Arch. Immunol. Ther. Exp. (2012) 60:373–382
DOI 10.1007/s00005-012-0186-x

ORIGINAL ARTICLE

Toll-Like Receptors Expression and NF-κB Activation in Peritoneal Leukocytes in Morphine-Mediated Impairment of Zymosan-Induced Peritonitis in Swiss Mice

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Received: 15 July 2011 / Accepted: 28 May 2012 / Published online: 23 August 2012

Abstract Zymosan-induced peritonitis represents a well-described model of acute inflammation. The binding of zymosan with its specific Toll-like receptors (TLR2 and TLR6) on leukocytes initiates activation and phosphorylation of nuclear factor (NF)-κB, which leads to accumulation of NF-κB p65 subunits in the nucleus and subsequently up-regulation of the proinflammatory cytokine genes expression. Intraperitoneal co-administration of zymosan and morphine significantly inhibits peritonitis in several strains of mice by decreasing the influx of exudatory cells; however, mechanisms of this action still remain unclear. We aimed to verify the effects of morphine on NF-κB and TLRs expression at messenger RNA and protein levels during the early stages of zymosan-induced peritonitis. Peritonitis was induced by a single injection of zymosan A or zymosan supplemented with morphine in Swiss mice. At selected time points, after stimulation, peritoneal leukocytes were harvested. The TLRs and NF-κB expression was assessed by real-time PCR and flow cytometry. In comparison with the mice injected with zymosan only, morphine co-injection significantly decreased the expression of phospho-NF-κB and TLR2 in all investigated immunocompetent cells as well as up-regulated the levels of nitric oxide (NO) in peritoneal fluid. Moreover, supplementation of zymosan with morphine altered the TLR, NF-κB and some proinflammatory cytokines (keratinocyte-derived chemokine, tumor necrosis factor-α) gene expression during ongoing inflammation. We may postulate that after morphine stimulation peritoneal leukocytes recognize less effectively zymosan antigens because of impaired TLRs expression. The lower TLR expression attenuates TLR-mediated signal transduction, which prevents NF-κB activation. Additionally, during zymosan-induced peritonitis, morphine may modulate the NF-κB expression, at least partially, by an up-regulated release of NO, as suggested by others.

Keywords Peritonitis · NF-κB · TLR · Morphine · Mice

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Introduction

Intraperitoneal injection of zymosan, a polysaccharide cell wall component derived from *Saccharomyces cerevisiae* represents a self-resolving model of acute inflammation and has been widely used for quantification of particular cell types and inflammation-related soluble factors (Ajuebor et al. 1999; Cash et al. 2009). Leukocytes infiltrating zymosan-inflamed peritoneum such as neutrophils (polymorphonuclear leukocyte, PMN) and macrophages (MFs) and also resident peritoneal mast cells (MCs) and peritoneal MFs are the critical cellular components involved in peritonitis. As demonstrated by selective depletion experiments, MFs and MCs produce the initial signals responsible for the accumulation of PMNs, eosinophils, and mononuclear cells in experimental models of inflammation (Ajuebor et al. 1999; Kołaczkowska et al. 2001), as well as are able to recognize conserved pathogen-associated molecular patterns (PAMPs) in invading pathogens by Toll-like receptors (TLRs) (Kawai and Akira 2010). The TLR family consists of more than 13 members, each detecting distinct PAMPs

derived from various microbial pathogens, such as viruses, bacteria, protozoa and fungi (Akira 2006, 2009).

Inflammatory signaling in response to zymosan requires TLR2 and TLR6 heterodimerization and their subsequent recruitment to the zymosan phagosome (Ozinsky et al. 2000). This action is essential for the nuclear factor (NF)-κB activation. NF-κB is a pivotal regulator in the expression of many proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and chemokines, e.g., keratinocyte-derived chemokine (KC) (Kawai and Akira 2007) detected at early time points in a murine model of peritonitis (Ajuebor et al. 1999; Kołaczkowska et al. 2001). NF-κB also mediates the expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production (Aktan 2004) released by infiltrating leukocytes and activated, resident tissue cells at sites of inflammation (Evans 1995). According to Ajuebor et al. (1998), in zymosan-induced peritonitis delayed response of PMNs intraperitoneal accumulation is dependent on the iNOS activation, as has been shown in iNOS knockout mice.

Zymosan-induced peritonitis may be modulated by supplementation of a proinflammatory agent with a high dose of morphine resulting in inhibition of the inflammatory response leading to the decreased PMNs influx (Natorska and Płytycz 2005). The inhibitory effects of morphine were shown to be completely reversed in naltrexone-pretreated animals implicating opioid receptor-mediated mechanisms. Furthermore, it was suggested that morphine might impair the level of chemoattractants either directly (by inhibiting their release from the peritoneal cells) and/or indirectly, by affecting the recruitment of inflammatory leukocytes from hemopoietic sites. The latter could result from their desensitization to some chemotactic factors (Grimm et al. 1998).

Moreover, it seems that morphine may invoke an intracellular molecular cascade and modulate the NF-κB activation (Welters et al. 2000a). Some previous reports demonstrated that in human blood neutrophils and monocytes morphine inhibited lipopolysaccharide (LPS)-induced NF-κB activation by naloxone-sensitive mechanism. This was mediated solely by the μ3 opiate receptor and coupled to NO release (Menzebach et al. 2003; Welters et al. 2000a). This inhibitory effect of morphine associated with an impairment of NF-κB activation was mainly observed during bacterial infections (Bonnet et al. 2008; Wang et al. 2008). Morphine treatment during *Streptococcus pneumoniae* lung infection was associated with NF-κB inhibition followed by decreased chemokine (MIP-2 and KC) and cytokine (TNF-α, IL-1, and IL-6) production by alveolar MFs (Wang et al. 2005). This suppression was correlated with reduced neutrophils recruitment and increased bacterial outgrowth (Wang et al. 2005). The latter study evidenced that morphine treatment impaired the activity of NF-κB through TLR9 signaling (Wang et al. 2008). Moreover, a novel study by Wang et al. (2011) showed that in dendritic cells morphine treatment modulates the MyD88-IRAK1/4-dependent TLR2 and Nod2 signaling, leading to a diminished IL-23 production, which plays a critical role in innate immunity against *S. pneumoniae* infection.

To our best knowledge, the effects of morphine on the NF-κB and TLRs expression during fungal in vivo infection have not been reported yet. Therefore, in the present study, we examined the effects of morphine on the NF-κB and TLRs expression at both messenger RNA (mRNA) and protein levels during the early stages of zymosan-induced peritonitis. To assess the opioid receptor-mediated mechanism, a well-known opioid receptor antagonist, naltrexone, was used.

Materials and Methods

Animals

Adult males of Swiss mice (4–6 weeks old, 25–30 g) purchased from the Unit of Laboratory Animals (Iłkowice, Poland) were used in the present experiments. All mice were housed five per cage under strictly controlled conditions (free access to food and water, 12-h dark–light cycle, temperature 22 °C). The ethical guidelines of the local committee on animal care (license no. 23/OP/2005 and 11/2010) were followed through the experiments.

Peritonitis Induction and Drug Treatments

After 1-week adaptation to the laboratory conditions, animals were either untreated (controls) or intraperitoneally (i.p.) injected with freshly prepared zymosan A [Z, 40 mg/kg body weight (b.w.), Sigma, London, UK] or zymosan supplemented with morphine hydrochloride (ZM, 20 mg/kg b.w., Polfa, Kutno, Poland). Some animals received intraperitoneal injections of naltrexone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA; 5 mg/kg), and 20 min later, they were injected i.p. with zymosan supplemented with morphine (NZM groups) (Kołaczkowska et al. 2001). The animals were sacrificed by cervical dislocation at time 0 (controls), 1, 2 or 4 h after i.p. injection, and their peritoneal cavities were lavaged with 1 ml of phosphate buffered saline (PBS). The retrieved exudatory leukocytes were stained with Turk's solution (0.01 % crystal violet in 3 % acetic acid) and counted in a hemocytometer (Kołaczkowska et al. 2001). The cell pellets were used for FACS staining or gene expression assays. Peritoneal exudate fluid was frozen at −20 °C prior to analysis. Experiments were repeated at least

three times with four to five animals per each experimental group.

Cells Staining

Peritoneum-derived leukocytes (2–3 × 10^6 of total cells) were washed in FACS buffer (2 % fetal calf serum in PBS), preblocked with an Fc block (anti-CD16/CD32 mAb blocking non-antigen-specific binding of immunoglobulins to the FcγRs receptors; BD Biosciences Pharmingen, San Diego, CA, USA) for 20 min on ice and after washing (5,000 rpm, 5 min, 4 °C) labeled by rat anti-mouse FITC-conjugated monoclonal antibodies, diluted 1:100 (BD Biosciences Pharmingen, San Diego, CA, USA) in anti-Ly-6G and LY-6C for neutrophils, anti-Mac-3 for MFs and anti-CD117 (c-kit) for MCs, in separated tubes on ice for 15 min (Kołaczkowska et al. 2008; Lafarge et al. 2007). Corresponding fluorescence labeled isotype control antibodies were used (BD Biosciences Pharmingen, San Diego, CA, USA). After repeated washings in FACS buffer, the cells were permeabilized by BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences, San Jose, CA, USA) on ice for 20 min. After two washes in BD Perm/Wash Buffer, cells were stained with phospho-NF-κB (ser536) rabbit monoclonal antibody Alexa Fluor 647 conjugate (Cell Signaling Technology, Danvers, MA, USA), which detects NF-κB only when phosphorylated at serine 536. The TLR2 and TLR6 expression was assessed upon leukocyte staining with a rat anti-mouse APC-conjugated TLR2 antibody (FAB1530A, R&D Systems, Minneapolis, MN, USA) or goat anti-mouse TLR6 antibody (E-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by donkey anti-goat IgG, F(ab′)-biotin conjugated (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and PerCP-Cy5.5-conjugated streptavidin on ice for 15 min (Kołaczkowska et al. 2008). After repeated washings in FACS buffer, the cells were subsequently suspended in 50 µl of FACS buffer for flow cytometry analysis.

Flow Cytometry Analysis

Samples were analyzed in a four-color FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA; Immunocytometry Systems, BD Biosciences, San Jose, CA, USA) by using CellQuest Pro software, and data were collected with 10,000 events being acquired. Leukocytes were initially gated by forward scatter/side scatter on the basis of their size and granularity. Subsequently, the data were collected in the FL1 flow cytometer channel (green channel, FITC), FL3 (PerCP-Cy5.5) or FL4 (APC and Alexa Fluor® 647).

In Vitro Study

The mouse monocyte–MF cell line RAW 264.7 was obtained from the European Type Culture Collection (ETCC, Chemical Co., St. Louis, MO, USA) and grown in DMEM (Sigma Chemical Co., St. Louis, MO, USA) supplemented with streptomycin (100 µg/ml), penicillin (100 U/ml) and 10 % fetal bovine serum (FBS, all reagents from PAA Laboratories GmbH, Austria). Cells were cultured at a density of 0.25 × 10^6 per ml in 12-well plates (Nalge Nunc International, Rochester, NY, USA) for 3–4 days to attain 80–90 % confluence. Subsequently, 1 day before experiment, the medium was replaced by DMEM without FBS. The RAW 264.7 cells were stimulated with zymosan (50, 100, 250 µg/ml) for 1 and 4 h. In some experiments, cells were preincubated for 2 h with morphine (50 nM, 50 µM, 1 mM). Untreated cells were used as controls. The TLRs expression was analyzed by flow cytometry as described above.

Analysis of mRNA Expression by Real-Time PCR

Total RNA was isolated by the RNeasy Mini kit, according to the manufacturer's protocols (Qiagen, Chatsworth, CA, USA). A total of 400 ng of RNA was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Life Technologies Co., Carlsbad, CA, USA) at 25 °C for 5 min, 42 °C for 30 min followed by 85 °C for 5 min. The cDNA was amplified with TaqMan Gene Expression Assays: TLR2 (Mm01213946_g1); TLR6 (Mm02529782_s1); TNF (Mm00443260_g1) and mouse IL-8 (KC, Mm00433859_m1) on an ABI PRISM® 7900HT Fast Real-Time PCR System (Life Technologies Co.). Assays were ordered from Life Technologies as Inventoried or Made-to-Order Assays. Glyceraldehyde-3-phosphate dehydrogenase (mouse GAPDH Endogenous Control, FAM/MGB Probe, Non-Primer Limited; Life Technologies Co.) or beta-actin (mouse ACTB Endogenous Control FAM/MGB Probe, Non-Primer Limited; Life Technologies Co.) was used as a housekeeping gene. To analyze the obtained data, the comparative threshold cycle (CT) method was applied (Giulietti et al. 2001). Briefly, the amount of a target gene, normalized to GAPDH, and relative to a calibrator (untreated group), is given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ of sample (Z, ZM or NZM groups) $-\Delta C_T$ of calibrator and ΔC_T is the C_T of the target gene subtracted from the C_T of GAPDH or ACTB. Expression of target genes of Z, ZM or NZM animals was normalized to GAPDH and/or ACTB and displayed as a fold-change relative to samples of untreated mice used as the calibrator.

Determination of NO Levels (Nitrite and Nitrate Assay)

The measurement of nitrate and nitrite levels in peritoneal fluid was used as an indirect method of determining NO

synthesis. Nitrite and nitrate are stable end products of NO metabolism and were measured in this study by a modified Griess reaction, described in detail elsewhere (Schmidt and Kelm 1996). Briefly, 145 µl aliquots of samples were incubated with 10 µl mixture of nitrate reductase, 5 µM flavin adenine dinucleotide, and reduced b-NADPH in concentration 1 mg/ml (all reagents Sigma Chemical Co., St. Louis, MO, USA) for 45 min at RT and followed by 10 min incubation with 10 µl mixture of 100 U/ml lactate dehydrogenase and 0.3 mM pirogronian acid in distilled water. The Griess reagent, containing equal parts of 1 % sulfanilamide in 5 % phosphoric acid (Sigma Chemical Co., St. Louis, MO, USA) and 0.0133 *N*-1-naphthylethylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO, USA) in distilled water, was added for 5 min. The nitrite and nitrate concentrations were calculated from a standard curve with sodium nitrite, and the sensitivity of this assay consistently reached 1 µM.

Statistical Analyses

All values are reported as mean ± SE in groups of three to five mice. Kinetic changes of each parameter were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test, to compare the values recorded at the individual time points with those at time 0 (in intact animals). Statistical comparisons were indicated by letters (e.g., A, B for Z group, A′, B′ for NZM and a, b for ZM group). Differences between zymosan (Z) and zymosan supplemented with morphine treated animals (ZM) were analyzed by Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

Effects of Morphine on Number of Exudate Cells in Zymosan-Induced Peritonitis

Zymosan injection induced significant increase of PMN at 4 h of inflammation. In animals co-injected with morphine (ZM), the number of neutrophils was significantly lower than in mice treated with zymosan only (Z) (Fig. 1a).

The number of MFs (Fig. 1b) and MCs (Fig. 1c) decreased significantly in both Z and ZM groups. In the latter group, the decrease in MC numbers was short-lasting and at 4 h returned to the control level (Fig. 1c). The inhibitory effect of morphine was completely reversed by naltrexone as evidenced by the similar number of PMN (Fig. 1a) and MC (Fig. 1c) in NZM (naltrexone-pretreated ZM) and Z groups. All groups had similar numbers of MFs (Fig. 1b).

Effects of Morphine on TLR2 and TLR6 mRNA Expression

Relative mRNA expression was estimated in the peritoneal cell pellets. The expression of TLR2 and TLR6 mRNA was

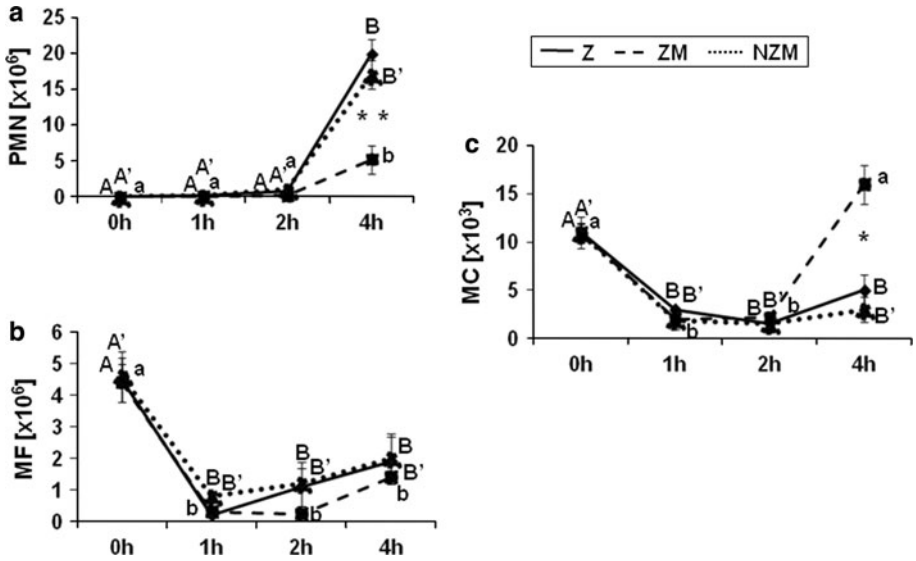

Fig. 1 Kinetic changes of leukocyte and mast cell numbers during the early stages of peritonitis in Swiss mice i.p. injected with zymosan (Z group) or zymosan supplemented with morphine (ZM group). Some animals were additionally pretreated with naltrexone (NZM group). Numbers of peritoneal polymorphonuclear leukocytes (PMNs, **a**), macrophages (MF, **b**) and mast cells (MCs, **c**) at time 0 (controls) or 1, 2 and 4 h after injection. All results are shown as the mean ± SE in groups of three to five mice. *Mean values not sharing letters* are statistically significantly different according to ANOVA ($p < 0.05$). Differences between Z and ZM groups are statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$

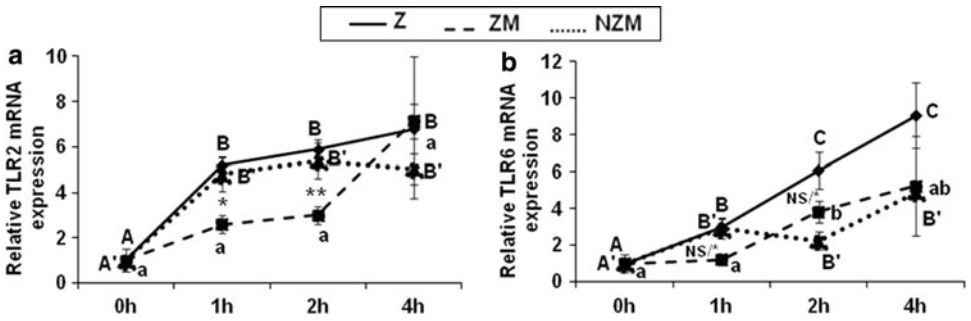

Fig. 2 Effects of intraperitoneal injection of morphine (ZM) and naltrexone (NZM) on TLR2 (**a**) and TLR6 (**b**) gene expression at 0 (control group), 1, 2 and 4 h after zymosan-induced peritoneal inflammation (Z) in Swiss mice. Relative mRNA expression was measured in the peritoneal cell pellets. Fold increase of TLR2 (**a**) and TLR6 (**b**) transcript is presented. All results are shown as mean ± SE in groups of three to five mice. Differences between Z and ZM groups are statistically significant at $*p < 0.05$, $**p < 0.01$, NS/$*p = 0.05$

4.5-fold and 2-fold up-regulated within the first hour of peritoneal inflammation, respectively (Fig. 2a, b). The expression of TLR6 mRNA was gradually increasing, reaching the highest value at 4 h of peritonitis (Fig. 2b). In contrast, no significant differences in the TLR2 mRNA expression between the selected time points were observed (Fig. 2a).

Morphine treatment significantly decreased TLR2 transcript levels at both 1 h (30 %; $p = 0.04$) and 2 h (40 %; $p = 0.004$) but not at 4 h of inflammation. This morphine action was reversed by naltrexone treatment. Morphine-inhibited TLR6 expression was on the border of statistical significance ($p = 0.05$) at 1 and 2 h of peritonitis.

Effects of Morphine on TLR2 and TLR6 Protein Expression

In physiological conditions (zymosan untreated mice) the expression of TLR2 and TLR6 on Mac-3^+ MFs reached 2 and 1 %, respectively (Fig. 3a, b) and it was below 1 % on Gr-1^+ neutrophils (Fig. 3c, d). The TLRs on MCs were expressed at very low to undetectable levels and non-significant fluctuations after zymosan or zymosan supplemented with morphine stimulation were observed (data not shown).

One hour after zymosan injection, both TLR2 and TLR6 expression on Mac-3^+ was significantly higher as compared to those in the control group (0 h). This situation was sustained for the next 2 h of inflammation (Fig. 3a, b). Expression levels of TLR2 and TLR6 on Gr-1^+ neutrophils were significantly up-regulated at each time point in comparison with the control animals (Fig. 3c, d).

Modulation of peritonitis with morphine resulted in a significant reduction of TLR2 expression in examined leukocytes at 1 and 2 h of inflammation (Fig. 3a, c). The morphine-induced inhibitory effect was completely reversed by naltrexone as evidenced by the similar TLR2 expression in NZM (naltrexone-pretreated ZM) and Z groups (Fig. 3a, c). Non-significant tendencies for morphine-induced down-regulation of TLR6 expression were observed in Mac-3^+ and Gr-1^+ cells (Fig. 3b, d).

In vitro studies showed that zymosan treatment induced strong up-regulation of TLR2 (Fig. 3e) and TLR6 (Fig. 3f) expression in RAW 264.7 cells at each time point. The TLR2 and TLR6 were expressed at similar levels after zymosan stimulation at dose 50 and 100 μg/ml (data not shown). The highest tested dose of zymosan (250 μg/ml) induced stronger expression of TLR2 than TLR6 (70 ± 2.8 vs. 31 ± 3 % at 1 h and 74 ± 3.3 vs. 28 ± 3.2 % at 4 h, both $p < 0.0001$). Morphine pre-incubation (at each dose) significantly impaired the expression of TLR2 but not TLR6 (Fig. 3e, f).

NF-κB Expression During Morphine-Modulated Peritonitis

Under basal conditions (0 h), the expression of active form of NF-κB was exhibited by 2.5 % of Mac-3^+ MFs, while Gr-1^+ neutrophils and c-kit$^+$ MCs showed very low levels of NF-κB expression (1 % and below) (Fig. 4).

During the first hour of zymosan-induced peritonitis, a significant increase in NF-κB expression was observed in Gr-1^+ and Mac-3^+ cells (up to 10 %). This enhancement was sustained till 4 h of inflammation. The NF-κB expression by c-kit$^+$ cells also increased rapidly during the first hour of inflammation (up to 5 %) and then begun to decline, reaching the control level at 4 h of inflammation.

Morphine treatment significantly decreased zymosan-induced NF-κB expression in investigated leukocyte subsets and in MC. The inhibitory effect was evidenced in Gr-1^+ cells and Mac-3^+ MFs during the entire time course of inflammatory response (Fig. 4a, b) and till the second hour of peritonitis in c-kit$^+$ MCs (Fig. 4c). Naltrexone reversed the effect of morphine.

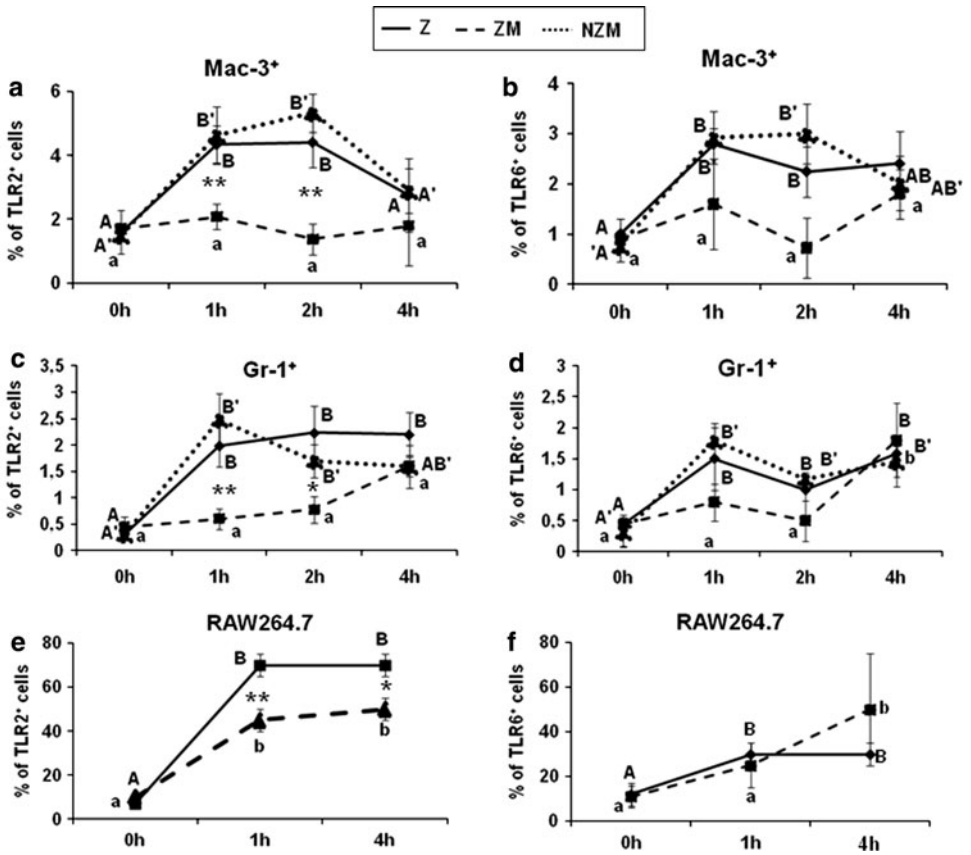

Fig. 3 Effects of intraperitoneal injection or in vitro pre-incubation of morphine (ZM) and naltrexone (NZM, only in in vivo study) on TLR2 (*left*) and TLR6 (*right*) expression in macrophages (Mac-3$^+$; **a**, **b**), neutrophils (Gr-1$^+$; **c**, **d**) and mouse monocyte-macrophage cell line RAW 264.7 (**e**, **f**) at 0 (control group) 1, 2 and 4 h after zymosan-induced peritoneal inflammation (Z) in Swiss mice or after in vitro stimulation. The ratio of peritoneal cells expressing TLR2 and TLR6 detected by flow cytometry analyses is presented. All results are shown as mean ± SE from of three to five experiments. *Mean values not sharing letters* are statistically significantly different according to ANOVA ($p < 0.05$). Differences between Z and ZM groups are statistically significant at $*p < 0.05$, $**p < 0.01$

The mRNA Level of Proinflammatory Mediators During Morphine-Modulated Peritonitis

Enhanced expression of KC mRNA in response to zymosan stimulation was detected at 1 h (over 200-fold), 2 h (500-fold) and 4 h (to 20-fold) of inflammation (Fig. 5a). Morphine significantly decreased zymosan-induced KC mRNA levels by 70 % ($p = 0.006$) at 2 h and 30 % ($p = 0.048$) at 4 h after treatment. Naltrexone preinjection did not reverse the action of morphine. The mRNA for TNF-α was up-regulated above 12-fold during the first 2 h of peritonitis and 7-fold at the 4 h of inflammation in comparison to the untreated group (Fig. 5b). Morphine treatment resulted in approximately a 30 % ($p < 0.03$) reduction of TNF-α mRNA content during the first 2 h of inflammation and a 40 % decrease ($p = 0.05$) at 4 h of inflammation. This action was completely reversed by naltrexone preinjection.

NO Levels in Peritoneal Fluid

Nitrite and nitrate levels in the peritoneal fluid from untreated mice reached concentration of approximately 7–10 μM (data not shown). After zymosan treatment, a significant increase in NO levels was observed in every time point in comparison to the untreated animals (Fig. 6). The morphine supplementation of inflammatory agent significantly enhanced NO production. This enhancement was abolished in NZM group at 1 and 2 h of inflammation. At 4 h of peritonitis, no differences between ZM and NZM groups were observed.

Discussion

Our previous papers have shown that morphine effectively inhibits the influx of PMNs in Swiss mice at 4 h after zymosan treatment (Natorska and Płytycz 2005). The anti-inflammatory effects of morphine were associated with the morphine-induced desensitization of leukocyte receptors for some chemotactic factors, as observed previously (Grimm et al. 1998; Szabo et al. 2001).

In the present study, we tried to elucidate if the anti-inflammatory mechanism of morphine action might be associated with the TLRs expression and the NF-κB transcription activation in peritoneal immunocompetent cells during the early stages of peritonitis. We have focused on the cells responsible for the course of the innate immune response, such as neutrophils (PMN), MFs and MCs.

In our study, zymosan induced an increase in the TLR2 and TLR6 expression in MFs and neutrophils almost immediately after the treatment. This rise was observed at both mRNA and protein levels and persisted till at least the

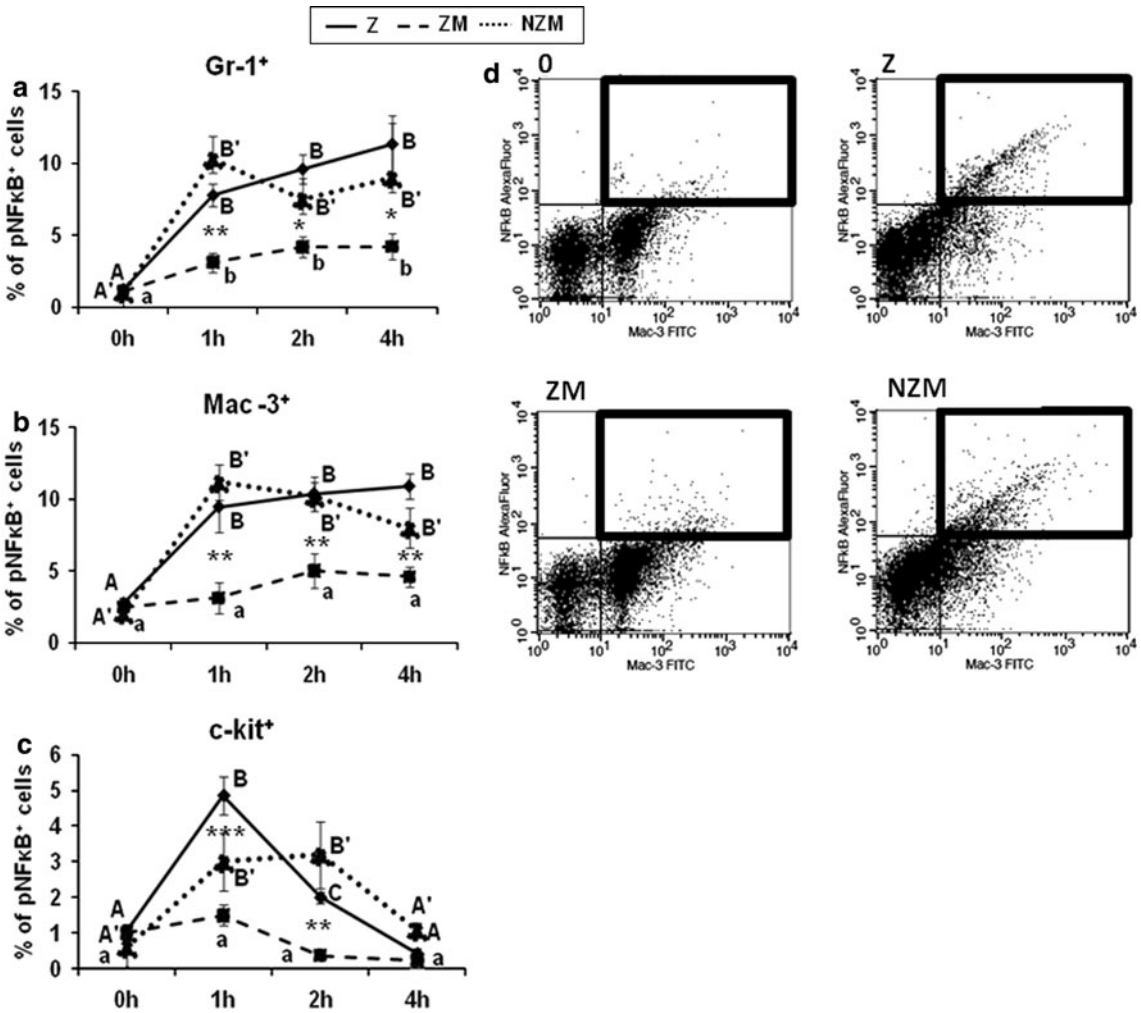

Fig. 4 Effects of intraperitoneal injection of morphine (ZM) and naltrexone (NZM) on the nuclear factor (NF)-κB expression in neutrophils (Gr-1$^+$; **a**), macrophages (Mac-3$^+$; **b**) and mast cells (c-kit$^+$; **c**) at 0 (control group), 1, 2 and 4 h after zymosan-induced peritoneal inflammation (Z) in Swiss mice. The ratio of peritoneal cells expressing the active form of NF-κB detected by flow cytometry analyses is presented. All results are shown as the mean ± SE in groups of three to five mice. *Mean values not sharing letters* are statistically significantly different according to ANOVA ($p < 0.05$). Differences between Z and ZM groups are statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Representative FACScan dot plots showing the NF-κB expression in macrophages (Mac-3$^+$) at 1 h after treatment from each experimental group. The quadrants of double positive cells are marked with *frame* (**d**)

fourth hour of inflammation. It has been shown previously that zymosan induces inflammatory signals in MFs through interactions with TLR2/TLR6 heterodimer, which is subsequently recruited to the phagosome (Ozinsky et al. 2000) and targeted to the Golgi apparatus independently of the clathrin-interacting endocytic machinery (Triantafilou et al. 2006). It was also demonstrated that the TLR expression on MFs and neutrophils was increased by proinflammatory cytokines (Kurt-Jones et al. 2002) or after exposure to a wide range of different bacteria species (Kobayashi et al. 2003).

Zymosan-induced up-regulation of TLR2 and TLR6 expression on peritoneal neutrophils and MFs may enhance their proinflammatory response associated with cytokine (TNF-α) and chemokine (KC) production (Kurt-Jones et al. 2002) followed by neutrophil influx that is a characteristic hallmark of zymosan-induced peritonitis (Kołaczkowska et al. 2001). The sustained increased TLRs expression showed in this study might be associated with the fact that the maximum of the inflammatory process linked with the intraperitoneal accumulation of PMNs is detected between the fourth and eighth hour of peritonitis (Kołaczkowska et al. 2001; Mullaly and Kubes 2007).

In contrast to MF and PMNs, the expression of TLRs on MCs was at very low levels. However, some studies provide evidence for TLR expression in various murine MC populations, thus potentially allowing pathogen recognition by those cells (Mrabet-Dahbi et al. 2009; Supajatura et al. 2002). Consequently, MC can produce different classes of mediators in response to distinct TLR activators (McCurdy

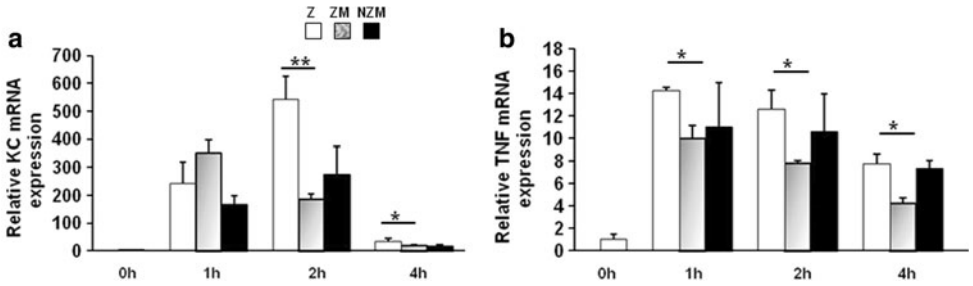

Fig. 5 Effects of intraperitoneal injection of morphine (ZM) and naltrexone (NZM) on KC (**a**) and TNF-α (**b**) gene expression at 0 (control group), 1, 2 and 4 h after zymosan-induced peritoneal inflammation (Z) in Swiss mice. Relative mRNA expression was measured in the pellets of peritoneal cells. All results are shown as the mean ± SE in groups of three to five mice. Some differences between Z and ZM groups are statistically significant at $*p < 0.05$, $**p < 0.01$

et al. 2003). Limited data are available for the TLR6 expression on MCs; nonetheless, the mRNA expression of TLR6 was detected in bone marrow-derived murine MCs (Ikeda and Funaba 2003).

All TLR signaling pathways lead to an activation of the transcription factor NF-κB. Upon its activation, the NF-κB-inhibitor IκBα is phosphorylated and proteolytically degraded (Tergaonkar 2006). Free NF-κB dimers translocate into the nucleus where they control an array of inflammatory cytokine genes expression (Kawai and Akira 2007) by initiating or regulating their transcription (Siebenlist et al. 1994). As shown, the intraperitoneal zymosan administration induced significant increase in phosphorylated NF-κB expression in peritoneal cells. This activation of NF-κB persisted for at least 4 h in PMN and MFs but not in MCs where it was short lasting. These differences between leukocytes in NF-κB activation may be associated with the function and/or timing of the cell types during the course of inflammation. In particular, the influx of exudatory leukocytes is mainly mediated by MC, and in this type of cells, NF-κB activation is followed by production of a wide range of proinflammatory mediators including biogenic amines (histamine and serotonin), proteases, cytokines (e.g., TNF-α) and chemotactic factors (e.g., IL-8, MCP-1), which are released at the beginning of the inflammatory response (Galli et al. 2005). Moreover, MCs are also known to store multiple mediators, immediately released upon their activation (Bischoff 2007).

It has been previously shown that the local morphine administration exerts antinociceptive and anti-inflammatory effects during zymosan-induced peritonitis in mice (Płytycz and Natorska 2002). It seems that local administration of exogenous morphine limits a chemotactic influx/accumulation of new leukocytes by desensitizing receptors for chemokines (Grimm et al. 1998). The present study has shown that modulation of peritonitis by morphine treatment resulted in a consistent reduction of TLR2, and NF-κB expression in all examined cell types at the mRNA and protein levels. In case of TLR2, this inhibition was reversed by naltrexone, which implicated the involvement of opioid receptor-mediated mechanisms. In line with our data, a study by Bonnet et al. (2008) demonstrated that morphine inhibited proinflammatory cytokine production in human monocytes after TLR2 stimulation. Moreover, the detailed experiments with opioid receptor antagonists revealed that this effect was mediated by the μ-opioid receptor (Bonnet et al. 2008). The inhibitory effects of morphine on LPS-induced TNF-α and IL-6 production have been reported in MFs (Roy et al. 1998). In this study, morphine treatment led to a decrease in NF-κB activation but this effect was not reversed by naloxone, suggesting the involvement of a non-classical opioid receptor. In fact, several groups have reported the existence of morphine binding sites, different from the classical μ-opioid receptors. These receptors, apart from being insensitive to naloxone, also have a relatively low affinity for morphine (Makman et al. 1995; Roy et al. 1992).

Further, we have shown that the inhibitory effects induced by morphine were accompanied by increased NO release to the peritoneal fluid. Previously, it was shown that morphine increases NO production in neutrophils, MFs and endothelial cells (Magazine et al. 1996). Moreover, up-regulation of NO production by constitutive isoforms of NOS was reported to inhibit the disassociation of the IκBα inhibitor complex and NF-κB binding to the respective DNA promoter region (Welters et al. 2000a, b). A study by Welters et al. (2000b) demonstrated that morphine-stimulated NO release down-regulated the expression of neutrophils receptor associated with phagocytosis and oxidative burst and this action was mediated by the μ3 opiate receptor. Additionally, it was shown that morphine down-regulated the expression of CD14 (a protein crucial for LPS recognition) in human blood monocytes and neutrophils (Welters et al. 2007). The decreased CD14 receptor expression correlates with inhibition of the other transcriptional factor: activator protein 1 by an NO-dependent process (Welters et al. 2007). We may speculate that the decrease in TLR and NF-κB expression during

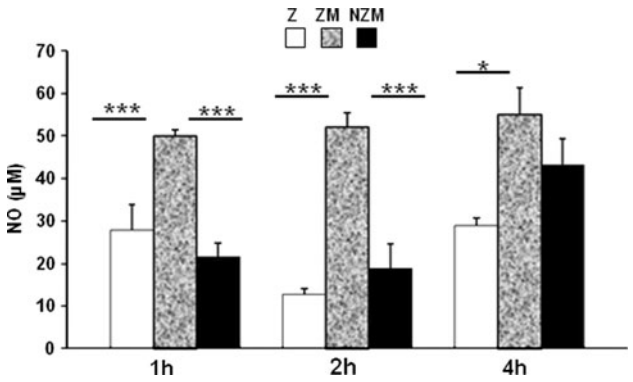

Fig. 6 Nitric oxide (NO) levels in the peritoneal exudate fluid after intraperitoneal injection of morphine (ZM) and naltrexone (NZM) at 0 (control group), 1, 2 and 4 h after zymosan-induced peritoneal inflammation in Swiss mice. All results are shown as mean ± SE in groups of three to five mice. Some differences between Z, ZM and NZM groups are statistically significant at $*p < 0.05$, $***p < 0.001$

ongoing zymosan peritonitis modulated with morphine might be dependent on NO mechanism associated with the regulation at the transcriptional levels. This, however, needs to be further clarified using NO scavengers or iNOS inhibitors in the presence of morphine.

Our previous study has shown that plasma chemotactic factors were significantly lower in animals with morphine-modulated peritonitis compared to those treated with zymosan or thioglycollate only (Chadzinska et al. 1999; Kołaczkowska et al. 2001). It can be explained by mechanism of the morphine-induced inhibition of the synthesis/release of chemotactic factors from the resident peritoneal cells. In the present study, the TNF-α and KC mRNA expression was diminished significantly after morphine treatment. In contrast, these cytokines levels in peritoneal fluid were comparable in groups treated with zymosan or zymosan supplemented with morphine (data not shown). It can be speculated that morphine may inhibit TNF-α and KC expression at the mRNA level in peritoneal leukocytes, but their elevated levels in peritoneal fluid may be originate from different cell types. It was showed that endothelial cells are capable of producing both TNF-α (Freyer et al. 1999; Ranta et al. 1999) and KC (Hol et al. 2010) in response to proinflammatory stimuli. One may speculate that morphine could influence the TNF-α and KC release by endothelium. This phenomenon should be further investigated.

The presented results reveal that supplementation of zymosan with morphine significantly decreased the expression of phosphorylated NF-κB as well as TLR2 in neutrophils and MFs in comparison to the zymosan-injected mice. Moreover, morphine co-injection with zymosan altered the gene expression of the TLR receptors, NF-κB and cytokines (KC, TNF-α) during ongoing inflammation. We may postulate that, after morphine stimulation, peritoneal leukocytes recognize less effectively zymosan antigens because of impaired TLRs expression. The lowered TLR expression attenuates TLR-mediated signal transduction that limits NF-κB activation. Additionally, during zymosan-induced peritonitis, morphine may modulate the NF-κB expression, at least partially, by a mechanism proposed by others, which is linked to up-regulated release of NO. If so, NO inhibits the disassociation of NF-κB from its inhibitor and prevents the translocation of NF-κB into nucleus; it might also act at the transcriptional level by decreasing receptor translation. The latter mechanism will be further investigated.

Acknowledgments This work was supported by Grant No. N N303 35 69 33 from the Polish Ministry of Science and Higher Education.

Conflict of interest The authors declare that they have no conflict of interest.

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