Differential Modulation of TLR3- and TLR4-Mediated Dendritic Cell Maturation and Function by Progesterone

Leigh A. Jones, Shrook Kreem, Muhannad Shweash, Andrew Paul, James Alexander and Craig W. Roberts

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Dendritic cells (DCs) play a pivotal role in immunity by linking the innate and adaptive immune systems and are vital in initiating protective responses to a number of pathogens (1–3). Ligation of TLRs with pathogen-associated molecular patterns induces maturation of DCs characterized by increased surface expression of CD40, CD80, and CD86 accompanied by the production of a number of cytokines. Following maturation and Ag uptake, DCs migrate to the lymphoid tissues to interact with B and T cells and initiate the development of the adaptive response (1, 4). Depending on the cytokine milieu created by the innate response to a particular pathogen, DCs can orchestrate the T cell response to either a Th1 or Th2 phenotype (1, 5).

The induction of a Th2 response has been demonstrated to occur in the vicinity of the placenta during mammalian pregnancy, and disruption of this has been associated with abortion (6, 7). In addition, during pregnancy, T regulatory cells specific for fetal alloantigens are involved in expression of tolerance to the fetus (8, 9). DCs have been demonstrated around the fetomaternal interface, and studies have shown that in this situation, their function is altered to favor production of regulatory cytokines, such as IL-10, and reduce secretion of inflammatory cytokines, such as IL-12 (10, 11). The microenvironment created during pregnancy differs from the normal physiological situation, particularly with regard to circulating hormones, and levels of glucocorticoids, estrogen, and progesterone are all increased. This change in the hormonal milieu is thought to play a major role in skewing the maternal immune response to a Th2 and T regulatory phenotype (12, 13), influenced in part by actions of hormones on a number of hematopoietic cells including DCs (14, 15).

Glucocorticoids have been reported to influence multiple aspects of DC function from initial development to mature cell stimulation. Consequently, glucocorticoids have been shown to limit the capacity of monocytes to develop into DCs, reduce Ag uptake, up-regulate adhesion molecule expression, downregulate maturation markers, such as CD40, CD80, CD86, and MHC class II, inhibit production of proinflammatory cytokines including IL-1α, IL-1β, IL-6, IL-12p70, IFN-γ, and TNF-α, reduce Ag presentation via the MHC class II pathway, and downmodulate T cell stimulatory capacity (16–22).

Given the paramount importance of DCs in linking, as well as driving, the innate and adaptive immune response and the well-documented ability of progesterone to modulate immune activity, there have been surprisingly few studies investigating how progesterone specifically modulates DC maturation and function. Progesterone has been shown to upregulate the differentiation of DCs as measured by increased endocytic activity from bone marrow precursors, although following LPS stimulation, an immature phenotype was maintained under the influence of progesterone as demonstrated by decreased MHC class II, CD40, CD54, and CD80 expression (23, 24). In contrast, a further study reported that progesterone treatment of resting murine splenic DCs resulted in increased expression of MHC class II and CD40 and enhanced T cell stimulatory activity (25). Progesterone is a ligand for the glucocorticoid receptor (GR) as well as the progesterone receptor (PR), and we have already demonstrated that progesterone can differentially regulate the signaling pathways employed by TLR3 and TLR4 agonists to affect costimulatory molecule expression and cytokine production. The Journal of Immunology, 2010, 185: 4525–4534.

Differential Modulation of TLR3- and TLR4-Mediated Dendritic Cell Maturation and Function by Progesterone

Leigh A. Jones, Shrook Kream, Muhammad Shweash, Andrew Paul, James Alexander, and Craig W. Roberts

The role of progesterone in modulating dendritic cell (DC) function following stimulation of different TLRs is relatively unknown. We compared the ability of progesterone to modulate murine bone marrow-derived DC cytokine production (IL-6 and IL-12) and costimulatory molecule expression (CD40, CD80, and CD86) induced by either TLR3 or TLR4 ligation and determined whether activity was via the progesterone receptor (PR) or glucocorticoid receptor (GR) by comparative studies with the PR-specific agonist norgestrel and the GR agonist dexamethasone. Progesterone was found to downregulate, albeit with different sensitivities, both TLR3- and TLR4-induced IL-6 production entirely via the GR, but IL-12p40 production via either the GR or PR. Of particular significance was that progesterone was able to significantly inhibit TLR3- but not TLR4-induced CD40 expression in bone marrow-derived DCs. Stimulation of the PR (with progesterone and norgestrel) by pretreatment of DCs was found to sustain IFN regulatory factor-3 phosphorylation following TLR3 ligation, but not TLR4 ligation. Overall, these studies demonstrate that progesterone can differentially regulate the signaling pathways employed by TLR3 and TLR4 agonists to affect costimulatory molecule expression and cytokine production.


Dendritic cell; GR, glucocorticoid receptor; IRF, IFN regulatory factor; poly I:C, polyinosinic-polycytidylic acid; PR, progesterone receptor; SAPK, stress-activated protein kinase.

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Address correspondence and reprint requests to Dr. Craig W. Roberts, Strathclyde Institute for Pharmacy and Biomedical Sciences, University of Strathclyde, 27 Taylor Street, John Arbuthnott Building, Glasgow G4 0NR, United Kingdom. E-mail address: c.w.roberts@strath.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; GR, glucocorticoid receptor; IRF, IFN regulatory factor; poly I:C, polyinosinic-polycytidylic acid; PR, progesterone receptor; SAPK, stress-activated protein kinase.

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therefore determined, using bone marrow-derived DCs (BMDCs), whether DC TLR-induced cytokine production and maturation was also GR or PR dependent. In addition, we determined whether different TLR ligands that used different signaling pathways were equally subject to progesterone modulation. Our results indicate significant plasticity in progesterone-influenced DC activity, whereby the hormone cannot only use either the PR or GR or both to mediate effects but also can selectively modulate the activities induced by distinct TLRs.

Materials and Methods

Compounds

LPSs from *Escherichia coli* 055:B5, polyinosinic-polycytidylic acid (poly I:C), progesterone, norgestrel, and dexamethasone were all obtained from Sigma-Aldrich (Dorset, U.K.). Progesterone and norgestrel were initially dissolved in 100% chloroform (BDH Laboratory Supplies, Poole, U.K.) at a concentration of 50 mg/ml. This stock solution was further diluted with complete medium to give a 250 μM solution for experimental use. Dexamethasone 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 mM solution for experimental use.

DC culture and treatments for cytokine and cell viability assays

Bone marrow cells were flushed from the femurs of 8-wk-old male BALB/c mice. These cells were cultured to produce BMDCs using GM-CSF–enriched media obtained from ×63 GM-CSF myeloma cells, which allows differentiation of bone marrow stem cells to immature DCs (27). BMDCs were grown in 75 cm² tissue culture flasks (TPP, Trasadingen, Switzerland) in culture medium containing RPMI 1640 (Life Technologies, Paisley, U.K.), 10% heat-inactivated FCS (Harlan Sera-Lab, Loughborough, U.K.), 10% GM-CSF–enriched medium, and 2 mM L-glutamine (GIBCO, Invitrogen, Paisley, U.K.), and incubated at 37°C and 5% CO₂ for 8 d. This method produces a large number of CD11c + DCs largely free from contamination with other cell types as previously described (27). BMDCs were then seeded at 5 × 10⁵ cells/ml on 96-well microtiter tissue culture plates at 100 μl volumes and incubated under standard conditions for 48 h. BMDCs were then activated with either LPS (50 ng/ml or 800 ng/ml) or poly I:C (50 ng/ml) in 50 μl volumes. Cells were simultaneously treated with medium alone, medium containing solvent vehicle controls (either chloroform or ethanol accordingly), or hormones. Progesterone and norgestrel were added in 50 μl volumes at 0.122–62.5 μM. Dexamethasone was added in 50 μl volumes at 0.122–62.5 nM. Cultures were incubated at 37°C and 5% CO₂ for 72 h. After this time, supernatants were collected from each triplicate well and stored at −20°C for subsequent determination of IL-6, IL-12p40, and IL-12p70.

DC culture and treatments for maturation marker assays

Bone marrow cells were collected from BALB/c femurs as previously described and centrifuged at 200 × g for 10 min at 4°C. Cell numbers were counted and diluted to 2.5 × 10⁵ cells/ml preosding at 2 ml volumes onto six-well plates (Nunc, Roskilde, Denmark). This procedure allows minimal disruption of DCs that could otherwise induce the nonspecific upregulation of maturation markers prior to experimental treatments. Following 7 d of culture, BMDCs were pretreated with progesterone (62.5 μM), norgestrel (62.5 μM), or dexamethasone (62.5 nM) for 24 h preaddition of LPS (100 ng/ml) or poly I:C (10 μg/ml). BMDCs were then left to incubate for 16 h at 37°C and 5% CO₂.

Abs

For ELISAs, the following capture Abs were used: anti-mouse mAb IL-6 (IgG1, clone MP5-20F3; catalog number 554400), anti-mouse mAb IL-10 (IgG1, clone JES5-2A5; catalog number 551215), anti-mouse mAb IL-12p40 (IgG1, clone 15.6; catalog number 551219), and anti-mouse mAb IL-12p70 (IgG2b, clone 9AG; catalog number 554658). Abs used for detection were biotin-labeled rat anti-mouse anti-cytokine mAbs for IL-6 (IgG2a, clone MP5-32C11; catalog number 554402), IL-10 (IgG1, clone SXC-1; catalog number 554423), and IL-12p40 (IgG2a, clone C17.8; catalog number 554476). For flow cytometry analysis, the anti-mouse Abs used were PE-labeled anti-CD11c (IgG2b, clone S-HCL-3; catalog number 333149), FITC-labeled anti-CD40 (IgG2a, clone 3/23; catalog number 553790), FITC-labeled anti-CD80 (IgG2, clone 16-10A1; catalog number 553768), and FITC-labeled anti-CD86 (IgG2a, clone GL-1; catalog number 553691). All Abs were purchased from BD Pharmlandingen (San Diego, CA).

Cytokine assays

The concentration of IL-6, IL-10, IL-12p40, and IL-12p70 present in cell culture supernatants was assayed by sandwich ELISA. Plates were coated with capture anti-mouse mAbs IL-6, IL-10, IL-12p40, and IL-12p70 as described above. Reombinant murine cytokines IL-10, IL-12p40, and IL-12p70 were purchased from BD Pharmlandingen, whereas reombinant murine IL-6 was purchased from Bender MedSystems (Vienna, Austria). The detecting Abs used were biotin-labeled rat anti-mouse mAbs (BD Pharmlandingen). The conjugate used for IL-6, IL-10, and IL-12p40 detection was streptavidin–alkaline phosphatase (BD Pharmlandingen). IL-12p70 was detected by addition of streptavidin–HRP (BD Pharmlandingen). IL-6, IL-10, and IL-12p40 ELISAs were completed by addition of p-nitrophenyl phosphate (Sigma-Aldrich) in glycine buffer and absorbances measured at 450 nm using a SpectraMax 190 microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA) and SoftMax Pro 3.0 software (Molecular Devices). The substrate used for IL-12p70 detection was 1% 3, 3', 5', 5'-tetramethylbenzidine (Sigma-Aldrich) in sodium acetate buffer at pH 5.5 containing 0.0075% hydrogen peroxide (BDH, Toronto, Ontario, Canada). This reaction was stopped by addition of 10% sulphuric acid.

Surface marker staining

Postincubation, cells were washed and stained with the Pan marker anti-CD11c and incubated with the appropriate surface marker at 4°C for 1 h. Abs used are as described above. Cells were washed, and fluorescence analysis was performed using the BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Positive cells were gated on obtained dot plots and data analyzed for mean fluorescence intensity of CD11c + cells expressing the chosen maturation marker using FACS Diva software (BD Biosciences). Isotype-matched negative controls were used to confirm staining specificity.

Assessment of cell viability

BMDCs plated in triplicate at 5 × 10⁵ cells/ml and treated with hormones/stimulants as previously were tested for cell viability using alamarBlue assay (Biosource). Reagent was added at 10% of the total well volume. Wells containing medium only were used as a blank. After 48 h, well contents were assayed for alamarBlue reduction by measuring the absorbance of the wells at 570 and 600 nm on a SpectroMax 190 plate reader (Molecular Devices) and the percentage reduction of alamarBlue calculated as previously described (28).

Western blotting

Abs used in Western blotting studies, including phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185; catalog number 9251S), phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; catalog number 4377S), p44/42 MAPK (ERK1/2; catalog number 4695S), phospho-IFN regulatory factor (IRF)-3 (Ser²³⁶; catalog number 4974S), IRF-3 (catalog number 4962), and phospho-NFκB (Ser³⁸⁹; p65; Ser³⁸⁹; catalog number 3031L), were purchased from Cell Signaling Technology (Beverly, MA). Abs raised against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA; catalog number 2607) and p65 (Santa Cruz Biotechnology; catalog number L0607) were purchased from Insight Biotechnology, Middlesex, U.K., and anti-phospho-p38 (catalog number 44-684G) was purchased from Invitrogen. All other materials used during gel electrophoresis and Western blotting were of the highest commercial grade available and purchased from Sigma-Aldrich.

Analysis of protein expression and/or phosphorylation in BMDCs

BMDCs (1 × 10⁶ cells; 1 ml) prepared on 12-well plates were exposed to vehicles, hormones, LPS, or poly I:C as appropriate and whole cell lysates prepared for Western blotting as described previously (29).

Data analysis, scanning densitometry, and statistical analyses

Scanning densitometry was performed on Western blots using an Epson Perfection 164054 Scan jet and Epson twain 55.52 (32.32) Scanjet Picture software (Epson, Long Beach, CA). All values are mean ± SE of n = 3 experiments. Statistical significance of differences was calculated by the one-way ANOVA followed by a Newman-Keuls test using GraphPad Prism Version 4.0 software (GraphPad, San Diego, CA). A p value <0.05 was accepted as significant. All other sets of data were analyzed using a Mann–Whitney U test using Statview software (SAS Institute, Marlboro, U.K.) with a value of p < 0.05 taken as significant.
**Results**

**Progesterone uses the GR to inhibit TLR3- and TLR4-induced IL-6 production by BMDCs**

The ability of BMDCs to produce IL-6 following stimulation with LPS or poly I:C was first confirmed using a simple dose-response experiment (0.012–25 μg/ml LPS/6.25–100 μg/ml poly I:C; data not shown). Totals of 800 ng/ml LPS and 50 μg/ml poly I:C were identified as the most suitable stimulant concentrations for use in studies with hormones because they allowed both potentiation and inhibition of IL-6 production to be assessed.

Once the assays had been standardized, we determined whether progesterone, norgestrel, and dexamethasone could modulate LPS- or poly I:C-induced IL-6 production by BMDCs. Over a number of experiments (n = 3), progesterone caused a significant inhibition of LPS-induced IL-6 production at 62.5 μM and 31.25 μM (Fig. 1A). Progesterone also significantly inhibited poly I:C-induced IL-6 production, but at lower concentrations >0.5 μM (IC50 of 0.5–1 nM; Fig. 1D), indicating that IL-6 production via TLR3 ligation was more sensitive to progesterone treatment than TLR4 ligation. Whereas norgestrel, the PR agonist, did not modulate IL-6 production by LPS- or poly I:C-stimulated BMDCs (Fig. 1B, 1E), the GR agonist dexamethasone demonstrated a greater capacity to reduce IL-6 levels compared with progesterone. Dexamethasone treatment using concentrations above 2 nM resulted in significant downregulation of LPS-induced IL-6 (IC50 of 7.8–15.7 nM; Fig. 1C), and at all concentrations tested, dexamethasone reduced poly I:C-induced IL-6 production by BMDCs (IC50 of 1–2 nM; Fig. 1F), again indicating that the TLR engaged determines sensitivity to hormone treatment and activity via the GR.

**Progesterone uses both the GR and PR to inhibit TLR3- and TLR4-induced total IL-12 production (p40) by BMDCs**

The ability of BMDCs to produce total IL-12 (p40) following stimulation with LPS or poly I:C was first confirmed using a simple dose-response experiment (0.012–25 μg/ml LPS/6.25–100 μg/ml poly I:C; data not shown). Totals of 50 ng/ml LPS and 50 μg/ml poly I:C were identified as the most suitable stimulant concentrations for use in studies with hormones because they allowed both potentiation and inhibition of IL-12 (p40) production to be assessed.

Progesterone induced a significant reduction of IL-12p40 production by LPS-stimulated BMDCs at all concentrations tested and had an IC50 of 1–2 μM (Fig. 2A). Progesterone also significantly reduced IL-12p40 production from poly I:C-stimulated BMDCs at concentrations >1 μM and had an IC50 of 0.5–1 μM (Fig. 2D). Norgestrel, the PR agonist, also downmodulated LPS-induced IL-12p40 production at all concentrations tested and had an IC50 of 0.5–1 μM (Fig. 2B). Norgestrel also significantly inhibited IL-12p40 production by poly I:C-stimulated BMDCs. At concentrations >31.3 μM (Fig. 2E), this time indicating that engagement of different TLRs is subject to different hormonal sensitivity via PR. Dexamethasone inhibited LPS-induced IL-12p40 levels from BMDCs, with significant downregulation occurring at concentrations >2 nM, demonstrating an IC50 of 1–2 nM (Fig. 2C).

![FIGURE 1.](http://www.jimmunol.org/) The influence of progesterone, norgestrel, and dexamethasone on IL-6 production by BMDCs stimulated with 800 ng/ml LPS or 50 μg/ml poly I:C for 72 h. Cells were cultured with LPS or poly I:C in the presence of progesterone (A, D), norgestrel (B, E), or dexamethasone (C, F). Unstimulated BMDCs failed to produce detectable levels of IL-6 (nd). Unstimulated cells treated with the solvent vehicle of 0.04% chloroform (progesterone and norgestrel) or 0.0001% ethanol (dexamethasone) produced undetectable or very low levels of IL-6 (data not shown). Stimulated control cell cultures were treated with 800 ng/ml LPS or 50 μg/ml poly I:C (closed bars). Stimulated control cell cultures treated with 800 ng/ml LPS or 50 μg/ml poly I:C and either 0.04% chloroform or 0.0001% ethanol produced levels of IL-6 that were comparable to stimulated cells treated with LPS or poly I:C 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.
caused significant downregulation of poly I:C-induced IL-12p40 production at all concentrations tested and had an IC$_{50}$ of 0.2–0.5 nM (Fig. 2F).

**Progesterone, norgestrel, and dexamethasone have no effect on the viability of stimulated BMDCs**

alamarBlue, a nonspecific stable dye that is used to assess cell viability and cell proliferation by measuring the reducing environment of the proliferating cell, was employed to determine whether the effects of hormones on BMDC cytokine production were due to a specific effect on signaling pathways or a nonspecific mechanism caused by cell death. Although stimulation with LPS and poly I:C significantly upregulated mitochondrial activity as compared with control cultures treated with medium alone, treatment with hormones had no effect on cell viability or proliferation as compared with LPS- or poly I:C-stimulated controls (data not shown). In addition, BMDCs stimulated with LPS or poly I:C and treated with progesterone, norgestrel, or dexamethasone failed to produce detectable levels of IL-10 (not shown).

**The ability of progesterone to downmodulate CD40, CD80, and CD86 expression depends on the TLR engaged**

The ability of BMDCs to express the costimulatory molecules CD40, CD80, and CD86 following stimulation with LPS or poly I:C was first confirmed using a simple dose-response experiment (62.5–1000 ng/ml LPS/6.25–100 μg/ml poly I:C; data not shown). Totals of 100 ng/ml LPS and 10 μg/ml poly I:C were identified as the most suitable stimulant concentrations for use in studies with hormones because they allowed both potentiation and inhibition of CD40, CD80, and CD86 expression to be assessed.

BMDCs were pretreated with progesterone, norgestrel, or dexamethasone for 24 h to investigate whether these hormones had any effect on expression of CD40, CD80, and CD86 by BMDCs (Fig. 3) following stimulation with LPS (Fig. 3) or poly I:C (Fig. 4). Progesterone, norgestrel, and dexamethasone did not have an effect on LPS-induced expression of CD40 by BMDCs (Fig. 3A). However, progesterone had a slight but significant inhibitory effect on BMDC expression of CD80 and CD86 in comparison with LPS-stimulated control cultures (Fig. 3B,3C). Norgestrel had no significant effect on LPS-induced BMDC expression of CD40 (Fig. 3A), CD80 (Fig. 3B), or CD86 (Fig. 3C). Dexamethasone, in contrast, induced a significant reduction in expression of CD86 (Fig. 3C) in comparison with LPS controls but not CD40 or CD80 (Fig. 3A,3B). By comparison with TLR4 ligation, progesterone demonstrated significant downregulation of poly I:C-induced BMDC expression of CD40, CD80, and CD86 (Fig. 4). Dexamethasone also inhibited poly I:C-induced BMDC’s CD40, CD80, and CD86 (Fig. 4) expression in comparison with poly I:C-treated controls. Norgestrel had no significant effect on poly I:C-induced expression of CD40 (Fig. 4A). In contrast to TLR4 ligation, it did...
significantly downregulate expression of CD80 and CD86 (Fig. 4B, 4C). Plots showing the mean fluorescence intensities for Figs. 3 and 4 are shown in Supplemental Fig. 1.

Ligation of the PR sustains IRF-3 phosphorylation following TLR3 ligation

TLR3- and TLR4-stimulated cellular responses are well recognized as being mediated via recruitment of a number of signaling cascades: classical p42/44 MAPK, p38 MAPK, JNK, NF-κB, and IRF-3/7 pathways. To examine further the effects of steroid hormones on TLR3- and TLR4-mediated signaling events that may precede cytokine expression, experiments were constructed to measure the expression and/or phosphorylation of key protein components of these cascades following exposure of cells to LPS or poly I:C over an acute time frame of 0–8 h. Although both LPS and poly I:C were effective activators (5–120 min) of the p42/44 MAPK, p38 MAPK, and JNK phosphorylation and parallel IκBα degradation and p65 RelA phosphorylation in BMDCs, cells pretreated (1 h) with the steroid hormones progesterone, norgestrel, and dexamethasone did not influence phosphorylation of any of the MAPK/SAPKs as assessed by Western blotting (data not shown). Furthermore, no modulation of p65 phosphorylation and IκBα degradation within the NF-κB cascade were observed in cells pretreated with the same hormones and exposed to poly I:C or LPS for 5–120 min (data not shown).

It was observed that both poly I:C and LPS were also able to activate, in parallel to the pathways detailed above, the IRF-3 cascade, as measured by Western blotting using antisera specific for the phosphorylation of Ser396 in the C terminus of IRF-3. Pretreatment of cells with progesterone or norgestrel for 1 h prior to addition of agonist prolonged poly I:C-stimulated IRF-3 phosphorylation in BMDCs significantly at 2 h postexposure to poly I:C (Fig. 5). Pretreatment of cells with dexamethasone, in contrast, had minimal effect upon poly I:C-stimulated IRF-3 phosphorylation over a similar time course but inhibited agonist-stimulated IRF-3 phosphorylation at later time points (4 and 8 h; Fig. 5). Pretreatment (1 h) with progesterone, norgestrel, or dexamethasone did not influence LPS-stimulated IRF-3 phosphorylation in BMDCs, again, as assessed by Western blotting (Fig. 6).

Discussion

It is well documented that much of the immunomodulation occurring during pregnancy is due to alterations in sex hormone levels, particularly progesterone (6, 30). Given the critical role that DCs play in orchestrating immune responses and their significant pre-
sense at the fetal–maternal interface (11, 31), the current study investigated the role of progesterone in modulating DC function. As a result, a number of key findings have arisen from our investigations: first, that the ability of progesterone to downregulate BMDC inflammatory cytokine production and costimulatory molecule expression depends in large part on what TLR is engaged; second, progesterone downmodulates some BMDC TLR-induced inflammatory mediators via the GR alone and others via the PR and the GR; and third, PR agonists sustain IRF-3 phosphorylation following TLR3 ligation but not TLR4 ligation. That DC TLR-induced activity can be differentially sensitive to progesterone depending on the TLR engaged is likely a result of the subtly different signaling pathways associated with TLR4 and TLR3 (32). Thus, although both TLRs can use MyD88-independent pathways, TLR3-induced NF-κB activation is entirely TRIF dependent (34). In addition, the TRIF-related adaptor molecule TRAM is an essential part of and restricted to the TLR4 MyD88-independent pathway (35). Recent studies have demonstrated that not only does TLR3 mediate a more potent antiviral response than TLR4 (33), but also that TLR3 contributes critically to the debilitating effects of a damaging host inflammatory response as a result of influenza A infection (36), and, despite a higher viral production in the lungs, mice deficient in TLR3 had a significant survival advantage. Consequently, with regard to the well being of the fetus, there could well be an evolutionary advantage in the apparent enhanced sensitivity of TLR3-induced inflammatory processes to downmodulation by progesterone.

In the current study, we demonstrate that TLR3- but not TLR4-induced CD40 expression can be downmodulated by progesterone. In addition, the induction of IL-6 via TLR3 ligation is an order of magnitude more sensitive to inhibition by progesterone than IL-6 produced via TLR4 ligation. That DC TLR-induced activity can be differentially sensitive to progesterone depending on the TLR engaged is likely a result of the subtly different signaling pathways associated with TLR4 and TLR3 (32). Thus, although both TLRs can use MyD88-independent pathways, TLR3-induced NF-κB activation is entirely TRIF dependent (33), and, unlike other TLRs that use IL-1R–associated kinase to activate NF-κB, TLR3-induced NF-κB activation is receptor interacting protein kinase dependent (34). In addition, the TRIF-related adaptor molecule TRAM is an essential part of and restricted to the TLR4 MyD88-independent pathway (35). Recent studies have demonstrated that not only does TLR3 mediate a more potent antiviral response than TLR4 (33), but also that TLR3 contributes critically to the debilitating effects of a damaging host inflammatory response as a result of influenza A infection (36), and, despite a higher viral production in the lungs, mice deficient in TLR3 had a significant survival advantage. Consequently, with regard to the well being of the fetus, there could well be an evolutionary advantage in the apparent enhanced sensitivity of TLR3-induced inflammatory processes to downmodulation by progesterone.

It has been reported that women who use the progesterone contraceptive depot medroxyprogesterone acetate are at greater risk of contracting the HIV and show increased incidence of HSV reactivation and viral shedding (37, 38). A recent study has suggested that progesterone could be limiting the antiviral response by inhibiting TLR7- and TLR9-induced IFN-α production by plasmacytoid DCs (39). TLR3 is also known to be a vital component of antiviral responses by binding dsRNA and ssRNA molecules to induce not only IFNs but also proinflammatory cytokines, which have a key role to play in antiviral defense (32, 40,
41). We believe that our data demonstrate a further mechanism through which progesterone could be functioning to limit the antiviral response by reducing TLR3-induced inflammatory responses.

The release of proinflammatory cytokines, such as IL-6 and IL-12, as induced by infectious disease is incompatible with successful pregnancy, and, consequently, we examined the role of progesterone on DC release of these cytokines (6). Progesterone reduced the production of IL-6 and IL-12 by murine BMDCs following stimulation with either LPS or poly I:C. Estrogen has previously been shown to reduce cytokine and chemokine production following TLR3 stimulation in a human cell line (42). However, to our knowledge, this is the first report to demonstrate the role of progesterone in modulating TLR3-induced pathways of IL-6 and IL-12 induction. Previous reports have described a reduction in IL-12 levels following progesterone treatment of lymphocytes from pregnant mice and murine splenic-derived DCs (25, 43). However, the observed reduction in IL-12 production is in contrast to studies carried out by Huck and colleagues (23), who failed to show any significant effects of progesterone on production of this cytokine. Indeed, Yang et al. (25) reported increased levels of intracellular IL-6 in murine DCs following progesterone treatment, although this study differed from ours in that cells were left unstimulated and were derived from spleen and not bone marrow. However, with regard to a successful pregnancy, it is the regulation of excessive inflammatory responses that is crucial.

Progesterone functions by binding to both the GR and PR (44–46). Using the receptor-specific agonists dexamethasone and norgestrel, we demonstrate in this study the ability of progesterone to differentially use these receptors to cause downregulation of IL-6 and IL-12 production. Whereas progesterone mediated a reduction in TLR4- and TLR3-induced IL-6 production exclusively through the GR, inhibition of IL-12 production was mediated through the GR and PR. Although it has been shown that glucocorticoids and progesterone can induce upregulation of the anti-inflammatory cytokine IL-10 in human DCs (46), our studies (results not shown) as well as one using rat-derived DCs treated with progesterone did not show an upregulation of IL-10 (47). The timing of LPS treatment relative to the seeding of DC cultures in preparation of stimulation experiments is critical in defining secretion patterns of IL-12 and IL-10 that likely explain the differences between such studies (48). Nonetheless, we show that progesterone can reduce IL-12 levels in a manner independent of IL-10.

Our studies provide further evidence of immunomodulatory activity through the PR by using the PR-specific agonist norgestrel. In addition, the action of hormones had no effect on cell metabolism in the current study as shown by a lack of any alteration to alamarBlue reduction in treated cells. Therefore, progesterone is directly modulating signaling pathways, leading to the production of IL-6 and IL-12 by differential usage of the GR and PR. Immunomodulatory actions through the GR have been well documented. These include its ability to interact with the transcription factor NF-κB (49). The PR has also been reported to physically interact with NF-κB, linked to cytokine production (49, 50). However, it would seem unlikely that the PR is inhibiting IL-12 production in this manner, as it would then be expected to induce downregulation of IL-6. It has been suggested that steroid hor-
mone receptors can differentially modulate genes at the promoter level, which could explain our observations (50, 51). Alternatively, divergences upstream of NF-κB in the individual signaling pathways leading to the synthesis of IL-6 and IL-12 may be targeted by norgestrel to result in the specific inhibition of IL-12.

Related to these points, we sought to characterize further the molecular mechanisms that underlie PR-mediated inhibition/suppression of LPS- and poly I:C-stimulated cytokine production. In this study, as a first step toward pursuing the potential role of signaling cascades in these processes, we examined the modulation of key signaling molecules recognized to be sensitive to TLR3/4 activation (52). PG, norgestrel, and dexamethasone collectively did not influence the phosphorylation status of the MAPKs and the activation of the NF-κB cascade, commonly engaged by this receptor superfamily. This suggested these pathways were not influenced by steroid hormone pretreatment. It must be noted, however, that in this study, experiments were limited to examination of the signaling cascades at the level of the MAPK/SAPKs and the p65/IκB complex directly. Further investigation is required to determine whether agonists at the GR and PR influence events further downstream in the cascade—in particular, nuclear functions.

It was noted, however, that progesterone and norgestrel pretreatment led to sustained IRF-3 phosphorylation that was specific to the poly I:C–TLR3 response; this was not apparent for LPS–TLR4 signaling. Numerous studies have described that elevated IRF-3 activation leads to enhanced antiviral responses, including gross expression of IFNs (53). Therefore, sustained phosphorylation may imply an expected elevation of cytokine production. More recently, however, experiments in macrophages (54) have detailed reduced expression of IL-12 p35 in response to viral infection due to constitutive activation of IRF-3 and its function as a repressor of transcription via binding to bp2172 to -122 of the p35 promoter (53). Whether this regulatory function is apparent in BMDCs and can be correlated with IRF-3 protein phosphorylation status remains to be determined.

The mechanism by which poly I:C-stimulated IRF-3 phosphorylation is sustained following progesterone and norgestrel pretreatment also remains to be characterized thoroughly. This may be a consequence of PR/GR-stimulated elevation of cellular kinase activity; potentially via the inducible isoform of IκB kinase and/or TRAF-associated NF-κB activator-binding kinase 1 as the recognized upstream kinase regulators of this transcription factor (52). Alternatively, there may be a decrease in phosphatase-catalyzed dephosphorylation of IRF-3. Again, relevant candidate phosphatases remain to be identified. GR-mediated immunosuppression has also been reported to be reliant on a glucocorticoid receptor-interacting protein 1–IRF-3 interaction (54) in the formation of a protein complex capable of repressing, although the influence of IRF-3 phosphorylation status upon this interaction has not been detailed. Overall, from our studies to date, it appears that these effects are specific to TLR3-mediated responses and as such may represent a mechanism by which progesterone and norgestrel mediate modulation of IRF-3 phosphorylation and therefore may potentially influence transcription factor activation, downstream cytokine production, and costimulatory molecule expression.

In addition to the vital role that DCs play in initiating the innate immune response by production of cytokines and chemokines, these cells are critically important in triggering an effective adaptive immune response. During the maturation process, DCs up-
regulate the expression of cell surface molecules, such as CD40, CD80, and CD86, that are important for interaction with B and T cells and subsequent development of the adaptive immune response (1, 4). Expression of these maturation markers is significantly upregulated following treatment of BMDCs with the TLR3 ligand poly I:C and the TLR4 ligand LPS. Of particular note in addition to the different sensitivities of these maturation factors to progesterone depending on the particular TLR engaged was that progesterone, but not dexamethasone or norgestrel, significantly inhibited TLR4-induced CD80, whereas progesterone inhibited TLR3-induced CD40 and CD80 to a significantly greater level than ligation of either PR or GR alone. This would indicate that progesterone can downregulate these molecules either by PR- or GR-independent mechanisms. A non-PR and GR mechanism could result from progesterone binding to membrane-associated receptors resulting in rapid, nongenomic effects on intracellular signaling that can subsequently affect nuclear translocation of transcription factors and protein synthesis (55, 56). This has not yet been shown in DCs but has been demonstrated in T cells, where progesterone inhibits both Ca2+ and K+ channels and thus gene expression as induced by NFAT (57).

Therefore, overall, this study provides further verification that progesterone will play a role in fine-tuning the immune response. However, more importantly, by targeting directly the inflammatory armory of DCs, progesterone is likely vital in creating a favorable immunological environment for fetal survival.

Disclosures

The authors have no financial conflicts of interest.

References


Mean Fluorescence Intensity plots demonstrating the effect of progesterone, norgestrel and dexamethasone on BDC CD40, CD80 and CD86 expression following LPS (A) or poly I:C stimulation. The data derived from these plots were used to construct the histograms in Figures 3 and 4 respectively of the manuscript.