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**References**

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Cutting Edge: Progesterone Regulates IFN-α Production by Plasmacytoid Dendritic Cells

Grant C. Hughes, Sunil Thomas, Chang Li, Murali-Krishna Kaja, and Edward A. Clark

Use of the progesterone (Pg) birth control depot medroxyprogesterone acetate (DMPA) increases a woman’s risk for sexually transmitted infection with HIV or HSV-2 via unknown mechanisms. Plasmacytoid dendritic cells (pDCs) are circulating and tissue-resident sentinels capable of making large quantities of IFN-α upon recognizing viruses through TLRs 7 and 9. In this study, we show that Pg inhibits TLR9-induced IFN-α production by human and mouse pDCs and that DMPA impairs TLR9- and virus-induced IFN-α production by pDCs in mice, providing a potential explanation for how DMPA impairs innate antiviral immunity in women. Pg failed to inhibit the Mda-5 pathway of IFN-α induction in dendritic cells, suggesting that Pg regulates select antiviral DC programs. This may occur through selective blockade of IFN regulatory factor-7 activation, a novel steroid action. Thus, through inhibition of TLR-mediated IFN-α production by pDCs, Pg may regulate antiviral immunity. The Journal of Immunology, 2008, 180: 2029–2033.

More than 100 million women worldwide use hormonal contraception (1). In sub-Saharan Africa, where an estimated 26 million people are infected with HIV (2), the long-acting form of progesterone (Pg), a depot medroxyprogesterone acetate (DMPA), is rapidly becoming the hormonal contraceptive of choice (3). However, DMPA use is associated with significantly increased risk of HIV (3) or HSV-2 (4) acquisition in women. In nonhuman primates DMPA increases vaginal transmission of SIV (5), and in mice DMPA causes a 100-fold increased susceptibility to vaginal HSV-2 infection (6). DMPA also blocks development of protective antiviral immunity in these models (7). How Pg impairs innate and adaptive antiviral responses is poorly understood (8).

Plasmacytoid dendritic cells (pDCs) are circulating and tissue immune sentinels capable of producing large amounts of IFN-α upon encountering viruses (9). IFN-α induces an antiviral state in nonimmune tissues and primes adaptive antiviral responses by directly activating APCs and T cells (10). pDCs sense viruses through TLRs 7 and 9, which recognize viral RNA and DNA, respectively. Myeloid DCs (mDCs) sense viral infection through additional cytoplasmic RNA receptors such as RIG-I (retinoic acid-inducible gene I) and Mda-5 but produce far less IFN-α per cell than do pDCs (10). Interestingly, the TLR pathway of IFN-α induction is abnormally activated in systemic lupus erythematosus, where immune complexes stimulate pDCs through TLRs 7 and 9 (11). How Pg regulates pDC functions in immunity and autoimmunity is largely unknown (8).

In this study we show that Pg blocks TLR-mediated and virus-induced IFN-α production by pDCs, providing a potential explanation for how Pg impairs innate antiviral defenses. We also show evidence suggesting that Pg may regulate select pathways of IFN-α induction, potentially through blockade of IFN regulatory factor (IRF)-7 translocation to the nucleus, a novel steroid hormone action. Together, these observations support epidemiological data linking DMPA use and the risk of viral infection. Understanding hormonal regulation of antiviral immunity is critical for health planning in sub-Saharan Africa, where DMPA use is rising in the face of HIV epidemics.

Materials and Methods

Cell cultures, hormone treatments, and stimulations

Human pDCs were purified with either anti-blood dendritic cell Ag (BDCA)-2 or anti-BDCA-4 magnetic beads (Miltenyi Biotech) from either American Red Cross donor PBMCs or donors mobilized with G-CSF (courtesy of the Fred Hutchinson Cancer Research Center, Seattle, WA). Human pDC isolates were 60–80% pure as measured by CD123 and BDCA-2/-4 costaining. Mouse spleens were treated with Liberase Blendzyme 2 and Gey’s lysis buffer and splenocytes used fresh or processed further. Splenic CD11c+ mPDCA1-
bone marrow pDCs were generated as previously described (13) with 100 ng/ml (both from RDI Division of Fitzgerald Industries). In vitro derived as previously described (12) in GM-CSF at 20 ng/ml and IL-4 at 10 ng/ml (both from RDI Division of Fitzgerald Industries). In vitro derived bone marrow pDCs were generated as previously described (13) with 100 ng/ml Escherichia coli ligand (R&D Systems). These cultures were 25–35% CD1c+ B220+ mPDCA1+ pDCs. Hormone treatments were with water-soluble Pg, estradiol (E2), or dexamethasone (Dex) complexed with β-cyclodextrin (vehicle) or with vehicle alone (all from Sigma-Aldrich). Unless otherwise stated, cells were stimulated with either polyinosinic-polycytidylic acid (poly(I: C)) at 50 μg/ml, Escherichia coli LPS at 0.1 μg/ml, imiquimod at 10 μg/ml (Sigma-Aldrich) or CpG ODN 2216 at 10 μg/ml. All cell cultures were in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin.

**Mice, hormone treatments, and virus infections**

Four- to 6-week-old female C57BL/6 mice were obtained from The Jackson Laboratory and housed in the University of Washington Medical Center animal facilities (Seattle, WA). Mice were injected s.c. with 2 mg of DMPA (Pfizer) diluted to 200 μl with vehicle (per DMPA product insert; Pfizer) or with 200 μl of vehicle alone. Fourteen days later, spleen cells were isolated or, in some experiments, mice were inoculated with 10⁹ PFU of vesicular stomatitis virus (VSV)-G fluorescent tail vein injection. All procedures used in this study complied with federal guidelines and institutional policies of the University of Washington Institutional Animal Care and Use Committee.

**Measurement of cytokine levels and flow cytometry**

Levels of the following cytokines were measured by ELISA kits for human IFN-α (PBL Biomedical) and mouse IL-12p40, IL-6, TNF-α, and IL-10 (R&D Systems). Mouse IFN-α was measured by ELISA using rat anti-mouse IFN-α (capture Ab; PBL Biomedical) followed by rabbit anti-mouse IFN-α (PBL Biomedical and donkey anti-rabbit HRP (Jackson ImmunoResearch Laboratories). Cell surface staining of human cells was performed with Abs against BDCA2, BDCA4 (both from Miltenyi Biotech), and CD123 (BD Biosciences). Cell surface staining of mouse cells was with Abs against CD11c, CD86, CCR7, MHC class II (BD Pharmingen), B220 (eBioscience), and mPDCA1 (Miltenyi Biotech). Flow cytometry was performed on a BD FACScan machine (BD Biosciences).

**Immunobots**

Total or nuclear cell lysates were obtained from in vitro derived pDCs as previously described (14). Lysate samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either rabbit anti-IRF-7 (Zymed Laboratories), or rabbit anti-lamin A/C (Cell Signaling), followed by HRP-conjugated anti-rabbit IgG (The Jackson Laboratory). Bands were visualized using an ECL Western Lighting chemiluminescence reagent (PerkinElmer).

**Results and Discussion**

**Progesterone inhibits IFN-α production by pDCs in vitro**

To examine the role of Pg in innate antiviral immunity, we purified human peripheral blood BDCA2+ or BDCA4+ pDCs, exposed them to Pg or estradiol (E2) as a hormonal control, and stimulated them with CpG. Although E2 had a modest inhibitory effect on IFN-α production in response to the TLR9 ligand CpG, Pg completely blocked it (Fig. 1A) in a dose-dependent manner (Fig. 1B). Inhibition of cytokine production was not due to cell death, because Pg had no effect on cell survival when compared with control cultures treated with vehicle (data not shown). Consistent with previous observations (15), CpG-treated human pDCs did not make detectible amounts of IL-12p40 or TNF-α or significant amounts of IL-6, although these cells produced these cytokines upon stimulation with CD40-ligand (data not shown). Cross-linking BDCA-2 or BDCA-4 (BDCA-2/4) on pDCs with magnetic beads did not significantly block IFN-α production (Fig. 1, A and B), as previously described in human pDCs (16). However, these earlier studies examined pDCs cultured for 6–24 h after cross-linking, whereas we cultured pDCs for 48 h after cross-linking. This difference suggests that the inhibitory effects of BDCA-2/4 cross-linking on IFN-α production may be transient. As with human pDCs, Pg impaired CpG-induced IFN-α production by mouse spleen pDCs (Fig. 1C) and, less potently, IL-12p40 production (Fig. 1D). Inhibition of cytokine production was not due to cell death, because Pg had no effect on cell survival when compared with control cultures treated with vehicle (data not shown). Thus, Pg regulates TLR-induced IFN-α production by human and mouse pDCs, providing a potential mechanism for how Pg impairs antiviral responses in vivo. Because Pg inhibited IFN-α at a dose that did not affect IL-12p40 production (Fig. 1, C and D), Pg may in some instances be a selective inhibitor of TLR-mediated IFN-α production. The doses of Pg required to inhibit IFN-α in vitro (Fig. 1) are higher than the serum levels during pregnancy but do occur within the human placenta (17).

**DMPA treatment selectively inhibits CpG-induced IFN-α production by spleen pDCs**

To assess the role of in vivo exposure to Pg on pDC functions, we treated mice with the same 2-mg dose of DMPA that blocks anti-HSV-2 immunity (6). This dose is expected to result in serum medroxyprogesterone acetate (MPA) levels of 20–80 ng/ml (18), similar to Pg levels in early murine and human pregnancy (19, 20). Treatment of mice with DMPA did not affect the total numbers of spleen T cells, B cells, pDCs, or mDCs (data not shown). However, DMPA significantly impaired CpG-induced IFN-α production by total splenocytes (Fig. 2A). In contrast, DMPA did not affect either poly(I:C)-induced IFN-α or CpG-induced IL-12p40 (Fig. 2A). CpG-induced but not poly(I:C)-induced IFN-α production by splenocytes required pDCs, because IFN-α responses were abrogated by pDC-depletion (Fig. 2B). To assess the pDC-intrinsic effects
of DMPA treatment, we sorted splenic pDCs from hormone-treated mice and found that they were significantly impaired in their production of IFN-α in response to CpG (Fig. 2C). Thus, DMPA acts in vivo to impair CpG-induced IFN-α production by pDCs. The fact that far lower serum concentrations of MPA (estimated 20–80 ng/ml) than Pg (0.2–2 μg/ml) were sufficient to inhibit IFN-α production suggests that the duration of exposure (2 wk vs 12–24 h) may play a role. MPA may also be a more potent suppressor of IFN-α than Pg. It is interesting that DMPA treatment did not inhibit IL-12p40 production by splenocytes (Fig. 2A), suggesting again that, under come conditions, DMPA may be a selective inhibitor of CpG-induced IFN-α. Furthermore, DMPA failed to inhibit poly(I:C)-induced IFN-α production by splenocytes (Fig. 2A), indicating that this pathway of IFN-α induction, which is not present in pDCs (9), is less sensitive to or not regulated by Pg. Selective inhibition of TLR-induced IFN-α may explain why DMPA predisposes to infection with certain viruses, e.g., HSV-2 or HIV.

DMPA treatment impairs serum IFN-α responses to viral infection

To study the effects of Pg on IFN-α responses by pDCs to viruses in vivo, we chose VSV, because early serum IFN-α responses to VSV infection rely on TLR7, MyD88 (21), and pDCs (22). DMPA-treated mice had significantly lower serum IFN-α levels 10 h after infection compared with vehicle-treated mice, although differences were less pronounced at 12 and 24 h (Fig. 3A). We did not observe significant amounts of IL-12p40 in the sera of infected mice (data not shown). This temporal pattern of IFN-α inhibition is strikingly similar to that seen in mice lacking either TLR7 or MyD88 (21, 23). Barchet et al. (22) showed that early IFN-α production after i.v. VSV infection was dependent on spleen pDCs. Taken together with our results (Figs. 2 and 3A), this indicates that Pg impairs pDC-dependent IFN-α responses to VSV but may have less of an effect on later pDC-independent IFN-α responses. Blunting of early pDC responses could explain the permissive effects of DMPA on vaginal HSV-2 infection in mice, where rapid (<12 h) TLR-dependent production of IFN-α by mucosal pDCs activates local host defenses to prevent viral dissemination (24).

The same mechanism may explain why DMPA increases the risk of HIV and HSV-2 acquisition in women and also enhances vaginal SIV transmission in nonhuman primates. Because HSV-2 infection is a risk factor for HIV acquisition (3), it is reasonable to hypothesize that the DMPA-associated risk for infection with these viruses is interrelated.

In nonhuman primates, DMPA treatment abrogates the generation of protective immunity to experimental HIV vaccines (7) and cellular responses to simian/human immunodeficiency virus (SHIV) infection (25). Therefore, we examined the effects of DMPA treatment on virus-induced APC activation. In uninfected mice, DMPA treatment had no effect on CD86 expression on CD11c+ spleen dendritic cells (DCs) (data not shown).

However, DMPA treatment inhibited CD86 up-regulation on these cells at 10 and 24 h after VSV infection (Fig. 3B). Similar effects were observed with the up-regulation of CCR7 and MHC II on spleen CD11c+ DCs and with CD69 expression on spleen CD3+ T cells, but these effects were less consistent (data not shown). Although it is unclear whether these observations represent direct or indirect hormone effects, inhibition of IFN-α-mediated APC and T cell activation represents a potential mechanism by which Pg can impair protective antiviral immunity and responses to vaccines relying on TLR ligand adjuvants.

**FIGURE 2.** Treatment of mice with DMPA impairs CpG-induced IFN-α production by pDCs ex vivo. A, CpG- and poly(I:C)-induced IFN-α and IL-12p40 production by total splenocytes from vehicle- and DMPA-treated mice. B, CpG- and poly(I:C)-induced IFN-α production by total splenocytes depleted of mPDCA1+ pDCs (pDC−). C, CpG-induced IFN-α production by purified spleen pDCs from vehicle- and DMPA-treated mice. All in vitro stimulations were for 24 h. Data show mean ± SD of duplicate determinations and are representative of 2–3 independent experiments. *, *p < 0.05; **, *p < 0.01, paired t test.

**FIGURE 3.** DMPA impairs early serum IFN-α responses and APC maturation after VSV infection. A, Serum IFN-α levels after VSV infection in vehicle and DMPA-treated mice. Values represent means ± SE of 3–9 mice per data point. B, CD86 up-regulation on spleen CD11c+ DCs after VSV infection. Data points represent mean ± SE of fold increase MFI CD86 surface staining compared with uninfected controls (3–6 mice per data point). *, *p < 0.05, paired t test.
DMPA by immunoblot. pDCs exposed to virus-induced IFN-α in spleen. This suggests that the suppressive effects of DMPA on IFN-α production may depend on the route of infection.

In summary, the female sex steroid hormone Pg inhibits CpG- and virus-induced IFN-α production by pDCs. This provides a potential mechanism for how DMPA birth control puts millions of women at risk for sexually transmitted HIV and HSV-2 infection. Our findings also indicate that steroid hormones may regulate inflammation and immunity not only at the level of gene transcription, but also by selectively regulating pattern recognition receptor signaling. Thus, Pg may regulate select antimicrobial programs. Finally, regulation of TLR-pathways by Pg may provide insight into the hormonal regulation of autoimmunity and pregnancy.

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References

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