number of lipid droplets in macrophages was observed after induction of pancreatitis, being the increase more evident in alveolar than in peritoneal macrophages (Figure 2 C).

Effect of lipids on macrophage activation

Figure 3 depicts the levels of IL-1 β , MRC-1 and the ratio of these M1 and M2 markers for macrophages treated for 2 h with epididymal lipid extracts, and then incubated with increasing concentrations of IFN γ +LPS for 6 h in the presence of lipid extracts. Since no changes were observed in the inflammatory response in retroperitoneal adipose tissue we only focused on the epididymal one. There were no differences in the ratio IL-1 β :MRC-1 when induction was carried on at low doses of IFN γ +LPS, but presence of lipids from pancreatitis resulted in a higher M1 polarization when cells were treated with high doses of IFN γ +LPS. This change was not related with the expression of IL-1 β , but with a higher inhibition of MRC-1.

Lipids from pancreatitis inhibit M2 phenotype

Additional phenotype markers were evaluated to verify the effect of lipids on M1 macrophages (Figure 4). The expression of TNF α was strongly induced when macrophages acquired the M1

phenotype and, as occurred with IL-1 β , lipids had no effect on its expression. On the other hand, CD36 and TGF β expression was inhibited and treatment with lipids from acute pancreatitis resulted in an additional inhibition of both M2 markers.

NF κ B activation

The effect of epididymal lipid extracts on the activation of NF κ B was evaluated by immunofluorescent analysis of p65 nuclear translocation (Figure 5A). In control macrophages, p65 staining was cytoplasmatic while in M1 macrophages, staining was observed only in the nuclei. This translocation was not modified by the presence of lipids from both control and pancreatitis. To confirm these results, a Western Blot analysis of I κ B α was performed. We observed that this protein was degraded when the M1 phenotype was induced and, as occurred with p65 translocation, no changes were observed in the level of degradation by the treatment with lipids (Figure 5B).

PPAR γ expression

Induction of an M1 phenotype was associated to an inhibition of the expression of PPAR γ RNA and, as occurred with MRC-1, the presence of lipids from acute pancreatitis resulted in a stronger inhibition of PPAR γ expression (Figure 6A).

Western blot analysis of PPAR γ (Figure 6B) confirmed that the levels of this nuclear receptor were reduced when cells acquired the M1 phenotype. As occurred with RNA expression, the reduction was reinforced when lipids from acute pancreatitis were present in culture medium. By contrast, control lipids did not modify neither the expression of PPAR γ RNA nor PPAR γ protein levels

DISCUSSION

The progression of the inflammatory response is strongly dependent on the activation status of macrophages. These cells show a remarkable versatility and have the ability to express different genetic programs in response to microenvironment, resulting in specific phenotypes that allow them to deal with infections and tissue damage with great effectiveness. Although microbial products or inflammatory mediators as cytokines are the best known activators, macrophage phenotype could also be influenced by lipids present in the microenvironment. This could be of particular importance in acute pancreatitis due to strong affectation of adipose tissue by the action of lipase and other lipolytic enzymes released by pancreas.

We have previously reported the involvement of damaged white adipose tissue as a source of inflammatory mediators that promotes the progression of inflammation in acute pancreatitis [11]. However, not all areas of adipose tissue show the same response to pancreatic damage, being the role of epididymal and mesenteric similar and more involved in inflammatory response than the retroperitoneal [13]. It is known that in the intraabdominal fat, there are differences between visceral (omental, mesenteric and epididymal) and retroperitoneal fat in lipid uptake and lipolytic sensitivity [19]. In this work we confirmed the differences in the response to inflammatory process between visceral and retroperitoneal areas of adipose tissue. After induction of experimental acute pancreatitis in rats, a strong inflammatory response was detected in both epididymal and mesenteric but not in the retroperitoneal adipose tissue (Figure 1). These results are coincident to that observed in patients, in which visceral fat-related obesity was associated with the development of systemic complications of severe acute pancreatitis while it was not observed in patients with non-central fat distribution [14].

Since different areas of adipose tissue do not have the same fatty acid composition [20] it could be hypothesized that the specificity of the response is related to differences on the degree of absorption of lipids or in the activation induced by them on the inflammatory cells. Initially, we evaluated the lipid uptake by macrophages treated with extracts obtained from epididymal, mesenteric or retroperitoneal adipose tissues. However, we found that there are not significant differences related with the origin of the extract (Figure 2). Lipid uptake was higher when

samples were obtained after induction of pancreatitis, but this could be explained by the higher levels of free fatty acids present in the samples. In fact, there was a good correlation between the concentration of fatty acids present in the extracts and the lipid uptake observed in macrophages (Figure 2).

These results suggest that there is a particular characteristic of lipids released by visceral adipose tissue during pancreatitis that increases the inflammatory response. This effect is not observed in the retroperitoneal adipose tissue. This is in line with some studies that have compared the epididymal and mesenteric fat depots and found a number of similarities ranging from lipid metabolism to the role they play in the inflammatory process, while other areas of non-visceral adipose tissue clearly show a different behavior [13,19].

The reason for these differences could be in the fatty acid composition, which is not the same in the different areas of adipose tissue [21]. In addition, the expression of enzymes, as cyclooxygenase, involved in the synthesis of bioactive lipids, also shows different levels depending on the area of adipose tissue [22]. Oxidative balance is also depending on the distribution of fat [23], thus resulting in different levels of oxidative modifications of lipids. Altogether result in the generation of a particular profile of lipid mediators in each type of adipose tissue with specific effects on the activation of macrophages and inflammatory response.

Since the systemic inflammation induced by acute pancreatitis is associated with a switch to M1 phenotype in macrophages, we go in depth in the effect of visceral fat by evaluating *in vitro* the effect of lipids from epididymal adipose tissue on the induction of this phenotype. Cells were preincubated with lipid extracts obtained from control or pancreatitis animals and then treated with increasing concentrations of IFN γ and LPS to induce the M1 phenotype. As expected, the M1 marker IL-1 β was upregulated while the M2 marker MRC-1 was inhibited. The main change induced by the presence of lipids from pancreatitis was the increase in the M1/M2 ratio observed at high IFN γ +LPS doses. Interestingly, this increase was not related with changes in the induction of IL-1 β but a higher inhibition of MRC-1 (Figure 3B). This effect was confirmed by measuring the expression of other markers related with M1 or M2 phenotypes (Figure 4).

Lipid pre-incubation did not modify the increased expression of the M1 cytokine TNF α , but resulted in additional inhibition of TGF β and CD36, which are related with M2 phenotypes.

Although M1/M2 balance is regulated by different transcription factors, two of them are particularly relevant. In the induction of M1 phenotype NF κ B plays a central role, while the M2 phenotype is associated with PPAR γ activation.

The lack of changes observed in the expression of IL-1 β and TNF α suggests that lipids do not have a significant effect on the mechanisms of activation of M1 phenotype. To confirm this, we evaluated the NF κ B status by measuring by Western Blot the amount of the inhibitory I κ B α subunit as well as by following the nuclear translocation of p65 subunit by immunofluorescence.

The immunofluorescent assay of p65 revealed the nuclear translocation of this transcription factor when cells were polarized to an M1 phenotype by IFN γ +LPS treatment. This activation was not modified by the addition of lipids. Moreover, lipids had no effect on the degradation of I κ B α induced by M1 inducers (Figure 5). These results indicate that the effect of lipid microenvironment on macrophage polarization is not related with changes in pro-inflammatory pathways.

By contrast, the presence of lipids from pancreatitis promoted relevant changes in PPAR γ levels. When macrophages acquired the M1 phenotype RNA expression of PPAR γ was reduced, but this inhibition became significantly more pronounced by the presence of lipids (Figure 6A). A similar pattern was observed in the Western Blot of PPAR γ . The induction of the M1 phenotype resulted in low levels of protein that were even lower in cells treated with lipids (Figure 6B). Both effects were restricted to lipids obtained from animals with pancreatitis, while the lipids from control animals had no effect.

These changes in PPAR γ could be relevant since this nuclear factor plays an important role controlling the M1/M2 balance by regulating the expression of a number of M2-related genes, including itself, as well as transrepressing the activity of many M1-related transcription factors including NF κ B [24]. Furthermore, in experimental models of acute pancreatitis in obese mice, administration of rosiglitazone, a PPAR γ agonist, improves the survival of mice fed with a high

fat diet [25]. Finally, the administration of lipase inhibitors that prevent the release of free fatty acids reduces the systemic inflammation and the severity of the disease [4].

PPAR γ is a transcription factor that can be activated by a variety of fatty acids and related species [26], therefore is not surprising that it was sensitive to lipid mediators generated in areas of fat necrosis. During acute pancreatitis, the fatty acids released undergo modifications, including oxidation and halogenation, which can modify their biological activity [12]. In this line, we have previously reported that lipids present in the ascitic fluid generated during acute pancreatitis have the ability to interfere the binding of PPAR γ to DNA [10].

In the clinical practice it has been observed that obesity in acute pancreatitis patients increases the risk of the process to evolve to a severe form of the disease. However, this fact is restricted to particular types of fat accumulation. Our results indicate that lipid microenvironment could play a role on the activation of macrophages, but it remains to identify the identity of the lipids involved in these effects. Free fatty acids, oxidized lipids, halogenated lipids and bioactive lipid mediators have been reported to be generated during acute pancreatitis [4,10,12,27]. The potential roles of each of these lipid species on the final phenotype of macrophages and their final implications for the progression of the disease remain to be elucidated.

In conclusion, our results indicate that during acute pancreatitis, some areas of adipose tissue generate lipid mediators that have the capability to interfere on the M2 response of macrophages resulting in a marked polarization to an M1 pro-inflammatory phenotype.

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Figure 1: Lipase levels in plasma (A) and myeloperoxidase (MPO) activity in pancreas, epididymal (EWAT), mesenteric (MWAT) and retroperitoneal (RWAT) white adipose tissue (B). The histological analysis of adipose tissue (C) revealed that pancreatitis results in a strong cell infiltration in epididymal and mesenteric adipose tissues, but has no relevant changes in retroperitoneal adipose tissue. $\ast=p<0.05$ vs control.

Figure 2: Lipid uptake in THP1 macrophages (A). After incubation with 500 µg/ml of lipids from epididymal (EWAT), mesenteric (MWAT) and retroperitoneal (RWAT) adipose tissue for 24 hours, cells were washed, fixed and stained with Oil Red O. Lipid samples from acute pancreatitis (AP) contained higher concentrations of free fatty acids, and there was a good correlation between lipid uptake and fatty acid concentrations (B). *In vivo* it could be observed an increase in the lipid uptake in alveolar (Alv) and peritoneal (Perit) macrophages after induction of acute pancreatitis (C)

Figure 3: Effect of control or AP lipids on RNA expression of (A) IL-1 β (M1 marker), (B) MRC-1 (M2 marker) and (C) the ratio M1/M2 in macrophages treated with IFN γ +LPS. Treatment with IFN γ +LPS increased the expression of IL-1 β and inhibited the expression of MRC-1, achieving its maximum effect at 25 ng/ml. Treatment with lipids from pancreatitis resulted in an additional inhibition of MRC-1 and a more pronounced M1/M2 ratio at higher doses of IFN γ +LPS. *=p<0.05 vs control

Figure 4: Effect of control or AP lipids on RNA expression of CD36, TGF β and TNF α . Macrophages treated with 125 ng/ml of IFN γ +LPS increased the expression of TNF α , and lipids had no effect on this induction. By contrast, CD36 and TGF β were inhibited by IFN γ +LPS and lipids from pancreatitis promoted an additional inhibition. *= p<0.05 vs control; + = p<0.05 vs M1.

Figure 5: Immunofluorescent analysis of p65 subcellular location (A) and Western Blot of I κ B α (B). In control macrophages, p65 was found mainly in the cytoplasm although also it was also detected in the nuclei. Induction of an M1 phenotype promoted the activation, and almost total nuclear translocation, of p65. Neither control lipids nor pancreatitis lipids modified this translocation. This result was confirmed analyzing the degradation of I κ B α in the cytoplasmic protein fraction by Western Blot.

Figure 6: RNA expression (A) and Western Blot (B) of PPAR γ . As expected, the induction of an M1 phenotype in macrophages inhibited the expression of PPAR γ , thus resulting in lower levels of RNA and protein. Lipids from acute pancreatitis (AP), but not lipids from control adipose tissue, induced an additional inhibition of both RNA expression and protein levels. *= p<0.05 vs control; + = p<0.05 vs M1.