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Contribution of CR3 to Nitric Oxide Production from Macrophages Stimulated with High-Dose of LPS

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The contribution of the complement receptor type 3 (CR3) to nitric oxide (NO) production from macrophages stimulated by LPS was investigated. When thioglycollate-elicited mouse peritoneal macrophages were stimulated with a high dose of LPS (10 μg/ml) in both the presence and absence of fetal calf serum, a source of LPS binding protein (LBP) necessary for the binding of LPS to CD14, NO production was observed. These findings suggest that CD14-dependent and CD14-independent signaling pathways for NO production are present in macrophages. Because binding and phagocytosis of bacteria by macrophages through the CR3 has been previously reported, we investigated whether the CR3 acts in CD14-independent signaling pathway for NO production. By flow cytometric analysis, the binding of FITC-labeled anti-CR3 monoclonal antibody (anti-CR3 mAb) to macrophages was inhibited by LPS. Anti-CR3 mAb induced iNOS protein and produced NO in a dose dependent manner. Further, NO production induced by anti-CR3 mAb was also inhibited by zymocel, β-glucan with a high affinity to CR3. These results suggest that the CR3 molecule acts in a CD14-independent signaling pathway, and contributes to NO production by macrophages stimulated with high doses of LPS.

LPS, a structural component of the outer membrane of Gram-negative bacteria, is a potent activator of macrophages [1]. LPS triggers abundant secretions of cytokins such as TNF-α, IL-1 and IL-6, which contribute to the pathophysiology of septic shock [2-4]. In this trigger, CD14, a 55 kDa glycosylphosphatidylinositol-linked membrane protein, acts as the major surface receptor for LPS. A complex formed with the serum protein, LPS-binding protein (LBP), is necessary for the binding of LPS to CD14 [5]. At low doses of LPS (<10 ng/ml), LPS-induced signaling and macrophage functional activation is completely blocked by anti-CD14 monoclonal antibody (mAb), indicating that CD14 predominantly acts as the LPS receptor for macrophage activation. On the other hand, at higher doses of LPS (>10 ng/ml) anti-CD14 mAbs did not block LPS-induced signaling and macrophage functional activation, suggesting that a CD14-independent mechanism of LPS stimulation exists [6-9].

Many studies have indicated that CD14 is the primary binding site of LPS for nitric oxide (NO) production from macrophages [10, 11]. However, little attention has been paid to the possibility of a CD14-independent pathway for NO production from macrophages stimulated by LPS.

In this report, we examined the effect of fetal calf serum (FCS), a source of LBP, on NO production from mouse peritoneal macrophages induced by high dose of LPS. Surprisingly we found that NO was produced in the absence of FCS, suggesting that a CD14-independent pathway exists for NO production by LPS. Thus we examined the contribution of complement receptor type 3 (CR3, CD11b/CD18, Mac-1) to NO production from mouse peritoneal macrophages. CR3 is a member of the leukocyte β_2 integrin family and is involved in the binding and phagocytosis of bacteria by macrophages [12, 13]. Anti-CR3 (α-chain) mAb induced iNOS and enhanced NO production from mouse peritoneal macrophages stimulated by LPS, suggesting that CR3 acts in CD14-independent pathway for NO production.

MATERIALS AND METHODS

Chemicals and reagents. Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4) and fetal calf serum (FCS) was purchased from Sigma Co., Ltd. (St. Louis, MO) and Moregate Co. Ltd. (Australia), respectively. Anti mouse inducible NO synthase (iNOS) polyclonal antibody was obtained from Affinity BioReagent Inc. (Neshanic Station, NJ). Anti-mouse CD14 monoclonal antibody (mAb) was purchased from PharMingen (San Diego, CA). Zymocel was purchased from Alpha-Beta technology (Wercester. MA).

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Preparation of anti-CR3 antibody. M1/70 cells which produce anti-CR3 (α chain) mAb were purchased from American Type Culture Collection (Bethesda, MD) and cultured in serum-free medium (Kohjin Bio., Japan). The culture supernatant was condensed by ultrafiltration, and used as anti-CR3 (α-chain) mAb. FITC-labeled anti-CR3 mAb was prepared by according to the method of Coligan et al. [14]. LPS contamination of anti-CR3 (α-chain) mAb preparations was estimated using Endospecy kit (Seikagaku Co., Ltd., Tokyo, Japan), and was 831 pg/μg protein of antibody.

Preparation of macrophages. C3H/HeN mice (male, 6-8 weeks old, Japan SLC Inc., Shizuoka, Japan) were injected with 1.0 ml of 3% thioglycollate (Difco Laboratory, Detroit, MI) intraperitoneally. On day 4, the peritoneal exude cells (PEC) were obtained by peritoneal lavage with 10 ml of ice-cold Hank's balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free) supplemented with 10 U/ml of heparin. PEC were washed twice and resuspended in RPMI-1640 medium supplemented with 10 % fetal calf serum, and overlaid on plastic plates (96-well culture plate, Corning). The plates were incubated in humidified 5% CO_2 at 37 °C for 2 hr to allow macrophage adherence. Each plate was washed with gentle agitation by warmed RPMI-1640 to dislodge non-adherent cells, and a macrophage monolayer was obtained.

Nitrite determination. Macrophages (1×10^5/well) were incubated with LPS or anti-CR3 mAb for 48 hr to elicit the production of NO. NO production was estimated by measurement of nitrite in the culture supernatant using Griess reagent as described by Stuehr and Nathan [15]. Macrophage viability subsequent to this treatment was assessed by the Trypan blue dye exclusion test, and was shown to have not changed.

Flow cytometry. The binding of FITC-labeled anti-CR3 mAb to macrophages was determined by FACSCalibur (Becton Dickinson). Thioglycollate-elicited mouse peritoneal macrophages (1×10^6 cells/ml) suspended in RPMI-1640 medium supplemented with 10% FCS were incubated with FITC-labeled anti-CR3 mAb (1 μg/ml) in the presence or absence of LPS (100 μg/ml) at 4°C for 30 min. Macrophages were washed with the medium and then fluorescence was quantified using a FACSCalibur.

Western blot analysis. Macrophage (1×10^6 /dish) were incubated with LPS (10 μg/ml) or various concentrations of anti-CR3 mAb for 48 hr. Cells were then scraped and lysed with lysis buffer (10 mM Tris-buffer (pH 7.2), 150 mM NaCl, 1 % Triton X-100, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl-fluoride, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin) at 4 °C for 1 hr. Samples (15 μg as protein) were electrophoresed on 7.5% SDS-PAGE and transferred onto Immobilon P membrane (Nihon Millipore, Tokyo, Japan). The membrane was blocked in 2% BSA in PBS containing 0.1 % Tween 20 and then incubated with anti-iNOS antibody. After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody, washed, and specific bands were detected with an ECL assay kit (Amersham Japan, Tokyo, Japan).

RESULTS

Effects of FCS on NO production. Macrophages were treated with 10 μg/ml of LPS for various times in the presence or absence of FCS, then the medium was changed to a FCS supplemented one and incubated for 48 hr to elicit NO production (Fig. 1). Irrespective of the addition of FCS, NO production was observed, but higher NO levels were observed from macrophages treated with LPS in the presence of FCS.

CD14 is considered to be the primary binding site for LPS in the nitric oxide (NO) production in macrophages stimulated with LPS [10, 11]. Therefore, the effect of an anti-CD14 mAb on NO production was examined (Fig. 2). Treatment of macrophages with anti-CD14 mAbs (10 μg/ml) resulted in partialy inhibition of LPS-induced NO production, indicating a CD14-independent pathway(s) is involved in NO production stimulated with a high dose of LPS.

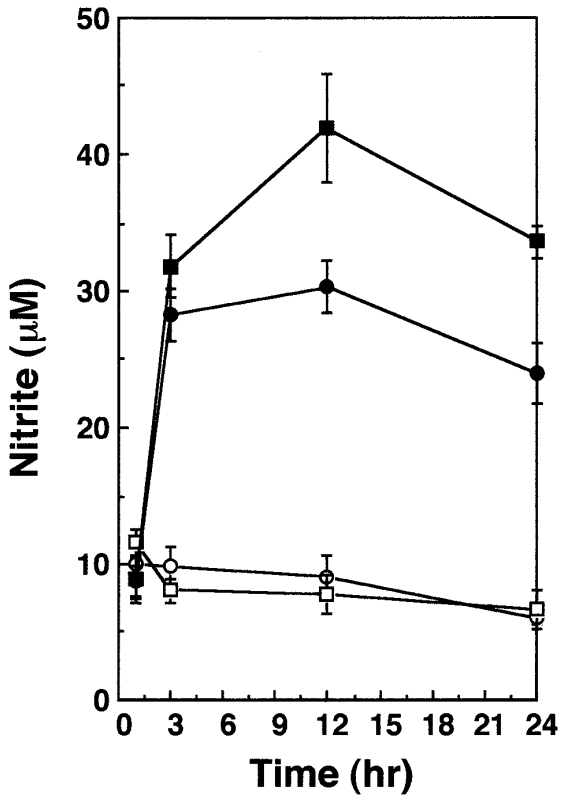

FIG. 1. Effects of FCS on NO production from macrophages. Macrophages were treated with 10 μg/ml of LPS for indicated times in the presence or absence of 10 % FCS, the medium was then changed to a FCS supplemented one and macrophages were further incubated for 48 hrs to elicit NO production. The values are means ± SD of triplicate cultures from three independent experiments. closed square; FCS+/LPS+, closed circle; FCS−/LPS+, open square; FCS−/LPS−, open circle; FCS−/LPS−.

NO production by anti-CR3 antibody. It is reported that there are many LPS receptors such as CD14, CR3, CD11c/CD18 (CR4) and scavenger receptors on macrophages [16-18]. Any of these receptors might contribute to NO production from macrophages stimulated with LPS. In this report, we investigated whether CR3 acts as a receptor for NO production in response to LPS stimulation. The binding of FITC-labeled anti-CR3 (α-chain) mAb to macrophages was inhibited by high doses of LPS (Fig. 3), indicating that LPS binds to CR3 on the surface of macrophages. We further treated macrophages with anti-CR3 mAb in the presence of FCS and absence of LPS stimulation, and measured the nitrite levels of the culture supernatant with Griess reagent to estimate NO production. Although nitrite lev-

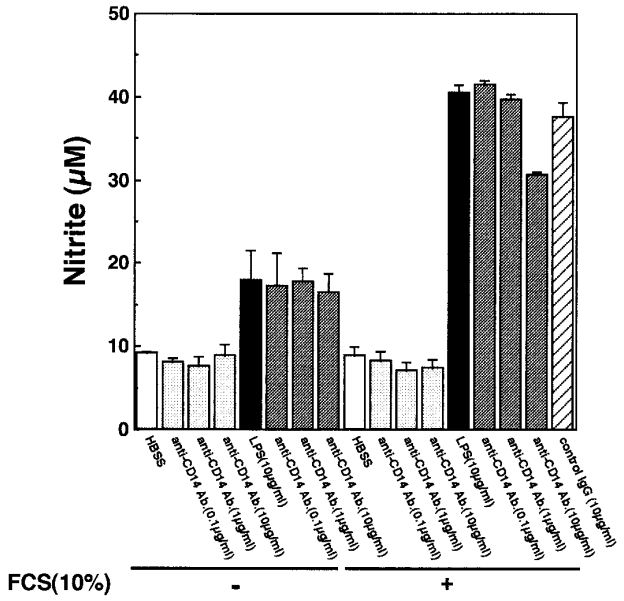

FIG. 2. Effects of anti-CD14 mAb on NO production from macrophages stimulated by LPS. Macrophages were treated with anti-CD14 mAb and LPS in the presence or absence of FCS for 48 hrs, and nitrite concentrations were measured by Griess reagent to evaluate NO production. The values are means ± SD of triplicate cultures from three independent experiments.

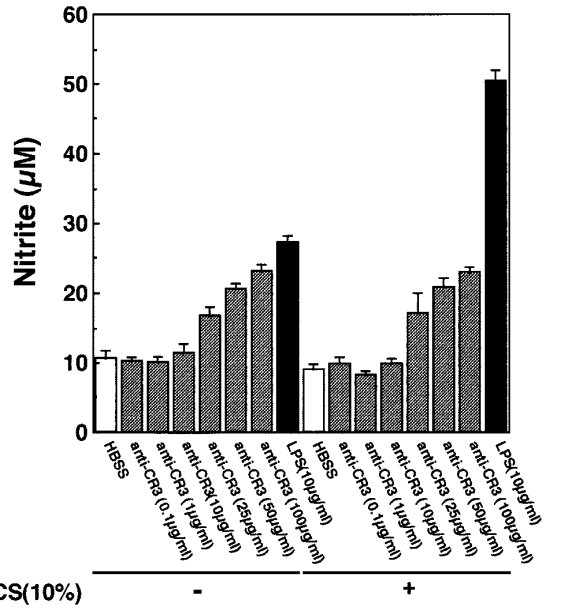

FIG. 4. Effects of anti-CR3 mAb on NO production from macrophages. Macrophages were incubated with anti-CR3 mAb for 48 hrs in the presence or absence of FCS, and nitrite concentrations were measured by Griess reagent to evaluate NO production. The values are means ± SD of triplicate cultures from three independent experiments.

els were lower than in LPS stimulated macrophages, the levels increased with the increase in anti-CR3 mAb concentrations (Fig. 4). In the absence of FCS, nitrite elevation also occurred with anti-CR3 mAb treatment. The nitrite level at 100 μg/ml of anti-CR3 mAb treatment was similar to levels induced by LPS stimulation (Fig. 4). To clarify the contribution of CR3 to NO production from macrophages by high dose of LPS, the effects of zymocel, which is β-glucan obtained from yeast and is a ligand for CR3, on NO production induced by anti-CR3 mAb and LPS. As shown in Fig. 5, NO productions induced by anti-CD3 mAb and LPS were inhibited dose-depenently by the addition of zymocel, and NO production induced by anti-CR3 mAb was reduced to the control levels at 25 μg/ml of zymocel. These findings suggest that CR3 participates in a NO production pathway in macrophages.

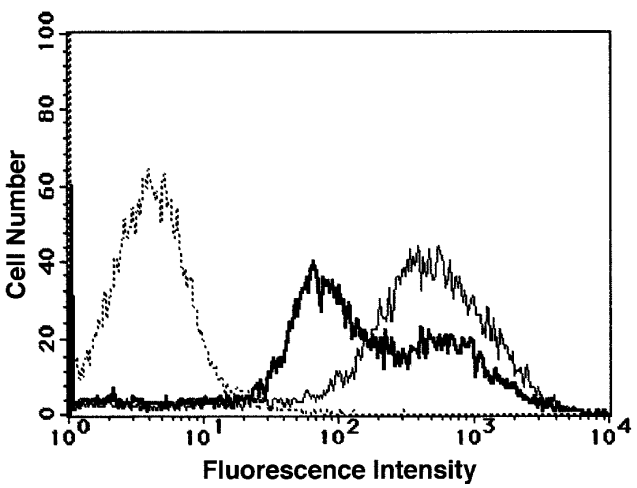

FIG. 3. Flow cytometry of FITC-labeled anti-CR3 mAb binding to macrophages. Macrophages were stained with FITC-labeled anti-CR3 mAb (1 μg/ml) in the presence or absence of LPS (100 μg/ml), and flow cytometry was conducted as described in Materials and Methods. dotted line; control serum staining, thin solid line; FITC-labeled anti CR3 mAb staining, thick solid line; FITC-labeled anti-CR3 mAb staining in the presence of LPS.

Anti-CR3 mAb used in this experiment is IgG2b, thus a contribution of Fcγ receptor (FcγR) to NO production was expected. NO production by control IgG was not observed as shown in Fig. 6, suggesting that FcγR participation in NO production by anti-CR3 mAb is eliminated.

Western blot analysis. The induction of iNOS following the anti-CR3 mAb treatment was examined by Western blot analysis. As shown in Fig. 7, a 130 kDa immunoreactive band was observed in the extract of both anti-CR3 mAb treated and LPS treated macrophages. Immunoreactive band intensity for anti-CR3 mAb treated macrophages was proportional to the concentrations of anti-CR3 antibody. These results indicate that the ligation of CR3 molecule on the surface of macrophages by anti-CR3 mAb induces iNOS.

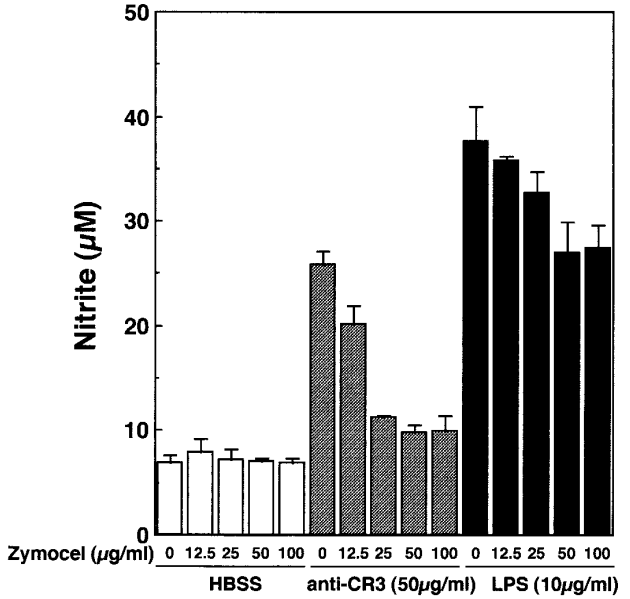

FIG. 5. Effects of zymocel on NO production from macrophages stimulated by anti-CR3 and LPS. Macrophages were incubated with anti-CR3 mAb or LPS for 48 hrs in the presence of zymocel, and nitrite concentrations were measured by Griess reagent to evaluate NO production. The values are means ± SD of triplicate cultures from three independent experiments.

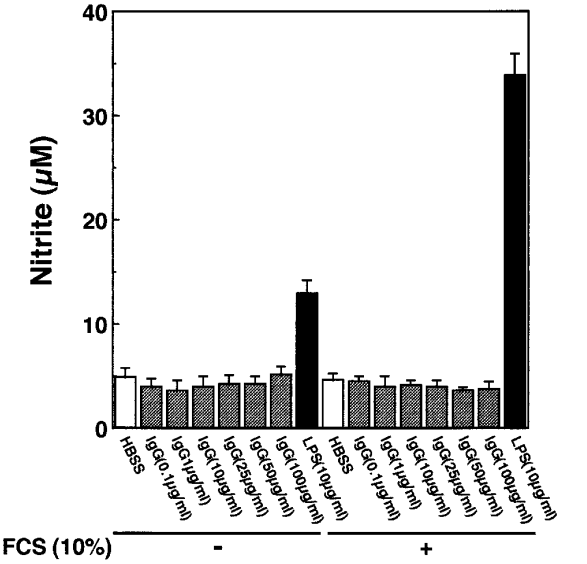

FIG. 6. Effects of IgG on NO production from macrophages. Macrophages were incubated with indicated concentrations of control IgG molecule for 48 hrs to clarify the contribution of FcγR on NO production. The values are means ± SD of triplicate cultures from three independent experiments.

DISCUSSION

It is well known that LPS triggers many pathophysiological events in mammalian cell. In macrophages the abundant secretions of interleukins such as IL-1, IL-6 and IL-8 are observed following LPS treatment [2-4]. In this trigger, CD14, a 55 kDa glycosylphosphatidylinositol-anchored protein expressed on the surface of macrophages, act as the major surface receptor for LPS. A complex formed with the serum protein, LPS-binding protein (LBP), is necessary for the binding of LPS to CD14 [5]. CD14 predominantly acts as a LPS receptor for macrophage activation at lower doses of LPS (<10 ng/ml), whereas a CD14-independent mechanisms participate in LPS-induced signaling and macrophage activation at higher doses of LPS (>10 ng/ml) [6-9]. In NO production from macrophages stimulated with LPS, CD14 is considered to be a primary binding site for LPS [10, 11]. We [19] and other researchers [20, 21] have reported that NO production from macrophages was also found at high doses of LPS. Consequently, a contribution of CD14-independent pathway(s) for NO production is expected. To investigate this possibility, the effect of FCS, which is a source of LBP supply being necessary for the binding of LPS to CD14, on NO production was investigated. As shown in Fig. 1, NO production was observed in macrophages treated with LPS in the absence of FCS. Further, NO production induced by a high dose of LPS could not be blocked completely by anti-CD14 mAb (Fig. 2). These results suggest that a CD14-independent pathway(s) contributes to NO production from macrophages stimulated with a high dose of LPS.

There are many LPS receptors on macrophages, such as CD14, CR3, CR4, and scavenger receptors [16-18]. Any of these receptors might contribute to NO production from LPS stimulated macrophages. CR3 is a $\beta 2$ integrin expressed on the surface of monocytes, macrophages, and neutrophils, and is involved in the binding and phagocytosis of bacteria [12, 13]. To investigate whether the CR3 molecule may act as a receptor for NO production from macrophages stimulated by LPS, we examined the effects of anti-CR3 (α-chain) mAb on NO production from macrophages. Binding of anti-CR3 mAb to macrophages was inhibited by high dose of LPS, but not complete (Fig. 3). This incomplete inhibition may come from following reason; macrophages express various kinds of LPS binding sites such as CD14, CR3, CR4, and scavenger receptor (16-18). Does-dependent induction of NO production was observed in macro-

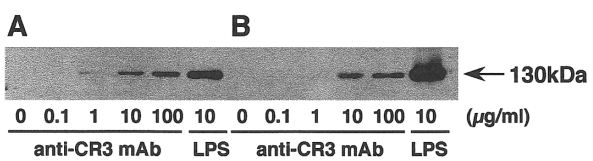

FIG. 7. Immunoblot analysis of iNOS. Macrophages were treated with indicated concentrations of anti-CR3 mAb or 10 μg/ml of LPS for 48 hrs in the absence (A) or presence (B) of FCS. Macrophage lysates were subjected to Western blot analysis as described in Materials and Methods.

phages treated with anti-CR3 mAbs, and the level of NO production increased was nearly equal in the presence and absence of FCS (Fig. 4). Further, Thornton et al. [22] reported that CR3 serves as β-glucan receptor through lectin sites of CD11b I-domain, thus the effect of zymocel, which is 1,3-β-glucan obtained from yeast and is a ligand for CR3, on NO productions from macrophages induced by anti-CR3 mAb and LPS was investigated to clarify the contribution of CR3 to NO production. As shown in Fig. 5, 25 μg/ml of zymocel showed complete inhibition of NO production induced by anti-CR3, and 40% of inhibition was observed in LPS induction. Finally, Western blot analysis suggest that the increase in NO production by anti-CR3 mAb was the result of the induction of iNOS expression (Fig. 7). These findings suggest that CR3 serves as a receptor for NO production from macrophages.

Recently, the role of CR3 for NO production was demonstrated. Pendio et al. [23] have reported that anti-CR3 (β-chain) antibody, but not anti-CR3 (α-chain) antibody, induced NO production in rat lung lavage cells. Goodrum et al. [24] have also reported that CR3 mediated the Gram-positive pathogens-induced signal for NO production in interferon-treated murine peritoneal macrophages and macrophage cell line J774.1. These findings, along with our own, suggest that CR3 molecule contributes to NO production.

Mozaffarian et al. [25] and Bayon et al. [26] recently reported that IgG immune complexes induced NO production in macrophages, indicating that FcγR can trigger NO production. Because the isotype of anti-CR3 mAbs used in this studies was IgG2b, the possibility that NO production was caused by anti-CR3 mAb may have been from the ligation of FcγR with IgG was considered. Thus, we examined the effects of IgG on NO production from thioglycollate-elicited mouse peritoneal macrophages, no NO production was observed (Fig. 6). This result negates the possibility that FcγR is connected with NO production from macrophages stimulated by anti-CR3 mAbs.

In conclusion, NO production was observed when thioglycollate-elicited macrophages were stimulated by a high dose of LPS in the absence of FCS, suggesting a CD14-independent signaling pathway for NO production is present in macrophages. Further, by flow cytometric analysis, we observed that the binding of anti-CR3 (α-chain) mAb to macrophages was inhibited by LPS. Finally, anti-CR3 (α-chain) mAbs induced NO production and expression of iNOS in a dose dependent manner. These results suggested that the CR3 acts as in a CD14-independent signaling pathway, and induces NO production when macrophages are stimulated with a high dose of LPS.

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