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Apoptotic neutrophils and nitric oxide regulate cytokine production by IFN-γ-stimulated macrophages

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ABSTRACT

Early apoptotic neutrophils but not secondary necrotic ones down-regulate LPS-induced proinflammatory cytokine production of macrophages, thereby contributing to the resolution of inflammation. IFN-γ is also a well-known stimulant of macrophages, but how the apoptotic neutrophils affect IFN-γ-stimulated macrophages remains largely unexplored. Since IFN-γ induces the expression of inducible nitric oxide (NO) synthase, we examined the production of NO and various cytokines, including MIP-2, TNF-α, IL-12p40, IL-6, IL-10, and TGF-β, by IFN-γ-stimulated murine macrophages, the effect of coculturing the macrophages with early apoptotic or secondary necrotic neutrophils, and the regulatory role of NO in such cocultures. IFN-γ induced significant production of NO, IL-12p40, and IL-6 by macrophages, but not other cytokines. Early apoptotic neutrophils but not secondary necrotic ones promoted NO production, whereas secondary necrotic ones and their supernatants promoted TNF-α production. In contrast, both early apoptotic and secondary necrotic neutrophils suppressed IL-12p40 and IL-6 production. Furthermore, macrophages from inducible NO synthase-deficient mice produced significantly higher levels of MIP-2 than those from wild-type mice. Consistent with this, treatment of macrophages with L-NAME, an NO synthase inhibitor, also induced the production of a large amount of MIP-2. In conclusion, this study suggests that early apoptotic neutrophils are critical in the resolution of inflammation, but that secondary necrotic neutrophils may not cause an inflammatory response. Apoptotic neutrophils, however, appear not to modulate cytokine production via NO.

1. Introduction

Neutrophils infiltrate into sites of bacterial infection to kill bacteria and then undergo apoptosis.

At sites of infection/inflammation, monocytes/macrophages are also recruited. Neutrophils and monocytes/macrophages cluster and interact, including at the resolution phase when macrophages quickly remove apoptotic neutrophils [1–3]. If this removal fails, or if massive apoptosis overwhelms the available scavenging capacity, progression to secondary necrosis ensues, and may result in leakage of the cell contents with induction of tissue injury and inflammatory and autoimmune responses [4,5]. The contents released from secondary necrotic cells include damage-associated molecular pattern molecules, such as high mobility group box-1 (HMGB1), S100A protein, SAP130 and ATP [6–11]. In normal situation, on the contrary, macrophages phagocytose apoptotic cells at the early stage (early apoptotic cells) by a highly redundant system of receptors and bridging molecules [12], and they do not induce excessive inflammatory responses but rather assist in resolving inflammation. In this regard, it is reported that IL-10 and TGF-β suppress the production of proinflammatory cytokines by LPS-stimulated monocytes/macrophages in coculture with early apoptotic cells [13,14], but the in vivo mechanism remains elusive.

Bacterial infection induces IFN-γ production [15]. IFN-γ is produced by T cells, NK cells, and NKT cells and causes many effects [16,17]. IFN-γ is the best inducer of NO in macrophages, which contributes to their antimicrobial and antitumor activities. NO also regulates inflammatory responses, including cytokine production [18]. We have previously shown that NO is produced by macrophages that phagocytose early apoptotic cells, and that NO suppresses MIP-2 and keratinocyte-derived cytokine (KC) production of macrophages to which no stimulants were added [19,20].

Although the regulation of cytokine production of LPS-stimulated macrophages by apoptotic cells has been extensively studied, that of IFN-γ-stimulated macrophages remains largely unexplored.

Abbreviations: NO, nitric oxide; HMGB1, high mobility group box-1; MIP-2, macrophage inflammatory protein-2; KC, keratinocyte-derived cytokine; WT mice, C57BL/6 mice; iNOS, inducible NO synthase; KO mice, C57BL/6 mice deficient in iNOS; i.p., intraperitoneally; PECs, peritoneal exudate cells; AxV, Annexin V; PI, propidium iodide; L-NAME, N^G–nitro-L-arginine methyl ester.

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We therefore investigated the regulation of NO and cytokine production of IFN-γ-stimulated macrophages by apoptotic neutrophils and the regulation of cytokine production by NO.

2. Materials and methods

2.1. Mice

C57BL/6 mice (WT) were purchased from Sankyo Lab Service (Tokyo). Six-week-old male C57BL/6 mice deficient in inducible NO synthase (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. Induction of apoptosis in neutrophils

WT mice were injected with 2 ml of thioglycollate broth intraperitoneally (i.p.). After 6 h, peritoneal exudate cells (PECs) were collected. PECs included 90.0% of neutrophils, as judged by H&E staining. The cells were washed with PBS twice, then suspended in RPMI 1640 medium containing 7% FCS at a cell density of 2×10^6 cells/ml and cultured for 2 or 24 h at 37 °C. We designate apoptotic neutrophils obtained by culturing for 2 h as early apoptotic neutrophils, and those obtained by culturing for 24 h as secondary necrotic neutrophils (see Section 3).

2.3. Preparation of peritoneal macrophages and their coculture with apoptotic neutrophils

Mice were injected with 2 ml of thioglycollate broth i.p. After 96 h, PECs were collected, washed with PBS, and incubated in RPMI 1640 medium containing 7% FCS for 1 h at 37 °C. Non-adherent cells were removed and washed three times with warm PBS. The remaining adherent cells included 95.9% of macrophages, as judged by H&E staining. Apoptotic neutrophils were washed with PBS three times, and they were added to macrophages in a ratio of 1:1 and stimulated with 100 ng/ml of IFN-γ (PeproTech, NJ), followed by incubation for 24 h (Figs. 1 and 2).

2.4. Treatment of macrophages with IFN-γ and/or L-NAME

Macrophages were treated with 1 mM N^G–nitro-L-arginine methyl ester (L-NAME, Sigma–Aldrich, MO) for 15 min, followed by addition of 100 ng/ml of IFN-γ and coculture with early apoptotic or secondary necrotic neutrophils for 24 h.

2.5. Measurement of NO

NO was quantified as nitrite by means of the Griess reaction. Briefly, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (Sigma–Aldrich), and the mixture was

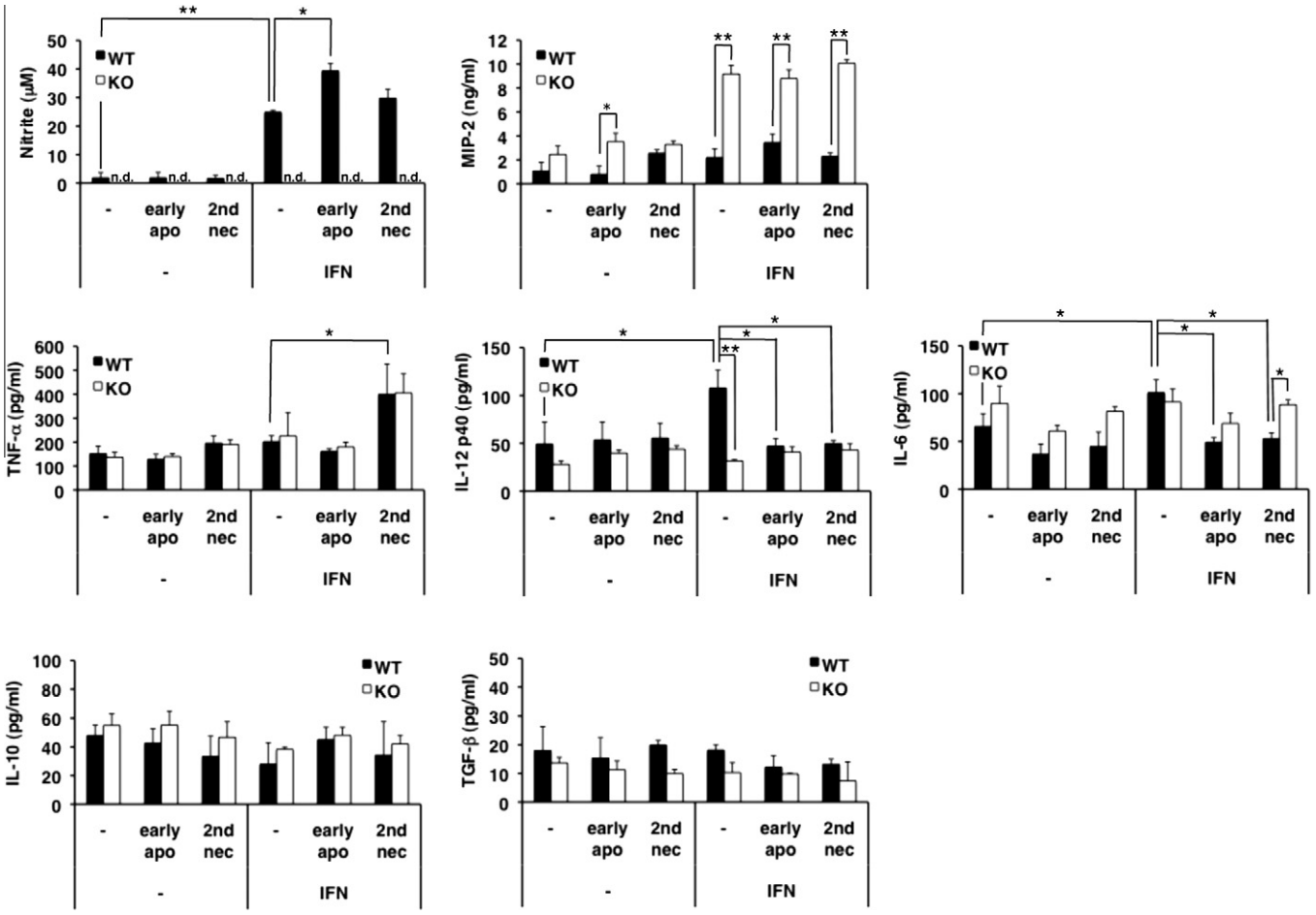

Fig. 1. NO and cytokine production of IFN-γ-stimulated macrophages from WT or KO mice after coculturing with apoptotic neutrophils. The levels of NO, MIP-2, TNF-α, IL-12p40, IL-6, IL-10 and TGF-β in the supernatants of 24-h cocultures of IFN-γ (100 ng/ml)-stimulated macrophages from WT or KO mice with early apoptotic or secondary necrotic neutrophils were determined. We carried out experiments twice and obtained essentially the same results. The results shown are representative of these two separate experiments. The data are expressed as the means ± SD of three independent cultures. The asterisks indicate significant differences ($^*p < 0.05$, $^{**}p < 0.01$).