Chlamydial infection in vitamin D receptor knockout mice is more intense and prolonged than in wild-type mice

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Abstract

Vitamin D hormone (1,25-dihydroxyvitamin D) is involved in innate immunity and induces host defense peptides in epithelial cells, suggesting its involvement in mucosal defense against infections. *Chlamydia trachomatis* is a major cause of bacterial sexually transmitted disease worldwide. We tested the hypothesis that the vitamin D endocrine system would attenuate chlamydial infection. Vitamin D receptor knock-out mice ($VDR^{-/-}$) and wild-type mice ($VDR^{+/+}$) were infected with 10^3 inclusion forming units of *Chlamydia muridarum* and cervical epithelial cells (HeLa cells) were infected with *C. muridarum* at multiplicity of infection 5:1 in the presence and absence of 1,25-dihydroxyvitamin D_3. $VDR^{-/-}$ mice exhibited significantly higher bacterial loading than wild-type $VDR^{+/+}$ mice ($P<0.01$) and cleared the chlamydial infection in 39 days, compared with 18 days for $VDR^{+/+}$ mice. Monocytes and neutrophils were more numerous in the uterus and oviduct of $VDR^{-/-}$ mice than in $VDR^{+/+}$ mice ($P< 0.05$) at d 45 after infection. Pre-treatment of HeLa cells with 10nM or 100nM 1,25-dihydroxyvitamin D_3 decreased the infectivity of *C. muridarum* ($P< 0.001$). Several differentially expressed protein spots were detected by proteomic analysis of chlamydial-infected HeLa cells pre-treated with 1,25-dihydroxyvitamin D_3. Leukocyte elastase inhibitor (LEI), an anti-inflammatory protein, was up-regulated. Expression of LEI in the ovary and oviduct of infected $VDR^{+/+}$ mice was greater than that of infected $VDR^{-/-}$ mice. We conclude that the vitamin D endocrine system reduces the risk for prolonged chlamydial infections through regulation of several proteins and that LEI is involved in its anti-inflammatory activity.

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Keywords

Chlamydial infection; 1,25-Dihydroxyvitamin D_3; Vitamin D receptor knock out mouse; HeLa cells; Leukocyte elastase inhibitor

1. Introduction

The vitamin D hormone (1,25-dihydroxyvitamin D, 1,25-$(OH)_2D$)[1] is a fat-soluble secosteroid and a major regulator of mineral homeostasis through its actions in the kidney, intestines, bone, and parathyroid glands. The biological effects of 1,25-$(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the super family of nuclear hormone receptors that function as agonist-activated transcription factors. Recent studies indicate that vitamin D hormone regulates the immune system through induction of natural host defense peptides, activation of pattern recognition toll-like receptors, and anti-inflammatory activity [1, 2]. Liu et al. [3] reported that toll-like receptor activation of human macrophages up-regulated expression of VDR and the 25-hydroxyvitamin D-1-hydroxylase (required for synthesis of 1,25-$(OH)_2D$), leading to induction of cathelicidin, an antimicrobial peptide, and killing of intracellular *Mycobacterium tuberculosis*. Subsequent animal and clinical studies have provided further evidence of the role of the vitamin D endocrine system in innate immunity [4–9]. It has also been reported that 1,25-$(OH)_2D_3$/VDR modulates the capacity of antigen presenting cells to induce T cell activation, cytokine secretion, and localized antigen-specific immune responses [10–13].

Chlamydia trachomatis is a major infectious bacterial agent of sexually transmitted disease (STD) in industrialized and developing nations. An estimated three million new cases of chlamydial infection occur annually in the United States [14]. Chlamydial infection, the most common STD in the United States, can cause severe health consequences for women, including pelvic inflammatory disease, ectopic pregnancy, chronic pelvic pain, and infertility [15]. The local mucosal epithelial cells of the genital tract are important in chlamydial infectivity, acting as sentinels to recognize pathogens and send signals to underlying immune cells [16, 17]. We report here on the use of the vitamin D receptor knock-out ($VDR^{-/-}$) mouse and HeLa cells (human cervical epithelial cells) to test the hypothesis that the vitamin D endocrine system attenuates chlamydial infection.

2. Materials and methods

2.1. Chemicals

1,25-Dihydroxyvitamin D_3 was obtained from MP Biomedicals (LLC, Solon, OH). Anti-mouse antibody to leukocyte elastase inhibitor (LEI) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody to *Chlamydia* conjugated with FITC was obtained from Bio-Rad (Hercules, CA).

2.2. Animals

Female $VDR^{-/-}$[18, 19] and $VDR^{+/+}$ mice on a C57BL/6J background (6 week old) were purchased from Jackson Laboratory (Bar Harbor, MA), fed food and water ad libitum, and maintained in laminar flow racks under pathogen-free conditions with a 12 h light and 12 h

dark cycle. All VDR$^{-/-}$ and VDR$^{+/+}$ mice were fed a rescue diet [20] high in calcium, phosphate, and lactose (Harlan Laboratories, Madison, WI) to achieve normal plasma calcium levels in the VDR$^{-/-}$ mice. The mice (n = 6/group) were infected at 8 week old and killed on d 45 after infection by cervical dislocation. The protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Morehouse School of Medicine.

2.3. Infectivity assay in VDR$^{-/-}$ and VDR$^{+/+}$ mice

Female VDR$^{-/-}$ and VDR$^{+/+}$ mice (n = 6/group, 8 week old) were subcutaneously administered 2 mg of medroxyprogesterone acetate (Sigma-Aldrich Co, St Louis, MO) 7 d prior to infection and infected intra-vaginally with 10^3 inclusion forming units (IFU) of *Chlamydia muridarum* in 20 μl of PBS. Bacterial shedding was assessed by performing vaginal swabs on d 3, 6, 9, 12, 15, 18, 25, 32, and 39 after infection. Tissue culture isolation of *C. muridarum* was by standard procedures and the number of inclusions per group per time point was determined [21].

2.4. Histology and immunohistochemistry

The entire genital tract was collected and fixed in 4% formaldehyde. The samples were embedded in paraffin, cut longitudinally into 4 μm sections, and stained with hematoxylin and eosin. The right and left uterine horns and oviducts were individually evaluated in a blinded method for the presence of acute inflammation (neutrophils), chronic inflammation (monocytes), and plasma cells. A four-tiered semiquantitative scoring system was used to quantitate the inflammation [22]: 0, normal; 1+, rare foci (minimal presence) of inflammatory cells; 2+, scattered (1–4) aggregates or mild diffuse increase in parameter; 3+, numerous aggregates (>4) or moderate diffuse or confluent areas of parameter; 4+, severe diffuse infiltration or confluence of parameter.

Tissue slides from the oviducts were incubated with a mouse anti-LEI (serpinB1a) primary antibody, followed by incubation for 30 min with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody in accordance with the manufacturer's directions. Images of representative fields were obtained using an Olympus Provis AX70 microscope equipped with a Leica DFC 320 Digital Camera system (Leica Camera AG, Solms, Germany).

2.5. Infectivity in HeLa cells

HeLa cells were pre-treated with 1,25-(OH)$_2$D$_3$ at different concentrations for 24 h. The treated and un-treated cells were washed and infected with *C. muridarum* at multiplicity of infection (MOI) 5:1 for 48 h. The intensity of infection was measured by staining with a chlamydial antibody conjugated with fluorescein isothiocyanate. The number of inclusions was calculated as previously described [23].

2.6. Proteomic analysis

HeLa cells that had been pre-treated with 1,25-(OH)$_2$D$_3$ (100nM) for 24 h or un-treated were infected with *C. muridarum* for 2 h. Proteins were extracted with a Bio-Rad protein extraction kit and cleaned with 2-D Clean Up Kit, according to the manufacturer's protocol. Protein concentration was determined by 2D Quant Kit from GE Healthcare (GE Healthcare,

Piscataway, NJ). Samples (80 μg) were labeled with Cy3 or Cy5 fluorescence dyes, mixed in a rehydration buffer, and subjected to two-dimensional fluorescence differential gel electrophoresis analysis (2D-DIGE) [24, 25]. The spots corresponding to differentially expressed proteins were digested and analyzed by nanocapillary LC-MS/MS (Xevo G2 Tof, Waters, Milford, MA). Protein candidates were identified with automated database searching against a NCBI database using MASCOT Daemon software (Matrix Sciences, Boston, MA).

2.7. Western blot analysis

HeLa cells that had been pre-treated with $1,25\text{-}(OH)_2D_3$ (100 nM) or un-treated for 24 h were pulsed with *Chlamydia muridarum* agent (MOI 5:1) for up to 2h. Protein expression of LEI at 0.5, 1, and 2 h after infection with Chlamydia was determined by Western blot analysis. The cell lysates were subjected to electrophoresis for protein separation on 4–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) in running buffer (Tris/glycine/SDS). Proteins were then transferred for 1 h onto nitrocellulose membranes in transfer buffer (Tris/glycine/methanol). Non-specific binding was blocked by incubating the membranes in 5% non-fat dried milk, 0.05% (v/v) Tween 20 in 1X TBS for 1 h at room temperature. After several washes with buffer (TBS Tween 0.05%), membranes were incubated with the primary antibodies overnight at 4°C. The polyclonal antibodies used were: serpinB1 (1:200) (Santa Cruz Biotech. Inc., Santa Cruz, CA); glyceraldehide-3-phosphate dehydrogenase (GAPDH) (1:200) (R&D Systems, Minneapolis, MN). Horseradish peroxidase-conjugated secondary antibodies were utilized in accordance with the manufacturer's directions (Santa Cruz Biotechnology, Santa Cruz, CA).

2.8. Statistical analysis

A mean ± SD was calculated for each group and statistical significance ($P< 0.05$) evaluated (SAS 9.2, SAS Institute Inc., Cary, NC). Repeated measures ANOVA was used to compare intensity and duration of infection in $VDR^{-/-}$ and $VDR^{+/+}$ mice and one way ANOVA to assess the effect of $1,25\text{-}(OH)_2D_3$ on intensity of infection in HeLa cells. A *t*-test was used to compare the pathology scores of $VDR^{-/-}$ and $VDR^{+/+}$ uterus and oviducts.

3. Results

3.1. Chlamydial infection and inflammatory response in VDR$^{-/-}$and VDR$^{+/+}$ mice

$VDR^{-/-}$ mice that were genitally infected with *C. muridarum* had significantly higher bacteria loads at d 6, 9, 12, 15 post-infection than $VDR^{+/+}$ mice, indicating a greater intensity of infection (Fig. 1). $VDR^{-/-}$ mice also exhibited a slower rate of clearance of the genital chlamydial infection than $VDR^{+/+}$ mice. Whereas $VDR^{+/+}$ mice had cleared the infection by d 18, $VDR^{-/-}$ mice cleared the infection by d 39. The uterus of uninfected $VDR^{+/+}$ and $VDR^{-/-}$ mice revealed normally observed branched folia with intact epithelial lining composed of both secretory and ciliated cells (Fig. 2). Significantly higher numbers of monocytes (pathology scores: 2.5 ± 0.3 vs. 0.7 ±0.7, *P*<0.01) and neutrophils (pathology scores: 1.2 ±0.6 vs. 0.2 ±0.2, *P*<0.05) were observed in the infected uterus of $VDR^{-/-}$mice, compared with that of infected $VDR^{+/+}$ mice. No inflammation was observed in the oviduct of un-infected $VDR^{-/-}$ and $VDR^{+/+}$ mice (Fig. 3). Significantly higher numbers of

monocytes (pathology scores: 3.2 ±0.6 vs. 1.7 ±0.7, $P<0.01$) and neutrophils (pathology scores: 1.5 ± 0.3 vs. 0.3 ± 0.3, $P< 0.01$) were observed in the oviduct of infected $VDR^{-/-}$ mice, compared with that of infected $VDR^{+/+}$ mice.

3.2. Effect of 1,25-(OH)₂D₃ on chlamydial infection in HeLa cells

Chlamydial infectivity of HeLa cells pre-treated with 10nM or 100nM 1,25-(OH)$_2$D$_3$ for 24h was significantly less than chlamydial infectivity of un-treated HeLa cells or HeLa cells pre-treated with 1.0nM 1,25-(OH)$_2$D$_3$(Fig. 4). Vitamin D hormone treatment of chlamydial-infected HeLa cells resulted in numerous differentially expressed protein spots, of which 25 have been identified, using LC-MS/MS ion search software and associated databases (Table 1). Fewer differentially expressed protein spots were observed for pre-treated un-infected HeLa cells (Supplemental Fig. 1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/jjsbmb.2012.11.002.

3.3. Leukocyte elastase inhibitor expression

Western blots indicated significantly more LEI in infected HeLa cells pre-treated with 1,25-(OH)$_2$D$_3$ than in un-treated infected HeLa cells (Fig. 5). Pre-treatment with 1,25-(OH)$_2$D$_3$ did not, however, significantly increase LEI in un-infected HeLa cells. LEI was detected in the oviduct and ovary of infected $VDR^{+/+}$ mice by immunohistochemistry, but not in the oviduct and ovary of infected $VDR^{-/-}$ mice (Fig. 6).

4. Discussion

In this study, vitamin D receptor knock-out mice and control mice ($VDR^{+/+}$) were used to evaluate the 1,25-(OH)$_2$D$_3$/VDR effect on genital chlamydial infection. Homozygous $VDR^{-/-}$ mice are phenotypically normal at birth and survive for at least six months [18]. These mice exhibit hypocalcemia, hyperparathyroidism, rickets and osteomalacia. One feature seen in $VDR^{-/-}$ mice, but not seen in mice made vitamin D deficient by dietary means, is alopecia, which develops progressively from the age of four weeks [18]. Feeding $VDR^{-/-}$ mice a rescue diet high in calcium, phosphorus, and lactose [20] normalized all of the symptoms above except alopecia [19]. In the present study, $VDR^{-/-}$ mice fed the rescue diet suffered a more intense and prolonged genital chlamydial infection compared with $VDR^{+/+}$ mice, suggesting that attenuation of genital chlamydial infectivity in the $VDR^{+/+}$ mice is not an indirect effect of maintaining normal calcium, but is a process which requires a functional VDR.

Intensive chlamydial infection and a prolonged infection course trigger inflammatory responses that are thought to be largely res,ponsible for the chlamydial induced pathology of the genital tract. Inflammation was not observed in the uterus and oviduct of un-infected $VDR^{-/-}$ and $VDR^{+/+}$ mice, but higher numbers of monocytes and neutrophils were observed in the infected uterus and oviduct of $VDR^{-/-}$ mice, compared with that of $VDR^{+/+}$ mice. The more intense and prolonged chlamydial infection in the $VDR^{-/-}$mice was thus accompanied by a more prolonged inflammatory response.

Genital epithelial cells are the first line of defense against microbial invasion, so we conducted chlamydial infection assays with HeLa cells, a genital epithelial cell line. Pre-treatment of HeLa cells with $1,25\text{-}(OH)_2D_3$ dramatically inhibited intracellular replication of *C. muridarum* and inclusion development. It is possible that $1,25\text{-}(OH)_2D_3$ mediates the protective response of epithelial cells to bacterial replication [26]. HeLa cells pre-treated with $1,25\text{-}(OH)_2D_3$ and infected with *C. muridarum* exhibited differential expression of 65 protein spots, whereas fewer differentially expressed protein spots were detected in un-infected HeLa cells pre-treated with $1,25\text{-}(OH)_2D_3$. Several categories of proteins were differentially expressed: cytoskeleton proteins that affect microfilament network and cell-cell junctions (tubulin, vimentin, tropomyosin chain, vinculin); stress response proteins related to protein folding and chaperones (heat shock proteins, protein disulfide isomerase, T-complex protein-1); proteins involved in oxidation/reduction (peroxiredoxin-2 and -6, thioredoxin-dependent peroxide reductase); proteins involved in growth and regulation of cell proliferation (tumor protein D54, 60S acidic ribosomal protein P0, proliferation-associated protein 2G4); proteins involved with ATP (ATP synthase subunit beta, transitional endoplasmic reticulum ATPase); calcium binding protein (protein S100-A7); anti-inflammatory protein (LEI); serum albumin precursor. A caveat to be considered in conducting complementary experiments with HeLa cells is that human monocytes and macrophages show vitamin D hormone induced expression of antimicrobial proteins (e.g. cathelicidin, beta-defensin), but these genes are not regulated by $1,25\text{-}(OH)_2D_3$ in mice.

We selected for further study LEI, an anti-inflammatory protein that was up-regulated in chlamydial infected HeLa cells pre-treated with $1,25\text{-}(OH)_2D_3$, compared with un-treated cells. LEI is a 42 kDa member of the serine protease inhibitor (serpin) superfamily [27] and is also known as monocyte/neutrophil elastase inhibitor (MNEI) and serpinB1. It inhibits neutrophil proteases (elastase, cathepsin G, proteinase-3) that are involved in killing phagocytosed microbes by forming irreversible covalent complexes with them. LEI is abundant in the cytoplasm of monocytes, neutrophils, and macrophages and attenuates the inflammatory response to microbial infection by limiting the activity of the proteases, thus limiting degradation of host defense and matrix proteins [28]. Recombinant MNEI administered daily by aerosolization to rats previously inoculated with *Pseudomonas aeruginosa* decreased the inflammatory injury and enhanced the clearance of bacteria from the infected rat lungs [29]. SerpinB1$^{-/-}$ mice exhibited higher mortality relative to wild type mice, associated with late-onset failed clearance of *P. aeruginosa*. Co-administration of recombinant serpinB1 with the *P. aeruginosa* inoculum normalized bacterial clearance in serpinB1$^{-/-}$ mice [30]. Gong et al. [31] reported that serpinB1$^{-/-}$ mice died earlier and in greater numbers than wild-type mice when infected with high-dose surfactant protein-D-sensitive influenza A/Philadelphia/82(H3N2). They concluded that serpinB1 plays a critical role in mitigating inflammation and restricting pro-inflammatory cytokine production in influenza infection. L-DNase II, an endonuclease involved in the degradation of genomic DNA during apoptosis, is derived from LEI by an acidic-dependent post-translational modification or by digestion with elastase [32–36]. Appearance of the endonuclease activity results in loss of the anti-protease activity [32]. Benarafa et al. [37] have indicated that human MNEI is encoded by a single SERPINB1 gene, whereas four murine genes have been

identified and fully sequenced, with one of them, EIA (SERPINB1a), being the mouse ortholog of MNEI.

In the present study, Western blots indicated up-regulation of LEI in infected HeLa cells pre-treated with $1,25\text{-}(OH)_2D_3$, but not in pre-treated un-infected HeLa cells (confirming the proteomics results). A greater amount of LEI was detected by immunohistochemistry in the oviduct and ovary of infected $VDR^{+/+}$ mice than in the oviduct and ovary of infected $VDR^{-/-}$ mice, although monocytes and neutrophils (the primary cellular sources of LEI) were more numerous in the oviduct of infected $VDR^{-/-}$ mice than in the oviduct of infected $VDR^{+/+}$ mice. The association of more prolonged chlamydial infection and inflammatory response with lesser LEI expression in the oviduct and ovary of $VDR^{-/-}$ mice, compared with $VDR^{+/+}$ mice, would suggest a role for LEI in countering the inflammatory response and for vitamin D hormone in LEI expression. Previous studies have demonstrated a role for $1,25\text{-}(OH)_2D_3$ in LEI expression. Protiva et al. [38] demonstrated, via global gene array analysis, activation of the VDR pathway in rectal mucosal biopsies from estradiol treated postmenopausal women and up-regulation of SERPINB1. Kovalenko et al. [39] identified numerous transcript changes, including up-regulation of SERPINB1, in response to $1,25\text{-}(OH)_2D_3$ treatment of the immortalized, non-transformed prostate epithelial cell line, RWPE1. Unlike the anti-microbial proteins, LEI appears to be regulated by $1,25\text{-}(OH)_2D_3$ in mice, as well as in cells of human origin. Wang et al. [40] have demonstrated that multiple DR3 elements in the serpinB1 gene are conserved between mouse and human. Consequently, the mouse can serve as a model for examining the relationship between the vitamin D endocrine system and LEI, a protein with anti-inflammatory effects.

In summary, we demonstrate here that genital chlamydial infectivity was more intense and prolonged in $VDR^{-/-}$ mice than in $VDR^{+/+}$ mice. The inflammatory response to chlamydial infection was also more prolonged in $VDR^{-/-}$ mice than in $VDR^{+/+}$ mice. The more prolonged chlamydial infection and inflammatory response in the $VDR^{-/-}$ mice coincided with lesser expression of LEI (an antiinflammatory protein) in the oviduct and ovary of $VDR^{-/-}$ mice, compared with $VDR^{+/+}$ mice, suggesting a role for LEI in countering the inflammatory response to chlamydial infection and for vitamin D hormone in LEI expression.

We conclude that the VDR is involved in immunoregulation of chlamydial infection. LEI, the protein examined in this study, was only one of numerous proteins differentially expressed in infected HeLa cells pre-treated with $1,25\text{-}(OH)_2D_3$. Additional studies are necessary to determine other mechanisms by which vitamin D hormone attenuates chlamydial infection and the anti-inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
1,25-(OH)$_2$D$_3$	1,25-dihydroxycholecalciferol
1,25-(OH)$_2$D	1,25-dihydroxyvitamin D
2D-DIGE	two-dimensional differential in gel electrophoresis
HE	hematoxylin and eosin
HeLa cells	human cervical epithelial cells
IFU	inclusion forming units
LEI	MNEI or serpinB1, leukocyte elastase inhibitor
MOI	multiplicity of infection
SERPINB1	leukocyte elastase inhibitor gene
VDR	vitamin D receptor
VDR$^{-/-}$	vitamin D receptor knock out mouse
VDR$^{+/+}$	C57BL/6J mouse

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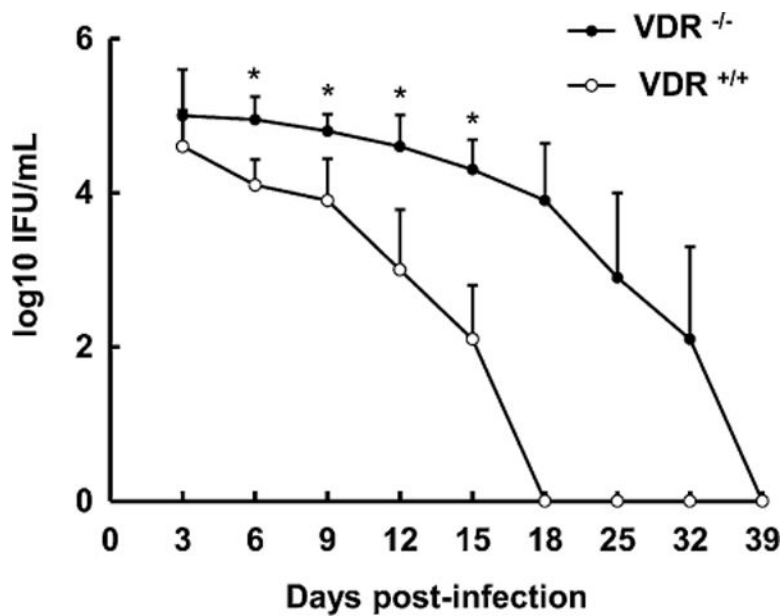
Fig. 1.
VDR$^{-/-}$ mice infected intra-vaginally with *Chlamydia muridarum* exhibit greater bacteria loads and slower rates of bacterial clearance than VDR$^{+/+}$ mice. VDR$^{-/-}$ and VDR$^{+/+}$ mice were infected intra-vaginally with 10^3 inclusion forming units (IFU) of *C. muridarum* as described in Section 2.3. The course of infection was monitored by isolation of *C. muridarum* from cervicovaginal swabs [21]. Values are mean $\pm$ SD, $n = 6$. Overall intensity different from VDR$^{+/+}$, $P < 0.01$. Rate of clearance different from VDR$^{+/+}$, $P < 0.001$. Intensity of infection different from VDR$^{+/+}$, *$P < 0.001$.

Un-infected VDR$^{+/+}$ | Infected VDR$^{+/+}$

Un-infected VDR$^{-/-}$ | Infected VDR$^{-/-}$
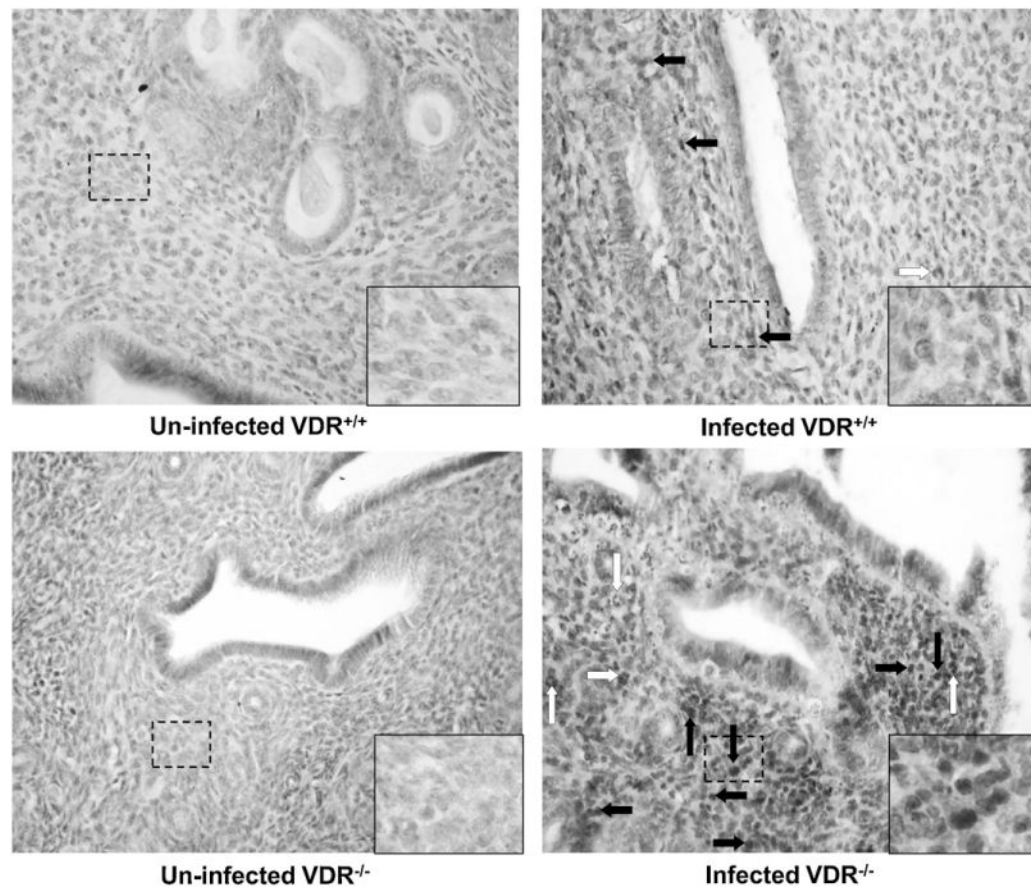
Fig. 2.
More monocytes and neutrophils are observed in the uterus of infected VDR$^{-/-}$ mice, compared with that of infected VDR$^{+/+}$ mice. The uterus horns of each mouse were removed at d 45 post-infection with *Chlamydia muridarum* and stained with hematoxylin and eosin (HE), as described in Section 2.4. HE-stained sections are at 40× magnification. Open arrows point to scattered neutrophils; closed arrows indicate monocytes/lymphocytes.
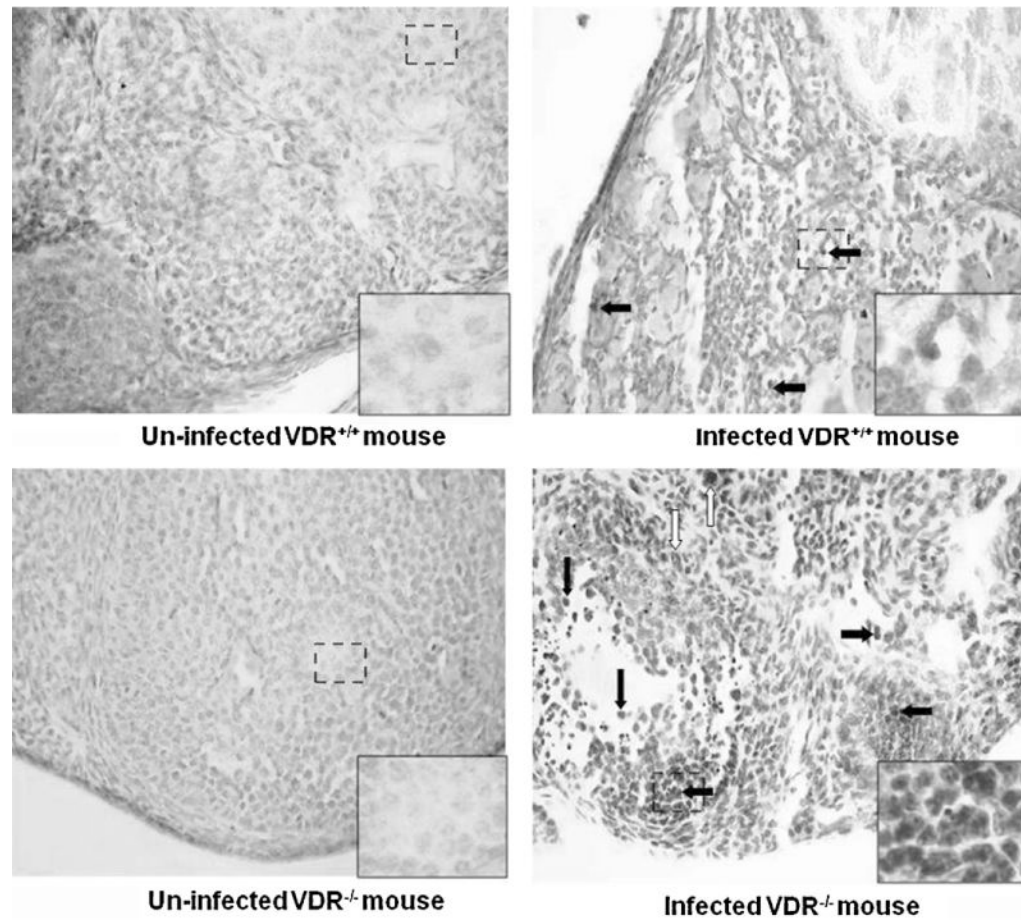
Fig. 3.
More monocytes and neutrophils are observed in the oviducts of infected VDR$^{-/-}$ mice, compared with that of infected VDR$^{+/+}$ mice. The oviducts of each mouse were removed at d 45 post-infection with *Chlamydia muridarum* and stained with hematoxylin and eosin (HE), as described in Section 2.4. HE-stained sections are at 40× magnification. Open arrows point to scattered neutrophils; closed arrows indicate monocytes/lymphocytes.
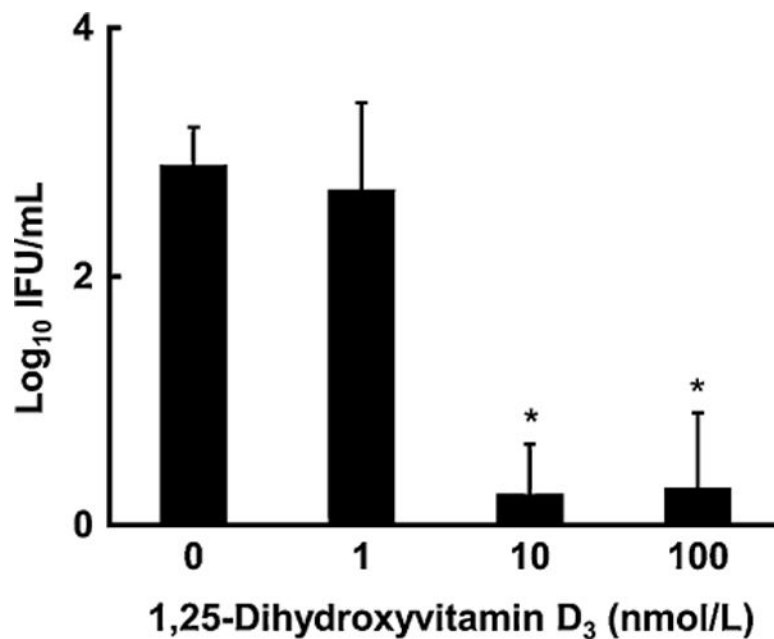
Fig.4.
Chlamydial infectivity of HeLa cells pre-treated with 1,25-(OH)$_2$D$_3$ is significantly less than chlamydial infectivity of un-treated HeLa cells. HeLa cells were pre-treated with 1,25-(OH)$_2$D$_3$ at different concentrations for 24h. Treated and un-treated cells were washed and infected with *Chlamydia muridarum* for 48 h as described in Section 2.5. The intensity of infection was measured by staining with a chlamydial antibody and determining the number of inclusions [23]. Values are mean ± SD, n = 6. Significant effect of 1,25-(OH)$_2$D$_3$ treatment, P< 0.001. Intensity of infection different from un-treated, *P< 0.001.
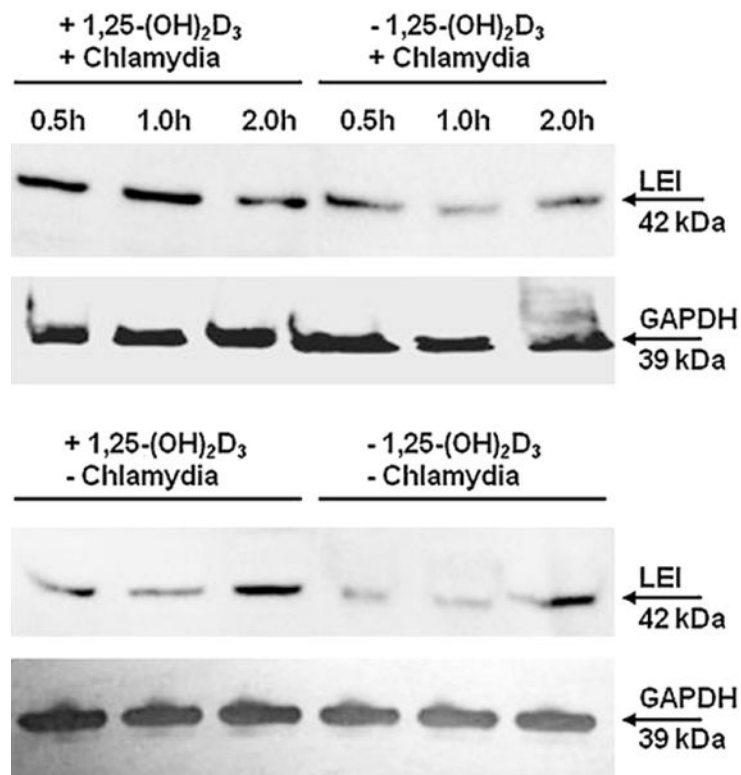
Fig. 5.
Leukocyte elastase inhibitor is up-regulated in infected HeLa cells pre-treated with 1,25-$(OH)_2D_3$, but not in pre-treated un-infected HeLa cells. HeLa cells that had been pre-treated with 1,25-$(OH)_2D_3$ (100nM) or un-treated for 24h were pulsed *with Chlamydia muridarum* (MOI 5:1) for up to 2 h. Total protein was extracted, subjected to SDS-PAGE, transferred onto nitrocellulose membrane, probed with primary and secondary antibodies, and imaged as described in Section 2.7.
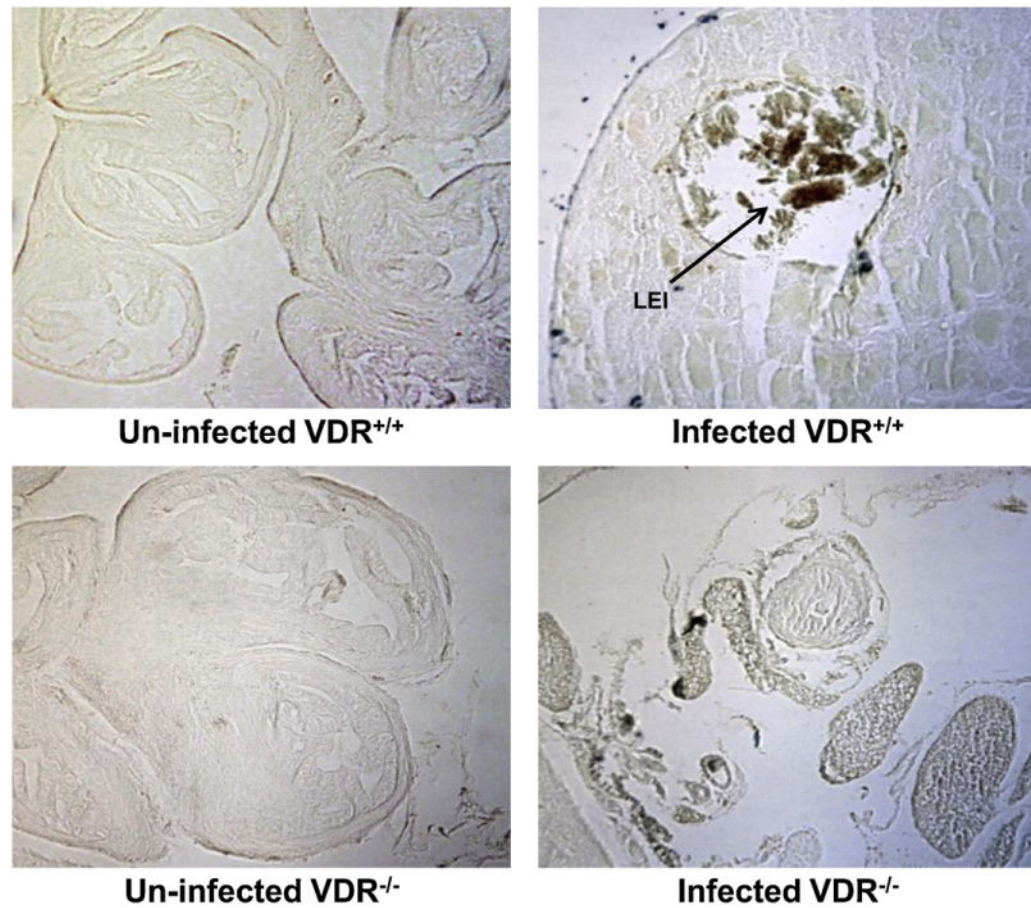
Fig. 6.
Leukocyte elastase inhibitor is detectable in the ovaries of infected $VDR^{+/+}$ mice, but not in the ovaries of infected $VDR^{-/-}$ mice. $VDR^{-/-}$ and $VDR^{+/+}$ mice were infected intra-vaginally with *Chlamydia muridarum* as described in Section 2.3. Tissue slides of ovaries from the mice were incubated with a mouse anti-leukocyte elastase inhibitor primary antibody and imaged as described in Section 2.4.

Table 1

Several spots were up-regulated in chlamydial-infected HeLa cells pre-treated with 1,25-dihydroxyvitamin D$_3$.

Spot[a]	Protein name	Accession ID[b]	MW(Da)[c]	pI[c]	Score[d]	Matched peptides[e]	Sequence coverage (%)
10	Protein S100-A6	S10A6_RABIT	10,147	5.30	54	1	20
13	Tropomyosin alpha-3 chain	TPM3_HUMAN	32,799	4.68	113	3	11
18	Tubulin beta chain	TBB5_HUMAN	49639	4.78	178	7	17
19	Hsc70-interacting protein	F10A1_HUMAN	41,305	5.18	91	2	6
20	ATP synthase subunit beta	ATPB_HUMAN	56,525	5.26	384	11	33
21	Vimentin	VIME_HUMAN	53,619	5.06	282	7	18
22	60 kDa heat shock protein	CH60_HUMAN	61,016	5.70	488	12	31
23	60 kDa heat shock protein	CH60_HUMAN	61,016	5.70	111	4	11
24	T-complex protein 1 subunit epsilon	TCPE_HUMAN	59,633	5.45	271	10	23
25	Heat shock cognate 71 kDa protein	HSP7C_HUMAN	70,854	5.37	393	11	22
26	Serum albumin precursor	ALBU_BOVIN	69,248	5.82	58	3	5
30	Protein disulfide-isomerase A3 precursor	PDIA3_HUMAN	56,747	5.98	223	8	21
34	T-complex protein 1 subunit beta	TCPB_HUMAN	57,452	6.01	183	4	9
36	Proliferation-associated protein 2G4	PA2G4_HUMAN	43,759	6.13	157	4	15
37	Proliferation-associated protein 2G4	PA2G4_HUMAN	43,759	6.13	41	1	7
39	Leukocyte elastase inhibitor	ILEU_HUMAN	42,715	5.90	103	3	8
42	60S acidic ribosomal protein P0	RLA0_HUMAN	34,252	5.71	173	4	13
46	Peroxiredoxin-6	PRDX6_HUMAN	25,019	6.00	130	4	20
48	Heat-shock protein beta-1 (HspB1)	HSPB1_HUMAN	22,768	5.98	122	3	18
49	Thioredoxin-dependent peroxide reductase	PRDX3_HUMAN	27,675	7.67	142	3	13
54	Peroxiredoxin-2	PRDX2_HUMAN	21,878	5.66	122	3	19
57	Tumor protein D54 (hD54)	TPD54_HUMAN	22,224	5.26	42	2	11
58	Transitional endoplasmic reticulum ATPase	TERA_HUMAN	89,266	5.14	144	7	9
64	Vinculin (Metavinculin)	VINC_HUMAN	123,722	5.50	60	3	2

[a] As indicated in Supplemental Fig. 1. Down-regulated protein spots were not identified fortechnical reasons.

[b] SwissProt protein accession number.

[c] Relative molecular mass of proteins and isoelectric point.

[d] Probability based mowse score that indicates the quality of the MS/MS peptide fragment ion matches. Ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

[e] Number of peptides that match the theoretical digest of the primary protein identified.