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Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors

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Summary

Macrophage function has been demonstrated to be subject to modulation by progesterone. However, as this steroid hormone can act through the glucocorticoid receptor as well as the progesterone receptor, the mechanism of action has not been precisely characterized. To determine the mode of action, we compared the ability of progesterone, norgestrel (a synthetic progesterone-receptor-specific agonist) and dexamethasone (a synthetic glucocorticoid receptor agonist) to modulate macrophage function following stimulation of the Toll-like receptor-4 (TLR-4) ligand lipopolysaccharide (LPS). The results demonstrate that following stimulation of TLR-4 with LPS and cotreatment with either progesterone or dexamethasone, but not norgestrel, there is a significant reduction in nitric oxide (NO) production, indicating that this progesterone-mediated effect is through ligation of the glucocorticoid receptor. In contrast, LPS-induced interleukin-12 (IL-12) production could be downregulated by all three steroids, indicating that ligation by progesterone of either the glucocorticoid or the progesterone receptors or both could mediate this effect. While progesterone downmodulated NO-mediated killing of *Leishmania donovani* by activated macrophages *in vitro*, most probably via the glucocorticoid receptor, it had little effect on *Toxoplasma gondii* growth in these cells. This would suggest that progesterone-mediated increased susceptibility to *T. gondii* during pregnancy is more likely to be related to the ability of the hormone to downregulate IL-12 production and a type-1 response utilizing the progesterone as well as the glucocorticoid receptors.

Keywords: *Leishmania donovani*; macrophage; nitric oxide; progesterone; Toll-like receptor; *Toxoplasma gondii*

Introduction

It is well documented that males and females can differ significantly in their response to infectious diseases as a consequence of sex hormone influences on immunity.[1–3] This sexual dimorphism is further emphasized during pregnancy when an increase in the concentration of not only estrogen but particularly progesterone is correlated with a shift in the maternal immune response to a T helper type 2 (Th2) phenotype characterized by increased levels of interleukin-4 (IL-4) and IL-5 along with the regulatory cytokines IL-10 and transforming growth factor-β (TGF-β).[4–8] This compromises the Th1 response required to resolve a number of intracellular parasitic infections.[9,10] This correlates with an increased severity of disease and deleterious effects on the outcome of pregnancy following infection with organisms such as *Toxoplasma gondii*, *Plasmodium chabaudi*, *Leishmania major* and *Leishmania*

Abbreviations: BMM, bone-marrow-derived macrophages; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte–monocyte colony-stimulating factor; IC_{50}, 50% inhibitory concentration; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M-CSF, monocyte colony-stimulating factor; NF, nuclear factor; NO, nitric oxide; PBS, phosphate-buffered saline; TGF, transforming growth factor; Th, T helper; TLR, TOLL-like receptor; TNF, tumour necrosis factor.

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donovani.[11–17] In addition, the existence of a Th2 cytokine milieu around the feto–maternal interface is likely to increase the likelihood of transplacental transmission. Consequently, the congenital route is a well described mode of transmission for *T. gondii* and there have also been reports of congenital *L. donovani* infections.[18–20]

The effects of progesterone in particular include inhibition of natural killer cell cytotoxicity via release of progesterone-induced blocking factor, increased production of IL-10 by dendritic cells, inhibition of Th1 development and induction of a Th2 response and increased antibody production.[21–25] There is also evidence to suggest that progesterone affects macrophage function. For example, it has been demonstrated that treatment of lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-stimulated RAW 264.7 macrophages with progesterone results in a dose-dependent decrease in nitric oxide (NO) production, which is the result of a reduction in inducible nitric oxide synthase (iNOS) gene promotor activity and iNOS gene expression.[26] It has also been shown that tumour necrosis factor-α (TNF-α) production by macrophages is inhibited by progesterone in a pretranscriptional manner, possibly through increased inhibitory-κB transcription which limits nuclear factor-κB (NF-κB) activity and thus decreases cytokine production.[27]

In particular, the influence of progesterone on macrophage function is likely to be of significance to the outcome of *T. gondii* and *L. donovani* infections as both parasitize these cells. Protection against both parasites is associated with macrophage IL-12 production driving a Th1 response to control parasite multiplication via macrophage activation and enhanced production of NO.[28,29] We therefore studied the ability of progesterone to modulate both afferent macrophage activity via measurement of IL-12 and efferent activity via measurement of NO production following TLR-4 ligation. Progesterone is known to signal via either progesterone or glucocorticoid receptors[30–32] so we compared the effects of progesterone with the effects of specific agonists of each of these receptors to identify the role of each receptor in its activity. Not only did we identify a dichotomy in the ability of the receptors to inhibit TLR-4-mediated NO and IL-12 production but also showed a difference in the ability of progesterone to inhibit macrophage killing of *L. donovani* and *T. gondii*.

Materials and methods

Cell culture

Bone marrow stem cells were removed from the bone marrow of 6- to 8-week-old male BALB/c mice bred in the University of Strathclyde under barrier conditions. These cells were cultured to produce bone-marrow-derived macrophages (BMMs) using medium enriched with monocyte colony-stimulating factor (M-CSF) and granulocyte–monocyte colony-stimulating factor (GM-CSF) obtained from supernatants of L929 cells (L-cell conditioned medium) grown to confluence. The BMMs were grown for 7 days in culture medium containing Dulbecco's modified Eagle's medium with sodium pyruvate, high glucose and pyridoxine hydrochloride (Gibco-BRL, Paisley, UK), 20% volume/volume (v/v) heat-inactivated fetal calf serum (HI-FCS; Harlan Sera-Lab, Loughborough, UK), 30% v/v L-cell conditioned supernatant, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at standard cell culture conditions of 37° and 5% CO_2. Cells were then harvested and counted using a haemocytometer before dilution to the appropriate concentration for experiments using complete medium (RPMI-1640; Gibco-BRL), 10% (v/v) HI-FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Maintenance of T. gondii

Tachyzoites of *T. gondii* RH strain were grown in the human foreskin fibroblast cell line in Iscoves's modified Dulbecco's medium (Invitrogen, Paisley, UK) containing 10% (v/v) HI-FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at standard conditions.

Maintenance of L. donovani

Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*; Harlan Olac, Bicester, UK) were used for the maintenance of the *L. donovani* strain 200016, which was clinically derived from a patient in India and collected under the regulations of the Bihar University Ethical Committee.[33] Promastigotes were maintained *in vitro* from spleen cell aspirates using TC-100 insect medium (Sigma, Poole, UK) containing 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin under standard conditions.

Cell culture treatments

For cell culture, 100 μl BMMs (2×10^6 cells/ml) was added to appropriate wells of 96-well microtitre tissue culture plates and incubated under standard conditions for 24 hr to allow adherence. The BMMs were then activated with 50 μl LPS (3·125 or 12·5 μg/ml; Sigma) from *Escherichia coli* 055:B5. Cells were simultaneously treated with 50 μl medium alone, medium containing solvent vehicle controls (either chloroform or ethanol accordingly) or medium containing hormones. Progesterone (Sigma) and norgestrel (Sigma) were initially dissolved in 100% chloroform (BDH Laboratory Supplies, Poole, UK) at a concentration of 50 mg/ml. This stock solution was further diluted with complete medium to give a 250-μM

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solution for experimental use. Dexamethasone (Sigma) was initially dissolved in 100% ethanol to give a 50-mM solution. This stock solution was further diluted with complete medium to give a 250-nM solution for experimental use. Progesterone and norgestrel were added in 50-μl volumes at 62·5, 31·3, 15·7 and 7·8 μM. Dexamethasone was added in 50-μl volumes at 62·5, 31·3, 15·7 and 7·8 nM. For experiments without parasites, cultures ($n = 3$ per treatment) were incubated under standard conditions for 72 hr, then supernatants were collected and stored at − 20° for subsequent determination of IL-12 or nitrite.

Cell culture infection with T. gondii

The BMMs (100 μl; 2×10^6/ml) in complete medium were added to appropriate wells of a 96-well tissue culture plate and incubated for 24 hr at standard conditions to allow adherence. The medium was then removed and cells were treated with 50 μl LPS (3·125 μg/ml) from *E. coli* 055:B5, with medium alone in the presence of progesterone at 4, 15·7 and 62·5 μM or with medium alone before being infected with *T. gondii* RH tachyzoites at a ratio of one parasite per host cell and incubated under standard conditions for 48 hr. After this incubation, 25 μl of ^{3}H-labelled uracil (1 μCi) was added to the appropriate wells and the cells were incubated at standard conditions for a further 24 hr. Cells from each well were harvested onto Titertek paper discs and placed into 5 ml beta vials (Hughes and Hughes Ltd., Romford, UK) containing 1 ml scintillation fluid (Fisher Scientific, Loughborough, UK). Beta emissions were analysed as counts per minute using a Beckman LS6500 multipurpose scintillation counter (Beckman Coulter, High Wycombe, UK).

Cell culture infection with L. donovani

One hundred microlitres of BMMs (1×10^6/ml) in complete medium were added to each well of a 24-well tissue culture plate (Techno Plastic Products, Trasadingen, Switzerland) containing round 13-mm cover slips. Cells were infected by adding 100 μl *L. donovani* promastigotes (strain 200016) at a parasite : host cell ratio of 1 : 20 and then incubating the cells under standard conditions for 24 hr. The medium was then changed to remove unattached parasites and replenished with 100 μl fresh medium or medium containing IFN-γ and LPS [100 U IFN-γ/ml (Pharmingen, San Diego, CA), 100 ng/ml LPS from *Salmonella minnesota* (Sigma)] and 100 μl medium alone or medium containing progesterone at 4, 15·7 and 62·5 μM ($n = 4$). Cells were fixed in methanol (Banford Laboratories, Norden Rochdale, UK) and stained with 10% (v/v) aqueous Giemsa stain (BDH Laboratory Supplies) so that the percentage of cells infected could be determined by microscopy.

Griess assay

After cell culture treatments, supernatants were aspirated and analysed for NO production using the Griess reaction assay that detects the concentration of nitrite, a stable end product of NO reaction with oxygen.[34] Supernatants (50 μl) were incubated with 50 μl 2% w/v sulphanilamide (Sigma) in 5% v/v phosphoric acid (Sigma) plus 50 μl 0·2% w/v naphthylethylenediamine (Sigma) in distilled water for 5–10 min at room temperature. The absorbance at 540 nm was determined using a SPECTRAmax 190 microtitre spectrophotometer using SOFTMAX PRO 3·0 software (Molecular Devices, Hove, UK). Potassium nitrite (Sigma) dilutions were used as standards.

IL-10 assay

The concentration of IL-10 present in cell culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA).[35] Plates were coated with anti-mouse IL-10 made from immunoglobulin G1 (IgG1) clone JES5-2A5 (Pharmingen) at 2 μg/ml diluted in phosphate-buffered saline (PBS) pH 9·0 at a volume of 50 μl/well and incubated overnight at 4°. Plates were washed three times in wash buffer (0·05% Tween-20 in PBS pH 7·4). Blocking buffer [PBS pH 7·0, 10% (v/v) fetal calf serum] was then added to each well at a volume of 200 μl and the plate was incubated for 1 hr at 37°. After incubation, the plate was washed three times as before. Samples were then added to the plate in a 50-μl volume along with recombinant murine IL-10 (Pharmingen) titrated in doubling dilutions from 20 ng/ml to 387·5 pg/ml in 50-μl volumes and incubated for 2 hr at 37°. Plates were then washed and biotin-labelled rat anti-mouse anti-IL-10 (IgM clone SXC-1; Pharmingen) was added at 1 μg/ml in a 50-μl volume and incubated at 37° for 1 hr before addition of the conjugate. The plate was then washed before addition of the conjugate which was streptavidin–alkaline phosphatase (AKP; Pharmingen) added at a volume of 100 μl at a 1 : 2000 dilution in blocking buffer that was then incubated for 45 min at 37°. Following a wash, the ELISA was completed by addition of *p*-nitrophenyl phosphate (Sigma) in glycine buffer at 1 mg/ml in 100-μl volumes. Absorbances were measured at 450 nm using a SPECTRAmax 190 microtitre plate spectrophotometer and SOFTMAX PRO 3.0 software. The detection limit of the assay was 156 pg/ml. Variation between replicate samples was low and all standard curves yielded regression coefficients of $R > 0{\cdot}99$.

IL-12 assay

The concentration of IL-12 present in cell culture supernatants was measured by ELISA in the same manner as that described for IL-10 enumeration with the following

changes. Capture anti IL-12p40 monoclonal antibody was rat anti-mouse (IgG1, clone C15.6 expressed by CHO cells) used at a concentration of 2 μg/ml. For standard curve production, recombinant murine IL-12p40 (Pharmingen) was titrated at the same concentrations as recombinant IL-10. The secondary antibody was biotinylated rat anti-mouse IL-12 (IgG2a, clone C17.8 expressed from CHO cells) of the p40 and p70 subunits, used at 1 μg/ml. The same conjugate was used as that for IL-10 but with a shorter incubation time of 30 min. The detection limit of this assay was 156 pg/ml. Variation between replicate samples was low and all standard curves yielded regression coefficients of $R > 0{\cdot}99$.

Statistical analysis

All sets of data were analysed using a Mann–Whitney *U*-test with a value of $P < 0{\cdot}05$ taken as significant.

Results

Progesterone utilizes the glucocorticoid receptor but not the progesterone receptor to inhibit LPS-induced nitrite production by BMMs

The BMMs were tested for their ability to produce NO by determining supernatant nitrite levels following stimulation with 0·012–25 μg/ml LPS. This allowed an appropriate concentration to be chosen for subsequent hormone experiments (data not shown); 3·125 μg/ml LPS was selected as the most suitable concentration for immune studies with hormones because it allowed both potentiation as well as inhibition of nitrite production to be assessed. The BMMs were stimulated with LPS and treated with progesterone, norgestrel or dexamethasone for 72 hr to determine whether the steroid or steroid receptor agonists modulated LPS-induced nitrite levels. Progesterone induced a dose-dependent reduction in nitrite production by BMMs with maximal inhibition occurring at 62·5 μM (Fig. 1a, $P < 0{\cdot}05$). Progesterone significantly inhibited nitrite production at all concentrations tested and had an 50% inhibitory concentration (IC_{50}) of 31·25–62·5 μM (Fig. 1a, $P < 0{\cdot}05$). Norgestrel had no significant effect on levels of BMM nitrite production at any concentration (Fig. 1b, $P < 0{\cdot}05$). Dexamethasone caused significant downmodulation of nitrite production at all concentrations tested (Fig. 1c, $P < 0{\cdot}05$) and had an IC_{50} of 7·8–15·7 nM.

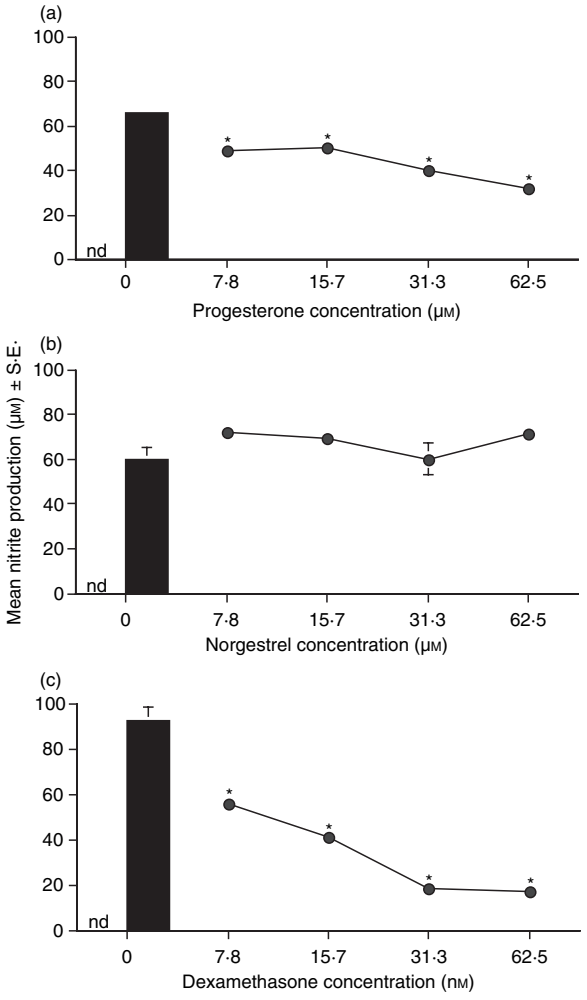

Figure 1. The influence of progesterone, norgestrel and dexamethasone on nitric oxide (NO) production by bone-marrow-derived macrophages (BMMs) stimulated with 3·125 μg/ml lipopolysaccharide (LPS) for 72 hr. Cells were cultured with LPS in the presence of progesterone (a), norgestrel (b) or dexamethasone (c). Unstimulated BMMs failed to produce detectable levels of NO (nd). Unstimulated BMMs treated with the solvent vehicle of 0·04% chloroform (progesterone and norgestrel) or 0·0001% ethanol (dexamethasone) failed to produce detectable levels of NO (data not shown). Stimulated control cell cultures were treated with 3·125 μg/ml LPS (closed bars). Stimulated control cell cultures treated with 3·125 μg/ml LPS and either 0·04% chloroform or 0·0001% ethanol produced levels of NO which were comparable to those produced by stimulated cells treated with LPS alone (data not shown). Results are the mean of $n = 3$ treatments from one of three representative experiments. Where SEs are not evident it is because they are too small to be visible. $^*P < 0{\cdot}05$ compared with stimulated controls using a Mann–Whitney *U*-test.

Progesterone utilizes both the glucocorticoid and the progesterone receptor to inhibit LPS-induced IL-12 production by BMMs

The BMMs were tested for their ability to produce IL-12 following stimulation with 0·012–25 μg/ml LPS (data not shown). A significantly higher concentration of LPS (12·5 μg/ml) than that used to induce nitrite production was required to measure hormonal influences on IL-12 production by BMMs.

Initial experiments showed that at concentrations between 7·8 and 62·5 μM progesterone consistently

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reduced BMM IL-12p40 production below background levels and therefore additional lower concentrations (0·006–8 μM) were included in the study of hormone-mediated effects on BMM IL-12p40 production so as to observe dose–response effects. Progesterone inhibited BMM IL-12p40 production at concentrations above 0·1 μM (Fig. 2a, $P < 0·05$) and had an IC_{50} of 0·1–0·2 μM. Norgestrel significantly reduced BMM IL-12p40 production at concentrations above 1 μM (Fig. 2b, $P < 0·05$) and had an IC_{50} of 1–2 μM. Dexamethasone also caused significant downmodulation of BMM IL-12p40 production above 0·025 nM, although this narrowly avoided reaching significance at 0·2 nM (Fig. 2c, $P < 0·05$) and had an IC_{50} of 2–4 nM. Therefore, all three treatments significantly reduced BMM IL-12p40 production. Values of IC_{50} for reduction in BMM IL-12p40 production by the three hormones revealed that dexamethasone exhibited the highest inhibitory action followed by progesterone then norgestrel.

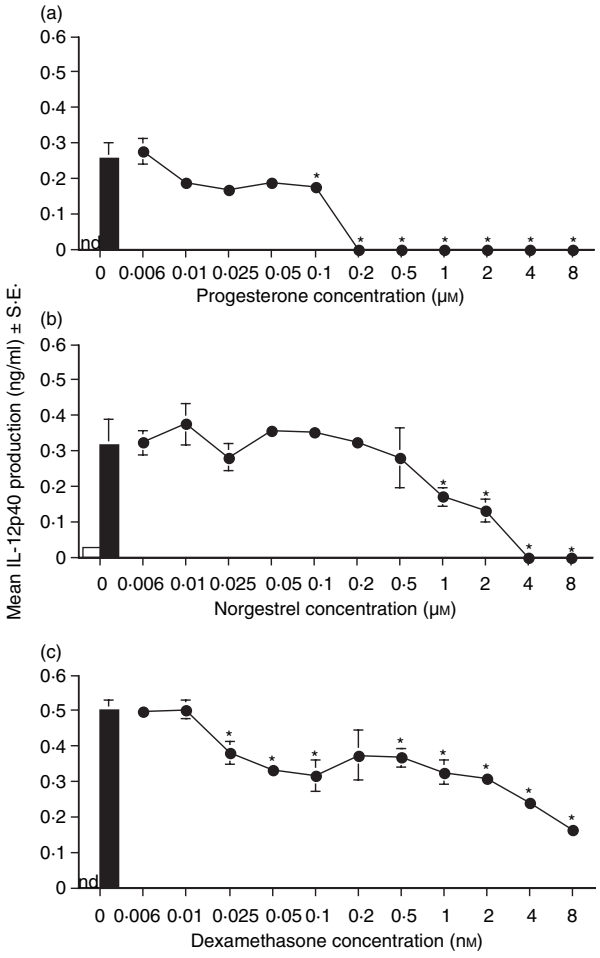

Figure 2. The influence of progesterone, norgestrel and dexamethasone on interleukin-12 p40 (IL-12p40) production by bone-marrow-derived macrophages (BMMs) stimulated with 12·5 μg/ml lipopolysaccharide (LPS) for 72 hr. Cells were cultured with LPS in the presence of progesterone (a), norgestrel (b) or dexamethasone (c). Unstimulated cells produced undetectable (nd) or very low levels of IL-12p40. Unstimulated cells treated with the solvent vehicle of 0·04% chloroform (progesterone and norgestrel) or 0·0001% ethanol (dexamethasone) failed to produce detectable levels of IL-12p40 (data not shown). Stimulated control cell cultures were treated with 12·5 μg/ml LPS (closed bars). Stimulated control cell cultures treated with 12·5 μg/ml LPS and either 0·04% chloroform or 0·0001% ethanol produced levels of IL-12p40 which were comparable to those produced by stimulated cells treated with LPS alone (data not shown). Results are the mean of $n = 3$ treatments from one of three representative experiments. Where SEs are not evident it is because they are too small to be visible. *$P < 0·05$ compared with stimulated controls using a Mann–Whitney *U*-test.

Downregulation of NO and IL-12 by progesterone, norgestrel and dexamethasone is not caused by non-specific effects on cell viability or potentiation of IL-10 production from BMMs

To investigate whether hormones were inhibiting BMM production of the proinflammatory mediators NO and IL-12 indirectly via effects on cell viability, LPS and hormone-treated BMMs were assayed for mitochondrial activity using the alamarBlue™ assay (Biosource, Nivelles, Belgium). Progesterone, norgestrel and dexamethasone treatment of LPS-stimulated BMMs had no effect on cell viability or proliferation compared with BMM cultures stimulated with LPS alone (Fig. 3).

To investigate whether progesterone was inhibiting LPS-induced BMM production of the proinflammatory mediators NO and IL-12 indirectly via production of the anti-inflammatory cytokine IL-10, levels were measured in the supernatants from hormone-treated macrophage cultures. Neither progesterone nor norgestrel had any significant effect on IL-10 production by LPS-stimulated BMMs (Fig. 4a,b). Dexamethasone treatment of LPS-stimulated BMMs actually caused a small but significant reduction in IL-10 at 62·5 nM concentrations in comparison to BMM cultures treated with LPS alone ($P < 0·05$, Fig. 4c). Therefore, the hormones are not inhibiting LPS-stimulated BMM IL-12 and NO production by an IL-10-dependent indirect mechanism.

Progesterone has no effect on LPS-induced killing of *T. gondii* by infected BMMs but reduces LPS/IFN-γ-induced killing of *L. donovani*

Production of nitric oxide by macrophages has been reported as an important killing mechanism during infection with *T. gondii* and *L. donovani*.[36–38] Therefore, the ability of progesterone to downmodulate nitrite production by LPS-stimulated BMMs in our studies would suggest that the hormone would aid parasite survival in host cells. However, progesterone has previously been reported to have no effect on *T. gondii* killing in a

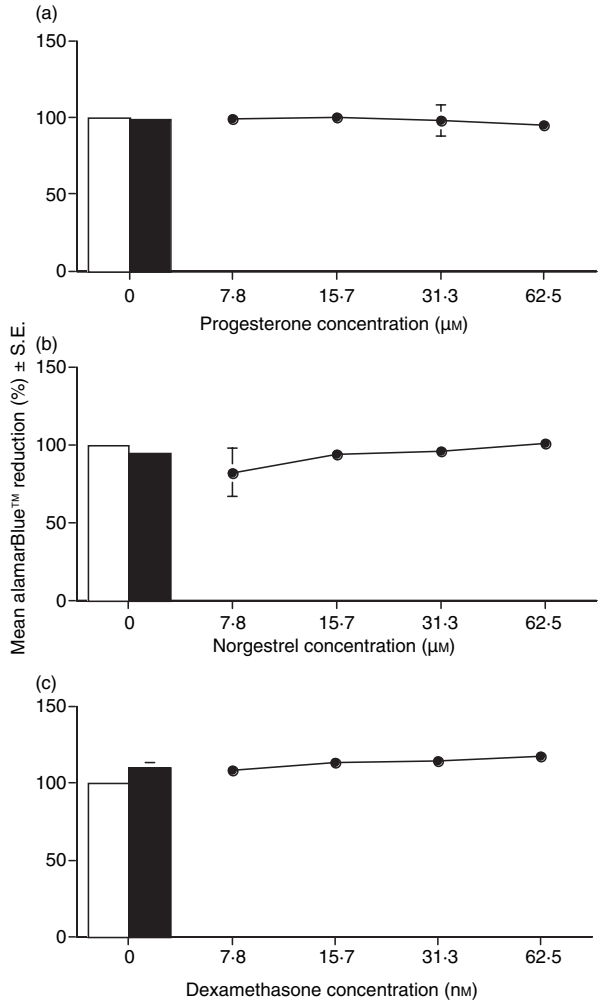

Figure 3. The influence of progesterone, norgestrel and dexamethasone on cell viability and proliferation of bone-marrow-derived macrophages (BMMs) stimulated with 12·5 μg/ml lipopolysaccharide (LPS) for 72 hr. Cells were cultured in the presence or absence of progesterone (a), norgestrel (b) and dexamethasone (c). The alamarBlue™ was added in 20-μl volumes after 48 hr. Unstimulated BMMs were treated with medium alone (open bars). Unstimulated BMMs treated with the solvent vehicle of 0·04% chloroform (progesterone and norgestrel) or 0·0001% ethanol (dexamethasone) reduced alamarBlue™ to the same extent as unstimulated controls receiving medium alone (data not shown). Stimulated control cells were treated with 12·5 μg/ml LPS (closed bars). Stimulated controls treated with 12·5 μg/ml LPS and either 0·04% chloroform or 0·0001% ethanol reduced alamarBlue™ to the same extent as stimulated cells receiving LPS alone (not shown). Where SEs are not evident it is because they are too small to be visible. Results are the mean ± SE of $n = 3$ treatments from two representative experiments.

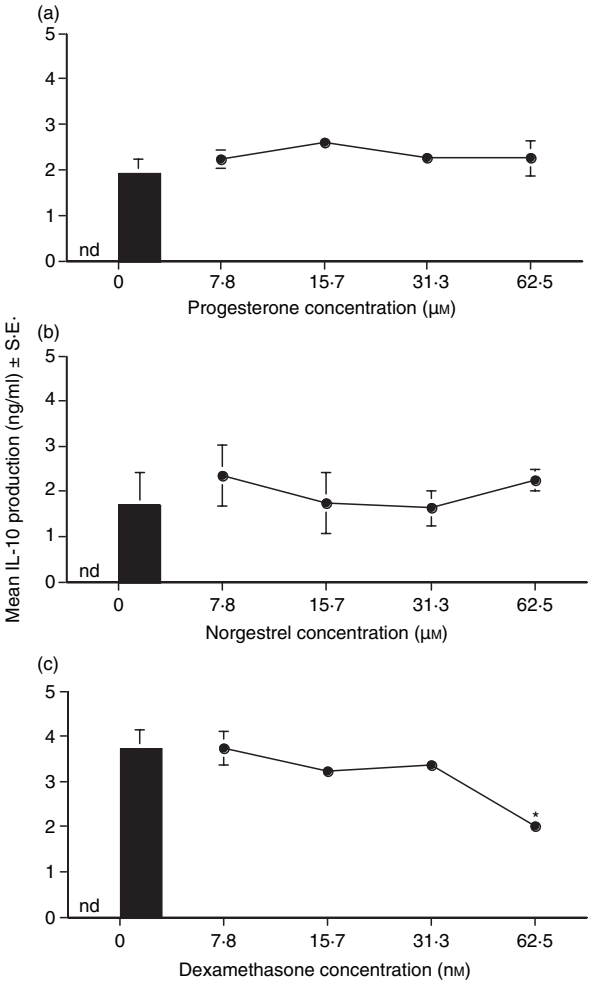

Figure 4. The influence of progesterone, norgestrel and dexamethasone on interleukin-10 (IL-10) production by bone-marrow-derived macrophages (BMMs) stimulated with 12·5 μg/ml lipopolysaccharide (LPS) for 72 hr. Cells were cultured with LPS in the presence of progesterone (a), norgestrel (b) or dexamethasone (c). Unstimulated BMMs treated with medium alone did not produce IL-10 (nd). Unstimulated BMMs treated with the solvent vehicle of 0·04% chloroform (progesterone and norgestrel) or 0·0001% ethanol (dexamethasone) did not induce detectable levels of IL-10 (data not shown). Stimulated control cell cultures were treated with 12·5 μg/ml LPS (closed bars). Stimulated control cell cultures treated with 12·5 μg/ml LPS and either 0·04% chloroform or 0·0001% ethanol produced levels of IL-10 which were comparable to those produced by stimulated cells treated with LPS alone (data not shown). Results are the mean of $n = 3$ treatments from two representative experiments. Where SEs are not evident it is because they are too small to be visible. $^*P < 0·05$ compared with stimulated controls using a Mann–Whitney *U*-test.

RAW 264.7 cell model regardless of its ability to down-regulate nitrite production by this cell line.[39] We aimed to further clarify this apparent anomaly in a primary macrophage cell line (BMM). Additionally, we investigated whether the ability of progesterone to inhibit NO production would influence the outcome of *L. donovani* infection of BMMs. In the latter study, BMMs were stimulated with IFN-γ and LPS because LPS alone was insufficient for the induction of parasite killing at the concentrations tested.

Activation of *T. gondii* tachyzoite-infected macrophages with 3·125 μg/ml LPS significantly reduced the number of infected cells compared with non-activated control cultures as measured by uptake of ^{3}H-labelled uracil (Fig. 5a, $P < 0{\cdot}05$). However, progesterone had no adverse effect on the ability of LPS-stimulated BMMs to limit parasite proliferation (Fig. 5a). Stimulation of *L. donovani* promastigote-infected macrophages with 100 U/ml IFN-γ and 100 ng/ml LPS significantly reduced the number of infected cells compared with non-activated control cultures (Fig. 5b, $P < 0{\cdot}03$). Cotreatment of stimulated BMMs with the highest dose of progesterone (62·5 μM) resulted in a significant reduction in parasite killing compared with activated control cultures (Fig. 5b, $P < 0{\cdot}03$).

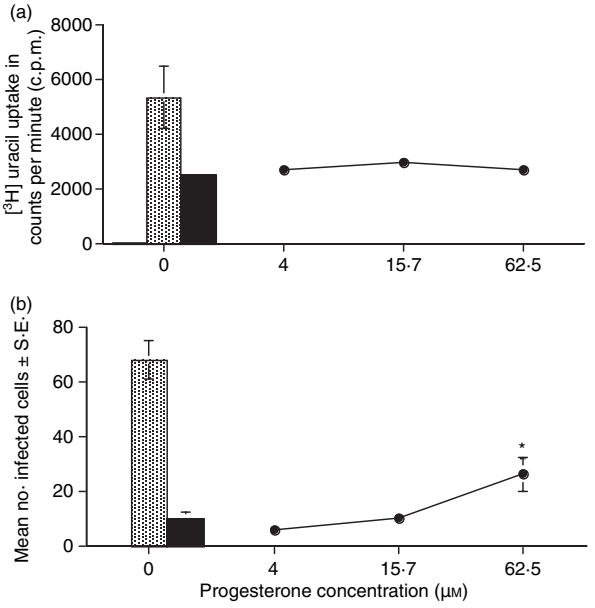

Figure 5. The effect of progesterone on the ability of activated bone-marrow-derived macrophages (BMMs) to kill *Toxoplasma gondii* and *Leishmania donovani*. The BMMs were infected with *T. gondii* tachyzoites of the RH strain at a parasite : host cell ratio of 1 : 1 and treated with 3·125 μg/ml lipopolysaccharide (LPS) in the presence or absence of progesterone (62·5, 15·7 or 4 μM) for 72 hr. Tachyzoite proliferation was measured by uptake of ^{3}H-labelled uracil added at 1 μCi after 48 hr (a). BMMs treated with medium alone = open bars; infected BMMs = shaded bars; infected cells stimulated with LPS = closed bars. Beta emissions are shown as mean counts per minute ± SE. Results are the mean of $n = 3$ treatments and are representative of three separate experiments. BMMs infected with *L. donovani* promastigotes at a parasite : host cell ratio of 1 : 20, were treated 24 hr postinfection with 100 U interferon-γ (IFN-γ) and 100 ng/ml LPS in the presence or absence of progesterone (62·5, 15·7 or 4 μM) for a further 72 hr. The mean infection (% ± SE) of cells is shown as assessed by Giemsa staining and microscopical examination (b). Uninfected BMMs = open bars; infected BMMs = shaded bars; infected cells stimulated with IFN-γ/LPS = closed bars. Results are the mean of $n = 4$ samples from three separate treatments. Where SEs are not evident it is because they are too small to be visible. *$P < 0{\cdot}03$ compared with IFN-γ/LPS activated controls.

Discussion

During the course of this study, the effect of progesterone on BMM activation via ligation of TLR-4 was investigated. Progesterone was found to downregulate the production of both IL-12, the major driver of a Th1 response, and NO, which plays a major role in the microbicidal activity of macrophages against intracellular parasites. The ability of progesterone to downmodulate IL-12 production by macrophages is to our knowledge a novel observation whereas the data on NO production confirmed work by Miller *et al.*,[26] who demonstrated that progesterone treatment caused a decrease in NO production as well as a reduction in the amount of iNOS messenger RNA transcripts.

Progesterone can function by binding to either the glucocorticoid receptor or the progesterone receptor but the progestin norgestrel exerts its action through the progesterone receptor alone.[30–32] Although the existence of a classical progesterone receptor in the macrophage is controversial, a few studies have described the existence of this receptor in human and mouse macrophages.[40,41] By studying the effects of progesterone, norgestrel and the glucocorticoid dexamethasone, on LPS-induced production of NO and IL-12, we aimed to discover which receptor progesterone was using to induce its immunomodulatory activity. The data in this study clearly demonstrated that the synthetic progestin had no effect on NO production following LPS stimulation. Therefore, progesterone downregulates LPS-induced NO production by binding to the glucocorticoid receptor and not the progesterone receptor. The glucocorticoid receptor ligand dexamethasone also significantly reduced LPS-induced BMM production of NO but was active at 100-fold lower concentrations than progesterone. It has been reported that dexamethasone can inhibit iNOS expression in LPS-treated murine macrophages by destabilizing the messenger RNA transcript.[42] The glucocorticoid receptor has also been demonstrated to physically interact with NF-κB, which has a role in induction of iNOS, therefore providing another possible means of inhibiting NO production.[43] A number of studies have highlighted similar activities for progesterone and glucocorticoid-mediated effects probably as a result of both signalling through the glucocorticoid receptor.[30,44] In addition, Miyaura and Iwata[23] reported that progesterone and the glucocorticoids corticosterone and dexamethasone, all inhibited thymocyte differentiation into IFN-γ-producing T cells. In contrast, Wan and Nordeen[44] describe genes that are differentially regulated by glucocorticoids and progesterone, regardless of their affinity for the same receptor, suggesting that signalling through the progesterone receptor is responsible for some of the latter hormone's immunoregulatory effects.

We have also demonstrated that IL-12 production by BMMs following LPS stimulation is inhibited by progesterone, norgestrel and dexamethasone. There were no significant differences in reduction of alamarBlue™ by hormone-treated BMMs compared with cells stimulated with LPS alone. Therefore, the inhibition of BMM NO and IL-12 production by progesterone, dexamethasone and norgestrel is the result of specific downregulatory effects on signalling pathways and not a non-specific effect on cell viability or proliferation. In addition, neither of the hormones had a stimulatory effect on IL-10 production by BMMs. This result is in contrast to a number of studies which demonstrate increased IL-10 production in dendritic cells generated in the presence of or pretreated with the hormone.[45–47] Conversely, it has been reported that dexamethasone can downregulate IL-10 production in human peripheral blood mononuclear cells and in the D10.G4.TH2 murine cell line when added at the same time as a stimulant such as LPS in a similar manner to our study.[48–50] Cell type and timing of dexamethasone treatment could therefore determine whether there is an inhibitory or stimulatory effect on IL-10 propagation following dexamethasone treatment. The lack of IL-10 induction by any of the hormones tested in our study shows that our observed reductions in NO and IL-12 were not the result of an indirect effect on IL-10 production. Consequently, progesterone signalling by either the glucocorticoid receptor or the progesterone receptor can regulate IL-12 production. Although the progesterone receptor has been demonstrated to physically interact with NF-κB it is not clear as to whether it has antagonistic properties similar to the glucocorticoid receptor.[51,52] It would seem unlikely that the progesterone receptor is inhibiting IL-12 production in this manner because it would then be expected to induce downregulation of iNOS. However, it cannot be ruled out that these two steroid hormones are differentially regulating IL-12 at the promoter level.[43,44,51,52] Alternatively, divergences upstream of NF-κB in the individual signalling pathways leading to synthesis of IL-12 and iNOS, for example differential usage of mitogen-activated protein kinases, are potential targets for the specific inhibition of IL-12 production by norgestrel following LPS stimulation.[53–55]

Macrophages are a vital component of the innate immune response and are known to control the growth of a number of intracellular parasites by utilizing a variety of cell killing mechanisms. In particular, NO has an essential role in protection against *L. donovani*. During *L. donovani* infection, NO production is required during the initial response as well as being essential in disease resolution.[37,56] The role of NO in control of *T. gondii* is somewhat controversial in comparison. Although macrophages from iNOS$^{-/-}$ mice infected with *T. gondii* demonstrate a reduced ability to kill parasites, these mice survive acute infection.[38] However, these mice also show an increased susceptibility to *T. gondii* during the chronic phase of infection that is related to a particular inability of iNOS$^{-/-}$ mice to control parasite multiplication in the brain resulting in severe necrotizing encephalitis.[36,38] As a result of the ability of progesterone to downmodulate BMM nitrite production, as described in this study, it was hypothesized that this would have a significant effect on parasite replication in BMMs. Although progesterone concentrations used in this study were higher than physiological serum levels of the hormone, they were similar to local placental levels.[57–59] Progesterone was shown in the course of this study to inhibit both NO production and killing of *L. donovani* parasites by IFN-γ- and LPS-stimulated BMMs. Consequently, progesterone-sensitive mechanisms, including NO reduction, are playing a role in controlling replication of this parasite. In contrast, killing of *T. gondii* parasites by LPS-stimulated BMM remained unaffected in cultures treated with progesterone. This is in agreement with studies by Gay-Andrieu *et al.*[39] who reported that progesterone had no effect on *T. gondii* multiplication in a macrophage cell line. Progesterone-treated, *T. gondii*-infected macrophages therefore seem to be able to control parasite replication through progesterone-insensitive, NO-independent methods that adequately compensate for the reduction in NO. Various NO-independent mechanisms could control *T. gondii* in activated macrophages, including production of reactive oxygen intermediates, TNF-α, chelation of iron and degradation of tryptophan.[60–66] Further studies are required to define the NO-independent mechanism responsible. An important caveat for the apparently disparate effects of the ability of progesterone to ablate macrophage killing of *T. gondii* and *L. donovani* is the difference in the experimental systems employed. Whereas macrophages in the *L. donovani* studies were incubated with both IFN-γ and LPS, those used in the *T. gondii* studies were incubated only with LPS. A direct comparison between parasites using LPS alone as stimulant was not possible because this was found not to induce *L. donovani* killing.

Macrophages also play a pivotal role in antiparasitic immunity by releasing cytokines such as IL-1, IL-6, IL-12, IL-18 and TNF-α to promote inflammation and aid direction of the adaptive response to a Th1 bias.[9,28,67] IL-12 has a central role in protection of the host during parasitic infection with either *T. gondii* or *L. donovani*.[28,67–71] In particular, the importance of progesterone as an exacerbatory factor during *in vivo T. gondii* infection may be a consequence of IL-12 inhibition and subsequent downregulation of the protective type-1 response. This may be a major factor in explaining why *T. gondii* is a common congenital infection.[72]

In conclusion, these results demonstrate that progesterone can downregulate production of NO and IL-12 by macrophages through a glucocorticoid receptor-mediated

mechanism and additionally production of IL-12 through a progesterone receptor-mediated mechanism following ligation of TLR-4. Whilst progesterone treatment resulted in inhibition of the leishmanicidal activity of activated BMMs infected with *L. donovani*, this was not the case in *T. gondii*-infected macrophages, where parasite killing was unaffected by the hormone. Although progesterone was shown in this study to have no direct effect on *T. gondii* killing in macrophages *in vitro*, the reduction witnessed in IL-12 production is likely to have downstream effects on the induction of a Th1 response and/or natural killer activation *in vivo* and thus be the cause in part for the increased susceptibility to *T. gondii* infection during pregnancy.

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