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Role of inducible nitric oxide synthase in the regulation of neutrophil migration in zymosan-induced inflammation

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SUMMARY

In the present study, by comparing the responses in wild-type mice and mice lacking the inducible (or type 2) nitric oxide synthase (iNOS), we investigated the role played by iNOS in the regulation of polymorphonuclear granulocyte (PMN) accumulation and chemokine production in the mouse peritoneal cavity in response to administration of zymosan (0·2 mg). Zymosan injection induced the production of nitric oxide, and triggered a time-dependent PMN immigration into the peritoneal cavity. This response was associated with increases in the level of the chemokines macrophage inflammatory protein (MIP)-1α, MIP-2, monocyte chemo-attractant protein (MCP)-1 and cytokine-induced neutrophil chemo-attractant (KC), as measured in the peritoneal cavities. Injection of zymosan also induced a time-dependent increase in the production of the anti-inflammatory cytokine interleukin-10 (IL-10) in the peritoneal cavity. When comparing the response between wild-type and iNOS knockout (KO) mice, we observed that the low-level PMN accumulation measured at 1 hr was slightly but significantly increased in the absence of functional iNOS. On the other hand, the delayed response (2–4 hr after zymosan) of PMN accumulation was suppressed in the iNOS KO mice. The early enhancement of PMN infiltration in the iNOS-deficient mice was associated with increased peritoneal levels of MIP-2, KC and IL-10 proteins. The delayed suppression of PMN infiltration was associated with reduced MIP-2 and IL-10 levels in the peritoneal cavity. The lack of iNOS did not affect the release of MIP-1α and MCP-1 at any of the time-points studied. The current data demonstrate that iNOS regulates the production of certain CXC (but not CC) proinflammatory chemokines, the production of IL-10 and exerts a biphasic regulatory effect on PMN accumulation in zymosan-induced acute inflammation.

INTRODUCTION

The inducible isoform of nitric oxide synthase (iNOS) has been shown to play a role as a terminal effector pathway in various forms of shock and inflammation.[1–6] Furthermore, recent studies indicated that nitric oxide (NO) from iNOS can also modulate the expression of pro- and anti-inflammatory genes.[7–12] Recently, several groups have succeeded in generating genetically engineered animals which lack the gene for iNOS.[13–15] The use of these animals has yielded conflicting information on the role of iNOS in various inflammatory diseases because the effect of iNOS gene deletion ranges from protection,[13,14,16–18] to lack of effect[13,15,18,19] or enhancement[20,21] of the inflammatory response (see reference 6 for a recent review on this subject).

The aim of the current study was to investigate the role of iNOS in modulating the course of polymorphonuclear granulocyte (PMN) accumulation and chemokine production in the mouse peritoneal cavity in response to administration of a local inflammatory stimulus, zymosan. The role of iNOS in neutrophil recruitment was defined by comparing the responses in wild-type mice and mice lacking iNOS. In addition to measuring the kinetics of PMN accumulation, we also measured the production of the murine chemokines macrophage inflammatory protein (MIP)-1α, MIP-2, monocyte chemo-attractant protein (MCP-1) and cytokine-induced neutrophil chemo-attractant (KC), and the production of the anti-inflammatory cytokine, interleukin-10 (IL-10), in the peritoneal cavities, and we have attempted to correlate the modulation of PMN accumulation with the modulation of the production of these mediators. The results of the current study demonstrate that iNOS regulates the production of certain

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Abbreviations: EDTA, ethylenediaminetetra-acetic acid sodium salt; IL, interleukin; MCP, monocyte chemo-attractant protein; MIP, macrophage inflammatory protein; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PMN, polymorphonuclear granulocyte.

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MATERIALS AND METHODS

Animals

The investigations conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and were performed with the approval of the Institutional Review Board of the Children's Hospital Research Foundation, Cincinnati, OH. Wild-type animals and iNOS knockout (KO) mice (C57BL10) were generated as previously described.[15] Breeding pairs of these mice were the generous gift of Dr V. Laubach.[15] Mice were housed, bred and maintained under standard conditions in the animal facility of the Children's Hospital Research Foundation, Cincinnati, OH. The iNOS KO animals appeared healthy and behaved similarly to the wild-type animals under our housing conditions.[22] Mice (20–25 g), 6 to 8 weeks of age, were used in the studies.

Model of inflammation

Zymosan peritonitis was induced as previously described.[23] Briefly, mice were injected intraperitoneally (i.p.) with zymosan A (0·2 mg in 500 μl of saline). At selected time-points, animals were killed by carbon dioxide exposure and peritoneal cavities were lavaged with 3 ml of phosphate-buffered saline (PBS) containing 3 mM ethylenediaminetetra-acetic acid sodium salt (EDTA). Aliquots of the lavage fluid were then stained with Turk's solution (0·01% crystal violet in 3% acetic acid) and differential countings performed using a Neubauer haemocytometer and a light microscope (Olympus BX40, Lake Success, NY). The lavage fluids were centrifuged at 400 ***g*** for 10 min and cell-free supernatants stored at −20° prior to chemokine and cytokine evaluation by enzyme-linked immunosorbent assay (ELISA; see below) or for measurement of nitrite and nitrate concentrations (the breakdown products of NO, see below).

Measurement of nitrite/nitrate concentration in the lavage fluids

Nitrite/nitrate production, an indicator of NO synthesis, was measured in lavage fluids as previously described.[24] First, nitrate in the peritoneal lavage fluids was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 mM) at room temperature for 3 hr. Nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μl of Griess reagent (0·1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; 1:1 vol/vol) to 100-μl samples. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrate concentrations were calculated by comparison with the OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

Chemokines and cytokine ELISA

Immunoreactive murine MCP-1, MIP-2, IL-10, MIP-1α and KC were quantified as described previously,[25] using a commercially available ELISA according to the manufacturer's protocol. In brief, for MCP-1 detection, lavage fluids (100 μl) were assayed per sample and compared with a standard curve constructed with 0–2·5 ng/ml murine MCP-1. Lavage fluids were tested similarly for the murine chemokines KC (standard curve ranging from 0 to 1 ng/ml), MIP-1α (standard curve ranging from 0 to 300 pg/ml), MIP-2 (0–500 pg/ml) and the cytokine IL-10 (standard curve ranging from 0 to 1 ng/ml). The ELISA method consistently detected KC, MIP-2, MIP-1α and murine IL-10 at >1·5 pg/ml and MCP-1 at >9 pg/ml. The ELISAs showed negligible (<1%) cross-reactivity with several murine cytokines and chemokines (data supplied by the manufacturer).

Reagents

Quantikine™ ELISA kits for murine IL-10, MIP-1α, MIP-2 and KC were purchased from R&D Systems (Abingdon, UK) whereas the specific murine MCP-1 ELISA Cytoscreen™ was from BioSource International (Canarillo, CA). Zymosan A and all other chemicals were from Sigma Chemical Co. (Poole, UK or St. Louis, MO).

Statistical analysis

Data are presented as mean ± SE of n mice per group, and statistical differences were evaluated by one-way analysis of variance once Bartlett's test had confirmed the homogeneity of the variances. *Post hoc* comparisons were made with the test of Bonferroni, using Instat™ software (version 2·04) on a Macintosh PC. A threshold value of $P<0·05$ was taken as significant.

RESULTS

Characterization of the inflammatory model

Zymosan injection induced a time-dependent accumulation of PMN in the peritoneal cavity, with a maximal cell influx at the 4 hr time-point (Fig. 1). PMN accumulation did not

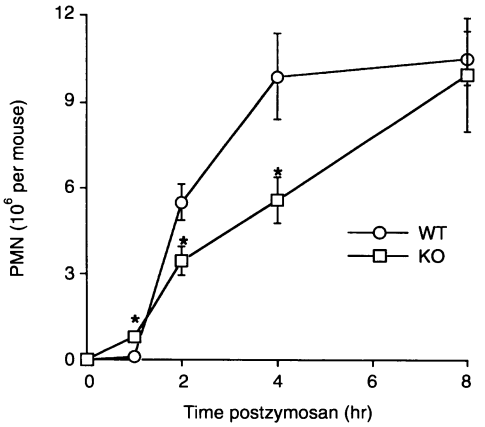

Figure 1. Role of iNOS in the zymosan-induced PMN extravasation in the murine peritoneal cavity. Mice (wild-type or iNOS-deficient, KO) were treated i.p. with zymosan (0·2 mg), at time 0 or left untreated (time 0, control group). Peritoneal cavities were washed at the reported time-points and lavage fluids handled as described in the Materials and Methods for quantification of PMN cell numbers. Data are expressed as the mean ± SE of $n=10$ mice per group. *$P<0·05$ indicates a significant difference between the response of wild-type and iNOS KO animals at a given time-point.

significantly increase further between 4 hr and 8 hr (Fig. 1), gradually returned towards baseline levels at 16–24 hr, and the cellular response was almost resolved by 48 hr postzymosan administration (data not shown). As almost all of the increase in PMN accumulation occurred between 0 and 4 hr in the wild-type animals, we selected the period of 0–8 hr for further analysis. The large predominance of neutrophils (99%) in the PMN population was confirmed in cytospin preparations stained with May–Grünwald and Giemsa (data not shown). In accordance with previous studies,[26,27] zymosan induced the production of nitrite and nitrate from the inducible NO synthase in the lavage fluids. For instance, 4 hr after zymosan administration, nitrite/nitrate concentrations in the lavage fluid had increased from $2{\cdot}3 \pm 0{\cdot}6$ μM (baseline) to $5{\cdot}3 \pm 0{\cdot}8$ μM ($P < 0{\cdot}01$, $n = 10$).

Injection of zymosan also induced the release of chemokines and cytokines into the peritoneal cavity. The level of these mediators in the peritoneal lavage fluid was determined by specific ELISA. No detectable amounts of murine MIP-1α, MIP-2, MCP-1, KC or IL-10 protein were found in basal conditions (Figs 2–4). As previously reported,[25] i.p. administration of zymosan resulted in a rapid release of murine MIP-1α, MIP-2, MCP-1, KC and IL-10 into the lavage fluids, in significant amounts, generally between 1 and 4 hr (Figs 2–4), returning towards basal levels at later time-points. The cytokine IL-10 was also detected in the cell-free inflammatory exudate as early as 1 hr postzymosan injection: its levels remained elevated up to 8 hr (Fig. 4), returning to basal values by 24 hr postzymosan (data not shown).

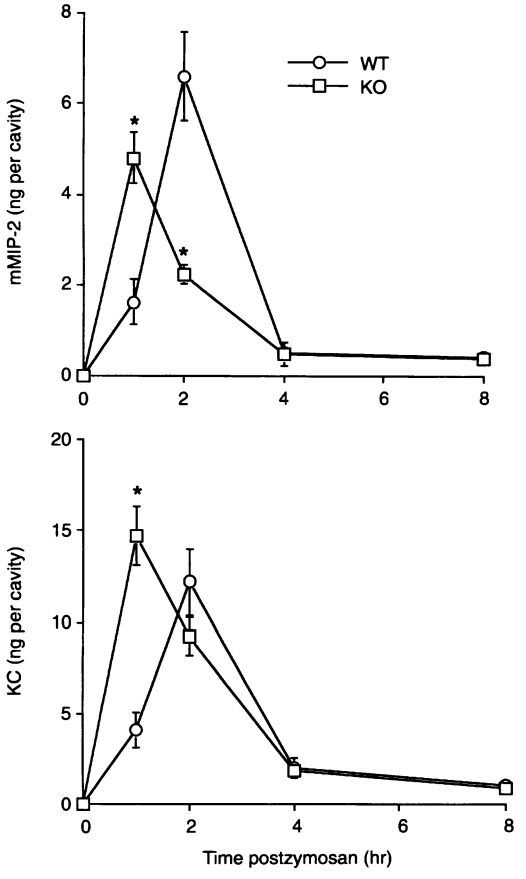

Figure 2. Role of iNOS in the zymosan-induced MIP-2 and KC release in the murine peritoneal cavity. Mice (wild-type or iNOS-deficient, KO) were treated i.p. with zymosan (0·2 mg), at time 0 or left untreated (time 0, control group). Peritoneal cavities were washed at the reported time-points and lavage fluids handled as described in the Materials and Methods for the measurement of MIP-2 (a) and KC (b) protein levels. Data are expressed mean ± SE of $n = 10$ mice per group. *$P < 0{\cdot}05$ indicates a significant difference between the response of wild-type and iNOS KO animals at a given time-point.

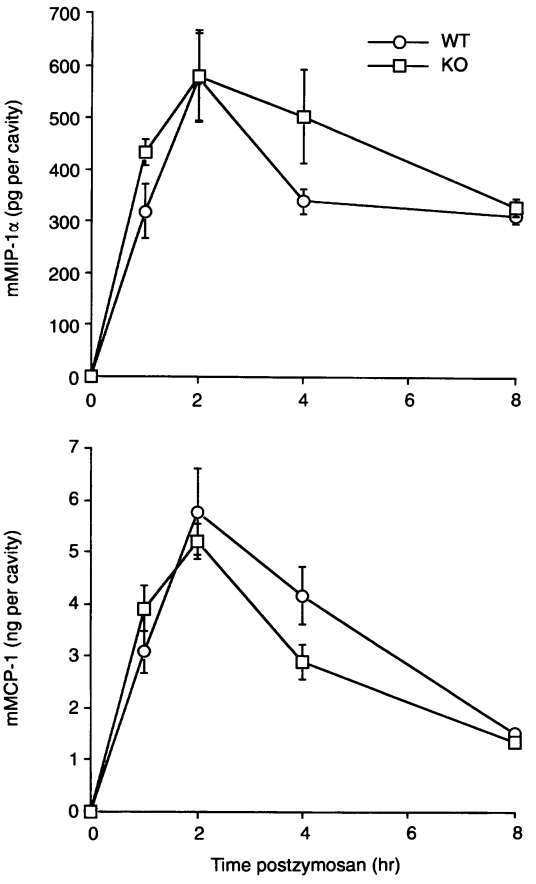

Figure 3. Role of iNOS in the zymosan-induced MIP-1α and MCP-1 release in the murine peritoneal cavity. Mice (wild-type or iNOS-deficient, KO) were treated i.p. with zymosan (0·2 mg), at time 0 or left untreated (time 0, control group). Peritoneal cavities were washed at the reported time-points and lavage fluids handled as described in the Materials and Methods for the measurement of MIP-1α (a) and MCP-1 (b) protein levels. Data are expressed as mean ± SE of $n = 10$ mice per group.

Modulation of the zymosan-induced inflammation by iNOS

Zymosan injection in iNOS knockout (KO) mice did not produce significant increases in nitrite/nitrate concentrations above basal values: for instance, at 4 hr postzymosan, nitrite/nitrate concentrations were $1{\cdot}4 \pm 0{\cdot}3$ μM (significantly lower than nitrite/nitrate in the wild-type animals at the same time-point postzymosan; $P < 0{\cdot}01$, $n = 10$). When comparing the response between wild-type and iNOS KO animals, we observed that the early (1 hr), low-level PMN accumulation was slightly, but significantly, higher in the absence of functional iNOS (Fig. 1). Furthermore, the delayed response

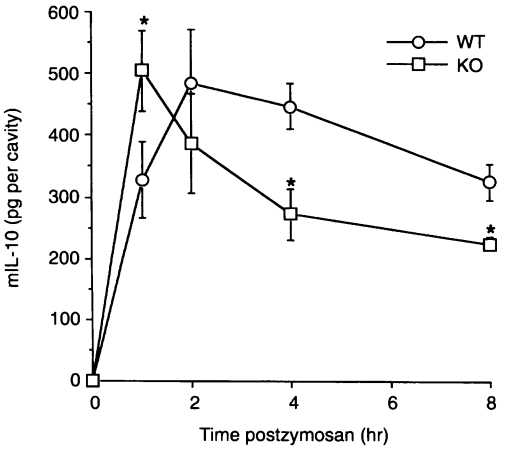

Figure 4. Role of iNOS in the zymosan-induced IL-10 release in the murine peritoneal cavity. Mice (wild-type or iNOS-deficient, KO) were treated i.p. with zymosan (0·2 mg), at time 0 or left untreated (time 0, control group). Peritoneal cavities were washed at the reported time-points and lavage fluids handled as described in the Materials and Methods for the measurement of IL-10 protein levels. Data are expressed as mean ± SE of $n = 10$ mice per group. $^*P < 0{\cdot}05$ indicates a significant difference between the response of wild-type and iNOS KO animals at a given time-point.

(2–4 hr after zymosan) of PMN accumulation was suppressed in these animals (Fig. 1). However, there was no significant difference in PMN accumulation between the two types of mice at the 8 hr time-point (Fig. 1). The early enhancement of PMN infiltration in the iNOS-deficient animals was associated with increased peritoneal levels of MIP-2 (Fig. 2a), KC (Fig. 2b) and IL-10 (Fig. 4). On the other hand, the delayed suppression of PMN infiltration was associated with reduced MIP-2 (Fig. 2a) and IL-10 (Fig. 4) levels in the peritoneal cavity. The lack of iNOS did not affect the release of MIP-1α or MCP-1 production (Fig. 3) throughout the experimental period.

When comparing the time course of MIP-2 and KC production (Fig. 2), one can conclude that in the absence of iNOS, the pattern of chemokine production remains largely unaffected (rapid increase in the levels of the chemokine, followed by a rapid decline), but the peak of the MIP-2 and KC production shifts to an earlier time-point.

DISCUSSION

In this study we provide evidence for a modulatory role of iNOS in the regulation of PMN accumulation in a zymosan-induced model of inflammation. The present study revealed a biphasic pattern of the regulation of PMN accumulation by iNOS: an early suppression followed by a delayed enhancement of PMN recruitment was seen. As a low level of NO production (by constitutive or inducible NO synthases) is known to suppress neutrophil adhesion to endothelial cells,[28,29] it is perhaps not surprising that a low level of iNOS, expressed in the early phase of the inflammatory response, also suppresses zymosan-induced neutrophil recruitment. The other main finding, i.e. the decrease of PMN infiltration in the later phases of inflammation in the absence of iNOS-derived NO, may also have been predicted by previous pharmacological studies. In fact, administration of pharmacological inhibitors with selectivity towards iNOS, such as S-methyl-isothiourea[30] and mercaptoethylguanidine[31] to rodents, suppresses neutrophil infiltration in the later phases of local inflammation.[32,33]

The mechanism(s) by which iNOS-derived NO promotes the recruitment of PMNs into the inflammatory site in the later stages of inflammation is presently unclear. One possibility, which was previously proposed, is that iNOS-derived NO (or its cytotoxic reaction product, peroxynitrite), triggers endothelial dysfunction, thereby promoting mononuclear cell recruitment.[33] Indeed, peroxynitrite can cause endothelial cell injury and dysfunction.[34,35] Another novel possibility, which can be put forward based on the results of the present study, is the following: iNOS-derived NO enhances the production of the CXC chemokines MIP-2 and KC in the delayed phase of inflammation, thus enhancing PMN recruitment. It is well established that these CXC chemokines play a role in the promotion of PMNs into the inflammatory site.[36–38] There is recent evidence indicating that iNOS-derived NO can up-regulate the production of a number of proinflammatory mediators: for example, pharmacological inhibition or inactivation of iNOS has been demonstrated to down-regulate the production of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) in allergic encephalomyelitis,[7] the production of IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF) and the activation of the signal transduction pathways nuclear factor kappa B and Stat-3 in haemorrhagic shock[12] and the expression of TNF-α, collagenase and stromelysin in a rat model of collagen-induced arthritis.[39] The suppression of the expression of these proinflammatory mediators may be either direct, i.e. related to the inhibition of a direct transcriptional effect of NO, or indirect, i.e. a consequence of the down-regulation of the inflammatory response.

The present data indicate that the regulation of chemokine production by iNOS-derived NO is a time-dependent phenomenon, and also shows specificity towards certain chemokines: in our study the production of the CC chemokines MIP-1α and MCP-1 was unaffected by the absence of iNOS. The mechanism of the biphasic regulation of CXC chemokine production by iNOS reported in the current study requires further investigation. In the literature, there are controversial reports regarding the regulation of chemokine production by iNOS and NO. In *in vitro* experiments, NO donors reduced MCP-1 production in rabbit smooth muscle cells,[40] while a non-isoform selective inhibitor of NOS increased MCP-1 production in cultured alveolar and peritoneal macrophages stimulated with bacterial lipopolysaccharide.[11] Inhibition of NOS also enhanced MCP-1 production in human endothelial cells.[41] However, inhibition of NOS failed to affect MCP-1 production in human adherent peripheral blood mononuclear cells (PBMC) stimulated with bacterial lipopolysaccharide.[10] In the latter study, inhibition of NOS reduced, while NO donors enhanced, MIP-1α production.[10] However, in another study in rat alveolar macrophages, the production of MIP-1α was unaffected by a NO donor.[42] In *in vivo* studies, inhibition of NO synthesis by a NOS inhibitor (with selectivity towards the constitutive isoforms of NOS) enhanced MCP-1 levels in a faecal peritonitis model,[11] and in the aortae of cholesterol-fed rabbits,[40] whereas in a rat model of pulmonary granulomatous inflammation, the same inhibitor reduced the expression of MCP-1.[43] In a model of carrageenan-induced local

inflammation in rats, a non-selective NOS inhibitor failed to affect, while an iNOS-selective inhibitor reduced MCP-1 production.[32] It is likely that the regulation of chemokine production by NO is a time-dependent process that is also linked to the cell target; in addition, variability of results may also be related to the stimulus, animal species and experimental model employed. Importantly, one has to keep in mind that inhibition of NO synthesis is likely to exert haemodynamic effects in most models, which may well have a secondary effect on the production of inflammatory mediators.

We have also observed that the production of IL-10 was affected by the absence of iNOS in the current model. In a study using human adherent PBMC stimulated with bacterial lipopolysaccharide, inhibition of NOS increased IL-10 production.[10] With regard to *in vivo* studies, previous reports have demonstrated that inhibition of NO synthesis by a NOS inhibitor with selectivity towards the constitutive isoforms of NOS enhanced IL-10 levels in a faecal peritonitis model.[11] These findings are consistent with our findings only for the early phase of inflammation. Although IL-10 plays a role in suppressing the infiltration of PMNs into inflammatory sites,[44–47] in the current study, IL-10 levels were elevated in the iNOS KO mice at the early time point, together with an increased PMN infiltration, while IL-10 levels were suppressed at later time points when PMN infiltration was also reduced. We conclude, therefore, that the modulation, by iNOS, of IL-10 production does not play a primary role in the regulation of PMN infiltration under the current experimental conditions. Nevertheless, it is conceivable that the early increase of IL-10 production in the iNOS KO mice may contribute to the more rapid down-regulation of MIP-2 and KC when compared to the time courses of the respective chemokine responses to zymosan in the wild-type animals.

In conclusion, the current data demonstrate that iNOS regulates the production of certain CXC, but not CC, proinflammatory chemokines. An action on the production of IL-10 was also seen, together with a biphasic regulatory effect on PMN accumulation in zymosan-induced acute inflammation. Suppression of PMN infiltration and suppression of the production of some of the CXC chemokines in the late phase of inflammation may contribute to the anti-inflammatory effects of inhibition or genetic inactivation of iNOS.

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