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Vitamin D and the Regulation of Placental Inflammation

Nancy Q. Liu,* Amber T. Kaplan,† Venu Lagishetty,* Yuxin B. Ouyang,* Yi Ouyang,‡§ Charles F. Simmons,‡ Ozlem Equils,** and Martin Hewison*,†

The vitamin D-activating enzyme 1α-hydroxylase (CYP27B1) and vitamin D receptor (VDR) support anti-inflammatory responses to vitamin D in many tissues. Given the high basal expression of CYP27B1 and VDR in trophoblastic cells from the placenta, we hypothesized that anti-inflammatory effects of vitamin D may be particularly important in this organ. Pregnant wild type (WT) mice i.p. injected with LPS showed elevated expression of mouse Cyp27b1 (4-fold) and VDR (6-fold). Similar results were also obtained after ex vivo treatment of WT placentas with LPS. To assess the functional impact of this, we carried out ex vivo studies using placentas −/− for fetal (trophoblastic) Cyp27b1 or VDR. Vehicle-treated −/− placentas showed increased expression of IFN-γ and decreased expression of IL-10 relative to +/+ placentas. LPS-treated −/− placentas showed increased expression of TLR2, IFN-γ, and IL-6. Array analyses identified other inflammatory factors that are dysregulated in Cyp27b1−/− versus Cyp27b1+/+ placentas after LPS challenge. Data highlighted enhanced expression of IL-4, IL-15, and IL-18, as well as several chemokines and their receptors, in Cyp27b1−/− placentas. Similar results for IL-6 expression were observed with placentas −/− for trophoblastic VDR. Finally, ex vivo treatment of WT placentas with the substrate for Cyp27b1, 25-hydroxyvitamin D3, suppressed LPS-induced expression of IL-6 and the chemokine Ccl11. These data indicate that fetal (trophoblastic) vitamin D plays a pivotal role in controlling placental inflammation. In humans, this may be a key factor in placental responses to infection and associated adverse outcomes of pregnancy. The Journal of Immunology, 2011, 186: 5968–5974.

Although vitamin D insufficiency is increasingly recognized as a health problem across the world, inadequate vitamin D status appears to be particularly prevalent in certain populations such as the elderly (1) and pregnant women (2–4). With respect to the latter, impaired vitamin D status during gestation is associated with poor skeletal growth in childhood (3), consistent with the classical functions of vitamin D. However, low maternal levels of the major circulating form of vitamin D, 25-hydroxyvitamin D3 (25OHD3), have also been linked to adverse outcomes in pregnancy such as pre-eclampsia, a disorder involving dysregulated placental vascularization that affects up to 10% of pregnancies (5, 6). In this way, the physiological impact of maternal vitamin D status may not be restricted to the endocrine regulation of skeletal homeostasis, but may also extend to more localized events in the placenta.

More than 30 y ago, the placenta was identified as a major site for conversion of 25OHD to the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), with both maternal decidua and fetal trophoblast demonstrating activity of the enzyme 1α-hydroxylase (CYP27B1) (7, 8). However, since then, the precise function of this extrarenal CYP27B1 has remained unclear. Co-expression of the receptor for 1,25(OH)2D3 (vitamin D receptor [VDR]) in both decidua (9) and trophoblast (10) suggests that 1,25(OH)2D3 produced by the placenta may function in an autocrine or paracrine fashion. In support of this, recent studies by our group and others have shown that autocrine metabolism of 25OHD3 to 1,25(OH)2D3 promotes antibacterial and anti-inflammatory responses in maternal decidua (11) and fetal trophoblast (12, 13). Such immunomodulatory actions of vitamin D are likely to be compromised under conditions of low maternal 25OHD3 status, with potentially detrimental consequences for placental physiology. In this study, we have used in vivo and ex vivo mouse models to show that dysregulated vitamin D metabolism (CYP27B1 knockout) or function (VDR knockout) in fetal cells from the placenta promotes aberrant inflammatory responses to immune challenge. Conversely, the major circulating form of vitamin D, 25OHD3, is a potent suppressor of placental inflammation. Collectively, these data suggest that vitamin D plays an important role in controlling fetal–placental immune responses during pregnancy.

Materials and Methods

Animals

Three types of mice were used for breeding in the study: 1) wild type (WT) C57BL/6J mice; 2) C57BL/6J mice heterozygous (+/−) for the mouse CYP27B1 gene (Cyp27b1) (gift from Dr. H.F. DeLuca, University of Wisconsin, WI (14)); and 3) C57BL/6J mice heterozygous (+/−) for the VDR gene (Jackson Laboratories, Sacramento, CA). Breeding and genotyping of mice Cyp27b1+/+, Cyp27b1+/−, and Cyp27b1−/− mice was carried out using previously documented protocols (14). Genotyping of VDR+/+, VDR−/−, and VDR−/− mice was carried out using a protocol provided by the suppliers in which cycling conditions were as follows: 94˚C for 3 min; 35 cycles of 94˚C for 30 s, 61˚C for 1 min, and 72˚C for 1 min; then 72˚C for 10 min. Primers were as follows: oMR0297, 5′-CAGGGACACTAGTACGTGCTG-3′; oMR219, 5′-CCATCATCCCACTGTCCT-3′; and oMR5220 5′-TTCCTGCAAGCGCAGCCCTTT-3′. All animals were maintained on LabDiet 5015 formula chow containing 0.8% calcium and 3.3 IU/g vitamin D3 (Purina, Richmond, IN). All procedures were reviewed and approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee (#1369) and the University of California, Los Angeles, Los Angeles, CA 90095; †Department of Pathology, Cedars-Sinai Medical Center, Los Angeles, CA 90048; ‡Department of Pathology, Veterans Affairs Medical Center, Long Beach, CA 90822; §Department of Pathology and Laboratory Medicine, University of California, Irvine, CA 92697; ††Medical Division, Pfizer, Inc., New York, NY 10017; and ‡‡Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA 90095

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Abbreviations used in this article: 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; 25OHD3, 25-hydroxyvitamin D3; qPCR, quantitative real-time RT-PCR; S-F, serum-free; VDR, vitamin D receptor; WT, wild type.

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of California Los Angeles Office of Protection of Research Subjects (#2009-132-02). Immune challenge of day 14 pregnant mice in vivo was carried out by i.p. injection of 25 μg LPS. Mice were then euthanized 24 h after LPS challenge.

Generation of placentas for ex vivo analysis

Male and female mice were mated using timed pregnancy protocols to generate placentas at identical stages of development. In brief, female mice were introduced to males for 1–2 days, after which the male was removed and the female observed twice daily for another 10 days for a vaginal plug. By maintaining a large cohort of females in this fashion and staggering the introduction of males, it was possible to define the day of conception, thereby minimizing experimental variation in fetal and placental tissues from one pregnancy to another. At gestational day 14, pregnant females were euthanized and dissected. Each placenta was then washed briefly in defined serum-free (S-F) medium (monocyte S-F medium; Invitrogen, Carlsbad, CA) and placed in a single well of a 24-well culture plate containing 1 ml S-F medium for 24 h. For matings involving Cy27b1+/− or VDR+/− mice, placentas were removed in conjunction with paired fetal tissue and the latter used for DNA extraction and genotyping as described earlier.

Immunohistochemistry

Immunohistochemical analysis of Cy27b1 and IL-6 protein expression in paraffin-embedded tissue sections was carried out using methods adapted from previous studies (15). In brief, sections were deparaffinized and hydrated through a series of xylene and graded ethanol washes, and antigen retrieval was carried out in 0.01 M sodium citrate buffer at 95°C for 2 min. The slides were then incubated in 3% methanol-hydrogen peroxide at room temperature for 15 min to quench endogenous peroxidase activity and washed in TBS, pH 7.6. Nonspecific binding was blocked with 1.5% of normal blocking serum in TBS, pH 7.6, and slides were then incubated in 3% methanol-hydrogen peroxide at room temperature for 20 min. Slides were then washed for 15 min in TBS, then incubated for 30 min with blocking serum in TBS, and slides were then incubated with either sheep anti-mouse Cy27b1 polyclonal antibody (1:10,000 dilution; The Binding Site, Birmingham, U.K.) or rabbit antihuman IL-6 polyclonal Ab (1:5000 dilution; Abcam, Cambridge, MA) at 4°C overnight. After a 15-min TBS wash, biotinylated rabbit anti-sheep or goat anti-rabbit IgG (1/200 diluted in 1.5% normal blocking serum/TBS; Vector Laboratories, Burlingame, CA) was added to sections for 60 min. Slides were then washed for 15 min in TBS, then incubated for 30 min with horseradish peroxidase-conjugated streptavidin (Vector Laboratories) and diaminobenzidine tetrahydrochloride was used to visualize protein expression, and Mayer’s hematoxylin was used to counterstain sections. Finally, the slides were dehydrated and mounted with glycerol mounting medium.

Quantitative real-time RT-PCR amplification of candidate gene cDNAs

RNA was extracted from mouse placenta using the RNeasy Total RNA extraction kit as detailed by the manufacturer (Qiagen, Valencia, CA), and 1.5 μg aliquots were used for cDNA synthesis by Superscript III reverse transcriptase (RT) and random primers (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Quantitative real-time RT-PCR (qPCR) was performed using a Stratagene Mx3005P qPCR system (La Jolla, CA), using TaqMan probes and primers (Applied Biosystems, Foster City, CA). Expression of mRNAs for specific mouse target genes was assessed using the following TaqMan mouse gene expression assays: Cy27b1 (Mm01165922_g1), VDR (Mm00437297_m1), Cy24a1 (Mm00487244_m1), IL-1α (Mm00439620_m1), IL-6 (Mm0044191_m1), IL-10 (Mm00439616_m1), IL-17 (Mm00439619_m1), TLR2 (Mm00442346_m1), TLR4 (Mm00445274_m1), IFN-γ (Mm008001788_m1), cathelicidin gene-related peptide (Cramp) (Mm00438285_m1), inducible NO synthase (Mm00440485_m1), Ccl11 (Mm00443238_m1), and GAPDH (4352339E) (ABI). Aliquots (50 ng of cDNA) were amplified under the following conditions: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate, and each target gene expression was normalized to GAPDH mRNA expression. The relative amount of target gene in each sample was estimated using the ΔΔCt method as described previously (16).

PCR array analysis

The RT2 Profiler PCR array for mouse inflammatory cytokines and their receptors (PAMM-01; SuperArray Bioscience (SAB) Corporation, Frederick, MD) was used to compare inflammatory responses to LPS (100 ng/ml, 24 h) in placentas with a Cy27b1+/− or Cy27b1−/− fetal genotype. Total RNA was extracted from placenta tissue by using the TRIzol protocol (Invitrogen). After the ethanol precipitation step, RNA was then further cleaned using SABiosciences’ RT2 qPCR-Grade RNA Isolation Kit. A 1-μg aliquot of total RNA was used to synthesize the first-strand cDNA using the SABiosciences’ RT2 First Strand Kit. The cDNA was mixed with the RT2 SYBR Green/ROX qPCR Master Mix, and 25 μl of the mixture was added to each of the wells containing different primers in each PCR array plate. The plate was run at 95°C for 10 min, 1 cycle; then at 95°C for 15 seconds and 60°C for 1 min, 40 cycles by using Stratagene Instruments Mx3005p. The outcome was normalized against the set of reference genes used for the qPCR. Analysis using the references genes present on the array yielded a comparable outcome.

Statistics

Experimental means were compared statistically using an unpaired Student t test. Where indicated, multifactorial data involving Cy27b1+/− and Cy27b1−/− treatments were compared using one-way ANOVA, with the Holm–Sidak method used as a post hoc multiple-comparison procedure. Statistical analyses were carried out using raw ΔCt values.

Results

Imune challenge in vivo and ex vivo enhances placental expression of Cy27b1 and VDR

i.p. LPS (25 μg, 24 h) injection of 14-d pregnant WT BL/6 mice induced placental expression of mRNA for Cy27b1 (4.3-fold increase relative to vehicle control-injected mice; p < 0.001) and VDR (5.9-fold increase relative to vehicle, p < 0.001) (Fig. 1A). There was no change in the expression of vitamin D catabolic enzyme Cy24a1 (Fig. 1). To confirm these in vivo findings, we next developed an ex vivo model. In this case, 14-d timed pregnancy WT BL/6 mice were euthanized, and placentas were removed carefully and treated with LPS (100 ng/ml, 24 h) in organ culture (Fig. 1B). Consistent with the in vivo data, expression of mRNA for Cy27b1 (2.7-fold, p < 0.01) and VDR (2.9-fold, p < 0.01) increased in LPS-treated organ cultures relative to vehicle-treated placentas. As with the in vivo injection studies, data from cultured placentas showed no change in expression of Cy24a1 after treatment with LPS (Fig. 1B). In addition to stimulating Cy27b1 and VDR, in vivo and ex vivo treatment with LPS enhanced the expression of the pathogen-recognition receptor TLR2 and the cytokines IL-1α (ex vivo), IL-6, and IL-10 (in vivo), confirming LPS induction of an inflammatory response in this organ (Fig. 1C, 1D). Immunohistochemical analyses confirmed the presence of protein for Cy27b1 in WT mouse placentas (Fig. 2A, 2C, 2E), with expression being enhanced in trophoblastic giant cells (Fig. 2B), labyrinth cells (Fig. 2D), and infiltrating inflammatory cells (Fig. 2F) of pregnant mice receiving i.p. injection of LPS. Histological analyses confirmed that injection of LPS was associated with increased infiltration of inflammatory cells (mainly neutrophils and some lymphocytes) within the choric placent and labyrinth regions of the mouse placenta (Supplemental Fig. 1).

Fetal knockout of Cy27b1 alters placental responses to LPS challenge

Male and female mice heterozygous for the Cy27b1 gene (Cy27b1+/−) were bred according to timed pregnancy protocols to generate gestational day 14 placentas with Cy27b1+/−, Cy27b1−/−, and Cy27b1+/− fetal genotypes (Fig. 3). Analysis of total tissue from the resulting placenta confirmed that expression of Cy27b1 mRNA diminished 4- and 5-fold, respectively, in placentas with a Cy27b1+/− or Cy27b1−/− genotype relative to Cy27b1+/+ placentas (Fig. 4A). However, mRNA for the enzyme was still detectable in these placenta because of expression of Cy27b1 in +/- maternal tissues. Treatment of Cy27b1+/−/− placentas with LPS induced expression of the enzyme, but this did not reach the same levels obtained with +/- placentas treated with LPS. This lack of Cy27b1 induction

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after LPS challenge was even more pronounced in −/− placentas, suggesting that the regulation of the enzyme by LPS primarily involves fetal trophoblastic cells. Expression of mRNA for the VDR and Cyp24a1 in Cyp27b1+/− and Cyp27b1−/− placentas was similar to that described for +/+ WT placentas. In the case of Cyp24a1, treatment with LPS had no significant effect on expression of this enzyme in placentas with +/+ or −/− genotypes for Cyp27b1. By contrast, in both Cyp27b1+/− and Cyp27b1−/− placentas, LPS-induced expression of VDR was significantly higher than observed in Cyp27b1+/+ placentas.

Treatment with LPS also induced expression of TLR2, IL-1α, and IL-10 to a similar extent in both Cyp27b1+/− and −/− placentas, when compared with vehicle-treated controls (Fig. 4B). However, only IL-6 showed significantly higher levels of expression in Cyp27b1−/− placentas when compared with LPS-treated +/+ or −/− placentas. In contrast with other cytokines, expression of IL-10 was significantly decreased in Cyp27b1−/− placentas in the absence of LPS challenge. Expression on IFN-γ was also significantly different in Cyp27b1−/− placentas in the absence of LPS challenge. In this case, the cytokine showed 2.8-fold greater levels in vehicle-treated Cyp27b1−/− placentas relative to Cyp27b1+/+ placentas, and this increased further 3.5-fold after treatment with LPS (Fig. 4B). No significant changes in IFN-γ expression were observed for Cyp27b1+/− or Cyp27b1−/− placentas after treatment with LPS. Other candidate inflammatory genes analyzed in this part of the study included IL-17, which was not induced by LPS in either Cyp27b1+/− or Cyp27b1−/− placentas (data not shown).

Elevated expression of IL-6 in LPS-treated Cyp27b1−/− placentas was confirmed by immunohistochemical analysis of IL-6 protein, which showed stronger staining in trophoblastic cells at the boundary with the maternal decidua (Fig. 4C). Samples of mRNA from vehicle- and LPS-treated placental samples were also analyzed using PCR arrays specific for factors associated with inflammation (see complete data in Supplemental Table I). When treated with LPS, Cyp27b1−/− placentas showed abnormally high

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**FIGURE 1.** Immune challenge in vivo or ex vivo enhances mechanisms for placental vitamin D metabolism and function. Expression of mRNA for the VDR, 1α-hydroxylase (Cyp27b1), and 24-hydroxylase (Cyp24a1) (A), and expression of mRNA for TLR2, TLR4, IL-1α, IL-6, and IL-10 (C) in total placentas from 14-d timed pregnant WT BL6 mice 24 h after i.p. injection with 25 μg LPS. Data shown are fold change in gene expression relative to placentas from vehicle-injected mice. Expression of mRNA for the VDR, Cyp27b1, and Cyp24a1 (B), and expression of mRNA for TLR2, TLR4, IL-1α, IL-6, and IL-10 (D) in total placentas from day 14 pregnant WT BL6 mice 24 h after ex vivo culture with or without LPS (100 ng/ml). Data shown are fold change in expression relative to placentas treated with vehicle only; n = 4 separate mice/placentas per LPS treatment. Asterisks denote statistically different from mice injected with vehicle in vivo or placentas treated with vehicle ex vivo. *p < 0.05, **p < 0.01, ***p < 0.001.
expression of a range of cytokines, chemokines, and chemokine receptors relative to Cyp27b1+/+ placentas treated with LPS is also shown as a dashed line. n = 4 separate placentas per fetal Cyp27b1 genotype. Asterisks denote statistically different from equivalent placenta of IL-1α (10-fold) and IL-6 (5-fold) mRNA expression relative to VDR−/− placentas (Fig. 6). By contrast, expression of mRNA for IL-10 was significantly lower in VDR−/− placentas relative to VDR+/+ or VDR+/− equivalents after treatment with LPS. In the absence of LPS, expression of IL-1α, IL-6, or IL-10 was unaffected by VDR gene ablation (data not shown).

Treatment with 25OHD3 or 1,25(OH)2D3 abrogates inflammatory responses to LPS in the placenta

To determine whether ex vivo vitamin D supplementation modulates LPS-induced placental inflammation, placentas from day 14 timed pregnancy WT BL/6 mice were treated with LPS (100 ng/ml, 24 h) in the presence or absence of 25OHD3, the major circulating form of vitamin D, or 1,25(OH)2D3, the active form of vitamin D. RT-PCR analysis of the resulting placentas showed that treatment with either 25OHD3 or 1,25(OH)2D3, but not 1,25(OH)2D3, decreased expression of Ccl11 mRNA, both in the presence and absence of LPS (Fig. 7A). In the absence of LPS, 25OHD3 and 1,25(OH)2D3 had no effect on expression of the cytokine IL-6. However, 25OHD3, but not 1,25(OH)2D3, potently suppressed LPS-induced expression of the cytokine IL-6 (Fig. 7B). Conversely, only treatment with 1,25(OH)2D3 was able to suppress LPS-induced IL-1α (Fig. 7C).

Discussion

A pivotal observation from our study is that LPS induces placental expression of both Cyp27b1 and VDR when used either in vivo or ex vivo (see Fig. 1). Regulation of the vitamin D system after TLR activation has been reported previously for several cell types. In vitro, the TLR4 ligand LPS is known to stimulate 1α-hydroxylase expression or activity in macrophages (17), monocytes (18), and dendritic cells (19), as well as kidney distal tubule cells (20) and endothelial cells (21). In monocytes, the TLR2 ligand 19-kDa lipoprotein stimulates expression of both CYP27B1 and VDR, providing an intracrine mechanism for vitamin D responses after loading the vitamin D form (VDR+/−) were bred with male Cyp27b1+/+ mice (see Fig. 3). Ex vivo LPS treatment of the resulting VDR+/−/−14-d timed pregnancy placentas resulted in significantly greater levels of IL-1α (10-fold) and IL-6 (5-fold) mRNA expression relative to VDR+/+ placentas (Fig. 6). By contrast, expression of mRNA for IL-10 was significantly lower in VDR−/− placentas relative to VDR+/+ or VDR+/− equivalents after treatment with LPS. In the absence of LPS, expression of IL-1α, IL-6, or IL-10 was unaffected by VDR gene ablation (data not shown).

FIGURE 5. PCR array analysis of inflammatory gene expression in Cyp27b1+/+ and Cyp27b1−/− placentas after LPS challenge. Female BL6 mice +/+ for the Cyp27b1 gene were bred with male Cyp27b1+/+ mice as outlined in Fig. 3. Placenta from the resulting pregnancies (day 14) with Cyp27b1+/+ or Cyp27b1−/− fetal genotypes were then cultured ex vivo in the presence or absence of LPS (100 ng/ml) for 24 h. Cultured total placental tissue was then used to assess expression of mRNAs using PCR arrays for genes associated with inflammation. Data shown are fold change in mRNA for genes differentially expressed at a statistically significant level in LPS-treated placentas with a Cyp27b1−/− fetal genotype relative to LPS-treated Cyp27b1+/+ placentas with a value of 1 (dashed line). Asterisks denote statistically different from LPS-treated placentas with a Cyp27b1+/+ genotype. *p < 0.05, **p < 0.01, ***p < 0.001.

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To determine whether ex vivo vitamin D supplementation modulates LPS-induced placental inflammation, placentas from day 14 timed pregnancy WT BL/6 mice were treated with LPS (100 ng/ml, 24 h) in the presence or absence of 1,25(OH)2D3, the major circulating form of vitamin D, or 1,25(OH)2D3, the active form of vitamin D. RT-PCR analysis of the resulting placentas showed that treatment with either 25OHD3 or 1,25(OH)2D3, but not 1,25(OH)2D3, decreased expression of Il1b mRNA, both in the presence and absence of LPS (Fig. 7A). In the absence of LPS, 25OHD3 and 1,25(OH)2D3 had no effect on expression of the cytokine IL-6. However, 25OHD3, but not 1,25(OH)2D3, potently suppressed LPS-induced expression of the cytokine IL-6 (Fig. 7B). Conversely, only treatment with 1,25(OH)2D3 was able to suppress LPS-induced IL-1α (Fig. 7C).
activation of TLR2/1 (18, 22). However, to date, there has been no report of TLR-mediated activation of Cyp27b1 and VDR in vivo. Thus, data in Fig. 1 suggest first that the placental vitamin D system is highly sensitive to immune regulators. Second, the parallel induction of Cyp27b1 and VDR after exposure to LPS is consistent with a localized intracrine or paracrine mechanism by which vitamin D responds to immune challenge during pregnancy. Finally, the induction of Cyp27b1 expression in vivo by LPS indicates that placental synthesis of 1,25(OH)2D3 can be induced by pathogen-associated molecular patterns. Interestingly, although placental Cyp27b1 and VDR were potently induced by LPS, there was no parallel stimulation of the vitamin D catabolic enzyme Cyp24a1, consistent with previous reports describing the extensive methylation of this gene in placental tissue (23). Suppression of Cyp24a1 expression in this manner has been postulated as a mechanism for maximal local generation of 1,25(OH)2D3 from 25OHD3 in the placenta and may help to explain the potent effects observed after knockout of Cyp27b1 or VDR in this tissue.

Placental expression of CYP27B1 in humans (24) and Cyp27b1 in mice (14) is induced early in gestation. In humans, the enzyme is strongly expressed in both maternal decidualized stromal cells and fetal trophoblastic cells, but it is also detectable in decidual macrophages (24). Analysis of Cyp27b1 protein expression in placentas from WT mice indicated background expression of the enzyme in both decidual and trophoblastic cells of the placenta. However, in vivo immune challenge dramatically increased Cyp27b1 levels specifically in trophoblastic giant cells, indicating more pronounced immune induction of the enzyme within the fetal component of the placenta. Mating of mice +/- for Cyp27b1 enabled selective knockout of the enzyme in fetal tissues within the placenta whereas retaining the capacity for synthesis of 1,25(OH)2D3 within the maternal decidua. In the absence of an immune stimulus, fetal loss of Cyp27b1 resulted in an 80% decrease in total placental mRNA for the enzyme (see Fig. 4). This is slightly higher than expected given the fetal +/- placentas expressed one maternal allele for Cyp27b1 compared with three alleles in the fetal +/- mice (see Fig. 2), and presumably reflects the relative abundance of trophoblast tissue compared with decidua on day 14 of pregnancy.

Of the various candidate genes studied under basal (non-LPS) conditions, only IFN-γ and IL-10 showed altered expression in untreated Cyp27b1 +/- placentas relative to placentas with a +/- fetal genotype. Treatment in vitro with 1,25(OH)2D3 is known to suppress IFN-γ production by T cells as part of its modulatory effects on T cell phenotype (25). Thus, fetal knockout of Cyp27b1 may influence placental IFN-γ as a consequence of impaired local synthesis of suppressive 1,25(OH)2D3. Overabundance of IFN-γ, a type 1 Th (Th1) cell cytokine, has been linked to recurrent spontaneous fetal abortion in humans (26, 27) and mice (27), implicating Th1 cell adaptive immunity in the pathophysiology of miscarriage. In view of the well-recognized ability of 1,25(OH)2D3 to promote transition from Th1 cell activity to more tolerant Th2 cell responses (28), it is tempting to speculate that loss of 1,25(OH)2D3 synthesis by the placenta may contribute to...
a less favorable immune environment during pregnancy. Moreover, recent reports demonstrating direct effects of 1,25(OH)2D3 on the generation of regulatory T cells (29) suggest that impaired synthesis of this hormone in the placenta may have wider consequences for tolerogenic immune responses during pregnancy, even in the absence of an immune challenge. IL-10 is produced by regulatory T cells and other cells (including villous cytotrophoblasts) within the placenta, where it appears to function as a key facilitator of successful pregnancy (30). Analysis of placental tissue has shown reduced levels of IL-10 in pre-eclampsia pregnancies (31); thus, it was interesting to note the decreased expression of this cytokine in Cyp27b1−/− placentas.

For several gene products, the consequences of Cyp27b1 or VDR knockout in the placenta were more pronounced after exposure to LPS (see Figs. 4, 5). Placental expression of inflammatory cytokines is part of normal immune function during pregnancy (32), but overexuberant cytokine responses are also known to play a key role in adverse outcomes of pregnancy (33–36). Analysis of pregnant mice has shown that IL-4 is a major placental cytokine, with expression increasing across gestation (37). In nongestational tissues, IL-4 acts as an anti-inflammatory Th2 cytokine, but at the interface between chorion and decidua, it appears to fulfill the opposite function by stimulating production of inflammatory mediators such as PGs (38). However, expression of IL-4 does not appear to be essential for normal pregnancy (39), and elevated placental expression of IL-4 does not appear to be a feature of disorders such as pre-eclampsia (40). Expression of the proinflammatory cytokine IL-15 has been described in mouse (41) and human (41, 42) placentas. Levels of the cytokine are also known to be increased in amniotic fluid from preterm births (42), and this appears to be due to increased expression of IL-15 mRNA in fetal membranes (42). Like IL-15, IL-18 is expressed at the interface between fetal and maternal cells in placenta, and is secreted into the amniotic fluid (43). However, in contrast with IL-15, amniotic levels of IL-18 appear to be induced primarily in response to intraamniotic infection (44). This is somewhat paradoxical give the established function of vitamin D in promoting antibacterial activity (45), but it may simply reflect the use of a TLR4 agonist (LPS) rather than other potential pathological challenges. Moreover, recent studies have shown that expression of IL-18 is also increased in placentas from patients with pre-eclampsia (46), further underlining a link between dysregulation of the placental vitamin D system and this prevalent clinical problem.

Over the last 10 y it has become clear that networks of chemokines and their receptors are central to the cellular cross talk that occurs between mother and fetus during pregnancy (47). Chemokines and their receptors play a key role in the placental trafficking of immunomodulatory cells such as uterine NK cells (48), but they are also important factors in promoting invasion of maternal tissues by trophoblastic cells (49). Expression of chemokines and chemokine receptors varies considerably within the different compartments of the placenta and fetal membranes. Consequently, as yet, it is unclear how the spectrum of elevated chemokines and chemokine receptors described in Fig. 5 will affect placental function. However, it is worth noting that in almost every case, loss of fetal Cyp27b1 resulted in increased expression of chemokines, together with their cognate receptors (Ccr3/Ccl11, Ccr4/Ccl3/Ccl17, Ccr6/Ccl20, Ccr8/Ccl11). The precise impact of such dysregulation of ligand-receptor expression on cellular trafficking in the placenta has yet to be determined, but it is unlikely to be benign and may induce or enhance detrimental responses to the danger signals such as trophoblast apoptosis.

Data presented in this study indicate that loss of 1,25(OH)2D3 production in the fetal compartment of the placenta is sufficient to cause generalized dysregulation of placental inflammation after an immune challenge. At least some of these responses appear to be occurring in an autocrine fashion because dysregulation of IL-1α and IL-6 response to LPS was also observed in placentas with fetal knockout of either Cyp27b1 or the VDR (see Figs. 5, 6). Further studies are required to determine whether a similar mechanism applies to all of the cytokines, chemokines, and chemokine receptors with dysregulated expression in Cyp27b1−/− placentas. It is possible that loss of 1,25(OH)2D3 production in fetal trophoblast will impact on VDR-expressing cells in maternal decidua: ex vivo we have shown that vitamin D metabolites act to suppress cytokine synthesis by decidual uterine NK cells (11). Likewise, given the abundant expression of Cyp27b1 in human decidua (24), it is probable that some immune functions of vitamin D in the placenta are mediated via autocrine synthesis of 1,25(OH)2D3 in the maternal side of the placenta. This could be addressed by mating Cyp27b1−/− females with Cyp27b1−/− males, although this has the added complication of pregnancy occurring against a completely abnormal maternal vitamin D backdrop. Significantly, the ex vivo model used in this study allowed us to show that, although loss of vitamin D function in the placenta was detrimental to placental inflammation, supplementation with vitamin D acted in a beneficial fashion (see Fig. 7). The fact that exogenous addition of precursor 25OHD3 was at least as effective as 1,25(OH)2D3 in suppressing LPS-induced expression of IL-6 and Ccl11 underlines the efficacy of placental Cyp27b1 expression as a mechanism for local activation of 25OHD3.

The placenta appears to be the most abundant source of Cyp27b1 expression and activity outside the kidney. Although this has been recognized for >30 y, the precise significance of placental capacity to synthesize active 1,25(OH)2D3 has yet to be defined. We have previously hypothesized an immunomodulatory function for placental CYP27B1 in humans (50), and this is supported by observations made with mouse tissue in this study. Data highlight a role for vitamin D as a potent regulator of inflammation in the placenta, and this may be a key factor linking vitamin D with pathological conditions such as pre-eclampsia. However, it is important to recognize that vitamin D is also able to influence other aspects of immunity, notably the stimulation of antibacterial innate immune responses (18, 22). In previous studies using human tissue, we have shown that induction of the antimicrobial protein cathelicidin by vitamin D is not restricted to the immune system but can also be observed with cells from both the maternal and fetal sides of the placenta (11, 12). In future studies, it will be important to determine whether similar antibacterial mechanisms are also influenced by vitamin D in the placenta.

Disclosures
The authors have no financial conflicts of interest.

References
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