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Decidualized Human Endometrial Stromal Cells Are Sensors of Hormone Withdrawal in the Menstrual Inflammatory Cascade[1]

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ABSTRACT

Menstruation is a complex process dependent on premenstrual release of inflammatory mediators and proteolytic enzymes from endometrial cells. Endometrial leukocytes are traditionally considered to be the major source of the inflammatory factors. However, evidence is emerging to suggest a role for decidualized endometrial stromal cells in the premenstrual inflammatory cascade. We sought to determine if withdrawal of hormone support (estrogen and progesterone) from decidualized endometrial stromal cells, in a model mimicking the precise timing leading to menstruation, activated inflammatory signaling pathways and downstream release of inflammatory mediators. Human endometrial stromal cells decidualized gradually over 12 days of estradiol and progestin treatment as evidenced by an increase in prolactin secretion. Withdrawal of hormone support from decidualized stromal cells resulted in a decrease in cytoplasmic IkappaB and a progressive increase in nuclear accumulation of NF-kappaB, as demonstrated by Western immunoblot and immunocytochemical analyses. Concomitant with nuclear translocation of NF-kappaB, hormone withdrawal led to production of a host of inflammatory mediators by the decidualized stromal cells, including IFN-alpha, IL-6, CCL11, GM-CSF, CCL2, IL1-RA, CXCL10, CXCL8, IL-12, IL-15, VEGF, and CCL5. Elevation of inflammatory mediators was not observed, however, upon hormone withdrawal in cells treated with the NF-kappaB inhibitor BAY 11–7085. Decidualized stromal cells are likely highly sensitive sensors of changing hormone levels. This provides a mechanism by which decidualized stromal cells may recruit inflammatory leukocytes into the premenstrual endometrium and contribute to the intense inflammation underlying this unique physiological process.

cytokines, decidualization, endometriosis, inflammation, menstruation, NF-κB, signal transduction

INTRODUCTION

Menstruation is a physiological process that occurs in very few species [1], being at its most extreme in humans. Therefore, the study of the complex array of inflammatory and proteolytic interactions involved in this self-programmed tissue destruction is inherently difficult. It is clear, however, that withdrawal of hormone support, namely the rapid decline in progesterone and estrogen late in the menstrual cycle, acts as a master regulator of the destruction cascades controlling menstruation. Elegant studies in the rhesus macaque demonstrate that adding back progesterone after its initial withdrawal can stop frank menses only until a threshold of inflammatory events is achieved [2]. After this critical point, menses cannot be avoided. Thus, the continued presence of progesterone, which has known anti-inflammatory properties [3–6], clearly inhibits excessive inflammatory events in the endometrium.

The cellular source of the inflammatory factors associated with menstruation is still unclear. Just prior to menses, the endometrium has a complex composition, including a highly secretory epithelium, terminally differentiated (decidualized) stromal cells and a large number of leukocytes (neutrophils, macrophages, eosinophils, basophils, and uterine natural killer [uNK] cells) [7, 8]. Many of the inflammatory factors that contribute to menstruation are assumed to derive from these leukocytes, which become highly activated around menses. However, evidence is emerging to suggest that the decidualized stromal cells also produce inflammatory factors during the immediate premenstrual phase that may play a profound role in recruitment of leukocytes into the tissue and in menstruation.

The NF-kappa (κ)B signaling pathway is a major regulator of inflammatory responses in many cell types. Under basal conditions the two NF-κB subunits, p65 (RelA) and p50, are held within the cytoplasm by IκB. Upon activation by extracellular signals, IκB kinases phosphorylate IκB, leading to ubiquination and rapid destruction by the action of the 26S proteasome. Destruction of the inhibitory IκB-NF-κB complex allows NF-κB to translocate into the nucleus where it recruits coactivators, leading to transduction and translation of inflammatory genes, including, for example, interleukin (IL)-8 and cyclooxygenase (COX)-2. In this context, Sugino et al. [9] demonstrated nuclear NF-κB, COX-2, and prostaglandin F2α production by decidualized stromal cells in culture 11 days after withdrawal of steroid hormone support. We therefore hypothesized that hormone withdrawal leads to progressive activation of inflammatory transcription factors and downstream induction of inflammatory chemokines and cytokines by the decidualized stromal cells within the premenstrual endometrium.

To compare activation of these factors and initiation of inflammation with the expected timing of a normal menstrual cycle, we established a model to closely mimic this timing. Primary cultures of human endometrial stromal cells were decidualized with estradiol-17β (E2) and medroxyprogesterone acetate (MPA) for 12 days followed by a further 4 days of either maintained hormones or withdrawal of hormones. NF-κB and IκB localization and subsequent production of inflammatory factors were examined across this time course. To determine if NF-κB signaling is important in the production of inflammatory factors, cells were treated with the inhibitor of NF-κB nuclear translocation, BAY 11–7085. We demonstrate progressive nuclear accumulation of NF-κB and reduction in

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cytoplasmic IκB following hormone withdrawal accompanied by differential elevation of certain inflammatory chemokines and cytokines highly relevant to menstruation. Inflammatory factors were not elevated after hormone withdrawal upon incubation with BAY 11–7085. This study therefore supports the contention that the decidualized endometrial stroma is a major source of the inflammatory factors that drive menstruation and are the initial sensor of hormone withdrawal.

MATERIALS AND METHODS

Ethics Statement

Ethical approval was obtained from Institutional Ethics Committees at Southern Health and Monash Surgical Private Hospital for all the tissue collections. Written informed consent was obtained from all the subjects.

Endometrial Tissue

Human endometrium was obtained by curettage from normal cycling women following laparoscopic sterilization or assessment of tubal patency. All the women were under 40 yr of age. None of the women had used hormonal treatment in the preceding 3 mo, and none of the women had endometriosis or other uterine pathologies. All the women were determined to have a normal endometrium. Menstrual cycle stage in normal cycling women was determined by patient testimony and confirmed by histological dating. All the tissues used in this study were collected between Days 10 to 14 of regular 28- to 32-day menstrual cycles. Tissues were immediately processed for cell isolation.

Cell Culture

Endometrial stromal cells from five women (n = 11) were isolated from endometrial curettage samples according to standard protocols. Briefly, endometrial tissue was chopped and incubated in PBS (Invitrogen, Mulgrave, VIC, Australia) containing 7.5 international units (IU)/ml collagenase III (Sigma, Sydney, NSW, Australia) and 100 mg/ml DNase I (Worthington, Lakewood, NJ) at 37°C with shaking at 130 rpm for 40 min. Digestion was stopped by addition of excess DMEM/F12 (Invitrogen). Digested samples were sequentially vacuum filtered through 45- and 11-μM filters before collection of the stromal cell pellet by centrifugation. Cells were sequentially seeded in DMEM/F12 media containing 10% charcoal-stripped fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Sigma) into sterile cell culture flasks for 25 min to allow cell attachment before removal of the blood contamination. Cells were allowed to grow for 4 days before seeding into 9×3 cm dishes and then allowed to settle and achieve confluency for 2 days. Stromal cells were visually checked for the presence of contaminating epithelial cells and discarded if such cells were present. Decidualization (n = 11 cell preparations from different women) was then performed in all the dishes using DMEM/F12 media containing 2% charcoal-stripped fetal calf serum/1% penicillin/streptomycin and the decidualization stimulus—10^{-8} M E2 (Sigma) and 10^{-7} M MPA (Sigma)—with media change every 2 days for 12 days. Photographs of cellular morphology were taken at Days 2, 9, and 12 of decidualization and Day 2 of hormone withdrawal. One dish of cells was terminated on Day 12 of decidualization (designated Day 0 of withdrawal) by removal of media and lysis of cells for protein isolation (10 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl, 0.1% Triton X-100, plus protease and phosphatase inhibitors). Four of the remaining plates were maintained in the continued presence of hormones, while hormones were withdrawn from the remaining four plates (n = 5 different patient samples). Additionally, cells were subjected to the same conditions (hormone maintenance or hormone withdrawal) or treated with 2.5 μM BAY 11–7805 upon hormone withdrawal (n = 6 different patient samples). One decidualization plate, one withdrawal plate, and one withdrawal plate with 2.5 μM BAY 11–7085 was terminated each day for 4 days spanning Days 13–16 of decidualization and Days 1–4 of withdrawal. All the media removed from cells were clarified by centrifugation at 1000 rpm and stored at −20°C before analysis. Additional stromal cells were seeded into chamber slides for immunohistochemistry. After decidualization for 12 days, one slide was terminated and the cells fixed (4% paraformaldehyde, 10 min at −20°C). The remaining chamber slides were subjected to hormone withdrawal and one similarly terminated every 24 h.

Western Immunoblot Analysis

Cell lysates were separated into cytoplasmic and nuclear fractions by low- and high-speed centrifugation. Following centrifugation at 4°C, 5000 rpm for 10 min, the supernatant was retained (cytoplasmic fraction). The remaining pellet was resuspended in 20 μl of nuclear lysis buffer (25% glycerol, 20 mM HEPES, 500 μM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid) and incubated on ice for 30 min with vortexing every 5 min. The lysate was subsequently centrifuged at 4°C, 14 000 rpm for 10 min and the supernatant retained as the nuclear fraction.

The cytoplasmic fraction (10 μg) was separated on 12.5% acrylamide gels and transferred to polyvinylidene difluoride membranes (Amersham, Rydalmere, VIC, Australia). Immunoblots were blocked in 5% skim milk/Tris-buffered saline/0.2% Tween 20 (Sigma) before overnight incubation in goat anti-IκB (1:500 dilution; Santa Cruz, Sapphire Bioscience, Waterloo, NSW, Australia) at 4°C, followed by washing in Tris-buffered saline (0.1 M Tris, 150 mM NaCl; pH 7.2)/0.2% Tween 20, incubation with horse anti-goat peroxidase antibody (1:200; Dako, Campbellfield, VIC, Australia) and visualization of protein using ECL (Pierce, Thermofisher, Scoresby, VIC, Australia) and Chemidoc (BioRad, Gladesville, NSW, Australia). Immunoblots were then stripped of bound antibody, probed with peroxidase-labeled GAPDH (1:10 000; Cell Signalling, Genesearch PTY) to control for loading, and proteins visualized as described above. Protein intensity was determined by densitometry (Image Labs, BioRad), and the IκB normalized against GAPDH. Data are presented as relative expression (n = 7).

Nuclear lysates (the entire isolated 20 μl) was separated on 10% acrylamide gels, transferred to polyvinylidene difluoride, blocked as above, and then incubated overnight in goat anti-NF-κB (1:250; Santa Cruz) at 4°C. Immunoblots were washed, incubated in peroxidase-labeled horse anti-goat antibody (1:200; Dako), and visualized as described above. Blots were then stripped of bound antibody, probed for Lamin A/C (1:2000; Cell Signalling) to control for loading, and the protein was again visualized. Protein intensity was determined by densitometry, and NF-κB was normalized against Lamin A/C. The data are presented as relative expression (n = 5 withdrawal, n = 3 withdrawal with BAY 11–7085).

Immunocytochemistry

Fixed cells were permeabilized with Triton X-100 prior to immunocytochemical staining for NF-κB. Briefly, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 10 min. Cells were washed in PBS and blocked in 10% horse serum, 2% human serum, 0.1% fish skin gelatin, and 0.05% Tween 20 in PBS for 30 min at room temperature followed by incubation with goat anti-NF κB (1:100; Santa Cruz) or an equivalent concentration of goat IgG (Dako) overnight at 4°C. Cells were thoroughly washed with PBS and subsequently incubated with biotinylated horse anti-goat antibody (1:200; Dako) for 1 h at room temperature. Cells were washed again, incubated with Alexa Fluor streptavidin 568 (1:200; Molecular Probes, Invitrogen) for 2 h at room temperature and mounted in Vectashield containing the nuclear counterstain 4′,6-diamidino-2-phenylindole (Dako). Slides were stored in the dark at 4°C until visualization.

Prolactin Assay

Prolactin (PRL) assays were performed at Southern Health pathology using the access/DXI PRL assay, which is a simultaneous one-step immunoenzymatic (sandwich) assay carried out on a Beckman Coulter Unicel DXI 800. Briefly, the culture media sample was added to a reaction vessel along with polyclonal goat anti-PRL alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-PRL antibody. The sample PRL binds to the monoclonal anti-PRL on the solid phase, while the goat anti-PRL-alkaline phosphatase conjugate reacts with a different antigenic site on the cell culture PRL. After incubation in a reaction vessel, the sample is subjected to separation in a magnetic field and washing to remove materials not bound to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, is added to the reaction vessel, and light generated by the reaction is measured with a luminometer. The light production is directly proportional to the concentration of PRL in the sample. The amount of analyte in the sample is determined from a stored, multipoint calibration curve. The analytical range of the assay is from 5.3 to 4240 mIU/L.

Luminex Multiplex Assay

Media from hormone-maintained and hormone-withdrawn cell cultures was concentrated 10-fold using 3-kDa molecular weight cut-off filters (Millipore, Kilsyth, VIC, Australia), based on pilot studies to determine that levels of factors fell within the working range of the assay. The concentration factor was accounted for in the final calculations. The chemokine, cytokine, and growth factor composition of the media was determined using a human cytokine 30-plex panel (Invitrogen) to examine vascular endothelial growth factor (VEGF),

interleukin (IL)-1β, granulocyte colony stimulating factor (G-CSF), epidermal growth factor (EGF), IL-10, hepatocyte growth factor (HGF), fibroblast growth factor (FGF)-2, interferon (IFN)-α, IL-6, IL-12, CCL5 (regulated and normal T cell expressed and secreted [RANTES]), CCL11 (Eotaxin), IL-13, IL-15, IL-17, CCL3 (macrophage inflammatory protein [MIP]-1α), granulocyte macrophage colony stimulating factor (GM-CSF), CCL4 (macrophage inflammatory protein [MIP]-1β), CCL2 (monocyte chemotactic protein [MCP]-1), IL-5, IFN-γ, tumor necrosis factor (TNF)-α, IL1-receptor antagonist (RA), IL-2, IL-7, CXCL10 (IP-10), IL-2 receptor (R), CXCL9 (monokine induced by gamma interferon [MIG]), IL-4, and CXCL8 (IL-8). The 96-well filter plate was hydrated with wash buffer (Invitrogen 30-plex panel, LHC6003, used according to manufacturer's instructions) prior to initiation of the assay. The human cytokine 30-plex antibody bead solution was vortexed and sonicated to break down bead aggregates prior to addition to plates. The beads were thoroughly washed before addition of the standards and samples, followed by incubation for 2 h at room temperature with agitation at 500 rpm. Liquid was then aspirated from the wells, and the beads were washed and incubated with the human 30-plex biotinylated antibody followed by incubation at room temperature for 1 h at 500 rpm. Bead-antibody complexes were again thoroughly washed and detected with Streptavidin-RPE. Bead regions were assigned to each analyte with 100 events/bead region counted and analyzed using a Luminex 200 instrument. Data indicating detection limits for each factor within this kit are provided in Table 1. All the samples for comparison were tested within a single run to negate interassay variability.

Statistics

Statistical analyses were performed using Statpad Prism. A *t*-test was applied for the assessment of significance. For multiple comparisons, ANOVA with a post hoc test (Newman-Keuls) was applied. $P < 0.05$ was taken as significant.

TABLE 1. Limits of detection in multiplex assay. The limits of detection for factors examined by multiplex assays are specific to each assay. The limits of detection for each factor examined on the 30-plex assay are presented.

Factor	Maximum (pg/ml)	Minimum (pg/ml)
VEGF	415	<1
IL-1β	8200	<5
G-CSF	3590	<5
EGF	5850	<1
IL-10	19 700	<1
HGF	1700	<5
FGF-2	3500	<10
IFN-α	13 800	<10
IL-6	5150	<1
IL-12	13 400	<5
RANTES/CCL5	6600	<5
Eotaxin/CCL11	1300	<1
IL-13	21 700	<1
IL-15	26 600	<5
IL-17	25 600	<1
MIP-1α/CCL3	15 720	<5
GM-CSF	19 200	<1
MIP-1β/CCL4	6530	<1
MCP-1/CCL2	22 550	<1
IL-5	5250	<1
IFN-γ	16 200	<1
TNF- α	11 100	<1
IL1-RA	42 260	<5
IL-2	18 400	<1
IL-7	6700	<10
IP-10/CXCL10	240	<1
IL-2R	19 660	<15
MIG/CXCL9	1300	<1
IL-4	49 000	<1
IL-8/CXCL8	11 000	<1

RESULTS

Withdrawal of Steroid Hormone Support from Decidualized Stromal Cells Results in a Decline in PRL Release

To determine whether removal of the hormonal stimulus affected the decidualization status of the cultured cells, cellular morphology and PRL release were assessed progressively through decidualization (up to Day 12) and in the hormone-withdrawn cell cultures 2 days later (Day 2 of withdrawal). In the presence of the decidualization stimulus (10^{-7} M E2 and 10^{-8} M MPA), endometrial stromal cell morphology changed from the characteristic fibroblastlike morphology on Day 2 (Fig. 1Ai) to an enlarged epithelioid morphology by Days 9 and 12 of decidualization (Fig. 1A, ii and iii, respectively). After withdrawal of the hormones, the cells appeared to revert back to a fibroblastlike morphology (Fig. 1Aiv). In the presence of E2 and MPA, the stromal cells progressively produced increasing concentrations of PRL, a proxy indicator of decidualization, from Day 2 (1.16 ± 0.31 mIU/L) to Day 12 (44.8 ± 7.57 mIU/L) of decidualization (Fig. 1B). On Day 12 of decidualization, hormone treatments were maintained or withdrawn. When the hormones were withdrawn, PRL concentrations on Day 14 (Day 2 of withdrawal) were lower compared with those observed on Day 12 of decidualization (26.16 ± 5.07 mIU/L on Day 2 of withdrawal vs. 44.8 ± 7.57 mIU/L on Day 12; $P < 0.01$) (Fig. 1). Treatment with BAY 11–7085 concurrent with hormone withdrawal resulted in a maintenance of PRL on Day 14, which is Day 2 of withdrawal (45.25 ± 10.9 mIU/L) (Fig. 1).

Withdrawal of Steroid Hormones from Decidualized Stromal Cells Leads to Disappearance of Cytoplasmic IκB and an Increase in Nuclear NF-κB

To determine whether withdrawal of hormones from decidualized endometrial stromal cells affects signaling by the transcription factor NF-κB and its inhibitor IκB, their cellular localization was investigated by Western immunoblot analysis. Withdrawal of hormones resulted in a rapid decline in cytoplasmic IκB protein, with some decline noted on Day 1 (Fig. 2A, lane 2, not significant) and a significant decline observed from Days 2 to 4 (*$P < 0.05$; Fig. 2A, lanes 3–5).

The withdrawal of hormones resulted in quantifiable nuclear accumulation of NF-κB from Day 2 of withdrawal (*$P < 0.05$; Fig. 3A lane 3) to day 4 of withdrawal ($P = 0.08$; Fig. 3A, lane 5). Immunocytochemical analysis reinforced the Western immunoblot data, with progressive translocation of NF-κB from their cytoplasmic localization on Day 0 of withdrawal (white arrowhead, Fig. 3Ci) to a nuclear localization on Day 2 of withdrawal (white arrowhead, Fig. 3Ciii). Nuclear translocation of NF-κB was also clear on Day 1 of hormone withdrawal (white arrowheads, Fig. 3Cii); this translocation in a small number of cells was not detected by Western immunoblot analysis.

Treatment of Decidualized Stromal Cells with NF-κB Inhibitor BAY 11–7085 Concurrent with Steroid Hormone Withdrawal Prevents Nuclear Translocation of NF-κB

Withdrawal of steroid hormones resulted in nuclear accumulation of NF-κB as demonstrated by Western immunoblot (Fig. 3, A and B) and immunohistochemical (Fig. 3C) analyses. Incubation of decidualized stromal cells with 2.5 μM BAY 11–7085 upon withdrawal of the hormones prevented the translocation of NF-κB to the nucleus (Fig. 3, D and E; *$P <$ 0.05).

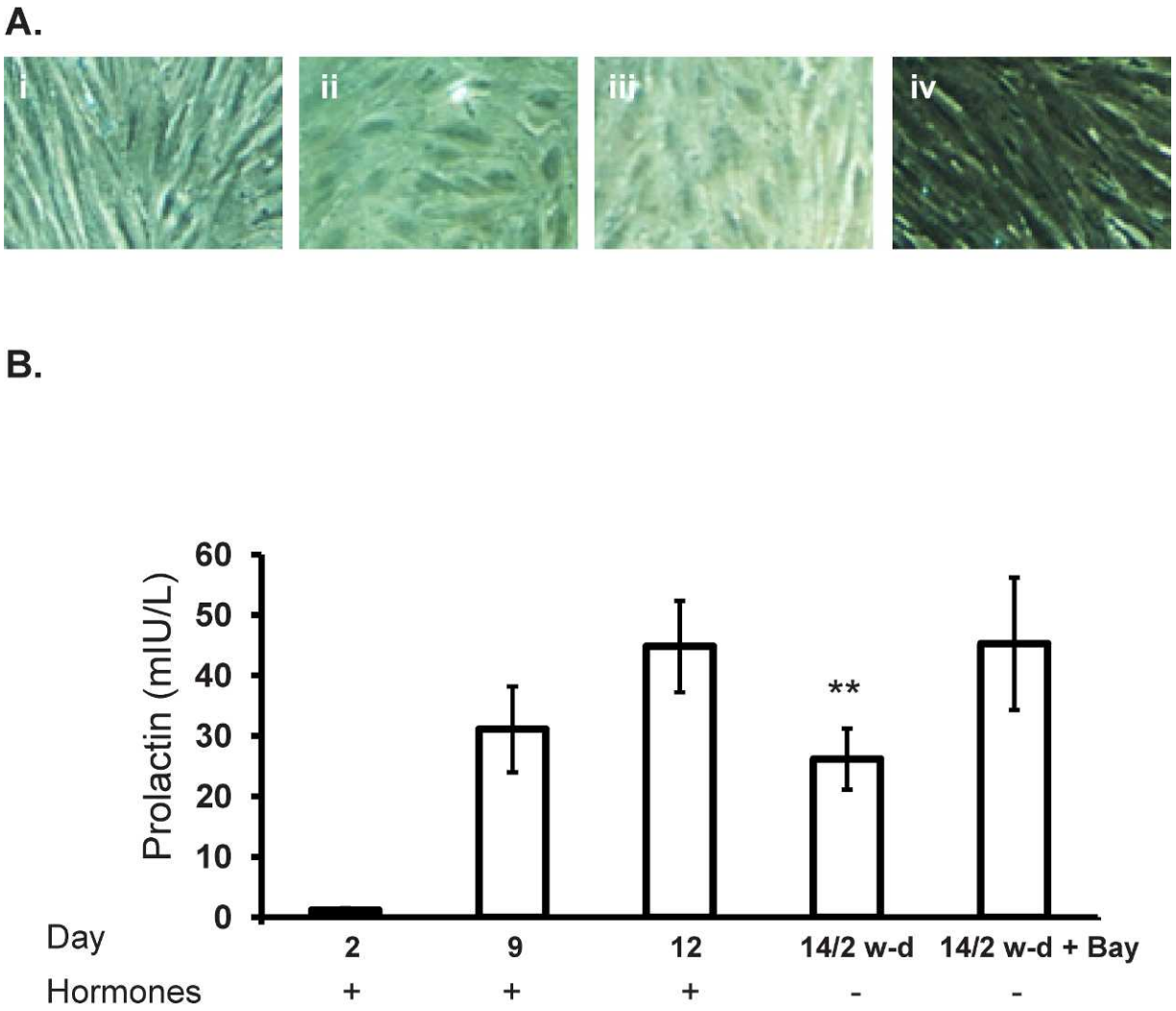

FIG. 1. Withdrawal of estrogen and progesterone leads to a reduction in PRL secretion. Incubation with 10^{-8} M E2 and 10^{-7} M medroxyprogesterone acetate (MPA), designated as hormones, resulted in an alteration in stromal cell morphology (**Ai–iii**, ×10 magnification) and a progressive increase in PRL secretion (**B**) from human endometrial stromal cells from Day 2 to Day 12 of hormone treatment concomitant with the appearance of morphological features of decidualization. However, withdrawal of hormones resulted in an alteration in cellular morphology (**Aiv**, ×10 magnification) and decreased PRL secretion on Day 14 ($P < 0.01$). Incubation with BAY 11–7085 upon hormone withdrawal maintained PRL secretion on Day 14. Data is presented as mean prolactin secretion (mIU/L) ± SEM of n = 11 individual experiments (n = 6 experiments using BAY 11–7085) using stromal cell preparations from different women.

Steroid Hormone Withdrawal Results in Release of Inflammatory Mediators

To determine if activation of inflammatory signaling mediated the transcription, translation, and secretion of downstream inflammatory factors, multiplex analysis of media collected from hormone-maintained and hormone-withdrawn cell cultures was performed. Maintenance of steroid hormones after Day 12 of decidualization mediated a steady state of inflammatory chemokine and cytokine production, with no significant difference between Day 12 of decidualization and Day 0 withdrawal (Fig. 4, white bars).

Withdrawal of steroid hormone support resulted in a selective increase in secretion of certain inflammatory chemokines and cytokines, which did not occur in cells that were maintained in the presence of hormones. The data for levels of individual factors that demonstrated changes from Days 0 to 4 of hormone withdrawal is shown in Figure 4.

A number of inflammatory factors showed increases from Day 1 to Day 3 after hormone withdrawal, including IFN-α, IL-6, CCL11, GM-CSF, CCL2, IL1-RA, CXCL10, and CXCL8 (Fig. 4, B, C, F, H, I, J, K, and L, respectively, gray bars). Other factors, including IL-12 and IL-15, appeared to exhibit a biphasic response with elevation on Days 1 and 3, while others, such as VEGF and CCL5, were elevated on one day only (Fig. 4, A, D, E, and G, respectively, gray bars).

This increased chemokine and cytokine secretion response did not appear to be due to a general inflammatory response to hormone withdrawal because IL-1β, G-CSF, FGF-2, CCL4, IL-7, and IL-2R were detected in all the samples but were not changed by hormone withdrawal (data not shown). Other chemokines and cytokines, namely, EGF, IL-10, IL-13, IL-17, CCL3, IL-5, IFN- γ, TNF-α, CXCL9, and IL-2, were below the detection limit of the assay.

Decidualized stromal cells that were treated with 2.5μM BAY 11–7085 upon hormone withdrawal did not exhibit elevation of chemokines, including IFN α, IL-6, IL-12, GM-CSF, CCL2, CXCL10, and CXCL8 (Fig. 5). This suppression after hormone withdrawal was not observed for CCL5, CCL11, or IL-1R α (data not shown).

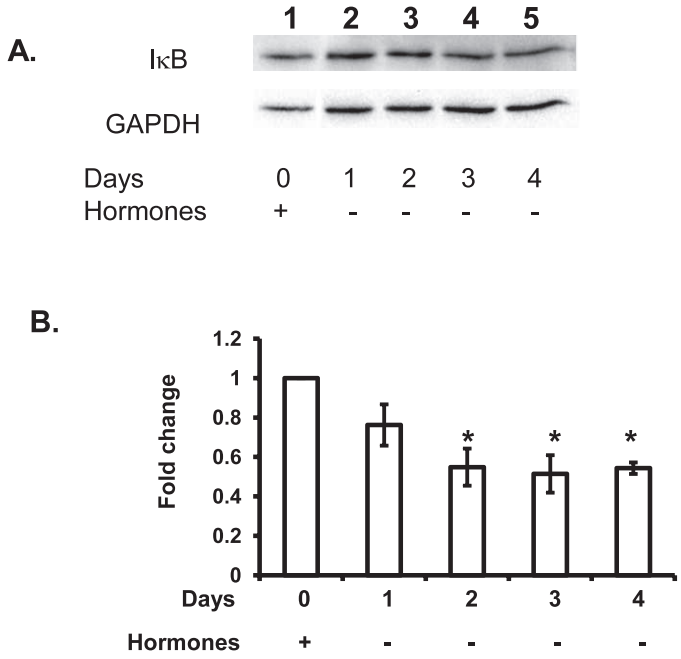

FIG. 2. Hormone withdrawal results in a decrease in cytoplasmic IκB. Withdrawal of 10^{-8} M E2 and 10^{-7} M MPA after Day 12 (**A**, lane 1) resulted in a slight reduction in cytoplasmic IκB on Day 1 of hormone withdrawal (**A**, lane 2) that was significantly reduced by Day 2 of withdrawal and maintained to Day 4 of hormone withdrawal (**A**, lanes 3–5, $*P < 0.05$). **B**) Data is representative of n = 7 individual experiments using stromal cell preparations from different women, presented as relative expression ± SEM. Statistics performed on normalized densitometry values.

DISCUSSION

Menstruation is a highly regulated inflammatory process, requiring local release of inflammatory mediators, influx and activation of leukocytes, and release and activation of matrix metalloproteinases (MMPs) and other degradative enzymes that lead to tissue breakdown. It is induced by the rapid fall in steroid hormones following the demise of the corpus luteum in a nonconception cycle. This study clearly demonstrates for the first time that withdrawal of steroid hormone support from decidualized endometrial stromal cells leads to activation of the inflammatory NF-κB-signaling pathway and selective stimulation of a host of inflammatory mediators that likely play important roles in the initiation and completion of menstruation.

Withdrawal of steroid hormones from decidualized endometrial stromal cells is known to decrease mechanisms that protect against damage by reactive oxygen species (ROS) [10] and to elevate the inflammatory signals COX-2 and prostaglandin F2α [9], thus contributing to the intense inflammation observed at menses. However, there are a large number of other inflammatory factors proposed to play a role in menses [7], whose cellular origin was unclear prior to this study. Certainly, the leukocytes that provide up to 40% of the total cell content of the premenstrual endometrium [11] are a rich source of inflammatory factors. But how the sudden influx of inflammatory cells is regulated is not well understood although it is clear that specific chemokines are needed for their recruitment, particularly in regions of the tissue close to blood vessels, which is where decidualization first occurs. Decidualized stromal cells produce many inflammatory cytokines and chemokines [12], but whether these are altered following loss of hormonal support, thus enhancing the inflammatory cascade associated with menstruation, was not known [7]. It is increasingly appreciated in other tissues that the stromal environment can establish important homing signals that tightly control the leukocyte subsets recruited to inflamed tissues [13], potentially reflected here in the controlled inflammation associated with menses. The endometrial epithelium is a site of intense inflammatory mediator secretion, and it is thus also likely that inflammatory mediators produced by the stroma signal in a paracrine manner to the epithelium to enhance chemokine and cytokine production in this compartment and exacerbate the inflammatory milieu at this time.

Models for appropriate study of menstruation are very limited [14]. This study was carefully designed to be physiologically relevant by application of a cell culture model that closely mimics the natural menstrual cycle. Stromal cells decidualize slowly as estrogen and progesterone levels rise throughout the early-mid secretory phase of the cycle. In a nonconception cycle, hormone levels then fall rapidly during the late secretory phase, resulting in menstruation. Our model thus provided steroid treatment to the stromal cells for 12 days, (slowly inducing decidualization as observed in vivo) followed by 4 days of hormone deprivation. The decrease in PRL production and alteration in cellular morphology just 2 days following hormone withdrawal, versus continually increasing PRL production with hormone maintenance, clearly demonstrates a response to removal of steroid support; PRL production is dependent on continued progestin.

After withdrawal of steroid hormone support, there was rapid loss of cytoplasmic IκB (within 24 h) and accumulation of NF-κB (within 48 h) in the nuclear compartment of decidualized stromal cells. Progressive translocation of NF-κB from the cytoplasm, where it is held captive by IκB under the influence of steroid hormones [6], to a nuclear localization was clearly visualized here by immunocytochemistry and extends published studies using later time points [9]. Interestingly, immunocytochemistry demonstrated nuclear translocation of NF-κB at early time points (Day1), which could not be detected by more global methods such as Western immunoblot analysis, which clearly requires large changes. Thus, the NF-κB pathway is rapidly released from inhibition following estrogen and progesterone withdrawal. Progesterone has been proposed to inhibit NF-κB-mediated inflammation by a number of mechanisms, including enhancing IκB expression and competing for binding sites on NF-κB target genes [2, 4–6]. Following progesterone removal, ROS are rapidly induced along with a decrease in protective mechanisms such as superoxide dismutases [9, 15]. The altered redox state caused by ROS activates the NF-κB pathway [16]. It may therefore be assumed that the initial increase in nuclear NF-κB after withdrawal of hormones follows an increase in ROS and release from IκB inhibition. The present study shows that while IκB decreases by Day 2 after withdrawal, it does not significantly decrease further. Therefore, continuing generation of NF-κB likely results from the action of the many inflammatory factors induced by hormone withdrawal that mediate a positive feedback loop for their own induction [16–18].

Multiplex analysis provided a powerful tool to further define the downstream inflammatory factors induced by hormone withdrawal of decidualized stromal cells. Of the thirty factors examined, eight increased from Day 1 to Day 3 following withdrawal, mirroring overall the increase in nuclear accumulation of NF-κB. A further four factors were increased but not within the same time frame. The majority of the regulated mediators have demonstrated NF-κB binding sites in their promoter regions; these include VEGF [19], IL6 [20],

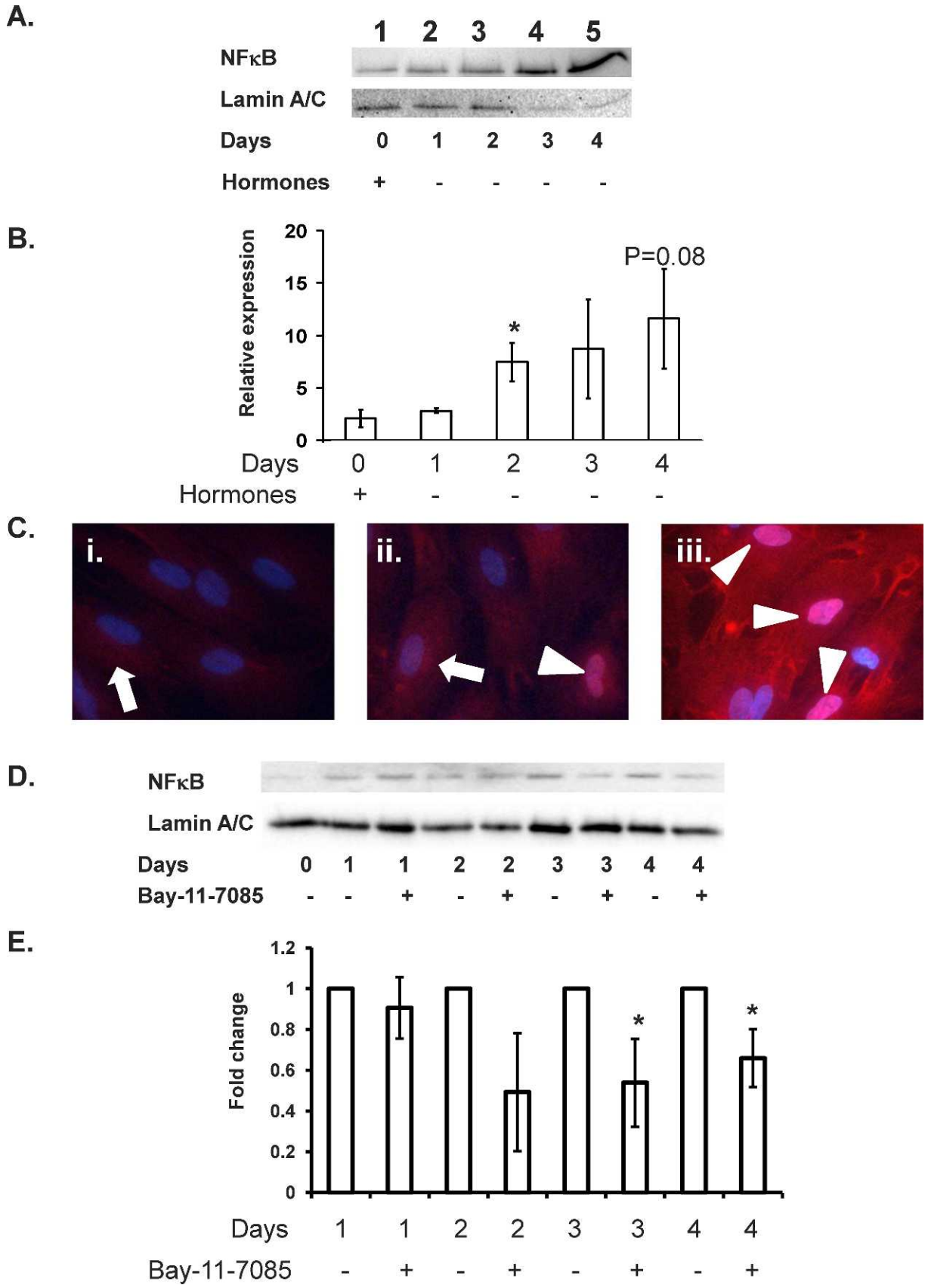

FIG. 3. Withdrawal of hormones from decidualized endometrial stromal cells leads to nuclear accumulation of NF-κB. On Day 12 of decidualization, nuclear NF-κB levels were low (**A**, lane 1), and NF-κB exhibited a cytoplasmic localization (**Ci**, ×40 magnification). Withdrawal of 10^{-8} M E2 and 10^{-7} M MPA mediated nuclear translocation of NF-κB within 24 h (**Cii**, white arrows), with progressive nuclear accumulation of NF-κB from Days 2 to 4 of hormone withdrawal (**A**, lanes 3–5; **B**, no hormone Days 2–4; **Cii** and **iii**, Days 1 and 2, respectively; white arrowheads mark location of NF-κB, x 40 magnification) as detected by Western immunoblot and immunocytochemical analyses. Incubation of hormone withdrawn decidualized stromal cells

TABLE 2. Localization and roles of chemokines and cytokines elevated after withdrawal of hormones from decidualized endometrial stromal cells.

Chemokine/cytokine	Localization/roles
IL-6	Role in acute inflammation [59]
	Elevated in glandular epithelium and stroma in late secretory phase [60]
CCL2	Localized to endometrial stroma [61]
	Recruits monocytes, macrophages, and dendritic cells into tissues [62]
	Regulates expression and activity of MT1MMP and MMP1 [63, 64]
CXCL10	Activates CXCR3 on NK cells, dendritic cells, and macrophages with homing functions to attract cells expressing this receptor [65–67]
	Elevated in peri-blood vessel stromal cells premenstrually [68]
GM-CSF	Localizes to and produced by endometrial epithelial cells [37, 38]
CXCL8	Mediator of neutrophil recruitment [69–71]
	Elevated in premenstrual endometrium close to blood vessels [12, 72, 73]
	Acts via CXCR2 to activate neutrophils to release cytotoxic contents and degradative enzymes, including MMP9 [39, 40]
	Truncated by MMP9, resulting in increased potency and enhanced chemotactic potential [41]
CCL11	Recruits eosinophils into tissues
	Elevated in endometrium during late secretory-menstrual phase [12, 42]
IL-12	Inhibits endometrial glandular epithelial and stromal cell survival [48, 49]
IL-15	Elevated peri-vascularly in premenstrual endometrium [50, 51]
	Influences recruitment, proliferation, and activation of uNK cells [74–77]
	Increases human uNK cell cytotoxicity [78, 79]

CCL11 [21], IL15 [22], GM-CSF [23, 24], MCP1 (CCL2) [25], IL1-Ra [26], IP10 (CXCL10) [27], and IL8 (CXCL8) [28]. Because several of the factors, such as FGF-2 and IL-7, which did not change upon hormone withdrawal, do not have NF-κB binding sites [29], we did not therefore expect any change in their levels in this system. We subsequently demonstrated by treatment of decidualized stromal cells with the NF-κb inhibitor BAY 11–7085 that elevation of key chemokines and cytokines, including IL-6, GM-CSF, CCL2, CXCL10, and CXCL8, were dependent on nuclear translocation of NF-κB. The majority of the regulated factors mediate recruitment and activation of the leukocytes, particularly macrophages, neutrophils, uNK cells, and eosinophils, which release a range of enzymes that mediate tissue destruction at menses [12]. Thus, we propose that decidualized stroma is a finely tuned sensor of hormonal stimuli. Importantly, it has been extensively demonstrated that chemokines and cytokines can activate endometrial MMPs [30–33] that mediate extracellular matrix turnover and hence decidual regression and tissue breakdown at menstruation. It is proposed that this local level of protease activity regulation within the endometrium by chemokines and cytokines accounts for the lack of direct inhibition of endometrial MMP expression in users of progestin-only contraceptives [34, 35]. Hence, local production of chemokines and cytokines by endometrial stromal cells after steroid hormone withdrawal elevate MMP expression and activity at the time of menstrual breakdown.

Roles for the factors stimulated in decidualized stromal cells in response to the rapidly falling estrogen and progesterone levels in the late secretory phase, in the events leading to menstruation, are strongly supported by published data that is summarized in Table 2. They will be further discussed in terms of their regulation after hormone withdrawal.

IL-6 is well known for its role in acute inflammation. A previous study found that hormone withdrawal does not alter IL-6 secretion by endometrial stromal cells [36]. However, the stromal cells in the previous study were exposed to estrogen and progesterone for only 5 days rather than the 12 days exposure in the current study, suggesting that the stromal cells must be well decidualized before an effect of hormone withdrawal can be observed.

Epithelial cells are stated to produce more GM-CSF in culture than stromal cells [37, 38]. However, its production by decidualized cells and under menstrual mimicking conditions have not been examined.

CXCL8 is elevated in the endometrium premenstrually, and after progesterone withdrawal, CXCL8, acting via CXCR2, activates neutrophils to release their cytotoxic contents and degradative enzymes, including MMP9 from intracellular granules [39, 40]. Neutrophil MMP9 truncates CXCL8 from CXCL8 (1–77) to CXCL8 (7–77) resulting in up to 27-fold higher potency in neutrophil activation, thus increasing the chemotactic gradient for further recruitment [41]. CXCL8 and neutrophils in concert thus initiate a positive feedback mechanism to enhance tissue inflammation and protease activity contributing to tissue instability and destruction at menses.

During most of the menstrual cycle, CCL11 localizes to the glandular and luminal epithelium, but it localizes to the stroma in the late secretory phase [42, 43]. It is likely that CCL11 production specifically by the decidual cells (that lie close to the vasculature) recruits eosinophils into the peri-menstrual tissue. Upon activation, eosinophils release intracellular granules containing eosinophil cationic proteins 1 and 2, which are seen extracellularly in endometrial tissue immediately prior to menses [44]. These granules also contain other cytotoxins and MMP3, MMP2, and MMP13 [45–47].

IL-12 inhibits endometrial glandular epithelial and stromal cell survival, which may be its mechanism of action in the lead up to menstruation [48, 49]. IL-15 is elevated peri-vascularly in secretory phase premenstrual endometrium [50, 51]. Progesterone withdrawal, presumably acting via IL15, results in uNK cell release of perforin [52].

with 2.5 μM BAY 11–7085 prevented nuclear translocation of NF-κB (**D** and **E**; $*P < 0.05$). Data presented as relative expression ± SEM. Statistics performed on normalized densitometry values. Data is representative of n = 5 (**A** and **B**) or n = 3 (**D** and **E**) individual experiments using stromal cell preparations from different women; $*P < 0.05$.

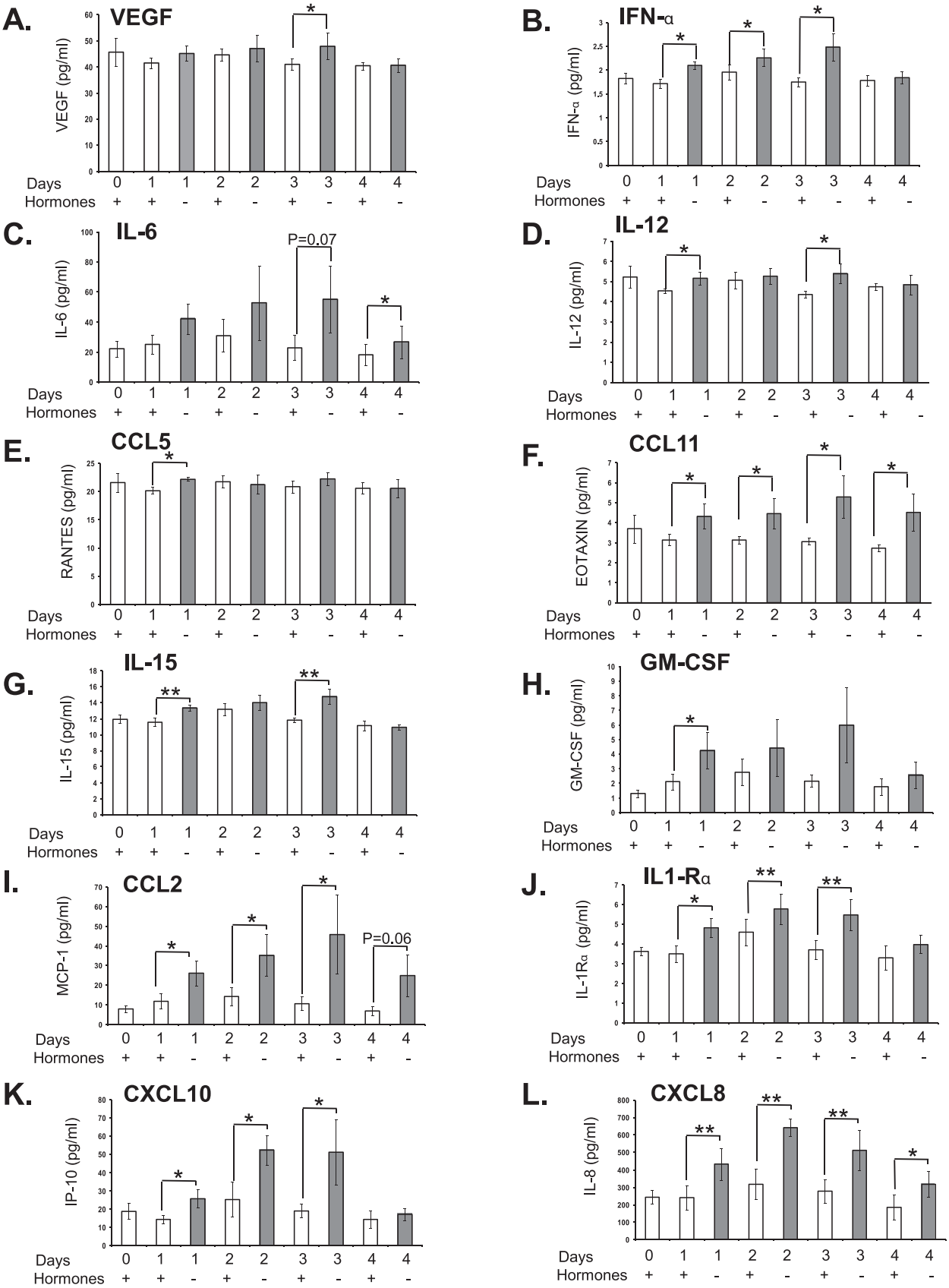

FIG. 4. Inflammatory mediators are released from decidualized endometrial stromal cells upon hormone withdrawal. Withdrawal of hormone support (gray bars) from decidualized endometrial stromal cells mediated production of VEGF (**A**), IFN-α (**B**), IL-6 (**C**), IL-12 (**D**), CCL5 (**E**), CCL11 (**F**), IL-15 (**G**), GM-CSF (**H**), CCL2 (**I**), IL1-RA (**J**), CXCL10 (**K**), and CXCL8 (**L**). Data are presented as mean ± SEM; $*P < 0.05$, $**P < 0.01$. Data is representative of n = 5 individual experiments using stromal cell preparations from different women. White bars indicate hormones maintained.

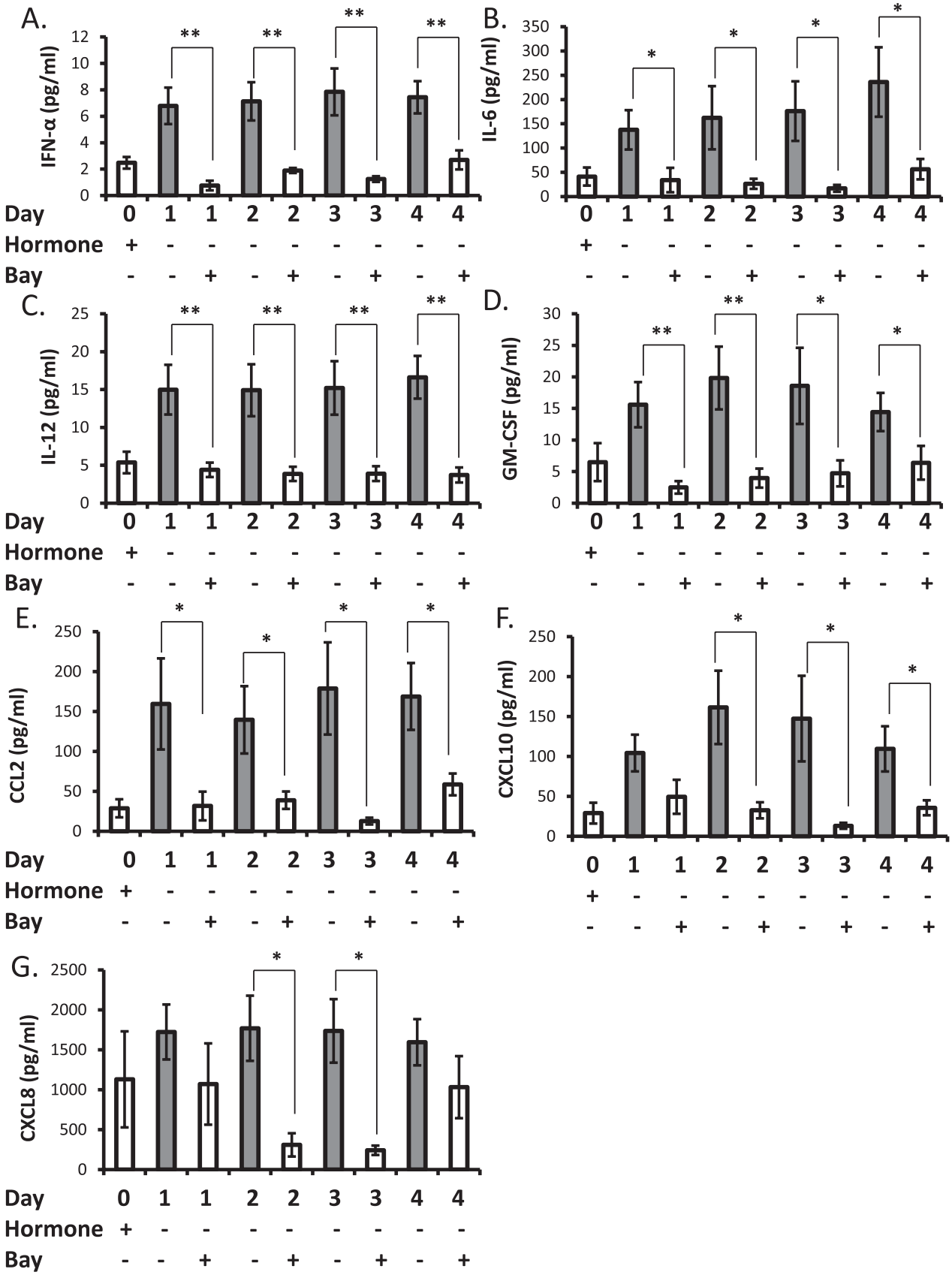

FIG. 5. Inhibition of NF-κB nuclear translocation inhibits elevation of inflammatory factors after withdrawal of hormone support from decidualized stromal cells. Incubation of decidualized stromal cells with 2.5 μM BAY 11–7085 upon withdrawal of hormone support the prevented production of IFN-α (**A**), IL-6 (**B**), IL-12 (**C**), GM-CSF (**D**), CCL2 (**E**), CXCL10 (**F**), and CXCL8 (**G**). Data are presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$. Data is representative of n = 6 individual experiments using stromal cell preparations from different women.

IL1-RA inhibits IL-1-mediated signaling. Its action may be to limit excessive inflammation because its absence is associated with increased leukocyte recruitment and excessive chemokine expression in a wound-healing model [53]. Given that menses is a normal situation of controlled inflammation, such control of the IL1 system may be one means by which restraint is applied.

The role of VEGF at menstruation is at present unclear. While it is proposed to play a role in postmenstrual endometrial repair [54], its major known role is to mediate blood vessel permeability. Excessive levels of VEGF may therefore result in endothelial leakiness, enhancing migration of inflammatory leukocytes into the tissue [55].

The importance of the inflammatory environment, including chemokines and cytokines and their actions on leukocyte recruitment and activation at menstruation, was reviewed recently [7]. This study has provided a critical missing piece of the jigsaw of events leading to menstruation, namely, the response of decidualized stromal cells to rapidly falling levels of ovarian steroid hormones. It explains to a large extent how the remarkable increase in leukocyte numbers and their activation in the endometrium is controlled, events that then drive the rapid and substantial tissue breakdown of menstruation. Additionally, because the epithelial compartment does not express the progesterone receptor in the secretory phase of the menstrual cycle [56, 57], it is likely that the endometrial stroma is the first sensor of alterations in hormone levels upon corpus luteum demise. Stromally derived inflammatory mediators may signal in a paracrine manner to the adjacent epithelium and endothelium to induce further inflammatory mediators, thus amplifying the menstrual inflammatory cascade. It has been demonstrated in tissue recombination experiments that many endometrial epithelial responses to hormone actions are dependent on activation of the stroma [58]. Understanding the key regulators of menstruation that could be targeted for treatment is important if we are to alleviate menstrual bleeding disorders and the breakthrough bleeding that limits the use of the highly effective long-term contraceptives.

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