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# Role of 5-lipoxygenase in the multiple organ failure induced by zymosan

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**Abstract** *Objective:* This study investigated the role of 5-lipoxygenase in the pathogenesis of multiple organ failure (MOF) induced by zymosan. *Design:* Male mice with a targeted disruption of the 5-lipoxygenase gene (5-LOKO) and littermate wild-type (WT) controls (5-LOWT) were used to evaluate the role of 5-lipoxygenase (5-LO) in the pathogenesis of MOF. *Setting:* University research laboratory. *Interventions and measurements:* MOF was induced by peritoneal injection of zymosan (500 mg/kg i.p. as a suspension in saline) in 5-LOWT and in 5-LOKO mice. MOF was assessed 18 h after administration of zymosan and monitored for 12 days (for loss of body weight and mortality). *Results:* A severe inflammatory process induced by zymosan administration in WT mice coincided with the damage of lung and small intestine, as assessed by histological examination. Myeloperoxidase activity indicative of neutrophil infiltration and lipid peroxidation were significantly increased in zymosan-treated WT mice. Zymosan in the WT mice also induced a significant increase in the plasma level of nitrite/nitrate. Immunohistochemical examination demonstrated a marked increase in the immunoreactivity to ICAM-1 and P-selectin in the lung and intestine of zymosan-treated WT mice. In contrast, the degree of (a) peritoneal inflammation and tissue injury, (b) up-regulation/expression of P-selectin and ICAM-1, and (c) neutrophil infiltration were markedly reduced in intestine and lung tissue obtained from zymosan-treated 5-LO deficient mice. Zymosan-treated 5-LOKO showed also a significantly decreased mortality. *Conclusions:* These findings clearly demonstrate that 5-LO exerts a role in zymosan-induced nonseptic shock.

**Keywords** 5-lipoxygenase · Multiple organ failure · Inflammation · Shock · Knock out 5-lipoxygenase · Zymosan

## Introduction

Multiple organ dysfunction syndrome is a cumulative sequence of progressive deterioration in function occurring in several organ systems, frequently seen after septic shock, multiple trauma, severe burns, or pancreatitis [1, 2, 3]. Zymosan is a nonbacterial, nonendotoxic agent that produces acute peritonitis and multiple organ failure characterized by functional and structural changes in liver, intestine, lung, and kidneys [4, 5, 6, 7]. The organ

dysfunction in zymosan-treated animals may in part be dependent on bacterial translocation [8, 9, 10]. We have recently reported that zymosan administration to mice causes within 18 h both signs of peritonitis and organ injury [11, 12]. The onset of the inflammatory response caused by zymosan in the peritoneal cavity was associated with systemic hypotension, high peritoneal and plasma levels of nitric oxide, maximal cellular infiltration, exudate formation, and cyclooxygenase activity [11, 12]. In addition, it has been demonstrated the generation of free fatty acids and eicosanoids in the zymosan-induced peritonitis model [13, 14, 15].

Leukotrienes (LTs) are potent lipid mediators of inflammatory responses and have been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma, arthritis, psoriasis, and inflammatory bowel diseases [16, 17]. The biological activities of LTs suggest that they are mediators of acute inflammatory and immediate hypersensitivity responses. Peptidyl LTs, which are released by leukocytes in response to inflammatory and immunological stimuli, cause contraction of endothelial cells, resulting in increased permeability of postcapillary venules [16, 17] and in increased adhesion of leukocytes to endothelial cells [18]. Activated polymorphonuclear leukocytes (PMN) are known to play an important role in tissue and organ damage [19, 20, 21]. It is now known that PMN migration and activation, occurring at the time of invasion, are controlled locally by cytokines [22, 23]. It has therefore recently been suggested that both the systemic inflammatory response syndrome and the multiple organ dysfunction syndrome associated with severe acute peritonitis may be secondary to the excessive activation of leukocytes [19, 20, 21], which in turn would result in the release of secondary proinflammatory cytokines including tumor necrosis factor $\alpha$, interleukin 6, and interleukin 8 [11, 12], all of which play an important role in the pathogenesis of zymosan-induced multiple organ failure (MOF).

In this study we investigated the role of 5-lipoxygenase (5-LO) in a model of zymosan-induced nonseptic shock using mice with a targeted disruption of the 5-lipoxygenase gene (5-LOKO) and littermate wild-type controls (5-LOWT). To characterize the role of 5-LO in this model of nonseptic shock we determined the following endpoints of the inflammatory response (a) exudate formation, (b) PMN infiltration, (c) lipid peroxidation, (d) adhesion molecules expression, (e) organ injury, and (f) mortality.

## Material and methods

### Animals

Male mice (4–5 weeks old, 20–22 g) were purchased from Jackson Laboratories (Harlan Nossan, Italy) and housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations (D.M. 116192) on protection of animals used for experimental and other scientific purpose and with the EEC regulations (O.J. of E.C. L 358/1 18 December 1986).

### Zymosan-induced peritonitis in mice

Animals were randomly divided into four groups ($n$=10 in each group). 5-LOWT group mice were treated with saline solution (0.9% NaCl) intraperitoneally and served as sham group. 5-LOWT group mice were treated with zymosan (500 mg/kg, suspended in saline solution, intraperitoneally). In the third and forth groups 5-LOKO mice received saline or zymosan administration. In another set of experiments animals ($n$=20 in each group) were randomized into four groups (as described above) monitored for loss of body weight and mortality for 12 days after zymosan or saline administration.

### Clinical scoring of systemic toxicity

Clinical severity of systemic toxicity (conjunctivitis, ruffled fur, diarrhea, and lethargy) in the mice was scored for 12 days after zymosan or saline injection on a subjective scale ranging of 1–3: 0=absence, 1=mild, 2=moderate, 3=serious. All clinical score measurements were performed by an independent investigator who had no knowledge of the treatment regimen received by each group of animals.

### Acute peritonitis assessment

Animals were killed 18 h after zymosan injection under ether anesthesia to evaluate the development of acute inflammation in the peritoneum. The abdominal cavity was carefully opened and the peritoneal cavity washed with 3 ml phosphate buffer saline (composition, in mM: NaCl 137, KCl 2.7, $NaH_2PO_4$ 1.4, $Na_2HPO_4$ 4.3, pH 7.4). The peritoneal exudate and washing buffer were removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (3 ml) from the total volume recovered. Peritoneal exudate was centrifuged at 7000 $g$ for 10 min at room temperature. Cells were suspended in phosphate buffer saline and counted by optical microscope with Burker's chamber after vital trypan blue stain.

### Measurement of nitrite/nitrate

Nitrite/nitrate production, an indicator of NO synthesis, was measured in plasma as previously reported [11].

### Immunohistochemical localization of P-selectin and ICAM-1

The intestine and lung were fixed 18 h after zymosan injection in 10% buffered formaldehyde, and 8-μm sections were prepared for localization of P-selectin, and intercellular adhesion molecule (ICAM) 1 as previously described [24]

### Quantification of organ function and injury

Blood samples were taken 18 h after zymosan or saline injection. The blood sample was centrifuged (1610 $g$ for 3 min at room temperature) to separate plasma. All plasma samples were analyzed

within 24 h by a veterinary clinical laboratory using standard laboratory techniques. The following marker enzymes were measured in the plasma as biochemical indicators of multiple organ injury/dysfunction: (a) liver injury was assessed by measuring the rise in plasma levels of bilirubine, alkaline phosphatase, alanine aminotransferase (ALT, a specific marker for hepatic parenchyma injury) and aspartate aminotransferase (AST, a nonspecific marker for hepatic injury) [25], (b) renal dysfunction was assessed by measuring the rises in plasma levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure), and (3) serum levels of lipase and amylase as an indicator of pancreatic injury.

### Myeloperoxidase activity

The use of measuring myeloperoxidase (MPO) activity to assess neutrophil infiltration has been previously reported [11].

### Lipid peroxidation measurement

The level of malondialdehyde (MDA), which is considered a good indicator of lipid peroxidation, was determined as previously described [26] in the intestinal and lung tissues.

### Light microscopy

Lung and small intestine samples were taken 18 h after zymosan injection. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room-temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, N.J., USA). Sections (thickness 7 μm) were deparaffinized with xylene, stained with hematoxylin/eosin and observed in Dialux 22 Leitz microscope.

### Data analysis

All values in the figures and text are expressed as mean ±standard error of the mean of $n$ observations; for the in vivo studies $n$ represents the number of animals studied. In the experiments involving histology or immuno-histochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparisons. Differences with a $p$ value less than 0.05 were considered significant. Statistical analysis of survival data was calculated by Fisher's exact probability test or by Hazard statistic. For such analyses the level of $p \leq 0.05$ was considered significant. The Mann-Whitney test was used to examine differences between the body weight and organ weights of control and experimental groups. When this test was used, $p \leq 0.05$ was considered significant.

### Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Milan, Italy). Primary monoclonal p-Selectin (CD62P) or ICAM-1 (CD54) for immunoistochemistry were purchases from Pharmingen (DBA, Milan, Italy). Reagents and secondary and non-specific IgG antibody for immunohistochemical analysis were from Vector Laboratories (DBA, Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Health-care., Thetford, UK).

## Results

### 5-LOKO phenotype reduces the development of zymosan-induced nonseptic shock model

Administration of zymosan caused a severe illness in the mice characterized by a systemic toxicity and significant loss of body weight (Fig. 1A, B). At the end of observation period (12 days) 80% of zymosan-treated 5-LOWT mice were dead (Fig. 1C). The absence of functional 5-LO in 5-LOKO mice prevented the development of systemic toxicity (Fig. 1A), the loss in body weight (Fig. 1B), and mortality (Fig. 1C) caused by zymosan. Sham animals injected only with saline appeared healthy and active throughout the observation period (data not shown).

### 5-LOKO phenotype reduces the development of acute peritonitis

5-LOWT mice developed acute peritonitis 18 h after zymosan administration, as indicated by the production of turbid exudate (Table 1). Trypan blue stain revealed significantly more PMN than in sham mice (Table 1). Sham animals demonstrated no abnormalities in the peritoneal cavity or fluid. The absence of 5-LO in mice (animals with the 5-LOKO phenotype) resulted in a pronounced reduction in exudate volume and significant attenuation in the zymosan-induced increase in leukocyte count in the peritoneum (Table 1).

#### *Nitric oxide production and lipid peroxidation*

The biochemical and inflammatory changes observed in the peritoneal cavity of zymosan-treated 5-LOWT mice were associated with a significant elevation in plasma nitrite/nitrate in comparison to control mice (Table 1). Intestine and lung were investigated for MDA levels 18 h after zymosan. As shown in Table 1, MDA levels were significantly increased in both the intestine and the lung of zymosan-treated 5-LOWT mice ($p<0.01$). The absence of 5-LO in mice did not modify the increase in NO production or the enhancement of MDA levels (Table 1).

#### *P-selectin and ICAM-1 expression and neutrophil infiltration is reduced in 5-LO deficient mice*

An indication of zymosan-induced MOF is the accumulation of neutrophils in the intestine and in the lung, which augments the tissue damage. Therefore 18 h after zymosan administration we evaluated the extent of the expression of P-selectin and ICAM-1, adhesion mole-

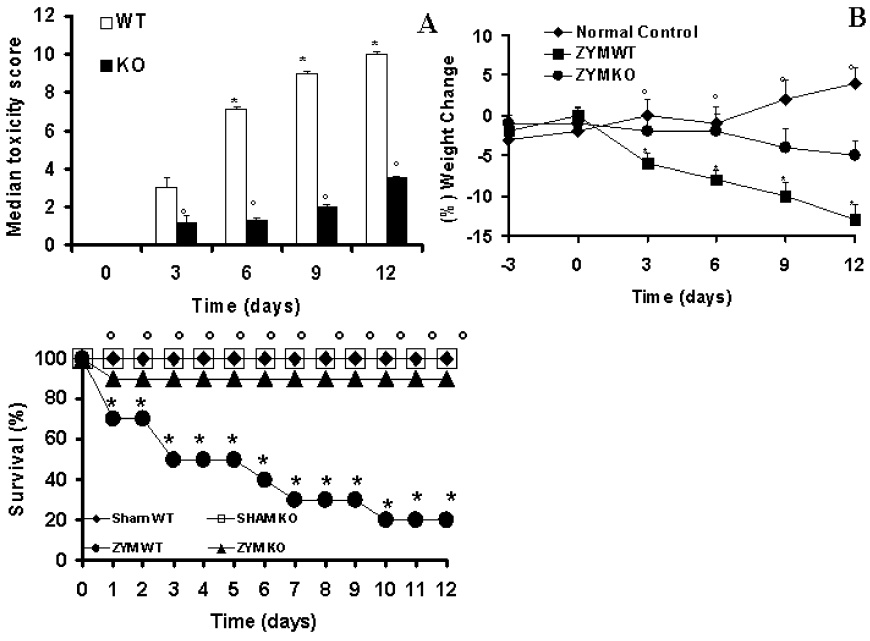


**Fig. 1** Toxicity score (**A**), body weight (**B**), and mortality (**C**) during 12 days after zymosan or saline injection. Responses in 5-LOWT controls and 5-LOKO animals are compared. Data are means ±SEM of 20 mice for each group. *$p$<0.01 vs. vehicle, °$p$<0.01 vs. 5-LOWT mice

**Table 1** Effect of absence of functional 5-LO on zymosan (*ZYM*) induced inflammation, NO plasma levels, and lipid peroxidation; data are means ±SEM of ten mice for each group

| | Volume exudate (ml) | Leukocytes ($10^6$ cells per rat) | Nitrite/nitrate (μM) | Lung MDA levels (μM/100 mg wet tissue) | Ileum MDA levels (μM/100 mg wet tissue) |
|---|---|---|---|---|---|
| Sham +5-LOWT | 0.1±0.04 | 1.1±0.2 | 13±1.8 | 28±2.2 | 27±1.2 |
| Sham +5-LOKO | 0.13±0.07 | 1.0±0.18 | 11±2.4 | 26±1.3 | 28±1.3 |
| ZYM +5-LOWT | 1.2±0.1* | 9.6±0.78* | 98±1.2* | 48±3.4* | 53±2.4* |
| ZYM +5-LOKO | 0.4±0.12** | 2.1±0.4** | 91±2.9* | 41±5.4** | 47±4.4** |

*$p$<0.01 vs. vehicle, **$p$<0.01 vs. 5-LOWT mice

cules that play a pivotal role in the rolling and firm attachment of neutrophils to the endothelium. Neutrophil infiltration into the intestine and in the lung was also assessed by measuring the activity of MPO, an enzyme that is contained in (and specific for) PMN lysosomes. MPO activity was significantly increased 18 h after zymosan administration in intestine (ESM figure 1A) and in lung (1B) from 5-LO wild-type mice. MPO activity was markedly reduced in intestine (ESM figure 1A) and lung (1B) from 5-LOKO mice. The increase in MPO activity in 5-LO wild-type mice was associated with the increase in imunohistochemical staining for ICAM-1 and P-selectin in the vessels wall of the inflamed ileum (Figs. 2C, 3C, Table 2) and in pulmonary tissue (Figs. 2A, 3A, Table 2). The immunostainings for ICAM-1 and P-selectin were markedly reduced in intestine (Figs. 2D, 3D, Table 2) and in the lungs (Figs. 2B, 3B, Table 2) from zymosan-treated 5-LO deficient mice. Please note that there was no staining for either ICAM-1 or P-selectin in tissue section obtained from sham-treated mice (data not shown).

To verify the binding specificity for ICAM-1 or P-selectin some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections, indicating that the im-munoreaction was positive in all the experiments carried out.

### *Multiple organ dysfunction syndrome caused by zymosan is reduced in 5-LO deficient mice*

*Effects on pancreatic injury.* In sham mice the administration of saline did not result in any significant alterations in the plasma levels of lipase and amylase (Fig. 4AB). Plasma levels of lipase and amylase were significantly higher in sham mice 18 h after zymosan administration, demonstrating the development of pancreatic injury in wild-type mice (Fig. 4AB). The absence of functional 5-LO in 5-LOKO mice abolished the pancreatic injury caused by zymosan (Fig. 4AB).

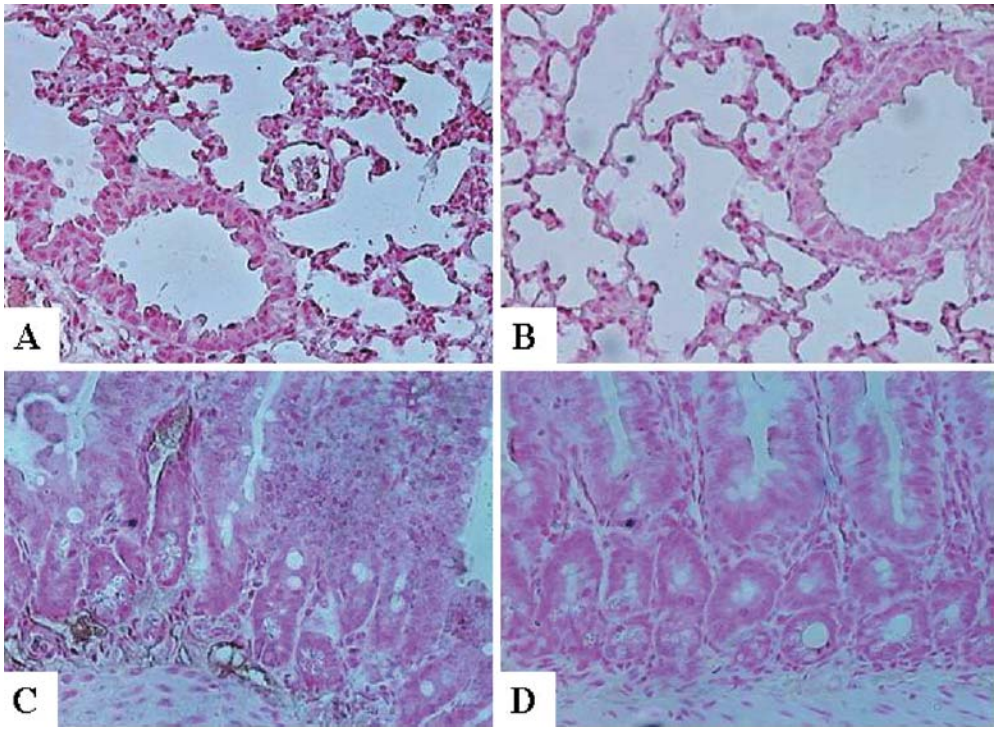


**Fig. 2** Immunohistochemical localization of ICAM-1 in the lung and intestine 18 h after zymosan injection. Positive ICAM-1 staining was found in the lung (**A**) and intestine (**C**) of zymosan-treated 5-LO wild-type mice. There was no detectable immunostaining in the lungs (**B**) or intestine (**D**) of zymosan-treated 5-LOKO mice. Original magnification ×125. Data are representative of at least three experiments performed on different experimental days

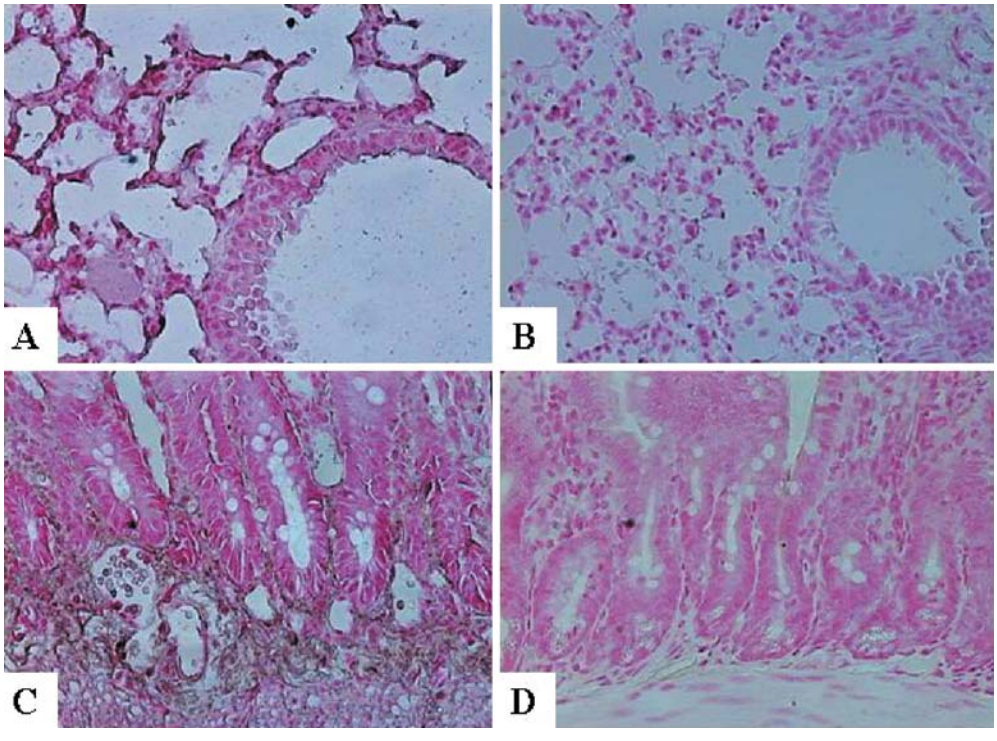


**Fig. 3** Immunohistochemical localization of P-selectin in the lung and intestine 18 h after zymosan injection. Positive P-selectin staining was found in the lung (**A**) and intestine (**C**) of zymosan-treated 5-LO wild-type mice. There was no detectable immunostaining in the lungs (**B**) or intestine (**D**) of zymosan-treated 5-LOKO mice. Original magnification ×125. Data are representative of at least three experiments performed on different experimental days

*Effects on the renal dysfunction.* In sham mice the administration of saline did not result in any significant alteration in the plasma levels of creatinine (Fig. 4C). Plasma levels of creatinine were significantly higher than in sham mice 18 h after zymosan administration, demonstrating the development of renal dysfunction in 5-LOWT mice (Fig. 4C). The absence of functional 5-LO in 5-

**Table 2** Typical densitometry evaluation: analysis of ICAM-1 and P-selectin from ileum and lung section. The assay was carried out using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as means of percentage of total tissue area ±SEM of five immunocytochemistry photographs for each group

| | ICAM-1 (% of total ileum) | P-selectin (issue area) | ICAM-1 (% of total ileum) | P-selectin (issue area) |
|---|---|---|---|---|
| Sham +5-LOWT | 0.9±0.03 | ND | 1.3±0.06 | ND |
| Sham +5-LOKO | 1.0±0.04 | ND | 1.1±0.07 | ND |
| ZYM +5-LOWT | 8.3±0.11* | 9.4±0.18* | 7.5±0.13* | 10.2±0.21* |
| ZYM +5-LOKO | 4.4±0.13** | 4.1±0.21** | 3.6±0.23* | 4.1±0.14** |

*$p$<0.01 vs. vehicle, **$p$<0.01 vs. 5-LOWT mice

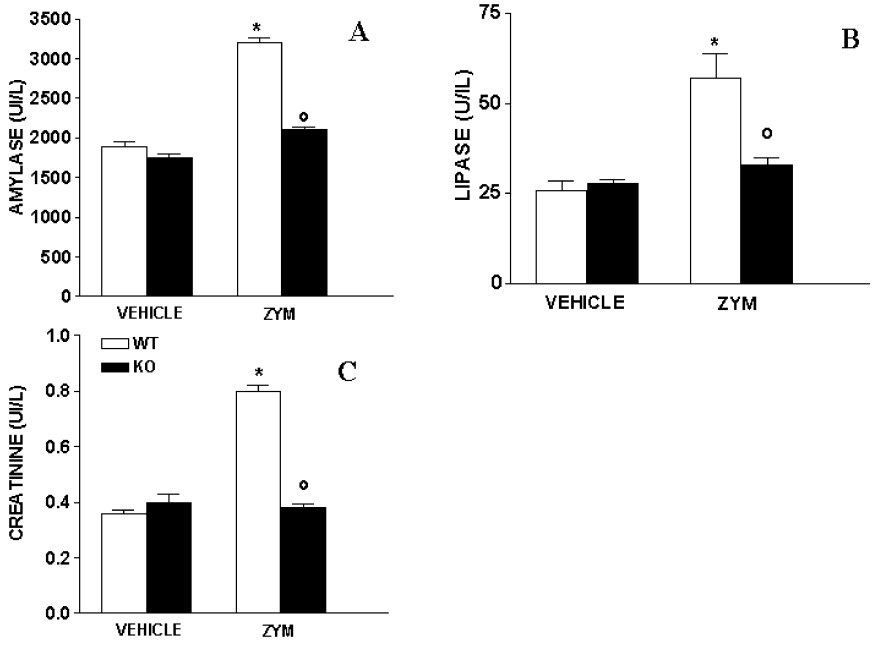


**Fig. 4** Amylase (**A**) and lipase (**B**) serum levels (U/l) and plasma levels of creatinine (**B**; U/l) 18 h after zymosan administration in 5-LO wild-type and 5-LO deficient mice. Data are means ±SEM of ten mice for each group. *$p$<0.01 vs. vehicle,°$p$<0.01 vs. 5-LOWT mice

LOKO mice abolished the renal dysfunction caused by zymosan (Fig. 4C).

*Effects on the liver injury.* In sham mice the administration of saline did not result in any significant alteration in the plasma levels of AST (ESM figure 2A), ALT (2B), bilirubin (2C), and alkaline phosphatase (2D). Plasma levels of AST, ALT, bilirubin, and alkaline phosphatase were significantly higher than in sham mice 18 h after zymosan administration, demonstrating the development of hepatocellular injury in wild-type mice (ESM figure 2). The absence of functional 5-LO in 5-LOKO mice abolished the liver injury caused by zymosan (ESM figure 2).

*Lung and intestine injury (histological evaluation) caused by zymosan is reduced in 5-LO deficient mice*

Histological evaluation of tissue injury in lung and small intestine 18 h after zymosan administration revealed pathological changes (see representative sections in Fig. 5AC). Lung biopsy specimens revealed extravasation of red cells and inflammatory cell infiltration (Fig. 5A). Sections from the distal ileum revealed significant edema in the space bounded by the villus and epithelial separation from the basement membrane (Fig. 5C). Absence of a functional 5-LO gene in 5-LO deficient mice resulted in a significant reduction in pulmonary and intestinal injury (Fig. 5BD).

## Discussion

This study provides important evidence that the absence of 5-LO (5-LO knock-out mice or 5-LOKO mice) attenuates: (a) the development of zymosan-induced peritonitis, (b) the infiltration of the lung and intestine with PMN (histology and MPO activity), (c) the degree of liver, kidney, and pancreas injury organ dysfunction (biochemical markers), and (d) the degree of lung and intestine injury (histology) caused by injection of zymosan. All of these findings support the view that 5-LO plays an important role in the degree of MOF induced by zymosan in the mice.

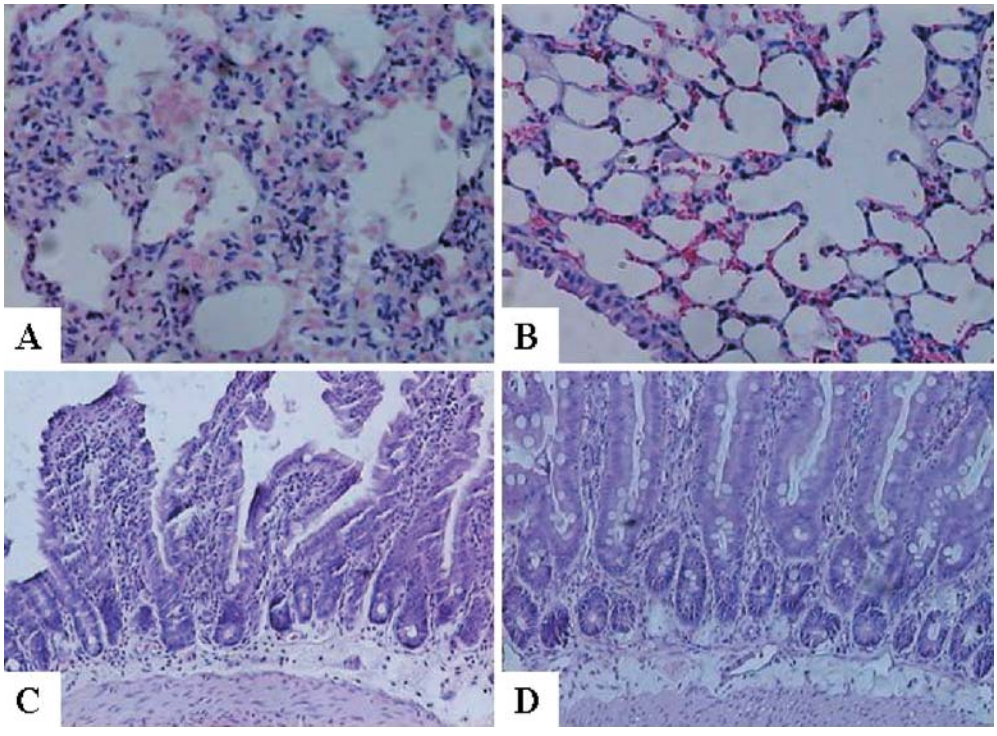


**Fig. 5** Morphological changes in lung and intestine 18 h after zymosan administration. **A** Representative lung sections from zymosan-treated 5-LOWT mice demonstrate inflammatory cells infiltration. **B** Lung sections from zymosan-treated 5-LOKO mice demonstrate reduced inflammatory cells infiltration. **C** Representative ileum sections from zymosan-treated 5-LOWT mice demonstrate edema in the space bounded by the villus and epithelial separation from the basement membrane. **D** Ileum sections from zymosan-treated 5-LOKO mice demonstrate reduced ileum injury. Original magnification ×125. Data are representative of at least three experiments performed on different experimental days

What, then, is the mechanism by which the inhibition of 5-LO protects the organ injury associated with zymosan-induced MOF? The role of oxidative stress in the pathophysiology of MOF has been recognized [12, 27, 28, 29, 30]. In the present study we confirmed that zymosan-induced MOF is characterized by a significant increase in NO plasma formation and an increase in lipid peroxidation in the intestine and lung. Therefore the absence of 5-LO did not reduce the production of NO or the increase in lipid peroxidation induced by zymosan administration. These data, in agreement with previously observations in various experimental models [31, 32], clearly demonstrate that inhibition of 5-LO does not effect the production of reactive oxygen species. On the contrary, it is well known that reactive oxygen species stimulate the formation of $LTB_4$ [33, 34, 35]. Therefore it has been pointed out that the contribution of LT to a specific inflammatory response depends both on the ability of cells present in the inflammatory lesion to produce a particular LT and on the response of the tissue to these bioactive lipids. It has been demonstrated that intraperitoneal injection of zymosan results in a marked increase in levels of LTB, in a biphasic manner, with a delayed peak response [36]. In addition, the role of eicosanoids in cellular influx has been demonstrated in the zymosan model, demonstrating that the selective 5-LO inhibitors zileuton and TZI-41127 attenuate LT biosynthesis and influx of neutrophils [36]. Endothelial adhesion molecules (e.g., P-selectin and ICAM-1) are major regulators of neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion, and emigration from the vasculature to the tissue [37, 38]. In the present study we observed that zymosan induced the expression of P-selectin on the endothelium of small vessels and upregulated the surface expression of ICAM-1 on endothelial cells in intestine and lung from 5-LO wild-type mice. In contrast, there was significantly less expression of P-selectin and ICAM- 1 in intestine and lung tissues from 5-LO deficient mice than in those from wild-type mice 18 h after zymosan administration. Interestingly, we found that the constitutive expression of ICAM-1 in the intestine and lung did not differ between sham-treated 5-LO deficient and wild-type mice (data not shown). Taken together with the finding of a marked reduction in the inflammatory cell infiltration in 5-LO deficient mice, these data suggest that by inhibiting the expression of P-selectin and ICAM-1 the absence of 5-LO regulates neutrophil recruitment both at the rolling and firm adhesion phase.

In conclusion, our findings demonstrate that the degree of MOF is significantly attenuated in 5-LOKO mice. The mechanisms of the anti-inflammatory effect of the absence of functional 5-LO are not entirely clear. However, the use of knock-out animals can conclusively define the role of a particular enzyme in pathophysiology of the disease. In the present study it appears that the genetic inhibition of 5-LO reduced the expression of adhesion molecules, the recruitment of neutrophils, and ultimately the degree of tissue injury. This effect is very likely secondary to the prevention, by absence of 5-LO, of endothelial injury and hence a preservation of endothelial barrier function. These results support the view that the

overproduction of LT contributes to the development of MOF. Finally, the discovery of the concept that 5-LO regulates neutrophil trafficking may provide new insights in the interpretation of the protective effect of 5-LO inhibition, which may be useful in the therapy of conditions associated with local or systemic inflammation.

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