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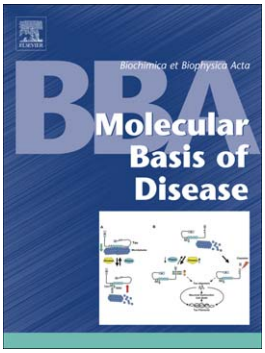

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Abstract

During inflammation, neutrophils infiltrate into the involved site and undergo apoptosis. Early apoptotic neutrophils are then cleared by phagocytes, leading to resolution of the inflammation, whereas if late apoptotic neutrophils are accumulated for some reason, they provoke pro-inflammatory responses such as TNF-α production. In order to determine how endogenously produced nitric oxide (NO) regulates neutrophil apoptosis and the resolution of inflammation, we compared peritoneal inflammation induced by *Staphylococcus aureus* bioparticles in wild type mice with that in inducible NO synthase (iNOS)-deficient ones. In this model, NO production was largely dependent on iNOS, the NO level peaking at 24 h. There were increases in the numbers of neutrophils and late apoptotic ones at 24 h in iNOS-deficient mice as compared with in wild type ones, and consequently TNF-α production at 36 h in iNOS-deficient mice. On the other hand, the administration of a NO donor to iNOS-deficient mice at 12 h decreased the numbers of neutrophils and late apoptotic ones at 24 h, and thereafter TNF-αproduction at 36 h. In addition, coculturing of macrophages with late apoptotic neutrophils caused TNF-α production and a NO donor inhibited the transmigration of neutrophils in a dose-dependent manner. Collectively, these results suggest a novel mechanism that endogenously produced NO suppresses neutrophil accumulation at a late stage of inflammation, thereby preventing the appearance of late apoptotic neutrophils and subsequent pro-inflammatory responses.

Key words: nitric oxide, apoptosis, neutrophils, TNF-α, iNOS, *Staphylococcus aureus* bioparticles

Abbreviations: NO, Nitric oxide; iNOS, inducible NO synthase; *S. aureus*, *Staphylococcus aureus*; WT mice, wild type mice/C57BL/6 mice; KO mice, iNOS-deficient mice/C57BL/6 mice deficient in iNOS; PECs, peritoneal exudate cells; TG, thioglycollate broth; PI, propidium iodide; DEA-NONOate, N-ethylethanamine : 1, 1-diethyl-2-hydroxy-2-nitrosohydrazine (1:1) ; MPO, myeloperoxidase.

1. Introduction

Neutrophils infiltrate into the involved site during inflammation. They perform functions such as bacterial killing and undergo apoptosis. Apoptotic neutrophils at an early stage are then cleared by phagocytes without further inflammatory responses including pro-inflammatory cytokine production being provoked. Therefore, early apoptotic neutrophils appear to be essential for the resolution of inflammation [1]. In support of this, the promotion of neutrophil apoptosis, perhaps early apoptosis, by PD98059 and R-roscovitine enhances the resolution of established inflammation [2, 3]. On the other hand, if late apoptotic neutrophils are accumulated for some reason, these cells provoke further inflammatory responses such as TNF-α production.

Nitric oxide (NO) is produced either by inducible NO synthase (iNOS) or by constitutive NO synthase such as endothelial NOS in a variety of cells, and is involved in physiological and pathological processes, including regulation of blood vessel dilatation, and functions as a neurotransmitter [4, 5]. Upon induction of iNOS by IL-1β, TNF-α, IFN-γ or LPS in macrophages, a large amount of NO is produced, which plays microbicidal and tumoricidal roles, and regulates inflammatory responses [6-8].

NO also regulates apoptosis positively or negatively in a concentration-dependent manner [9]. A large amount of NO derived from iNOS induces apoptosis, while a small amount of NO, such as that derived from constitutive NOS, inhibits apoptosis. Therefore, there is the possibility that NO produced *in vivo* under inflammatory conditions induces apoptosis and the resolution of inflammation [9].

To determine how endogenously produced NO regulates neutrophil apoptosis and the resolution of inflammation, we used *Staphylococcus aureus* (*S. aureus*) bioparticles,

which had been heat-treated and fixed with formalin, to construct a model of inflammation, because the inflammatory response to live *S. aureus* would be affected by the difference in bactericidal ability between wild type (WT) and iNOS-deficient (KO) mice. *S. aureus* bioparticles induced virtually no late apoptotic neutrophils in WT mice, whereas in KO mice, there were increases in the numbers of neutrophils and late apoptotic ones, and consequently TNF-α production. In addition, the administration of a NO donor to KO mice decreased the numbers of neutrophils and late apoptotic ones, and thereafter TNF-αproduction. Thus, our study suggested that endogenously produced NO suppresses neutrophil accumulation at a late stage of inflammation, thereby preventing an increase in the number of late apoptotic neutrophils and further inflammatory responses.

2. Materials and methods

2.1 Mice

C57BL/6 mice were purchased from Sankyo Lab Service (Tokyo). Six-week-old male C57BL/6 mice deficient in iNOS (KO) were purchased from Taconic and bred in the SPF facility of Toho University. This experiment was approved by the animal experiment committee of Toho University.

2.2 Model of acute inflammation, and detection of neutrophils, macrophages and apoptosis by flow cytometry

C57BL/6 (WT) or KO mice were injected with 5×10^7 *S. aureus* bioparticles (Molecular Probes, Eugene, OR), which had been heat-treated and fixed with formalin, in 0.5 ml of PBS. At different time points, peritoneal exudate cells (PECs) were obtained, washed and treated with anti-CD32/16 mAb and mouse IgG for 30 min on ice before staining with FITC-anti-Gr-1 or F4/80 mAb for 30 min on ice or staining with FITC-Annexin V and/or propidium iodide (PI) for 10 min at r.t. They were then analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, CA). For flow cytometric analysis of apoptosis, neutrophils were identified by means of FSC vs. SSC profiles. Annexin V single-positive cells are defined as early apoptotic ones, and Annexin V and PI double-positive cells as late apoptotic ones.

2.3 Immunohistology

PECs from WT mice were cytospun and then immersed in 0.3% H_2O_2 in methanol for 30 min. They were then incubated in a 3% BSA solution for 1 h at r.t. After washing with PBS

twice, they were reacted with anti-MPO Abs (1/400; Serotec, Oxford, UK) at 4°C overnight. They were then washed and reacted with biotinylated anti-rabbit IgG (1/200, Serotec). They were then washed and reacted with the reagent mixture in a VECTASTAIN ABC-PO Kit (Vector Laboratories, Burlingame, CA) for 1 h at r.t., followed by washing with PBS twice. The reagent mixture in a Diaminobenzidine Substrate Kit (Vector Laboratories) was added, followed by incubation for 10 min. They were then washed with PBS three times, and dehydrated by immersion in 75, 85 and 100% ethanol for 2 min, sequentially. As a control, some samples were incubated with normal rabbit IgG (Sigma-Aldrich, MO) instead of the anti-MPO Abs.

2.4 Measurement of NO

NO was quantified by means of a fluorometric assay specific to nitrite [10]. Briefly, 250 μl of peritoneal lavage fluid was added to 125 μl of 2,3-diaminonaphthalene (TCI, Tokyo) in 0.5 M H_2SO_4. Samples were then incubated for 5 min. at r.t. in the dark, followed by the addition of 125 μl of 10% NaOH. The intensity of the fluorescence was measured at an excitation wavelength of 365 nm and an emission one of 410 nm.

2.5 Induction of apoptosis in neutrophils

WT mice were injected with 2 ml of thioglycollate broth (TG) i.p. After 6 h, PECs were collected. The PECs included 90.0% of neutrophils, as judged on H&E staining. The cells were washed with PBS twice and then suspended in RPMI 1640 medium containing 7 % FCS at the cell density of 5×10^5 cells/ml for 2 or 24 h at 37°C. We designated apoptotic neutrophils obtained on culturing for 2 h as early apoptotic neutrophils, and ones obtained on culturing for 24 h as late apoptotic neutrophils. The early apoptotic neutrophils included 16.2

± 1.8% of Annexin V-positive/propidium iodide (PI)-negative cells and 2.0 ± 0.2% of Annexin V-positive/PI-positive ones, whereas late apoptotic neutrophils included 6.0 ± 0.7% of Annexin V-positive/PI-negative cells and 61.5 ± 3.3% of Annexin V-positive/PI-positive ones.

2.6 Treatment with DEA-NONOate in vitro and in vivo

Neutrophils were treated with 100 μM N-ethylethanamine:1, 1-diethyl-2-hydroxy-2-nitrosohydrazine (1:1) (DEA-NONOate; Sigma-Aldrich; half life 16 min in phosphate buffer at 22ºC, according to the manufacturer's information) or PBS for 15 min at 37°C prior to the addition of *S. aureus* bioparticles in the ratio of 1:5, followed by culture for various times and subsequent analysis of apoptosis by flow cytometry. KO mice were injected (i.p.) with 500 nmol of DEA-NONOate at 12 h after *S. aureus* bioparticle-injection, followed by preparation of PECs after a further 12 h.

2.7 Preparation of peritoneal macrophages (TG macrophages) and coculturing of them with apoptotic neutrophils

WT or KO mice were injected with 2 ml of TG i.p. After 96 h, PECs were collected then washed with PBS, followed by incubation in RPMI 1640 medium containing 7 % FCS at the cell density of 12.5×10^5 cells/well in 96-well plates for 1 h at 37°C. Nonadherent cells were removed and washed three times with warm PBS. The resultant adherent cells included 95.9 % of macrophages, as judged on H&E staining. The adherent cells were then treated with or without 100 ng/ml of IFN-γ, and cocultured with early or late apoptotic neutrophils in the ratio of 1:1 for 24 h, followed by determination of the TNF-α level by means of a specific ELISA.

2.8 Preparation of mouse neutrophils from bone marrow

Bone marrow cells were obtained and suspended in HBSS. They were laid on a five-layer Percoll gradient of 81%, 62%, 55%, 50%, and 45% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden), respectively, diluted in HBSS, and then centrifuged (1700 g, 30 min, r.t.) without braking. The neutrophils from the 55%/62% interface and the upper part of the 62% layer were harvested in BSA 1%-coated tubes. After washing with PBS, the red cells were eliminated by hypotonic lysis. 88±2.2% of the cells were morphologically mature neutrophils.

2.9 Neutrophil transmigration assay

Neutrophil chemotaxis was measured in a 24-well chemotaxis chamber (Becton Dickinson, Franklin Lakes, NJ). Polyethylene membrane filters (3 μm pore size) were coated with 1% gelatin (Nacalai, Kyoto) overnight, followed by culturing of endothelioma cells on them, which were provided by Dr. Sorimachi, for 24 h. The lower wells were filled with 1-100 μM DEA-NONOate and/or 25 ng/ml of MIP-2 (PeproTech) in RPMI 1640 medium. A filter membrane was positioned over each of the loaded wells, and 200 μl of bone marrow derived-neutrophils (1×10^6) was placed directly onto the filter. The chamber was incubated for 3 h. The cells that had crossed the endothelioma layer were collected from the lower compartment and then cytospun, followed by counting with software ImageJ.

2.10 Statistics

The significance of the data was evaluated by means of Student's *t* test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1 Leukocyte infiltration and phagocytosis of apoptotic neutrophils by macrophages upon acute inflammation

Upon acute inflammation, infiltrating neutrophils undergo apoptosis and the apoptotic neutrophils are then removed by phagocytes like macrophages, leading to resolution of the inflammation. In order to determine how neutrophil apoptosis is regulated by NO endogenously produced during inflammation, we employed a peritoneal inflammation model induced by *Staphylococcus aureus* bioparticles.

When mice were injected with *S. aureus* bioparticles, which comprised heat- and chemical-treated *S. aureus*, neutrophil infiltration peaked at 9 h post-injection, and then monocytes/macrophages infiltrated into the peritoneal cavity (Fig. 1A). As shown in Fig. 1B, at 6 h post-injection one neutrophil contained one *S. aureus* particle, whereas at 24 h post-injection two neighboring macrophages contained cells with round nuclei, possibly apoptotic neutrophils, the percentages of such macrophages being 35.2 ± 7.5%. Moreover, as shown in Fig. 1C, the cell inside a macrophage was stained with anti-myeloperoxidase (MPO) antibodies, whereas an adjacent macrophage was not, indicating that the cell inside the macrophage was a neutrophil. Macrophages at 48 h post-injection, on the other hand, did not contain such apoptotic neutrophils, suggesting that apoptotic neutrophils are digested by macrophages, as shown in Fig. 1B.

3.2 NO production *in vivo*

To confirm that NO is produced in the peritoneal cavity in this inflammation model, we measured NO as nitrite by means of a fluorometric assay specific to nitrite [10],

which is more sensitive than the Griess assay. As shown in Fig. 2, NO was much more significantly produced in WT mice than KO ones, suggesting that NO is mainly produced via iNOS. The NO level in the peritoneal cavity of WT mice peaked at 24 h. A small amount of NO was detected in the peritoneal cavity of KO mice, suggesting that NO is also constitutively produced in these two strains of mice.

3.3 Kinetics of the neutrophil infiltration and apoptosis, and TNF-α production upon injection of *S. aureus*.

Since NO is known to regulate apoptosis positively or negatively [9], we next examined the kinetics of neutrophil infiltration and apoptosis of neutrophils in WT and KO mice (Fig. 3A and B). Annexin V single-positive cells were defined as early apoptotic ones, and Annexin V and PI double-positive cells as late apoptotic ones. There were more neutrophils in KO mice than in WT mice at 12 and 24 h post-injection. Only a small number of late apoptotic neutrophils were detected in WT mice, suggesting that apoptotic neutrophils are phagocytosed by macrophages as soon as they appear and/or that NO prevents neutrophils from undergoing late apoptosis. In contrast, in KO mice, there was an increase in the number of late apoptotic neutrophils at 9, 18 or 24 h post-injection, the latter two increases being statistically significant. The number of early apoptotic neutrophils in KO mice was much lower than that expected from the number of late apoptotic neutrophils in KO mice. Fig. 3C shows representative results of flow cytometric analysis at 24 h post-injection.

Late apoptotic but not early apoptotic cells induce inflammatory responses such as TNF-α production by macrophages [11-13]. We therefore examined whether or not TNF-α is produced in KO mice (Fig. 3D). At 3 h post-injection, TNF-α was detected in WT and KO

mice, but virtually no late apoptotic neutrophils were present in the two strains of mice. At 9 h post-injection, although the number of late apoptotic neutrophils in KO mice was greater than that in WT mice (Fig. 3B), no TNF-α was detected. This is probably because there were no macrophages at 9 h post-injection (Fig. 1A). At 36 h post-injection, on the other hand, a large amount of TNF-α was detected in the peritoneal cavity of KO mice but not in that of WT ones.

3.4 The effect of DEA-NONOate on neutrophil apoptosis *in vitro*

In order to reveal the mechanism underlying the appearance of late apoptotic neutrophils at a late stage of inflammation in KO mice, we examined the effect of a NO donor, DEA-NONOate, on neutrophil apoptosis *in vitro*. When neutrophils were treated with *S. aureus* bioparticles, a large number of neutrophils underwent late apoptosis rather quickly, as compared with untreated neutrophils (Fig. 4 right, crosses vs. filled squares). In contrast, the presence of DEA-NONOate, a NO donor, inhibited the induction of late apoptosis by *S. aureus* bioparticles significantly at 1, 3 and 6 h (Fig. 4 right, filled squares vs. filled diamonds). These results suggest that NO prevents neutrophils from undergoing late apoptosis upon the clearance of *S. aureus* bioparticles in WT mice. On the contrary, when neutrophils were not treated with *S. aureus* bioparticles, DEA-NONOate did not affect apoptosis significantly (Fig. 4, crosses vs. open diamonds).

3.5 The effect of DEA-NONOate on the numbers of neutrophils and late apoptotic ones, and the level of TNF-α at a late stage of inflammation in KO mice

The above results suggest that NO plays a critical role in preventing late apoptosis of neutrophils and consequent TNF-α production. To confirm this, we examined the effect

of DEA-NONOate on the increases in the numbers of neutrophils and late apoptotic ones, and that on the increase in the level of TNF-α at a late stage of inflammation in KO mice.

Treatment with DEA-NONOate at 12 h post-injection significantly reduced the increase in the number of late apoptotic neutrophils at 24 h post-injection in KO mice (Fig. 5A). The treatment also significantly reduced the increase in the number of neutrophils at 24 h and that in the level of TNF-α at 36 h post-injection in KO mice (Fig. 5B and C).

3.6 TNF-α production by macrophages upon coculturing with late apoptotic neutrophils

The data in Figs. 3 and 5 indicate the parallelism between the appearance of late apoptotic neutrophils and TNF-α production. We then examined directly whether or not late apoptotic neutrophils caused TNF-α production in macrophages of KO mice (Fig. 6). When IFN-γ-stimulated macrophages, but not non-stimulated ones, were cocultured with late apoptotic neutrophils, TNF-α was significantly produced. Early apoptotic neutrophils did not induce TNF-α production by IFN-γ-stimulated macrophages, as might be expected. These results indicate causal relationship between the appearance of late apoptotic neutrophils and the production of TNF-α, but NO was not involved in the process, because TNF-α production by macrophages from KO mice was not different from that by macrophages from WT ones.

3.7 The effect of DEA-NONOate on the transmigration of neutrophils

Because DEA-NONOate reduced the number of neutrophils of KO mice at 24 h (Fig. 5B), we then examined whether or not NO suppresses neutrophil transendothelial migration *in vitro*. As shown in Fig. 7, DEA-NONOate significantly suppressed MIP-2-induced neutrophil transendothelial migration in a dose-dependent manner.

4. Discussion

It is important for the resolution of inflammation that, after neutrophils have performed their roles, such as the phagocytosis and killing of bacteria, they quickly undergo apoptosis and are then silently phagocytosed by macrophages [11]. If this mechanism fails, neutrophils might produce an excess amount of superoxide at the site of inflammation, which would cause tissue damage, or they might undergo late apoptosis to induce a further inflammatory response, i.e., a vicious cycle. Indeed, late apoptotic or necrotic cells induce inflammatory responses such as the production of IL-8 and TNF-α by macrophages [12, 13]. In addition, damage-associated molecular patterns, such as HMGB1, S100, uric acid, ATP, elastase, and SAP 130, are released from late apoptotic or necrotic cells, and they induce many inflammatory responses [14-17]. Furthermore, the excessive production of a pro-inflammatory cytokine like TNF-α upon induction of necrosis or failure of removal of apoptotic cells causes chronic inflammation [18, 19].

By employing a peritoneal inflammation model induced by *Staphylococcus aureus* bioparticles, we found in this study that there were many more late apoptotic neutrophils in KO mice than in WT ones at 24 h post-injection, and consequently TNF-α production at 36 h post-injection. TNF-α produced at 36 h post-injection in KO mice, however, did not induce an additional neutrophil accumulation for unknown reasons. Although further study is needed to clarify whether or not other proinflammatory cytokines are produced, it is possible that such TNF-α production results in autoimmune disease. These findings suggest that endogenously produced NO suppresses late apoptosis of neutrophils, thereby preventing inflammatory responses. Consistent with this notion, the administration of a NO donor to KO mice decreased the number of late apoptotic neutrophils

and thereafter TNF-αproduction. Thus, our study indicates the beneficial role of endogenously produced NO as to the resolution of inflammation. Determination of whether or not this also holds true for other inflammation models awaits further studies.

One of the mechanisms underlying the appearance of late apoptotic neutrophils in KO mice is that neutrophil transmigration is not adequately regulated in the absence of NO. NO-mediated suppression of neutrophil transmigration was reported previously [20]. We confirmed this in this study and further showed by administration of a NO donor to KO mice that NO suppresses the accumulation of neutrophils and the appearance of late apoptotic neutrophils. However, NO did not always cause suppression of neutrophil transmigration, because the total neutrophil numbers detected in WT and KO mice at 9 h were similar. Therefore, an increase in the neutrophil number may involve a mechanism other than de-regulation of neutrophil transmigration. Similarly, the appearance of late apoptotic neutrophils at a late stage of inflammation in KO mice may also involve a mechanism other than de-regulation of neutrophil transmigration, because the numbers of neutrophils and late apoptotic ones do not always correlate each other. It is unlikely, however, that MIP-2 and KC may contribute to the accumulation of neutrophils at a late stage of inflammation, thereby leading to the accumulation of late apoptotic neutrophils, because there were no differences between the levels of these chemokines in WT and KO mice at a late stage of inflammation (data not shown). On the other hand, the second possibility that phagocytosis of apoptotic cells is impaired in KO mice is rather remote, because macrophages in KO mice phagocytose apoptotic cells as efficiently as those in WT mice [21, and unpublished results]. The third possibility is that apoptosis proceeds to a late stage in the absence of NO. It is known that bacterial infection induces the necrosis of neutrophils, and the underlying mechanism is being clarified that pore-forming toxin, streptolysin O, and serine protease ScpC induce

necrosis of neutrophils [22]. We found that *S. aureus* bioparticle also induces late apoptosis of neutrophils, and that *S. aureus* bioparticle-mediated late apoptosis of neutrophils is suppressed by a NO donor. Therefore the first peak for accumulation of late apoptotic neutrophils in KO mice may be caused by a failure of such a control mechanism by which NO prevents *S. aureus* bioparticle-mediated late apoptosis of neutrophils. At a late stage of inflammation, however, *S. aureus* bioparticles may have already been removed by neutrophils. Taken together, the most probable mechanism underlying the appearance of late apoptotic neutrophils in KO mice is that neutrophil transmigration is not adequately regulated in the absence of NO.

IFN-γ-stimulated macrophages but not non-stimulated ones cocultured with late apoptotic neutrophils produced TNF-α in this study. This may be physiologically relevant, because IFN-γ was detected in the peritoneal cavity in this model. Other researchers have reported that coculturing of non-stimulated mouse bone marrow-derived macrophages with human lysed or late apoptotic neutrophils produced TNF-α [13]. Although the authors in Ref. 13 suggested that elastase in lysed human neutrophils is responsible for TNF-α production, it is not clear whether or not the effect of late apoptotic neutrophils is also due to elastase. If elastase is also involved in the response to late apoptotic neutrophils, then mouse activated neutrophils would induce TNF-α production even in the absence of IFN-γ. Consequently, late apoptotic human (resting) neutrophils may possess something that is absent from late apoptotic mouse (activated) neutrophils.

TNF-α production by IFN-γ-stimulated macrophages from KO mice cocultured with late apoptotic neutrophils was not different from that by IFN-γ-stimulated macrophages from WT ones, indicatingthatthisprocesswasNO-independent. In contrast, coculturing of macrophages with early apoptotic cells induced NO production, thereby suppressing MIP-2

production [23]. On the contrary, at 3 h post-injection, TNF-α was produced in KO mice as well as in WT mice, and the level in KO mice was lower than that in WT ones, although the difference was not statistically significant.

In summary, endogenously produced NO suppresses neutrophil accumulation at a late stage of inflammation, thereby preventing the accumulation of late apoptotic neutrophils and subsequent pro-inflammatory responses. Thus, this study revealed a critical role of NO in the resolution of inflammation.

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Figure 1

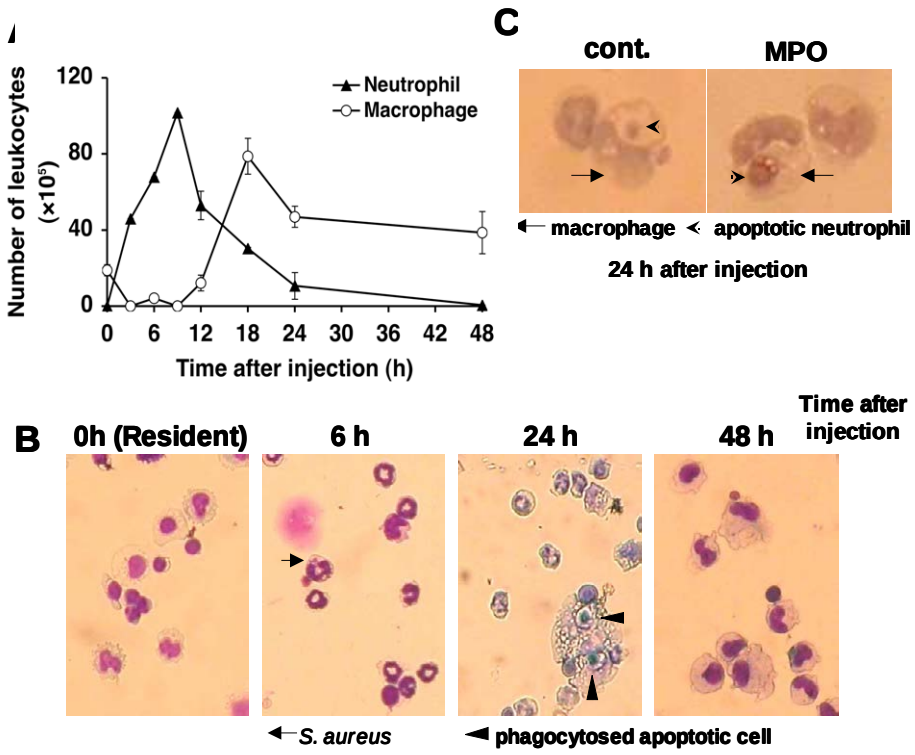

Fig. 1. Leukocyte infiltration and phagocytosis of apoptotic neutrophils by macrophages. A, WT mice were injected with *S. aureus* bioparticles, followed by determination of the numbers of Gr-1 highly positive cells (Neutrophil) and F4/80 positive cells (Macrophage) in the peritoneal cavity. The results are expressed as means ± SD for four independent experiments. B, resident peritoneal cells or peritoneal exudate cells (PECs), which were obtained at various time points, were stained with hematoxylin (24 h) and eosin (Resident, 6 h, 48 h). The results are representative of four independent experiments. C, PECs at 24 h after *S. aureus* bioparticle-injection were stained with an anti-MPO Ab (apoptotic neutrophil) and hematoxylin (nuclei). Rabbit IgG was used as a control (cont.) for the anti-MPO Ab. The data are representative of four experiments.

Figure 2

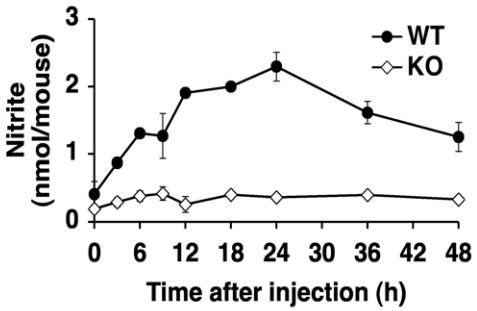

Fig. 2. NO production *in vivo*. The levels of NO in peritoneal exudates at different time points after *S. aureus* bioparticle-injection were determined as nitrite by means of a fluorometric assay, as described under Materials and Methods. The results are expressed as means ± SD for four independent experiments.

Figure 3

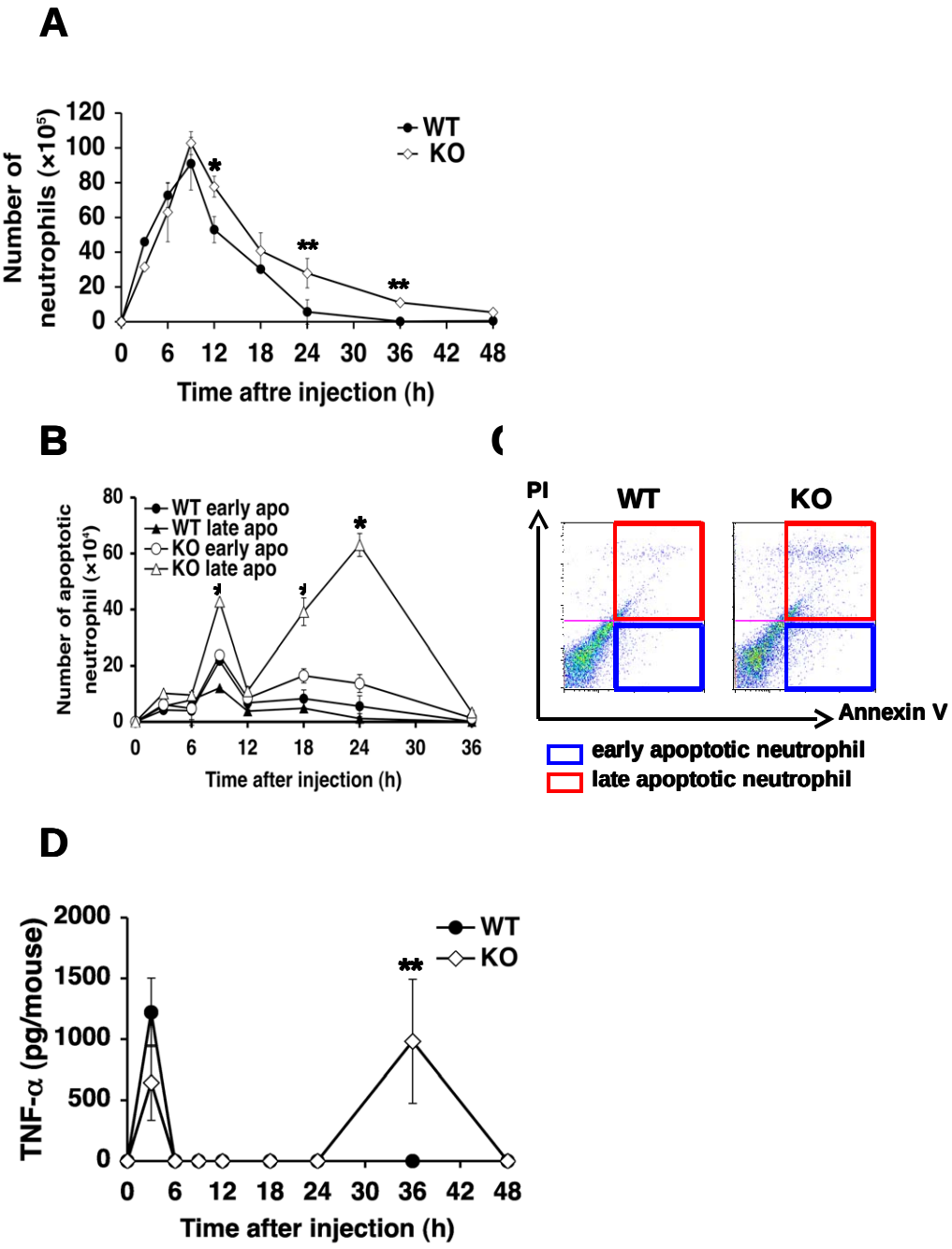

Fig. 3. Kinetics of the neutrophil infiltration and apoptosis, and TNF-α production upon injection of *S. aureus*. A, WT or iNOS KO mice were injected with *S. aureus*, followed by

determination of the number of neutrophils in the peritoneal cavity. The results are expressed as means ± SD for four independent experiments. *, $p<0.05$, **, $p<0.01$ vs. WT. B, WT or iNOS KO mice were injected with *S. aureus* bioparticles, followed by determination of the numbers of early and late apoptotic neutrophils in the peritoneal cavity. Annexin V single-positive cells are defined as cells at an early stage of apoptosis, and Annexin V and PI double-positive cells as ones at a late stage of apoptosis. The results are expressed as means ± SD for four independent experiments. *, $p<0.05$ vs. KO. C, Representative data of flow cytometric analysis of apoptosis at 24 h after *S. aureus* bioparticle-injection in B. D, WT or iNOS KO mice were injected with *S. aureus* bioparticles, followed by preparation of PECs by lavage of the peritoneal cavity. The level of TNF-α in a supernatant was determined by ELISA. The results are expressed as means ± SD for four independent experiments. **, $p<0.01$ vs. WT.

Figure 4

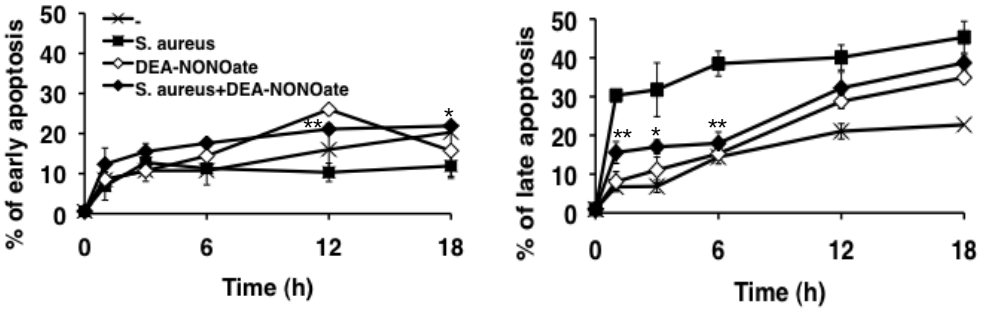

Fig. 4. The effect of DEA-NONOate on neutrophil apoptosis *in vitro*. TG neutrophils were treated with 100 μM DEA-NONOate or PBS for 15 min, followed by the addition of *S. aureus* bioparticles, culturing for various times, and then determination of the percentages of early (left panel) and late apoptotic neutrophils (right panel). The results are expressed as means ± SD for three independent experiments. The significance between no (crosses) vs. DEA-NONOate (open diamonds) or between *S. aureus* (filled squares) vs. *S. aureus* + DEA-NONOate (filled diamonds) was evaluated. *, $p<0.05$ vs. - DEA-NONOate, **, $p<0.01$ vs. - DEA-NONOate.

Figure 5

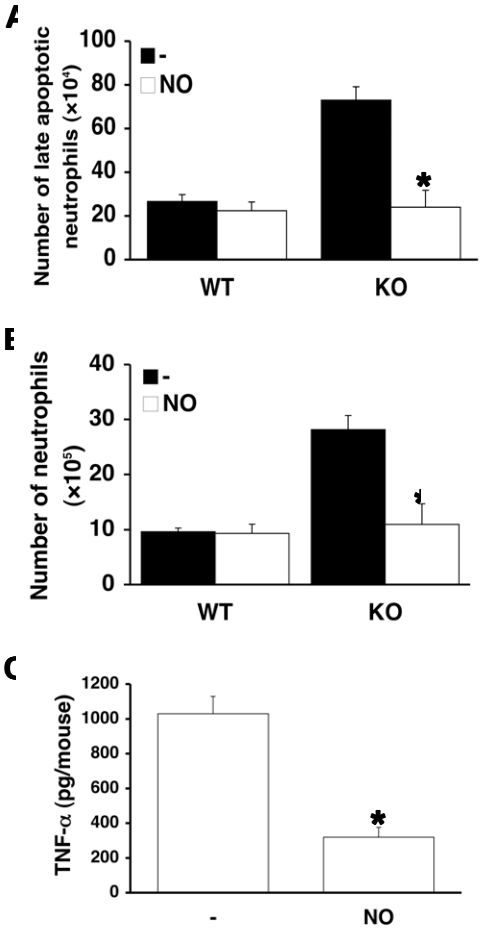

Fig. 5. The effects of DEA-NONOate on the numbers of late apoptotic neutrophils and neutrophils, and the level of TNF-α in a late stage of inflammation in KO mice. WT or KO mice were injected with 500 nmol of DEA-NONOate (NO) or PBS (-) at 12 h after *S. aureus* bioparticle-injection, followed by flow cytometric determination of the numbers of late apoptotic neutrophils (A) as well as neutrophils (B) in peritoneal exudates at 12 h after DEA-NONOate injection. The results are expressed as means ± SD for four independent

experiments. *, $p<0.05$ vs. - (control). C, KO mice were injected with 500 nmol of DEA-NONOate (NO) at 12 h after *S. aureus* bioparticle-injection, followed by ELISA determination of the levels of TNF-α in peritoneal exudates at 24 h after DEA-NONOate injection. The results are expressed as means ± SD for four independent experiments. *, $p<0.05$ vs. - (control).

Figure 6

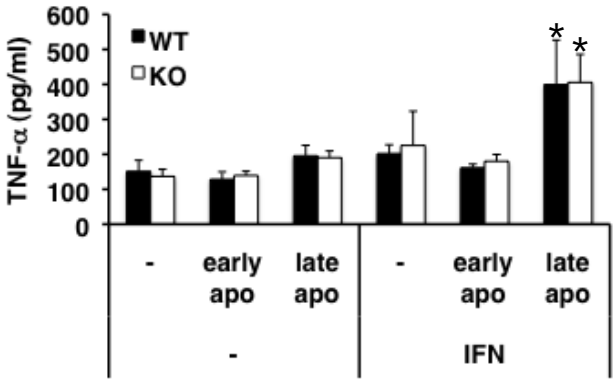

Fig. 6. TNF-α production by macrophages upon coculture with late apoptotic neutrophils. TG macrophages from WT or KO mice were treated with or without 100 ng/ml of IFN-γ, and then cocultured with early or late apoptotic neutrophils for 24 h, followed by determination of the TNF-α level by means of a specific ELISA. The results are expressed as means ± SD for three independent experiments. *, $p<0.05$ vs. macrophages only.

Figure 7

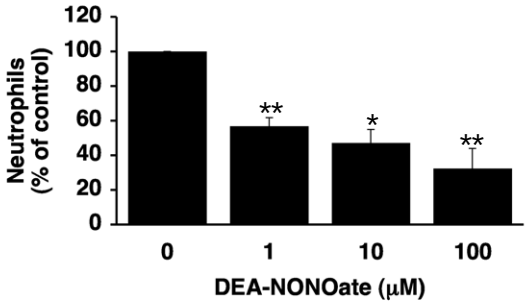

Fig. 7. Suppression of neutrophil transmigration by DEA-NONOate. The lower wells were filled with 25 ng/ml of MIP-2 and 0-100 μM DEA-NONOate in RPMI. A filter membrane coated with endothelioma was positioned over each of the lower wells, and then bone marrow-derived neutrophils were placed directly onto the filter. The chamber was incubated for 3 h. The cells that had crossed the endothelioma layer were collected from the lower compartment and cytospun, followed by counting with software ImageJ. The results are shown as percentages of the number of neutrophils with 0 μM DEA-NONOate. The results are expressed as means ± SD for three independent experiments. *, $p<0.05$ vs. - DEA-NONOate, **, $p<0.01$ vs. - DEA-NONOate.