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Lipopolysaccharide-induced cell cycle arrest in macrophages occurs independently of nitric oxide synthase II induction

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Abstract

Lipopolysaccharide (LPS, a Gram-negative bacterium cell wall component) is a potent macrophage activator that inhibits macrophage proliferation and stimulates production of nitric oxide (NO) via NO synthase II (NOSII). We investigated whether NO mediates the LPS-stimulated cell cycle arrest in mouse bone marrow-derived macrophages (BMM). The addition of the NO donor DETA NONOate (200 μM) inhibited BMM proliferation by approx. 80%. However, despite NO being an antimitogen, LPS was as potent at inhibiting proliferation in BMM derived from NOSII−/− mice as from wild-type mice. Consistent with these findings, LPS-induced cell cycle arrest in normal BMM was not reversed by the addition of the NOSII inhibitor *S*-methylisothiourea. Moreover, in both normal and NOSII−/− BMM, LPS inhibited the expression of cyclin D1, a protein that is essential for proliferation in many cell types. Despite inhibiting proliferation DETA NONOate had no effect on cyclin D1 expression. Our data indicate that while both LPS and NO inhibit BMM proliferation, LPS inhibition of BMM proliferation can occur independently of NOSII induction.

Keywords: Lipopolysaccharide; Cell cycle; Nitric oxide; Macrophage

1. Introduction

Macrophages are key players in the immune response against foreign organisms. Upon encountering Gram-negative bacteria, macrophages detect lipopolysaccharide (LPS) in the bacterial cell wall, resulting in the activation of many signalling pathways, which in turn regulate the expression of genes directing the plethora of macrophage responses to bacterial challenge [1]. One such response is the production of large amounts of the toxic gas nitric oxide (NO) via the enzyme NO synthase II (iNOS or NOSII) [2]. In addition, LPS blocks macrophage proliferation [3,4]. It is possible that LPS-stimulated NO production and cell cycle arrest are linked, as NO is known to inhibit proliferation in a wide range of cell types (e.g. [5–7]). Such a link could be viewed as advantageous as it would ensure that replicating macrophage DNA would not be exposed to the mutagenic free radical NO and its derivatives such as peroxynitrite [8].

The role, if any, of NO in LPS-stimulated cell

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cycle arrest is undefined. Evidence for a link between LPS-stimulated NO production and cell cycle arrest includes data from our recent studies examining LPS responses in macrophages from mice with a null mutation in a chain of the type I IFN receptor (IFNAR1−/−). In response to LPS, macrophages from IFNAR1−/− mice were not only resistant to cell cycle arrest, but were also unable to make NO [9]. Others have shown that the addition of the NOS inhibitor *N*-methyl-L-arginine (L-NMA) increased proliferation in an LPS-treated macrophage cell line [10]. In addition, it has been reported that the ability of urea to reverse the antiproliferative effect of LPS on a macrophage cell line was linked to its ability to inhibit NOSII expression [11]. Consistent with these findings, exogenously added NO is reported to inhibit proliferation of a macrophage-like cell line [12].

The above reports appear to support the idea that NO mediates LPS-stimulated cell cycle arrest. However, in contrast, others have shown that the NOS inhibitors L-NAME and L-canavanine had no effect on LPS-induced cell cycle arrest in a macrophage cell line, suggesting that NO does not mediate the antimitogenic actions of LPS [13].

While it is difficult to determine the exact reason(s) for the conflicting conclusions presented in the literature, there are possible explanations. Other than our studies [9], all of the above studies employed cell lines. Cell lines can develop altered characteristics upon long term culture, and can show abnormal perturbations in cell cycle machinery [14]. Also, in the studies summarised above, different pharmacological inhibitors of NOS were used, and these agents were not highly specific for inhibition of NOSII.

The present study investigating the role of NO in LPS-stimulated cell cycle arrest used approaches that sought to overcome some of the potential problems associated with previous studies. Firstly, rather than a cell line, primary macrophages were used, namely, mouse bone marrow-derived macrophages (BMM). Secondly, BMM from NOSII−/− mice [15] were used, which to our knowledge is the first time that such a 'genetic' approach has been applied to this biological question. Lastly, the NOSII inhibitor *S*-methylisothiourea (SMT) was chosen as it is reported to be highly selective for NOSII and to be far more potent than L-NMA [16,17].

2. Materials and methods

2.1. Reagents

Reagents were obtained from the following sources: RPMI medium from ICN-Flow Laboratories (Sydney, Australia); fetal calf serum (FCS) from CSL (Parkville, Australia); L-cell-conditioned medium was used as a source of CSF-1, and was prepared as previously described [18]; recombinant human CSF-1 was a gift from Chiron (USA); LPS was purchased from Sigma (St. Louis, MO, USA) (cat. No. L-2630), and was derived from *Escherichia coli* serotype 0111:B4; DETA NONOate (DN) was from Cayman Chemicals (Ann Arbor, MI, USA), and was prepared and stored as recommend by the manufacturer; *S*-methylisothiourea was purchased from Sigma; tritiated thymidine was from Amersham UK (cat. No. TRA61).

2.2. Antibodies

Anti-iNOS rabbit polyclonal antibody was from Transduction Laboratories (Lexington, KY, USA) (cat. No. 32030). Anticyclin D1 mouse monoclonal antibody was from Santa Cruz (sc450); HRPO-conjugated secondary antibodies were from Silenus (Hawthorn, Australia).

2.3. Cells

Primary cultures of mouse BMM were generated as previously described [18,19], and grown in RPMI, 10% FCS, and 30% L-cell-conditioned medium (source of CSF-1). BMM were rendered quiescent by incubation for approx. 18 h in CSF-1-depleted medium. Cell culture incubator conditions were 5% CO_2, 37°C.

Standard BMM used in this study were derived from CBA-C57Bl6 or Balb/c mice. NOSII−/− BMM were derived from NOSII−/− mice [15], and the wild-type ('WT') or '+/+' BMM were derived from a matching strain.

In all experiments BMM were examined by light microscopy at the end of the incubation periods and there was no indication of cytotoxicity (e.g. non-adherent cells) following incubation with LPS or DN.

2.4. Nitric oxide assay

BMM in 24-well plates were stimulated in 500 μl incubation volumes as described in the text for approx. 24 h. Cell-conditioned medium was collected for analysis of nitrites using the Griess reagent as previously described [20]. Briefly, 100 μl of Griess reagent (equal volumes of 0.1% naphthylethylenediamine (dissolved in water) and 1% sulphanilamide (dissolved in 5% phosphoric acid)) were added to 100 μl of cell-conditioned medium, incubated for 5 min at room temperature and absorbance at 550 nm measured using a Labsystems Multiscan RC microplate reader. A standard curve was constructed using a nitrite solution serially diluted in cell culture medium.

2.5. Western blotting

Quiescent BMM in six-well dishes (approx. 1×10^6 cells/well) were treated as described in the text and harvested for Western blotting using standard procedures previously described [4]. Membranes were probed with primary antibodies (at 100–125 ng/ml), then appropriate HRPO-conjugated secondary antibody and bands detected by chemiluminescence (ECL, Amersham, UK).

2.6. DNA synthesis assay

BMM in 24-well plates were incubated with the indicated agents for approx. 24 h in the presence of 0.5 μCi/ml tritiated thymidine. Cells were harvested by gently tipping off the medium, adding 0.2 M NaOH (0.5 ml) and aspirating onto glass fibre filters using an Inotech cell harvester. Filters were dried and radioactivity on filter discs detected by scintillation counting (Packard TopCount).

3. Results

To determine whether NO inhibited mouse BMM proliferation, quiescent BMM were stimulated to proliferate with CSF-1 (5000 U/ml), in the presence or absence of the NO donor DN for approx. 24 h. The conditioned medium from the cells was collected for determination of nitrite concentration (a stable end product of NO breakdown), and the cells were assayed for DNA synthesis. While 10 μM DN had no effect on CSF-1-stimulated DNA synthesis, 200 μM DN inhibited DNA synthesis by 78% (Table 1). Unstimulated or CSF-1-stimulated BMM did not produce detectable levels of nitrite, while the addition of 10 μM DN and 200 μM DN resulted in the detection of 6 μM nitrite and 89 μM nitrite, respectively.

Having established that BMM were sensitive to NO-induced cell cycle arrest, we next examined the effect of the NOSII inhibitor SMT [16] on the ability of LPS to inhibit proliferation in BMM. Quiescent BMM were stimulated to proliferate with CSF-1 (5000 U/ml), in the presence or absence of the indicated concentrations of LPS and SMT for approx. 24 h, after which the medium from the cells was collected for determination of nitrite concentration, and the cells were assessed for DNA synthesis. LPS at 0.1 ng/ml inhibited proliferation by about 50%, while 1–100 ng/ml completely inhibited proliferation (Fig. 1). The addition of the NOSII inhibitor SMT (50 μM) had no effect on the ability of LPS to inhibit proliferation (Fig. 1). As expected, SMT reduced the levels of nitrite detected in medium from LPS-stimulated BMM (Table 2). These data suggest that NO does not mediate LPS-stimulated cell cycle arrest.

Experiments were performed using BMM derived from NOSII−/− mice [15] in order to confirm the data generated with the pharmacological agent SMT.

Table 1
Effect of DN on proliferation and medium nitrite concentration in normal BMM

Treatment	DNA synthesis (dpm)	Nitrite (μM)
Unstimulated	1 853 ± 423	0 ± 2
CSF-1	29 250 ± 2 211	0 ± 2
CSF-1+10 μM DN	30 752 ± 2 243	6 ± 1
CSF-1+200 μM DN	6 502 ± 1 701	89 ± 8

Cells were incubated with the indicated agents and DNA synthesis and medium nitrite concentration were measured after 24 h. Data are representative of three such experiments and are mean ± S.D. of quadruplicate determinations.

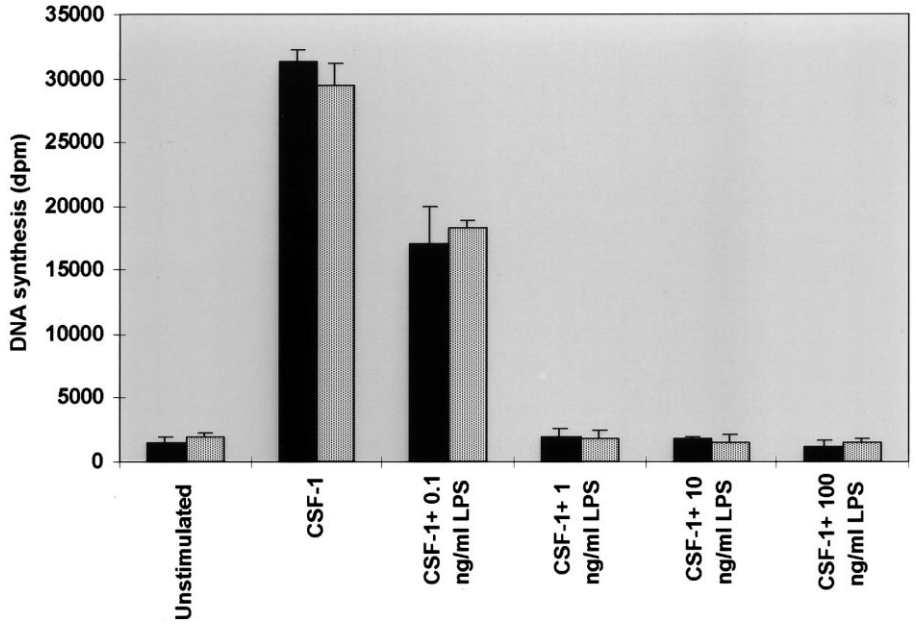

Fig. 1. The effect of SMT on LPS-stimulated cell cycle arrest. Quiescent BMM were left unstimulated, or stimulated with CSF-1 (5000 U/ml) and LPS (0.1–100 ng/ml) in the absence (black bars) or presence (grey bars) of SMT (50 μM), for approx. 24 h. Data show mean ± S.D. for quadruplicate determinations, and are representative of four similar experiments.

Quiescent BMM were stimulated to proliferate with CSF-1 (5000 U/ml), in the presence or absence of LPS or DN, as indicated. After 24 h, the medium from the cells was collected for determination of nitrite concentration, and the cells were assessed for DNA synthesis. LPS (1–100 ng/ml) inhibited proliferation in both +/+ and NOSII−/− BMM (Table 3). These data confirm that NO does not mediate the LPS-stimulated cell cycle arrest in BMM. The DN data in Table 3 also show that both +/+ and NOSII−/− BMM are sensitive to NO-induced cell cycle arrest.

Cyclin D1 is a protein critical for G_1 progression in many cell types [21]. To investigate why LPS was still able to block proliferation in NOSII−/− BMM the effect of LPS on cyclin D1 expression was determined. Quiescent BMM were stimulated with the indicated agents for approx. 8 h and cyclin D1 levels measured in lysates by Western blotting. The data in Fig. 2 show both +/+ and NOSII−/− BMM express cyclin D1 in response to 5000 U/ml CSF-1, and this expression is inhibited by 100 ng/ml LPS. In contrast, 100 μM DN was found to have no effect on CSF-1-stimulated cyclin D1 expression, even though it inhibited proliferation by about 50% (see Table 3). Other experiments using 200 μM DN, which led to approx. 80% inhibition of DNA synthesis (see Table 1), also showed there was no effect on CSF-1-stimulated cyclin D1 protein expression (data not shown).

4. Discussion

LPS, a potent macrophage activator, inhibits macrophage proliferation and stimulates production of NO. There are conflicting reports regarding the role of NO in LPS-stimulated cell cycle arrest. While some reports indicate LPS inhibits cell proliferation

Table 2
The effect of LPS and SMT on medium nitrite concentration (μM) in normal BMM

Treatment		plus SMT
Unstimulated	0 ± 2	0 ± 2
CSF-1	0 ± 2	0 ± 2
CSF-1+0.1 ng/ml LPS	0 ± 5	0 ± 2
CSF-1+1 ng/ml LPS	23 ± 3	3 ± 0
CSF-1+10 ng/ml LPS	35 ± 3	11 ± 1
CSF-1+100 ng/ml LPS	36 ± 4	17 ± 1

Cells were incubated with the indicated agents and medium nitrite concentration was measured after 24 h. Data are representative of three such experiments and are mean ± S.D. of quadruplicate determinations.

Table 3
Effect of LPS and DN on proliferation and nitrite production in +/+ and NOSII−/− BMM

Genotype		LPS (ng/ml)	DN (μM)	DNA synthesis (dpm)	Nitrite (μM)
+/+	Unstimulated	–	–	530 ± 101	4.0 ± 0.1
+/+	CSF-1	–	–	77 534 ± 7 030	4.1 ± 0.1
+/+	CSF-1+	1	–	436 ± 120	5.7 ± 0.7
+/+	CSF-1+	10	–	579 ± 231	16.1 ± 4.0
+/+	CSF-1+	100	–	330 ± 91	18.7 ± 3.1
+/+	CSF-1+	–	10	72 020 ± 11 091	8.1 ± 0.1
+/+	CSF-1+	–	100	35 101 ± 4 002	39.5 ± 3.9
−/−	Unstimulated	–	–	679 ± 222	4.4 ± 0
−/−	CSF-1	–	–	77 010 ± 7 211	4.0 ± 0
−/−	CSF-1+	1	–	551 ± 210	3.8 ± 0.1
−/−	CSF-1+	10	–	811 ± 501	3.8 ± 0.2
−/−	CSF-1+	100	–	532 ± 280	4.1 ± 0.3
−/−	CSF-1+	–	10	77 302 ± 7 101	9.1 ± 0.4
−/−	CSF-1+	–	100	34 020 ± 7 201	39.1 ± 8.2

Cells were incubated with the indicated agents and DNA synthesis and medium nitrite concentration were measured after 24 h. Data are representative of three experiments using two different primary BMM preparations. Data are mean ± S.D. of triplicate determinations.

by increasing NO production [9–11], others have shown this is not the case [13]. The aim of the current study was to further examine the role of NO in LPS-induced cell cycle arrest using both pharmacological and genetic approaches. The present work indicates that LPS inhibition of BMM proliferation is independent of NO production.

Although NO inhibits proliferation of a number of cell types (e.g. [5–7]) there are surprisingly few studies which have reported on the effect of NO donors on macrophage proliferation. One such study showed that NO donors inhibited proliferation of the macrophage-like Mm1 cells, arresting them in the G_2/M phase of the cell cycle [12]. In the present study we used 'quiescent' BMM, which are synchronised in G_0/early G_1 due to 18 h growth factor deprivation [4]. Upon stimulation with CSF-1, these cells re-enter the cell cycle and take around 24 h to complete a round of replication [4]. We found that addition of the NO donor DN inhibited the cells from entering S phase, indicating a G_1 phase arrest.

Having established that BMM were sensitive to NO-induced cell cycle arrest, we examined the role NO in LPS-stimulated cell cycle arrest. We found that co-addition of the NOSII inhibitor SMT significantly reduced LPS-stimulated NO production, as expected. However, SMT had no effect on LPS-stimulated cell cycle arrest. This was apparent even when the concentration of LPS used was submaximal in terms of inhibition of proliferation (0.1 ng/ml LPS, Fig. 1). Furthermore, we found that NOSII−/− BMM were as sensitive to LPS-stimulated cell cycle arrest as were normal BMM. These data indicate LPS inhibits BMM proliferation via NO-independent mechanisms.

It is noteworthy that 1 ng/ml LPS was still potently antimitogenic in the presence of SMT, even though the SMT reduced nitrite levels from 23 μM to 3 μM (Fig. 1 and Table 2). Therefore, LPS that generated 3 μM nitrite was more antimitogenic than 10 μM DN that generated 6 μM nitrite (Table 1). Thus, the NO level in response to an antimitogenic concentration of LPS remained below the threshold

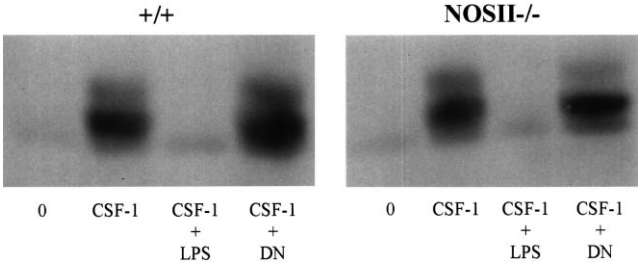

Fig. 2. The effect of LPS and DN on cyclin D1 expression in BMM. Quiescent BMM (0) from either wild-type (+/+) or NOSII−/− mice were stimulated with CSF-1 (5000 U/ml), LPS (100 ng/ml) or DN (100 μM), as indicated. Cells were harvested after 8 h for Western blotting and detection of cyclin D1 (approx. 36 kDa). These data are representative of three such experiments.

for NO-induced G_1 arrest, further indicating that NO does not play a role in LPS-stimulated cell cycle arrest.

D-type cyclins are growth factor sensors that are essential and rate limiting for G_1 progression in normal mammalian cells [21]. We have previously shown that following addition of CSF-1, cyclin D1 is the major D-type cyclin expressed in BMM [22]. We have also shown that LPS potently inhibits cyclin D1 expression in BMM [4,23]. In the present study we show that LPS also inhibits cyclin D1 expression in NOSII−/− BMM. This observation is likely to explain the NO-independent G_1 arrest by LPS in NOSII−/− BMM. Furthermore, DN did not inhibit CSF-1-stimulated cyclin D1 protein expression in BMM, indicating that NO inhibits BMM proliferation via mechanisms other than repression of D-type cyclin expression. This observation further separates antiproliferative mechanisms of NO and LPS, and is consistent with LPS inhibiting BMM proliferation through NO-independent pathways.

The present data appear to be the first reporting on the effect of NO on expression of a cell cycle protein in macrophages. They are similar to data generated using vascular smooth muscle cells which showed NO had no effect on cyclin D1 expression in [24], but that NO was linked to repression of cyclins A and E expression [24,25], inhibition of cyclin-dependent kinase 2 activity [24,26] and upregulation of expression of the cyclin-dependent kinase inhibitor p21 [24,27].

The present study indicates that LPS-induced cell cycle arrest is independent of NO production. This differs from the conclusions drawn by others (see Section 1). We are unsure as to the reasons for the apparently conflicting findings. The studies described here used primary cells, and to our knowledge are the first to use macrophages derived from NOSII−/− mice to explore this question. The design of our studies is therefore in contrast to others which used the macrophage-like cell line RAW264.7. It may be that this cell line has, or develops over time, altered LPS/NO responses compared to primary cells. Given that both NO and LPS alter expression of cell cycle proteins, it is pertinent to note that cell lines can show altered expression of cell cycle proteins [14], as can subcultured primary cells [28]. Thus, we contend the current findings are more likely to reflect the behaviour of macrophages in vivo.

The present work contributes to our understanding of mechanisms underlying macrophage-mediated host defence. Given both LPS and NO inhibit macrophage proliferation, it seemed reasonable to expect LPS-stimulated NO production and cell cycle arrest to be linked, especially since NO is a genotoxic agent and such a link would avoid exposing replicating DNA to NO. However, our data indicate this is not the case, and suggest that LPS invokes other mechanisms in order to inhibit proliferation. However, the present study may point toward an important backup mechanism in that if the primary NO-independent antiproliferative mechanisms of LPS somehow become disabled, high levels of NO will ultimately inhibit proliferation and prevent exposure of replicating DNA. That LPS and NO both block proliferation is probably also important in ensuring that the challenged macrophage directs its energy towards immediately producing compounds to fight invading bacteria, rather than towards cell division.

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