Volume 13, issue 3, September 2002 | Articles originaux

Absence of endogenous interleukin-10 enhances the evolution of acute lung injury

Abstract

Interleukin-10 (IL-10) exerts a wide spectrum of regulatory activities in the immune and inflammatory response. The aim of this study was to investigate the role of endogenous IL-10 on the modulation of the inflammatory response in mice subjected to carrageenan-induced lung injury. When compared to carrageenan-treated IL-10 wild-type (WT) mice, carrageenan-treated IL-10 knock-out mice (IL-10KO) mice experienced a higher rate of pleural exudation, and polymorphonuclear cell migration. Exudate levels of the pro-inflammatory

cytokines tumour necrosis factor, interleukin-1beta and interleukin-6 were also greatly enhanced in IL-10KO mice in comparison to wild-type mice. Lung myeloperoxidase (MPO) activity was significantly reduced in IL-10WT mice when compared to IL-10KO mice-treated with carrageenan. The degree of oxidative and nitrosative damage was significantly higher in IL-10KO mice than in wild-type littermates, as indicated by elevated malondialdehyde levels and formation of nitrotyrosine and poly (ADP-ribose) synthetase (PARS). Staining of lung tissue sections obtained from carrageenan-treated IL-10WT with an anti-COX-2 antibody showed a positive staining of the inflamed tissue. Furthermore, expression of inducible nitric oxide synthase (iNOS) was found mainly in the macrophages of the inflamed lungs from carrageenan-treated IL-10WT mice. The intensity and degree of the staining for COX-2 and iNOS were markedly enhanced in tissue sections obtained from carrageenan-treated IL-10KO mice. Most notably, the degree of lung injury caused by carrageenan was also enhanced in IL-10KO mice. Taken together, our results clearly demonstrate that endogenous IL-10 exerts an anti-inflammatory role during acute inflammation and tissue damage associated with carrageenan-induced pleurisy, possibly by regulating neutrophil recruitment, and the subsequent cytokine and oxidant generation.

Full text

ABBREVIATIONS

NO: nitric oxide

NOS: nitric oxide synthase

ecNOS: constitutive endothelial nitric oxide synthase

iNOS: inducible nitric oxide synthase

PARS: poly (ADP-ribose) synthetase

MPO: myeloperoxidase

PMN: polymorphonuclear cell

PBS: phosphate-buffered saline

INTRODUCTION

The balance between pro-inflammatory and other processes that serve to down regulate the inflammation factor, influences the degree and persistence of inflammatory process in the lung and other tissues [1, 2]. Pro-inflammatory cytokines including TNF-alpha, IL-1 and interleukin-6 (IL-6) contribute to the extension of the inflammatory process [3]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has immunosuppressive functions through down regulation of gamma interferon (IFN-gamma) production [4] and macrophage activation *in vitro*, *e.g.*, cytokine production [5], generation of nitric oxide (NO) [6], and surface expression of major histocompatibility complex class II [7]and co-stimulatory molecules [8], which also influences chemokine production and leukocyte infiltration [9].

Whereby the contribution of inflammatory leukocytes to the progression of experimental pleurisy is not well defined, neither are the mechanisms whereby these leukocytes are recruited to the lung are not fully understood. Recently, several chemotactic cytokines (chemokines), which demonstrate a high degree of specificity for the movement of leukocyte subpopulations, have been isolated and cloned. These chemokines include, MIP-Ialpha, MIP-1beta, RANTES, MCP-1, MCP-2, IL-8, and IP-10. The chemokines are chemotactic for neutrophils and for mononuclear cells [10-13].

PMNs moving out of the circulation into inflamed tissue play a crucial role in the destruction of foreign antigens and in the breakdown and remodelling of injured tissue [14]. PMNs can also be directly injurious to tissue during inflammation [15, 16]. Leukocyte-endothelial interaction involves a complex interplay among adhesion glycoproteins (*i.e.* integrins, immunoglobulin superfamily members and selectins). Leukocyte rolling is the first step in leukocyte-endothelial interaction and facilitates PMNs activation and adherence [17-19]. However, leukocyte accumulation is a complex phenomenon, which also involves endothelium-based adhesion molecules [20]. Intercellular adhesion molecule (ICAM-1) is an adhesion molecule normally expressed at a low basal level, but its expression can be enhanced by various inflammatory mediators such as interleukin-1 (IL-1) and TNF-alpha [21].

Furthermore, there is a great deal of evidence that the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage [22-24].

The purpose of this study was to investigate the role of endogenous IL-10 in a murine model of carrageenan-induced pleurisy. To address this question, the release of the pro-inflammatory cytokines and neutrophil infiltration were evaluated. Nitrotyrosine formation and lung levels of malondialdehyde were determined as indices of nitrosative and oxidative stress, respectively. Furthermore, we investigated whether the absence of IL-10 affects the lung activation of PARS and the expression of iNOS and COX-2. We observed that the absence of IL-10, exaggerated the lung injury induced by carrageenan, whereas maintenance of endogenous IL-10 production significantly attenuated the injury, indicating that IL-10 may mediate important features of acute inflammation.

MATERIALS AND METHODS

Animals

C57BLJ10 mice (4-5 weeks old, 20-22 g), with a targeted disruption of the IL-10 gene (IL-10KO) and wild-type controls littermates (IL-10WT) were purchased from Jackson Laboratories (Harlan Nossan, Italy). The animals were housed in a controlled environment and provided with standard rodent diet and water. Animal care was in compliance with Italian regulations for the protection of animals used for experimental and other scientific purposes (DM 116192), as well as with the EEC regulations (OJ of EC L 358/1 12/18/1986).

Carrageenan-induced pleurisy

Mice were anaesthetised with isoflurane and subjected to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.2 ml) or saline containing 1% gamma-carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 hours after the injection of the carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened and the pleural cavity rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 mug/ml). The exudate and washing solution were removed by aspiration and the total volume measured.

Any exudate, which was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in phosphate-buffered saline (PBS) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining. In a second group of experiments, pleurisy was induced in IL-10WT mice treated with anti-mouse IL-10 antibody (150 mug/day; Genzyme Corp) injected intraperitoneally (i.p.) at 24 and 1 hour before carrageenan administration. The dose of the IL-10 antibody used here has been previously used [25].

Measurement of nitrite/nitrate

Nitrite+nitrate (NOx) production, an indicator of NO synthesis, was measured in the supernatant samples as previously described [26]. Briefly, the nitrate in the supernatant was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 muM) at room temperature for 3 hours. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 mul of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100 mul samples. The optical density at 550 nm (OD_{550}) was measured using an ELISA microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison with the OD_{550} of standard solutions of DMEM.

Determination of nitric oxide synthase activity

The calcium-independent conversion of L-arginine to L-citrulline in the homogenates of lungs (obtained 4 hours after carrageenan treatment) served as an indicator of iNOS activity [26]. Lungs were scraped into a homogenate buffer composed of 50 mM Tris.HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) and homogenised in the buffer on ice, using a tissue homogeniser. Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described [27]. Briefly, homogenates (30 mul) were incubated in the presence of [^{3}H]-L-arginine (10 muM, 5 kBq per tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 muM) and EGTA (2 mM) for 20 min at 22° C. Reactions were stopped by dilution with 0.5 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the

eluted. [^{3}H]-L-citrulline activity was measured with a Beckman scintillation counter.

Measurement of cytokines and chemokines

IL-10, TNFalpha, IL-6, IL-1beta, MIP-2 and MIP-1alpha levels were evaluated in the exudate 4 hours after the induction of pleurisy by carrageenan injection. The assay was carried out using a colorimetric, commercial kit (Calbaiochem-Novabiochem Corporation, USA). The ELISA has a lower detection limit of 30 pg/ml.

Immunohistochemical localisation of nitrotyrosine, PARS, iNOS, COX-2, P-Selectin and ICAM-1

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described [28]. At 4 hours after carrageenan administration, the lungs were fixed in 10% buffered formaldehyde and 8 mum sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimised by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with primary anti-nitrotyrosine antibody (1:1000), anti-COX-2 antibody, anti-iNOS antibody, anti-P-selectin antibody, anti-ICAM-1 antibody or anti-poly (ADP-Ribose) (PAR) antibody (1:500), or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG (for nitrotyrosine, P-selectin, iNOS) or with a biotin-conjugated anti-goat IgG (for PAR and for COX-2), or with a biotin-conjugated anti-mouse (for ICAM-1) and avidin-biotin peroxidase complex. Immunocytochemistry photographs (n = 5) were assessed by densitometry. The assay was carried out using Optilab Graftek software on a Macintosh personal computer (CPU G3-266).

Measurement of prostaglandin E_2 in the pleural exudate

The amount of prostaglandin E_2 (PGE_2) present in the pleural fluid was measured by radioimmunoassay, without prior extraction or purification [29].

Assessment of COX activity

Lungs were obtained 4 hours after the induction of pleurisy by carrageenan injection. The material was homogenised at 4° C in a buffer containing protease inhibitors at the ratio of 5:1 (v/w). The protein concentration in the homogenates was measured by the Bradford assay [30], with bovine serum albumin (BSA) used as standard. Homogenates were incubated at 37° C for 30 min in the presence of excess arachidonic acid (30 muM). The samples were boiled and centrifuged at 10,000 g for ?? min. The concentration of 6-keto-PGF_{1alpha} present in the supernatant was measured by radioimmunoassay as previously described [31].

Light microscopy

Lung biopsies were taken 4 hours after injection of carrageenan. The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in phosphate-buffered saline) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Tissue sections (thickness 7 mum) were deparaffinized with xylene, stained with haematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz).

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described [32]. At 4 hours after intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each piece of tissue was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x g at 4° C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 mumol of peroxide/min at 37° C and was expressed in milli units per gram weight of wet tissue.

Determination of malondialdehyde (MDA) levels

The levels of malondialdehyde (MDA) in the lung tissue were determined as an indicator of lipid peroxidation [33]. Lung tissue, collected at the specified time, was homogenised in 1.15% KCl solution. An aliquot (100 mul) of the homogenate was added to a reaction mixture containing 200 mul of 8.1% SDS, 1,500 mul of 20% acetic acid (pH 3.5), 1,500 mul of 0.8% thiobarbituric acid and 700 mul distilled water. Samples were then boiled for 1 hour at 95° C and centrifuged at 3,000 x g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary anti-nitrotyrosine, anti-poly(ADP-ribose), anti-COX-2 antibodies and avidin-biotin peroxidase complex were obtained from 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 Healthcare Ltd, Thetford, Norfolk, UK).

Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean (s.e.m.) of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different days. The results were analysed by one-way ANOVA, followed by a Bonferroni *post-hoc* test for multiple comparisons. A *p*-value less than 0.05 was considered significant.

RESULTS

The development of carrageenan-induced pleurisy is increased in IL-10KO mice

All WT-mice, which had received carrageenan, developed an acute pleurisy, producing 0.95 ± 0.03 ml of turbid exudate (Figure 1A). When compared to the

number of cells collected from the pleural space of sham-operated mice (1.1 ± 0.2 x 10^6/mice, Figure 1B), injection of carrageenan induced a significant increase in the number of PMNs (54 ± 2 x 10^6/mice, Figure 1B). Histological examination of lung sections of IL-10WT mice treated with carrageenan showed tissue injury as well as inflammatory cell infiltration (Figure 2a). Pre-treatment of IL-10WT mice with anti-IL-10 antibody (*Table 1)*, as well as IL-10KO mice (Figure 2b), showed massive and more severe lung injury, inflammatory cell infiltration, as well as a significant increase in the pleural exudate. No histological alterations, inflammatory cell infiltration and exudate formation were found in sham-operated mice (data not shown).

Chemokine expression and neutrophil infiltration

The above histological pattern of lung injury appeared to be correlated with the influx of leukocytes into the lung tissue. Therefore, we investigate the role of IL-10 on the expression of chemokines in the inflamed lung during experimental pleurisy. MIP-lalpha and MIP-2 levels were significantly increased 4 hours after carrageenan administration in IL-10WT mice *(Table 1)*. MIP-1alpha and MIP-2 levels in IL-10KO mice and in the IL-10WT mice with anti-IL-10 antibody were significantly augmented in comparison to WT mice *(Table 1)*. Therefore, we next evaluated neutrophil infiltration by measurement of the activity of myeloperoxidase. Myeloperoxidase activity was significantly elevated at 4 hours after carrageenan administration in IL-10WT mice (Figure 3A). In IL-10KO mice and in the IL-10WT mice with anti-IL-10 antibody, lung myeloperoxidase activity was markedly increased in comparison to IL-10WT animals (Figure 3A).

Expression of adhesion molecules (ICAM-1, P-selectin)

Neutrophil infiltration in the lung tissues was associated with expression of adhesion molecules. In fact, 4 hours after the carrageenan injection, positive stainings for ICAM-1 (3.1 ± 0.08% of total tissue area; Figure 4A, A1) and for P-selectin (3.10 ± 0.09% of total tissue area; Figure 4C, C1) were found to be increased along the bronchial epithelium and in vessels from the carrageenan-treated IL-10WT mice. In carrageenan-treated IL-10KO mice, the staining for ICAM-1 (5.70 ± 0.1% of total tissue area) and P-selectin (5.62 ± 0.07% of total tissue area) was visibly and significantly increased (Figure 4B, B1, D, D1) in comparison with the IL-10WT mice. It is important to underline that no staining for ICAM-1 or P-selectin was observed in lungs of saline-treated mice (data not

shown). A similar increase of the positive staining of ICAM-1 and P-selectin was found in lungs from the IL-10WT mice with the anti-IL-10 antibody (data not shown).

Cytokine production

To test whether the endogenous IL-10 modulates the inflammatory process through the regulation of cytokine secretion, we analysed the exudate levels of pro-inflammatory cytokines TNFalpha, IL-1beta and IL-6 in IL-10KO and wild type mice. A substantial increase in TNF-alpha, IL-1beta, IL-6 and IL-10 production was found in the exudate from IL-10WT mice 4 hours after carrageenan administration *(Table 1)*. Levels of TNF-alpha, IL-1beta and IL-6 were significantly higher in IL-10-deficient mice than in IL-10WT animals *(Table 1)*. A similar enhancement of the pro-inflammatory cytokines was also observed in the exudate from IL-10WT mice treated with an antibody against IL-10 *(Table 1)*. In contrast, the levels of IL-10 were not detectable in IL-10KO mice and were significantly reduced in IL-10WT mice treated with an antibody against IL-10 (Table 1). No significant cytokine increased was observe in the exudate of sham-operated mice.

Nitric oxide production

The levels of NO were significantly ($p < 0.01$) increased in the exudate from carrageenan-treated IL-10WT mice in comparison to sham-treated mice (Figure 5A). In contrast, the levels of NO were significantly increased in the exudate of carrageenan-treated IL-10KO and carrageenan-treated IL-10WT mice treated with the anti-10 antibody (Figure 5A). In the lungs obtained from animals subjected to carrageenan-induced pleurisy, a significant increase of iNOS activity was detected at 4 hours (Figure 5B). The iNOS activity was significantly ($p < 0.01$) increased in IL-10KO mice and in IL-10WT mice treated with the anti-IL-10 antibody (Figure 5B). Immunohistochemical analysis of lung sections obtained from IL-10WT mice treated with carrageenan revealed a positive staining (3.2 ± 0.07% of total tissue area) for iNOS, which was primarily localised in bronchial epithelium (Figure 6A, A1). In carrageenan-treated IL-10KO mice, the staining for iNOS (4.92 ± 0.1% of total tissue area) was visibly and significantly increased (Figure 6B, B1) in comparison with the IL-10WT mice. It is important to underline that no staining for iNOS was observed in lungs of saline-treated mice

(data not shown). A similar increase in the positive staining of iNOS was found in the lung from the IL-10WT mice with anti-IL-10 antibody (data not shown).

Lipid peroxidation and nitrotyrosine formation

The formation of free radicals and oxidant molecules during lung inflammation has been suggested to contribute significantly to tissue injury [22]. At 4 hours after carrageenan administration, a substantial increase in the lung MDA levels, indicative of lipid peroxidation, was observed in IL-10WT (Figure 3B).

In IL-10KO mice and in the IL-10WT mice with anti-IL-10 antibody, lung MDA levels were markedly increased in comparison to those of IL-10WT animals (Figure 3B).

Furthermore, a positive staining for nitrotyrosine (2.8 ± 0.09% of total tissue area), a marker of nitrosative injury, was found in the lungs of IL-10WT mice (Figure 7A, A1). In carrageenan-treated IL-10KO mice, the staining for nitrotyrosine (5.1 ± 0.12% of total tissue area) was visibly and significantly increased (Figure 7B, B1) in comparison with the IL-10WT mice. It is important to underline that no staining for nitrotyrosine was observed in lungs of saline-treated mice (data not shown). Similar increase in the positive staining of nitrotyrosine was found in the lung from the IL-10WT mice with anti-IL-10 antibody (data not shown).

COX activity (PGE_2 and 6-keto-PGF_{1alpha})

Measured the increase in the formation of PGE2 in the exudate assessed the COX activity in carrageenan-induced pleural exudate and lung homogenates. The amount of PGE_2 found in the pleural exudate of carrageenan-treated IL-10WT mice was 335 ± 16 pg/mice (n = 6). The amount of PGE_2 was significantly enhanced in the exudate obtained from IL-10KO mice as well as from IL-10WT mice treated with the anti-IL-10 antibody (Figure 8A). In lungs from carrageenan-treated IL-10WT mice, the amount of 6-keto-PGF_{1alpha} was 216 ± 12 pg/mg/tissue (Figure 8B). The amount of 6-keto-PGF_{1alpha} was significantly increased in the lungs from carrageenan-treated IL-10KO mice as well as from IL-10WT mice treated with the anti-IL-10 antibody (Figure 8B). In lung sections obtained from IL-10WT mice treated with carrageenan, a positive staining for COX-2 (2.97 ± 0.12% of total tissue area) was observed in alveolar macrophages (Figure 6C, C1). In carrageenan-treated IL-10KO mice, the staining for COX-2 (3.98 ± 0.14% of

total tissue area) was visibly and significantly increased (Figure 6D, D1) in comparison with the IL-10WT mice. Note that there was no staining for COX-2 in lungs of saline-treated mice (data not shown).

COX-1 was also detected by immunohistochemistry analysis in the lung sections obtained from IL-10WT mice treated with carrageenan, but its positive staining remained almost the same as that in the tissue obtained from sham-operated control animals (data not shown). The lungs of carrageenan-treated IL-10KO mice shown similar amounts of positive staining for COX-1 as those found in the lungs of IL-10WT mice (data not shown).

PARP formation

To investigate the cellular mechanisms, by which endogenous IL-10 may attenuate lung injury, we evaluated the activation of PARP, an important mediator involved in the inflammatory process [24]. Positive staining (2.65 ± 0.1% of total tissue area) for poly ADP-ribosylated proteins (an indicator of PARP activation) was found in lung sections obtained from IL-10WT mice treated with carrageenan (Figure 7C, C1). A significant increase (4.78 ± 0.08% of total tissue area) in positive staining for PARP (Figure 7D, D1) was found in the lungs of carrageenan-treated IL-10KO mice. Similar increase in the positive staining of PARP was found in the lungs from the IL-10WT mice with anti-IL-10 antibody (data not shown). No staining for PARP was observed in lungs of saline-treated mice (data not shown).

DISCUSSION

Our data demonstrate that (i) the development of carrageenan-induced pleurisy, (ii) the infiltration of the lung with PMNs (histology and MPO activity), (iii) the degree of lipid peroxidation in the lung, and (iv) the degree of lung injury (histology) caused by injection of carrageenan is significantly exaggerated in IL-10 knock-out mice (when compared to wild-type mice treated with carrageenan). All these findings suggest that endogenous IL-10 reduced the inflammation associated with carrageenan-induced pleurisy. In order to support the hypothesis that endogenous IL-10 plays an anti-inflammatory role in the model of inflammation used here, we have also pre-treated wild-type mice with an antibody against IL-10, and subsequently subjected these animals to injection of carrageenan into the pleural space. Interestingly, pre-treatment of

wild-type mice with an antibody against IL-10 also increased the development of carrageenan-induced pleurisy, the infiltration of the lung with PMNs, the degree of lipid peroxidation and the degree of lung injury caused by injection of carrageenan. Taken together, these two studies strongly support the view that IL-10 plays an anti-inflammatory role in carrageenan-induced pleurisy in mice. What then, is the mechanism by which IL-10 reduces the inflammation caused by injection of carrageenan into the pleural cavity of the mice?

There is evidence that the pro-inflammatory cytokines TNF-alpha and IL-1 help to propagate the extension of a local or systemic inflammatory process [34, 35]. *In vitro* studies have shown that IL-10 can suppress synthesis of several pro-inflammatory cytokine, including IL-1, TNF-alpha, IL-6, as well as the chemokines (MIP-Ialpha and MIP-2) [36, 37]. We confirm here that the inflammatory process caused by injection of carrageenan into the pleural cavity leads to a substantial increase in the levels of TNFalpha, IL-6 and IL-1, MIP-Ialpha and MIP-2 in the exudate. Interestingly, the levels of these pro-inflammatory cytokines, as well as those of the the two chemokines are significantly enhanced in the exudate obtained from animals, which are unable to produce endogenous IL-10 (IL-10 knock-out mice) or from wild-type mice in which the effects of endogenous IL-10 have been attenuated by an antibody against this cytokine. These findings suggest that in the presence of endogenous IL-10, the degree of inflammation and, hence, the formation of pro-inflammatory cytokines and chemokines are significantly reduced.

Both chemokines and cytokines may, in concert, play a pivotal role in initiating the recruitment of monocytes and leukocytes to the site of lung injury. Endothelial adhesion molecules are major regulators of neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion, and emigration from the vessels to the tissue. P-selectin is rapidly released to the endothelial surface from preformed storage pools after exposure to certain stimuli such as hydrogen peroxide, thrombin, histamine or complement, and allows the leukocytes to roll along the endothelium [38]. ICAM-1, constitutively expressed on the surface of endothelial cells, is then involved in the neutrophil adhesion [21, 38]. We confirmed in the present study that carrageenan induced the appearance of P-selectin on the endothelium of small vessels, and up-regulated the surface expression of ICAM-1 on endothelial cells in both wild-type and IL-10KO mice. However, we demonstrated larger increments in the expression of P-selectin and ICAM- 1 in IL-10-deficient mice compared to wild-type mice 4 hours

after carrageenan administration. Interestingly, we found that the constitutive expression of ICAM-1 in the lung did not differ between sham IL-10- deficient and wild-type mice. Taken together with the finding of a marked reduction in the inflammatory cell infiltration in wild-type mice, these data suggest that endogenous IL-10 does not modulate the constitutive expression of adhesion molecules. IL-10, however, appears to inhibit the expression of P-selectin and ICAM-1 in acute lung inflammation, regulating neutrophil recruitment both at the rolling and firm adhesion phase. In fact, there was a good correlation between the significant reduction of leukocyte infiltration in wild-type mice (when compared to IL-10KO mice) and the reduced tissue damage, as evaluated by histological examination.

There is a great deal of evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contribute to tissue damage [22-24]. Inhibitors of NOS activity reduce the development of carrageenan-induced inflammation and support a role for NO in the pathophysiology associated with this model of inflammation [39-41]. We demonstrate here that the formation of nitrite and nitrate (metabolites of NO in water), as well as the induction of iNOS protein in macrophages and airway epithelial cells, caused by injection of carrageenan into the pleural cavity, is increased in lungs of IL-10 knock-out mice as well as in wild-type mice pre-treated with an antibody against IL-10. This finding suggests that endogenous IL-10 may regulate the induction of iNOS caused by carrageenan in the lung. The induction of iNOS caused *e.g.* by injection of endotoxin in rodents *in vivo*, is mediated by endogenous TNF-alpha, as polyclonal antibodies against this cytokine abolish the induction of iNOS caused by endotoxin in the rat [42]. Like TNF-alpha, endogenous IL-1 also plays an important role in the induction of iNOS, as the endogenous IL-1 receptor antagonist also reduces the degree of iNOS induction caused by LPS in rodents [43]. As the levels of TNF-alpha and IL-1 are significantly increased in the exudate obtained from IL-10 knock-out mice and from wild-type mice pre-treated with an antibody against IL-10, we propose that the increase of the induction of iNOS protein and activity observed in mice, which are unable to produce endogenous IL-10 (IL-10 knock-out mice) are secondary to an enhanced formation of endogenous TNF-alpha and IL-1. As for iNOS, TNF-alpha and IL-1, also mediated the expression of COX-2. As the levels of TNF-alpha and IL-1, as well as the induction of iNOS, are significantly enhanced in the exudate obtained from IL-10 knock-out mice (and from wild-type mice pre-treated with an antibody against IL-10), it is not surprising that the degree of

COX-2 induction is also significantly increased in macrophages located in the lungs of IL-10 knock-out mice subjected to carrageenan-induced pleurisy. There is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of local inflammation [44, 45], and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects [46-48]. We demonstrate here that the increase in the levels of PGE_2 caused by injection of carrageenan into the pleural cavity is exaggerated in the exudate of IL-10 knock-out mice, as well as in wild-type mice pre-treated with an antibody against IL-10. The enhanced formation of PGE_2 is secondary to the expression of COX-2 protein, as (i) there was no increase in the expression of COX-1 protein (detected by immunohistochemistry) after carrageenan injection and (ii) selective inhibitors of COX-2 activity including NS-398 (nimesulide) and SC-58125 (Celecoxib) markedly abolished the increase in PGE_2 caused by injection of carrageenan into the pleural space [49] Thus, we suggest that IL-10 may regulate the expression of COX-2 protein and activity caused by injection of carrageenan in the lung and that the subsequent enhanced formation of PGE2 (and other COX-2 metabolites) contributes to the observed inflammatory process. This conclusion is supported by the previous studies which showed that selective inhibitors of COX-2 activity reduced the inflammation caused by carrageenan in the rat [47, 48].

In addition to prostaglandins and NO, peroxynitrite is also generated in carrageenan-induced inflammation [24-26]. We demonstrate here that injection of carrageenan into the pleural cavity of wild-type mice leads to a substantial increase in the degree of nitrosylation of proteins in the lung. In contrast, the degree of staining for nitrotyrosine was significantly enhanced in IL-10 knock-out mice and in wild type mice pre-treated with an antibody against IL-10. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation (footprint) of peroxynitrite [49]. There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; *e.g.*, the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine [50]. Increased nitrotyrosine staining is considered, therefore, as an indication of "increased nitrosative stress" rather than a specific marker of the generation of peroxynitrite. Thus, our results suggest that the degree of "nitrosative stress" caused by injection of carrageenan is exaggerated in lungs from animals, which

are unable to produce endogenous IL-10 or those in which the effects of endogenous IL-10 have been attenuated/abolished by an antibody against this cytokine.

ROS and peroxynitrite produce cellular injury and necrosis *via* several mechanisms, including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA which trigger energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD *in vitro* and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed "the PARS suicide hypothesis" [50-52]. There is recent evidence that the activation of PARS may also play an important role in inflammation [51-54]. We also demonstrate here that the increase in PARS activity caused by carrageenan in the lung is increased in lungs of IL-10 knock-out mice as well as in lungs of wild-type mice pre-treated with an antibody against IL-10.

CONCLUSION

In conclusion, this study demonstrates that the degree of inflammation caused by injection of carrageenan in to the pleural cavity of the mouse is significantly exaggerated in IL-10 knock-out mice. Similarly, pre-treatment of wild-type mice with an antibody against IL-10 also enhanced the inflammatory response caused by subsequent injection of carrageenan. These findings support the view that endogenous IL-10 contributes to the regulation of the inflammatory response in the model of carrageenan-induced pleurisy used here. The mechanisms of the anti-inflammatory effect of IL-10 are not entirely clear. It appears that IL-10 reduces the formation of other pro-inflammatory cytokines such as TNF-alpha and IL-1, which in turn may augment the recruitment of neutrophils, the expression of iNOS and COX-2 protein and activity, and ultimately the degree of peroxynitrite formation and tissue injury. In addition, IL-10 appears to reduced the recruitment of PMNs. Finally, our findings suggest that interventions which may enhance the generation or the effects of IL-10 may be useful in conditions associated with local or systemic inflammation.

Acknowledgements. This study was supported by a grant from MURST. The authors would like to thank Giovanni Pergolizzi and Carmelo La Spada for their

excellent technical assistance during this study, Mrs Caterina Cutrona for secretarial assistance and Miss Valentina Malvagni for editorial assistance with the manuscript.

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Figures

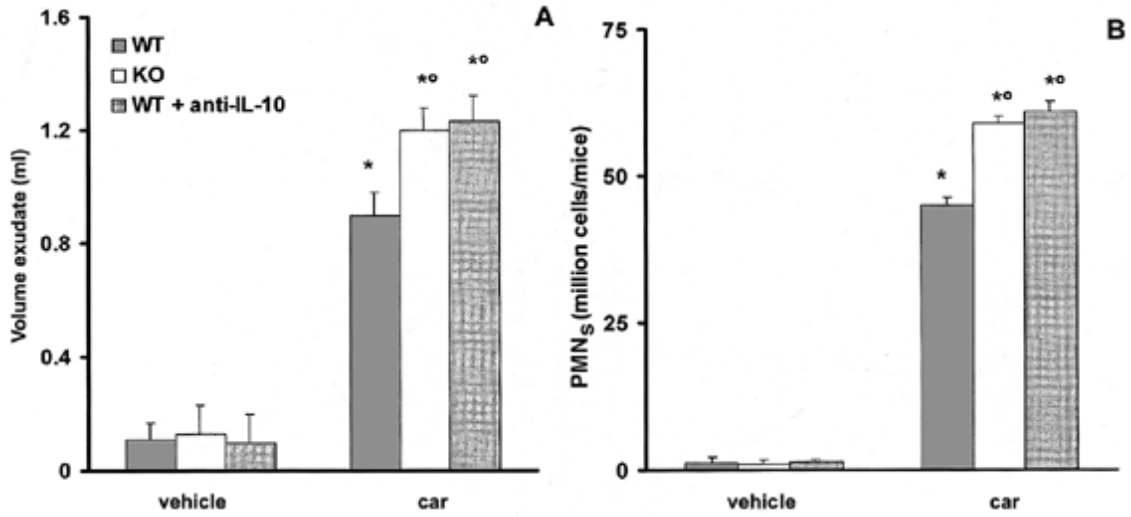

Figure 1

(A) **Exudate volume;** (B) **leukocyte accumulation.**

IL-10KO mice show a significant increase in peritoneal exudate, leukocyte migration and nitrate/nitrite levels. Responses in IL-10WT controls and IL-10KO animals were compared. Data are means ± SEM of 10 mice for each group. *p < 0.01 *versus* vehicle. °p < 0.01 represents significant reduction of the various parameters in the group in which IL-10 was inhibited.

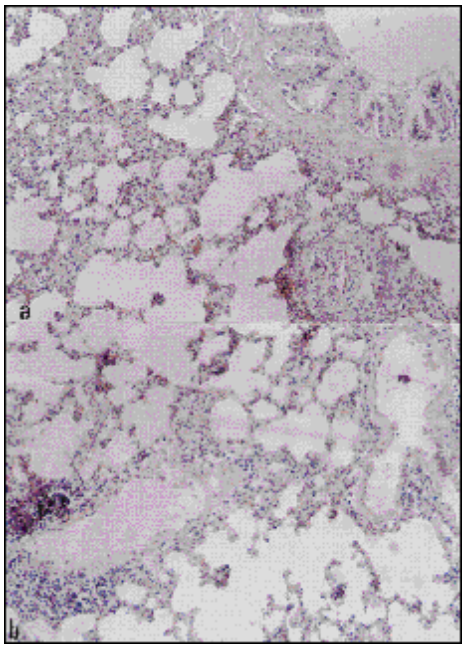

Figure 2

Effect of endogenous IL-10 on lung injury.

The lung sections from carrageenan-treated IL-10WT mice demonstrate inflammatory cells infiltration **(a)**. The lung sections from carrageenan-treated IL-10KO mice demonstrate a marked increase in cellular infiltration **(b)**. Original magnification: 125x. Figure is representative of at least 3 experiments performed on different days.

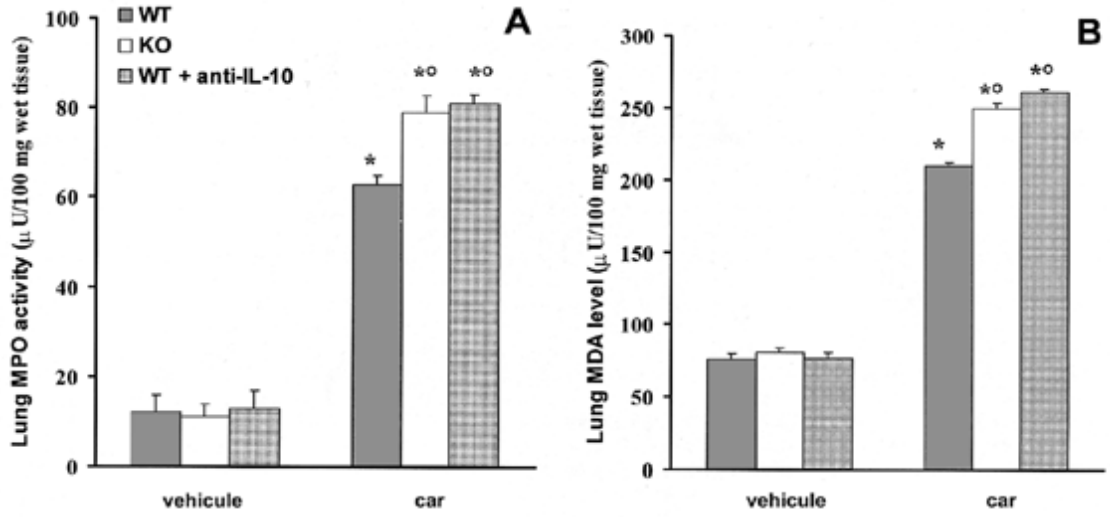

Figure 3

Effect of endogenous IL-10 on polymorphonuclear leukocyte migration and lipid peroxidation.

Myeloperoxidase (MPO) activity **(A)** and malondialdehyde (MDA) **(B)** in the lungs of carrageenan-treated mice sacrificed at 4 hours. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated IL-10WT mice in comparison to sham mice. IL-10 depletion significantly enhanced the carrageenan-

induced increase in MPO activity and MDA levels. Data are means ± SEM of 10 mice for each group. *p < 0.01 *versus* vehicle. °p < 0.01 represents significant reduction of the various parameters in the group in which IL-10 was inhibited.

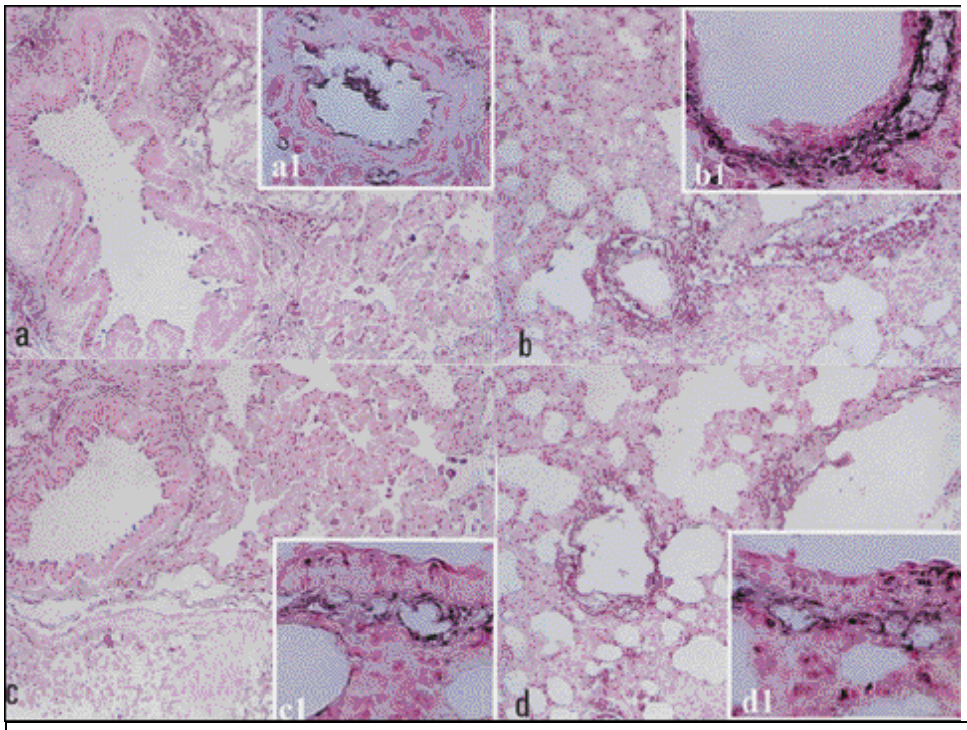

Figure 4

Immunohistochemical localization of ICAM-1 and P-selectin in the lung.

Sections obtained from carrageenan-treated IL-10WT mice showed intense positive staining for ICAM-1 **(a, a1)** and for P-selectin **(c, c1)** on bronchial epithelium. The degree of bronchial epithelium staining for ICAM-1 **(b, b1)** and for P-selectin **(d, d1)** was markedly increased in tissue sections obtained from IL-10KO mice. Original magnification: **a, b, c, d** 145x; **a1, b1, c1, d1** 375x. Figure is representative of at least 3 experiments performed on different experimental days.

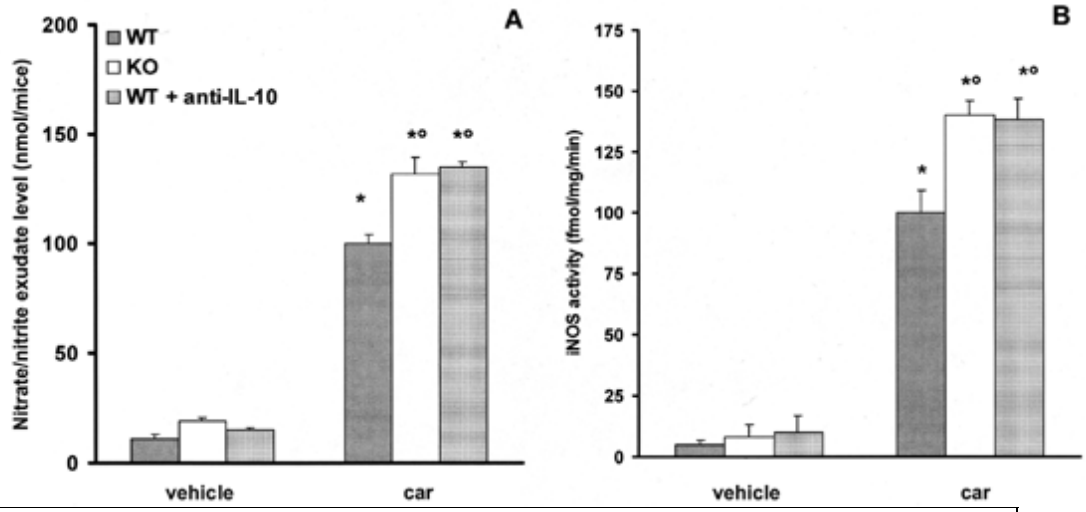

Figure 5

Nitrite and nitrate concentrations in pleural exudate (A) **and pulmonary iNOS activity** (B) **4 hours after carrageenan administration.**

Nitrite and nitrate levels and iNOS activity in carrageenan-treated IL-10WT mice were significantly increased *versus* sham group. IL-10KO mice show a significant

enhancement of the carrageenan-induced elevation of nitrite and nitrate levels and the expression of iNOS activity. Data are means ± SEM of 10 mice for each group. *p < 0.01 *versus* vehicle. °p < 0.01 represents significant reduction of the various parameters in the group in which IL-10 was inhibited.

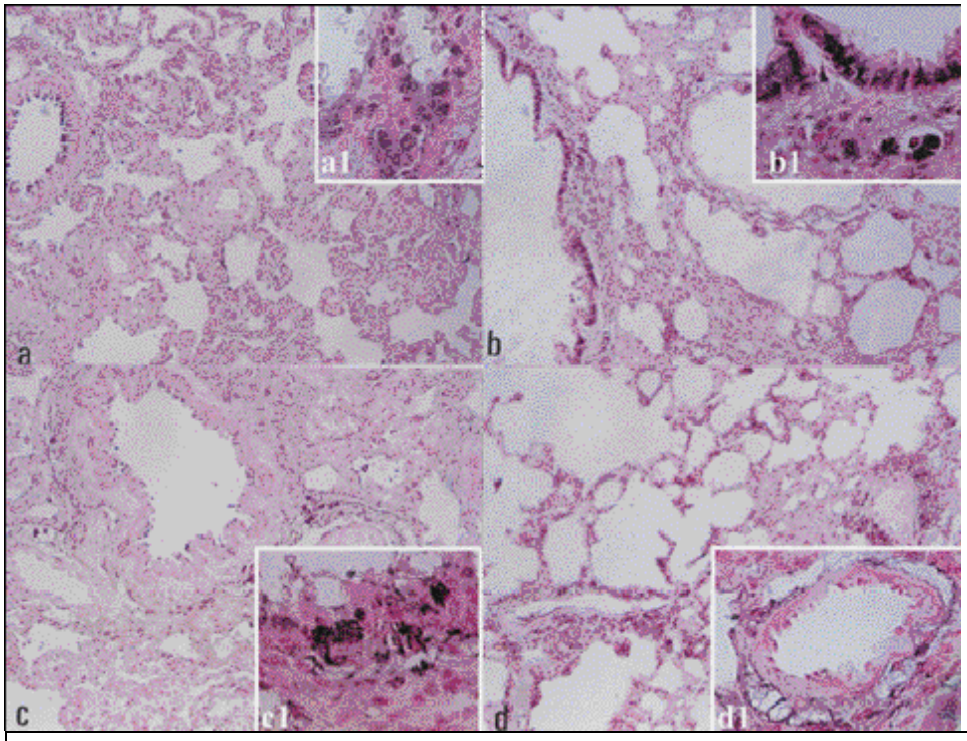

Figure 6

Immunohistochemical localization of iNOS and COX-2 in the lung.

Section obtained from carrageenan-treated IL-10WT mice showed intense positive staining for iNOS **(a, a1)** and for COX-2 **(c, c1)** in bronchial macrophages. The degree of bronchial epithelium staining for iNOS **(b, b1)** and for COX-2 **(d, d1)** was markedly increased in tissue sections obtained from IL-10KO mice. Original magnification: **a, b, c, d** 145x; **a1, b1, c1, d1** 375x. Figure is representative of at least 3 experiments performed on different days.

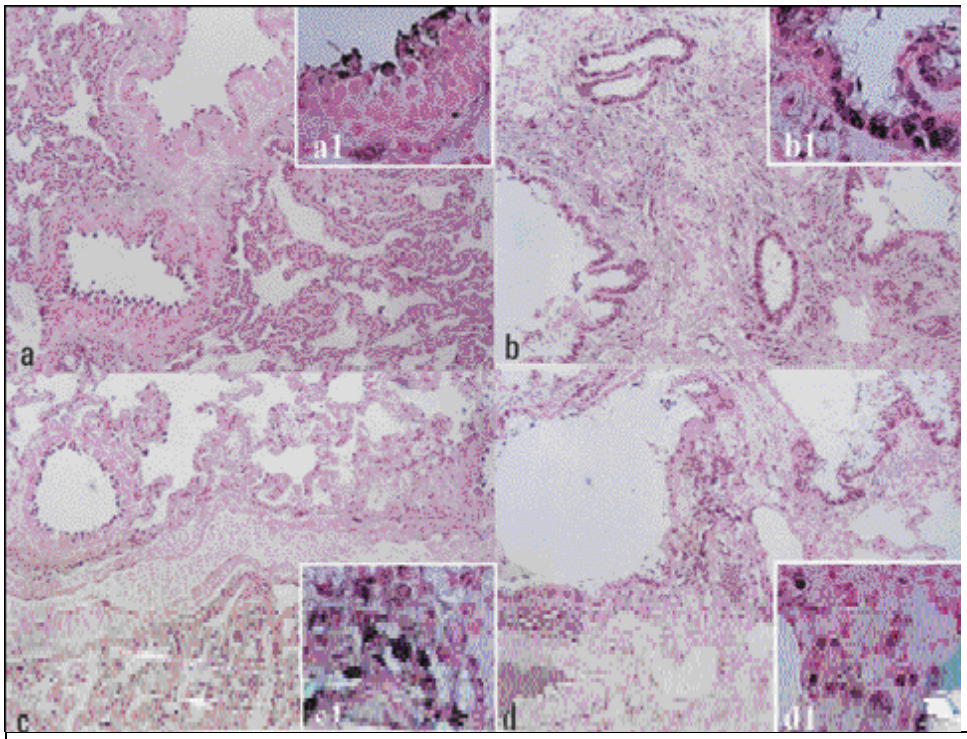

Figure 7

Immunohistochemical localization for nitrotytosine and for PARP in the lung.

Immunohistochemistry for nitrotyrosine **(a, a1)** and for PARP **(c, c1)** shows positive staining along the vessels and in the bronchial epithelium from carrageenan-treated IL-10WT mice. The intensity of the positive staining for nitrotyrosine **(b, b1)** and for PARP **(d, d1)** was markedly increased in tissue sections obtained from carrageenan-treated IL-10KO mice. Original magnification: **a, b, c, d** 145x; **a1, b1, c1, d1** 375x. Figure is representative of at least 3 experiments performed on different days.

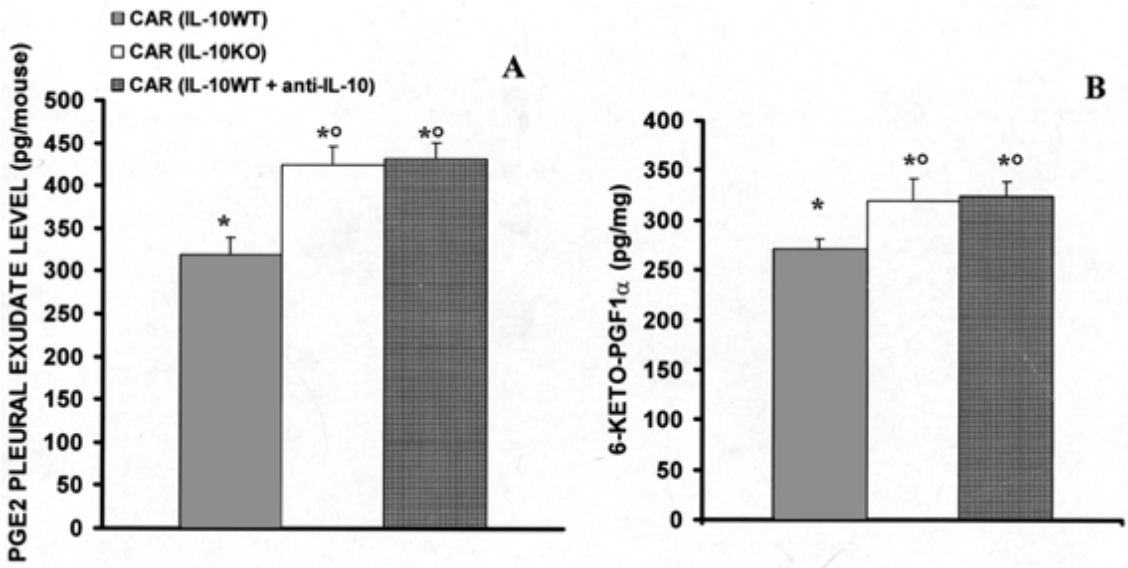

Figure 8

PGE_2 levels in the pleural exudate (A) **and 6-keto-PGF_{1alpha} in the lungs** (B) **from carrageenan-treated mice.**

Data are means ± SEM of 10 mice for each group. *p < 0.01 *versus* vehicle. ^{o}p < 0.01 represents significant reduction of the various parameters in the group in which IL-10 was inhibited.

Table 1
Effect of absence of IL-10 on chemokine and cytokine production 4 hours after carrageenan-induced inflammation

	MIP-Iα (pg/ml)	MIP-2 (ng/ml)	TNF-α (pg/ml)	Il-6 (unit/ml)	IL-1β (pg/ml)	IL-10 (pg/ml)
IL-10WT + vehicle	70 ± 5	7 ± 0.9	17 ± 1.9	ND	2 ± 0.9	ND
IL-10KO + vehicle	65 ± 4	9 ± 0.6	15 ± 1.6	ND	3 ± 0.6	ND
IL-10WT + anti-IL10 + vehicle	60 ± 4	12 ± 1	18 ± 1.3	ND	2.8 ± 1	ND
IL-10WT + car	580 ± 4.3*	38 ± 2.4*	420 ± 6.3*	32 ± 4.8*	230 ± 8*	450 ± 7.4*
IL-10WT + car	800 ± 12°	52 ± 2.2°	532 ± 3.5°	48 ± 3.2°	310 ± 4.2°	ND
IL-10WT + anti-IL10 + car	820 ± 14°	55 ± 1.9°	513 ± 3.1°	49 ± 3.1°	305 ± 3.9°	14 ± 2.9°

Data are means ± SEM of 10 animals for each group. *p < 0.01 *versus* vehicle. °p < 0.01 represents significant increase of the various parameters in the group in which IL-10 was absent.

Authors

Salvatore Cuzzocrea, Emanuela Mazzon, Laura Dugo, Ivana Serraino, Rosanna Di Paola, Tiziana Genovese, Angela De Sarro, Achille P. Caputi

Ph. D. Institute of Pharmacology, School of Medicine, University of Messina, Torre Biologica - Policlinico Universitario Via C. Valeria, Gazzi, 98100 Messina Italy.

In this issue

Methods to map protein interactions in mammalian cells: different tools to address different questions

Sven Eyckerman, Jan Tavernier
two-hybrid, enzymatic complementation, FRET/BRET, MAPPIT, protein splicing.

Absence of endogenous interleukin-10 enhances the evolution of acute lung injury

Salvatore Cuzzocrea, Emanuela Mazzon, Laura Dugo, Ivana Serraino, Rosanna Di Paola, Tiziana Genovese, Angela De Sarro, Achille P. Caputi
carrageenan, inflammation, nitric oxide, peroxynitrite, superoxide dismutase, IL-10.

Dose-effect of interleukin-10 and its immunoregulatory role in Crohn's disease

Alain Schmit, Monica Carol, Fabienne Robert , Patrick Bontems, Jean-Jacques Houben, André Van Gossum, Michel Goldman, Françoise Mascart
Crohn's disease, interleukin-10, tumor necrosing factor-alpha.

Expression of genes for cytokines and cytokine-related functions in leukocytes infected with Herpes simplex virus: comparison between resistant and susceptible mouse strains

Søren R. Paludan, Jesper Melchjorsen, Lene Malmgaard, Soren C. Mogensen
leukocytes, cytokines, cDNA arrays, Herpes simplex virus.

Mycophenolic acid and methotrexate inhibit lymphocyte cytokine production via different mechanisms

Sacha de Lathouder, Andreas H. Gerards, Els R. de Groot , Marijke Valkhof, Lucien A. Aarden
lymphocytes, apoptosis, whole blood, activation markers, drug effects, immunosuppression.

Cytokine pattern in cystic fibrosis patients during antibiotic therapy and gene therapy using adenoviral vector

Philippe Reix, Gabriel Bellon, Jacques Bienvenu, Andrea Pavirani, Hélène Levrey-Hadden
cytokine, cystic fibrosis, adenoviral vector, antibiotic.

Induction of interleukin-2 receptor alpha (IL-2Ralpha) expression by interleukin-2: important role of the interleukin-2 receptorv beta chain region between the two Stat5 docking sites

Véronique Imbert, Roger Rezzonico, Patrick Reichenbach, Markus Nabholz
Stat5, IL-2 receptor, T lymphocytes, CD25.

Bronchopulmonary hyperreactivity and lung eosinophil sequestration but not their migration to the alveolar compartment are independent of interleukin-5 in allergic mice

Barbara Proust, Claude Ruffié, Jean Lefort, B. Boris Vargaftig
bronchopulmonary hyperreactivity, eosinophilia, IL-5, TRFK-5.

Complex network of cytokines activating murine microglial cell activity against Acanthamoeba castellani

Nunzia Benedetto, Claude Auriault
A. castellani, cytokine, MGC.

The mechanism by which interleukin-1 beta reduces net fluid absorption from the rat colon

Sawsan Ibrahim Kreydiyyeh, Rana Al-Sadi
Interleukin-1 beta, Na+K+ ATPase, NaKCl2, Cl- secretion, Na+ absorption, colon.

Proinflammatory interferon-gamma - inducing monokines (interleukin-12, interleukin-18, interleukin-15) - serum profile in patients with systemic lupus erythematosus

Ewa Robak, Tadeusz Robak, Anna Wozniacka, Malgorzata Zak-Prelich, Anna Sysa-Jedrzejowska, Henryk Stepien
SLE, Interferon gamma, IL-12, IL-15, IL-18, disease activity.

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