Chlamydia trachomatis: TLR4-mediated recognition by human dendritic cells is impaired following oestradiol treatment

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ABSTRACT

Genital *Chlamydia trachomatis* infection creates a substantial reproductive health burden in women. The high incidence of asymptomatic infection often precludes timely antibiotic therapy to control the sequelae of infection, and therefore a vaccine is required. Dendritic cells (DC) are now being used as an adjuvant for vaccine development; however, the fate of *C. trachomatis* in human DC and differential regulation of cytokine secretion remains unclear. Hence, an *in vitro* study was performed using *C. trachomatis* (serovar D) elementary body (EB)-pulsed, monocyte-derived DCs co-cultured with autologous $CD4^+$ T cells. Secreted cytokines were measured to assess the protective/pathogenic immune response. The effect of β-oestradiol in the modulation of DC function and on Toll-like receptor (*TLR*) gene expression was also studied. Elementary body-pulsed DCs showed induction of protective Th1 immune response with upregulation of TLR4 expression, secretion of interleukin (IL)-6, IL-12 and interferon (IFN)-γ, together with upregulation of major histocompatibility complex (MHC) class II, CD83 and CD86. When co-cultured with autologous $CD4^+$ T cells, DCs presented chlamydial antigens efficiently, as shown by proliferation of T cells and secretion of IL-2 and IFNγ, which provide a protective immune response. However; pretreatment of cells with oestradiol significantly reduced TLR4 expression and upregulated IL-10 secretion, modulating the Th1 immune response to a Th2-type response, which may lead to pathogenesis.

KEY WORDS: Chlamydia trachomatis.
Dendritic cells.
Estradiol.
Genital tract infections.

Introduction

The number of genital tract *Chlamydia trachomatis* infections is steadily increasing worldwide. In women, chlamydial infection of the lower genital tract ascends to the upper genital tract. If left untreated this can lead to serious sequalae such as pelvic inflammatory disease (PID), which can cause ectopic pregnancy and tubal infertility.[1] Repeat or multiple infections with *C. trachomatis* increase the likelihood of these sequalae, with a two- to five-fold increase in the risk of ectopic pregnancy and approximately four- to six-fold increase in the chance of PID.[2] A major concern with *C. trachomatis* infection is that 70% of infected women are asymptomatic.[3] It is also believed that antibiotic intervention increases the longer-term rates of re-infection because of inability of the person to develop protective immunity.

Dendritic cells (DC) are likely to be the first antigen-presenting cells that encounter *C. trachomatis* following genital and ocular chlamydial infection. They are important for priming cell-mediated immune responses and innate inflammatory processes and therefore potentially play an important role in host immunity and chronic inflammation, the hallmark of chlamydial disease.[4] The fate of *C. trachomatis* in human DCs is less clear and previously two groups have reported on the ability of the L2 and E serovars to multiply in human monocyte-derived DCs and the differential regulation of inflammatory cytokine secretion.[5–7]

Current chlamydial vaccine efforts centre on the interaction between *Chlamydia* and dendritic cells to produce specific effectors and memory responses. Dendritic cells play a central role in the induction of T-cell and B-cell immunity *in vivo*[8] by largely depending on the upregulation of co-stimulatory and adhesion molecules and also on the secretion of inflammatory cytokines.[9–11] The current immunological paradigm maintains that antigen presentation by immature (non-activated) DCs leads to tolerance, whereas mature antigen-loaded DCs promote the development of antigen-specific protective immunity.[12] Thus, factors that influence the maturation of DCs and their ability to generate a protective Th1 immune response against *Chlamydia* will be key to developing an effective vaccine. Furthermore, differential expression and engagement of Toll-like receptors (TLRs) at the surface of dendritic cells and macrophages also influences the type of immune response induced by microbial pathogens.

In addition, oestradiol has been shown to have multiple effects on DC function, the regulation of TLR expression, and shifting helper T-cell cytokine production towards a Th2 profile by immune cells.[13] Studies in mice and other animal models have shown that oestrogen plays an important role

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in controlling immune responses to chlamydial infection and in determining the outcome of infection.[14] An association between recurrent chlamydial infection and high oestradiol level in women has been reported.[15] Oral contraceptives have also been shown to increase susceptibility to chlamydial infection and other sexually transmitted diseases.[16] However, the role of oestradiol in modulating human monocyte-derived DC function in *C. trachomatis* infection (serovar D) has not been studied.

Thus, the present study aims to characterise the effect of *C. trachomatis* infection on the expression of surface molecules by human monocyte-derived DCs and also the *in vitro* cytokine production in the presence of activated $CD4^+$ T cells. Further, *in vitro* studies are conducted to determine the role of oestradiol in increasing chlamydial susceptibility through its effect on TLR4-mediated recognition of *C. trachomatis* and the subsequent cytokine secretion pattern.

Materials and methods

Unless otherwise noted, all the chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plastic and glassware used in cell culture and immune assay was purchased from Greiner, Germany.

Cell line and Chlamydia *culture*

A human cervical epithelial cell line (HeLa 229) was procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained as described previously.[17] The cells were pretreated with diethylaminoethyl-dextran (DEAE-D). Thereafter, HeLa cell monolayers were infected with *C. trachomatis* serovar D (a human clinical isolate) by centrifugation at 2000 rpm for 1 h. Infected HeLa cells were cultured at 35°C in 5% CO_2. *C. trachomatis* was harvested from infected cells after 66 h and chlamydial elementary bodies (EBs) were purified by differential renografin density gradient centrifugation. To harvest, cells were removed by agitation with glass beads (4 mm) and cold phosphate-buffered saline (PBS; 2 mL). Cell suspensions were pooled and the cells were ruptured by sonication. This suspension was centrifuged at 3000 rpm for 10 min at 4°C. The EBs were harvested by removing debris in the supernatant by centrifugation at 16,000 rpm for 1 h at 4°C. The pellet was resuspended in 10 mmol/L sodium phosphate, 250 mmol/L sucrose, 5 mmol/L L-glutamine and 150 mmol/L sodium chloride buffer (SPGS). Cell line and bacterial stocks were tested for *Mycoplasma* contamination by a polymerase chain reaction (PCR) method prior to use and were found to be negative.

Culture of monocyte-derived DCs and expression of co-stimulatory molecules

Following informed written consent, non-heparinised venous blood was collected from women attending the gynaecology out-patient department, Safdarjung Hospital, New Delhi, India, The study received approval from the hospital's ethics review committee.

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque density gradient centrifugation and were washed in RPMI 1640 medium (x3). The PBMCs were labelled with anti-CD14 antibodies labelled with magnetic microbeads and then separated from other leucocytes using a magnetic cell sorter (Miltenyi Biotec, Germany).

The $CD14^+$ cells were inoculated (1–2x10^6/mL) into each well of a six-well plate and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 25 mmol/L HEPES, 0.02 mol/L 2-mercaptoethanol, 10 mg/mL gentamycin and 2 mg/mL amphotericin B, in the presence of 50 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF) and 20 ng/mL interleukin (IL)-4 at 37°C in a 5% CO_2 incubator for six to seven days. The GM-CSF and IL-4 were supplemented every two days. Subsequently, the immature monocyte derived DCs (MDDCs) were washed and analysed for CD14 and CD1a expression (markers for immature DCs) by flow cytometry.

Immature MDDCs obtained were stained with phycoerythrin (PE)-conjugated anti-CD14 and anti-CD1a antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR (Becton Dickinson, San Jose, USA) for 25 min on ice. Appropriate isotype-matched control antibodies were used to rule out non-specific fluorescence. Preparations were then washed with stain buffer (PBS supplemented with 0.1% NaN_3 and 2% FBS) and acquired using Caliber fluorescence-activated cell sorter (FACS; BD Biosciences, San Jose, USA). A total of 10,000 events were acquired and analysis was performed using the Cellquest software (Becton Dickinson).

Pulsing immature MDDCs with chlamydial EBs and oestradiol treatment

After 5–6 days of culture, 10^5 cells/mL immature MDDCs in 24-well cell culture plates were infected with live chlamydial EBs at a multiplicity of infection (MOI) of 2:1 (DC:EB). Infected MDDCs were then washed and incubated for 6 h at 35°C. In parallel experiments, DCs were treated with oestradiol (Sigma) at concentrations of 10 µg/mL, 1 µg/mL and 0.1 µg/mL for 24 h, washed (x2) and then pulsed with chlamydial EBs. *Escherichia coli* LPS was used as the positive control in all experiments.

Polymerase chain reaction array analysis

Dendritic cells were seeded in six-well plates at a density of 2.5x10^5 cells/well. The DCs were infected with chlamydial EBs for 12 h, while mock infected cells were taken as control. After exposure, total RNA was extracted from the cells using the RNAeasy Mini Kit (Qiagen, Valencia, USA). Complementary DNA (cDNA) was generated from RNA using the Reaction Ready First Strand cDNA synthesis kit (SABioscience, Maryland, USA) following the manufacturer's instructions. The human TLR signalling pathway RT^2-PCR-Profiler PCR array (SA Biosciences) was carried out according to the manufacturer's instructions using a real-time PCR detection system (Eppendorf). Data were uploaded into GE array analyser software (SA Bioscience) for analysis and verification of appropriate experimental procedures.

Co-culture of DCs with autologous $CD4^+$ T lymphocytes

Immature MDDCs were infected with live *C. trachomatis* EBs. Infected MDDCs were then washed with PBS and cultured in RPMI 1640 for 24 h at 37°C. Autologous human $CD4^+$ T cells were separated from PBMCs by positive selection in a magnetic cell sorter using anti-CD4 antibody labelled with magnetic microbeads and co-cultured with EB-pulsed DCs

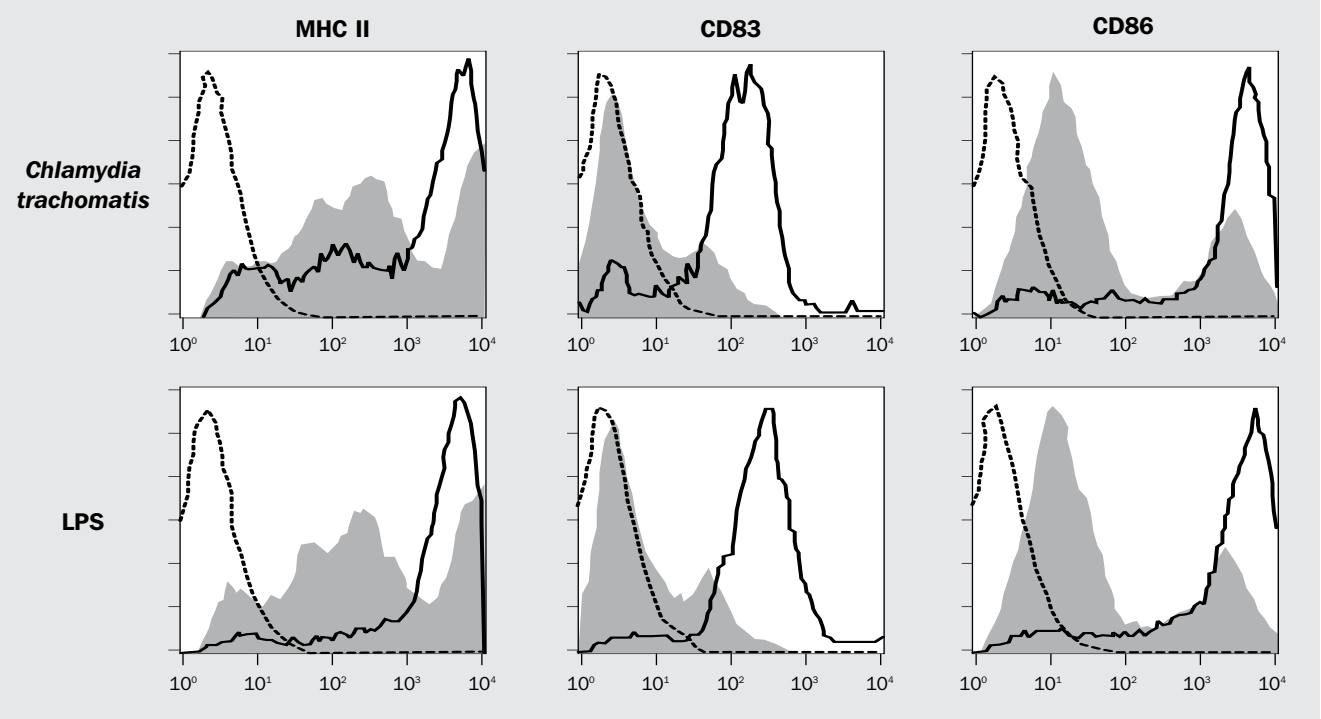

Fig. 1. Flow cytometric analysis showing expression of co-stimulatory molecules on dendritic cells pulsed with *C. trachomatis* elementary bodies (EBs) and LPS (positive control). Flow cytometric analysis revealed up-regulation in expresison of MHC-II, CD83 (DC maturation marker) and CD86 compared to unstimulated dendritic cells.

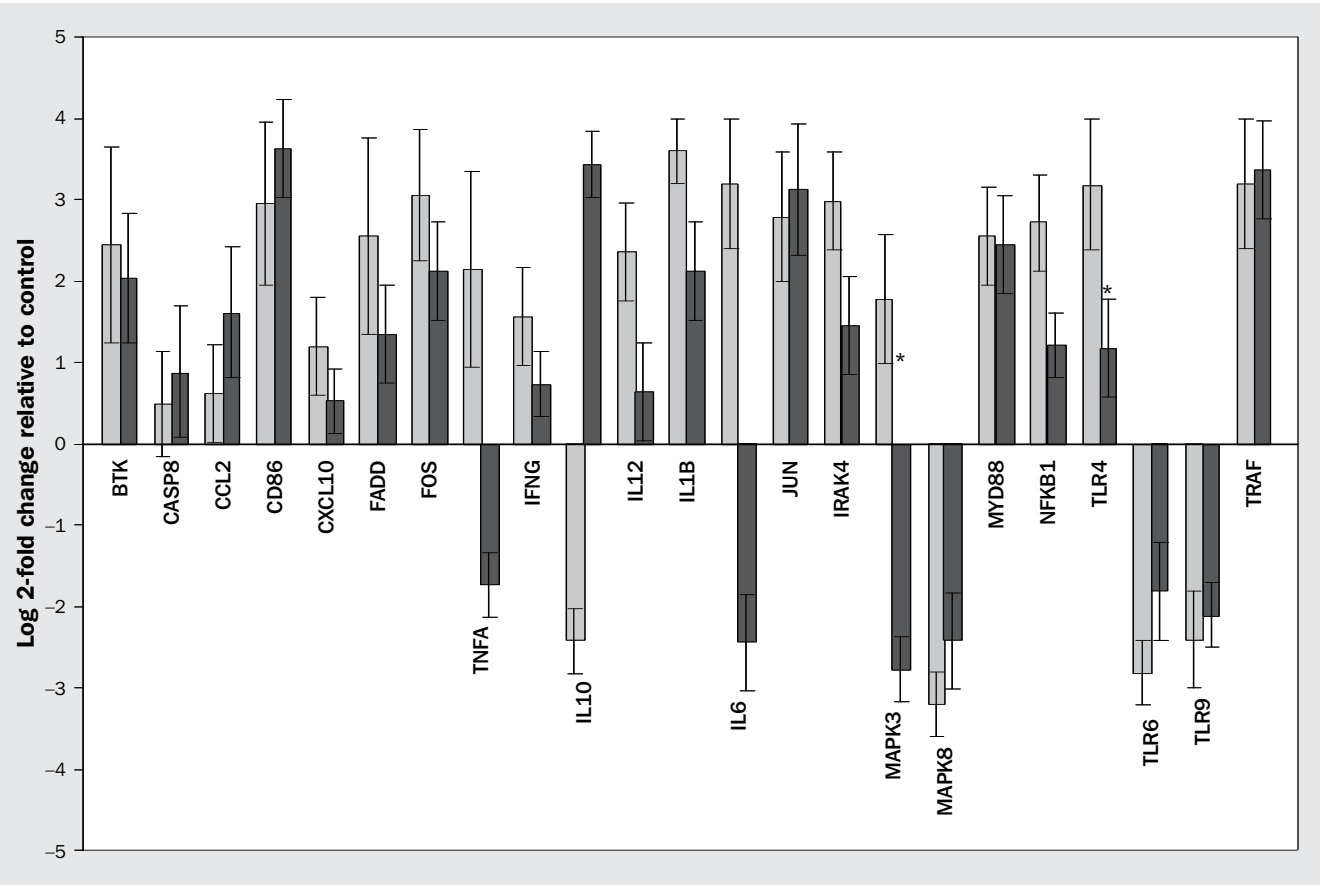

Fig. 2. Effect of oestradiol treatment on Toll-like receptor signalling pathway. Light columns indicate gene expression in dendritic cells untreated with oestradiol. Dark-coloured columns show gene expression in oestradiol-treated dendritic cells. Oestradiol-treated cells show significant ($P<0.05$) decrease in gene expression of *TLR4*. Data presented as mean (±SD) of three individual experiments in triplicate. Statistical analysis used two-tailed Student's *t*-test. $P<0.05$ was considered significant.

for a further four days at 37°C. Proliferation of $CD4^+$ T cells was studied by a cell proliferation kit (Promega), following the manufacturer's instructions. The culture supernatants were harvested and analysed for the production of various cytokines. In parallel experiments, the culture of dendritic cells with autologous T cells was performed in the presence of oestradiol.

Quantification of cytokines by ELISA

Quantification of cytokines (IL-6, IL-12, IL-4, IL-10, tumour necrosis factor-α [TNFα] and interferon-γ [IFNγ]) in culture supernatants was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (eBiosciennces, San Diego, USA), following the manufacturer's instructions.

Statistical analysis

Data were presented as mean (±SD) of three individual experiments in triplicate. Statistical analysis was determined using the two-tailed Student's *t*-test and $P<0.05$ was considered significant.

Results

Culture of MDDCs and expression of co-stimulatory molecules

After culture for five to seven days in recombinant GM-CSF and IL-4, monocytes transform into immature dendritic cells ($CD1a^+$ cells) as confirmed by flow cytometry. Analysis of EB-pulsed immature dendritic cells revealed upregulation in expression of major histocompatibility complex class II (MHC-II), CD83 (DC maturation marker) and CD86 compared to non-stimulated dendritic cells (Fig. 1).

Oestradiol down-regulates TLR4 and associated signalling **in vitro**

The expression of a number of Toll-like receptor signalling genes such as *IRAK4*, *MyD88* and *nF-κB* were upregulated following chlamydial infection. Significantly expressed genes in oestradiol-treated and -untreated conditions are shown in Figure 2. Oestradiol treatment significantly ($P<0.05$) reduced TLR4 expression compared to untreated cells. Gene expression of TLR4-associated downstream signalling molecules (IRAK4 and NF-κB) was also found to be down-regulated. Gene expression of the Th1-associated cytokines IL-12, IL-6, TNFα and IFNγ was also reduced ($P<0.05$); however, expression of IL-10 by DCs increased significantly after oestradiol pretreatment.

Secretion of cytokines by DCs with oestradiol treatment

Oestradiol treatment significantly ($P<0.05$) reduced the release of the pro-inflammatory cytokines IL-12 and TNFα at the highest concentration; however, a non-significant decrease in levels of IL-6 was observed. A significant ($P<0.01$) increase in secretion of IL-10 was observed with the highest oestradiol concentration. IL-4 was below the detection limit in all the experiments (Fig. 3).

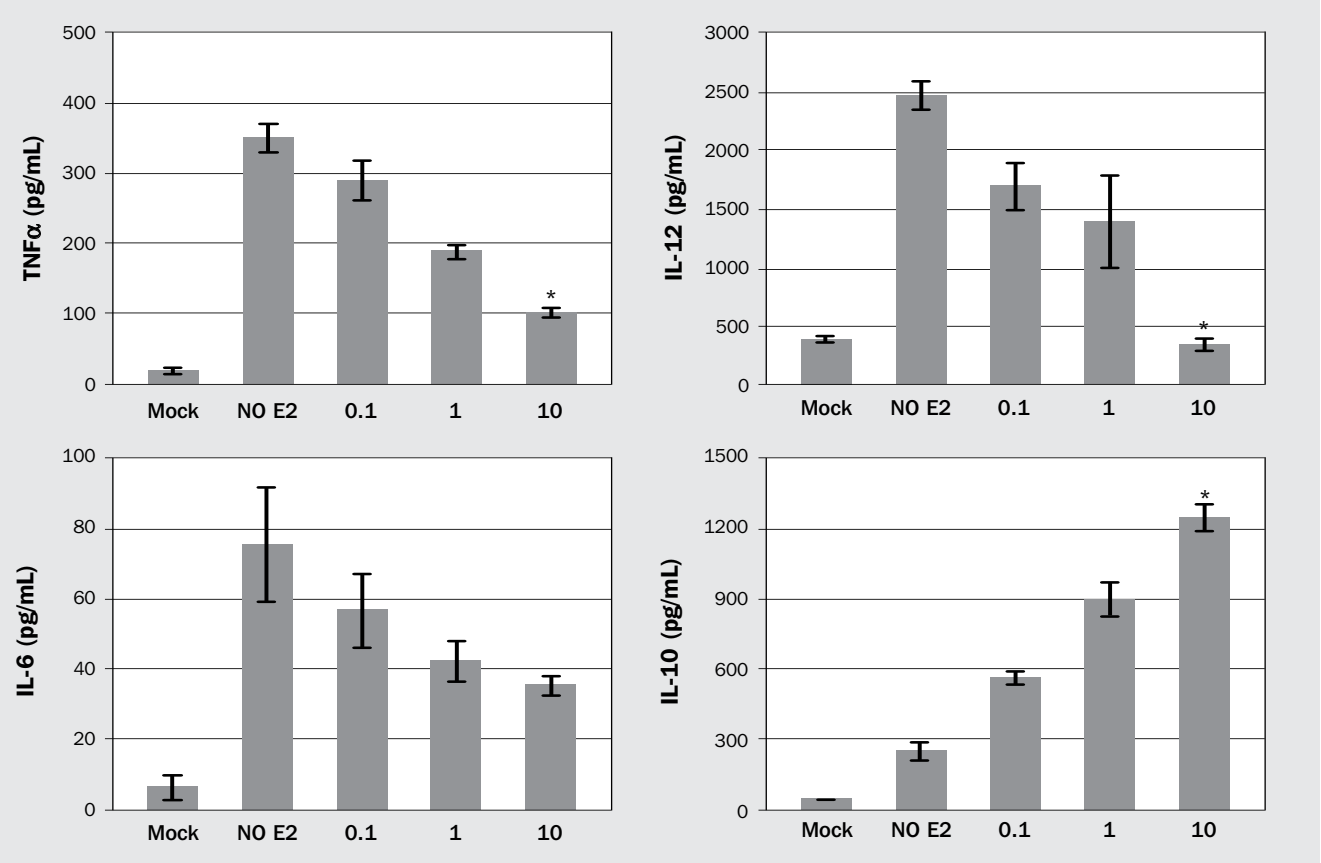

Fig. 3. Cytokine levels in the supernatant of dendritic cells pulsed with *C. trachomatis* EBs. Significant ($P<0.05$) reduction in secretion of TNFα and IL-12 was observed. IL-10 levels increased ($P<0.01$) with increasing oestradiol concentration. Statistical analysis used two-tailed Student's *t*-test. $P<0.05$ was considered significant. NO E2 = oestradiol-untreated dendritic cells.

Cytokine secretion and the effect of oestradiol

When *Chlamydia*-pulsed DCs were co-cultured with autologous CD4⁺ T cells it was found that dendritic cells efficiently presented chlamydial antigens to CD4⁺ T cells, as shown by the significant proliferation of CD4⁺ T cells (data not shown). The mean levels of IL-1β, IL-2, IL-6, IL-10, IL-12 and IFNγ in culture supernatants of EB-pulsed DCs cultured with activated autologous CD4⁺ T cells, together with controls, are shown in Figure 4. IL-2 and IL-12 levels were significantly higher ($P<0.01$) in the supernatants of EB-pulsed DCs cultured with activated autologous CD4⁺ T cells. Treatment of DCs with oestradiol significantly reduced secretion of IL-6 ($P<0.01$) and IL-12 ($P<0.05$) by CD4⁺ T cells; however, oestradiol increased the secretion of IL-10 from activated CD4⁺ T cells.

Discussion

The aim of this study was to examine the interaction between human DCs and *C. trachomatis* with respect to DC and CD4⁺ T-cell activation and induction of pro-inflammatory immune responses, together with the role of oestradiol in modulating DC function to increase host susceptibility to *C. trachomatis*.

Viable chlamydial EBs (serovar D) were used to induce the

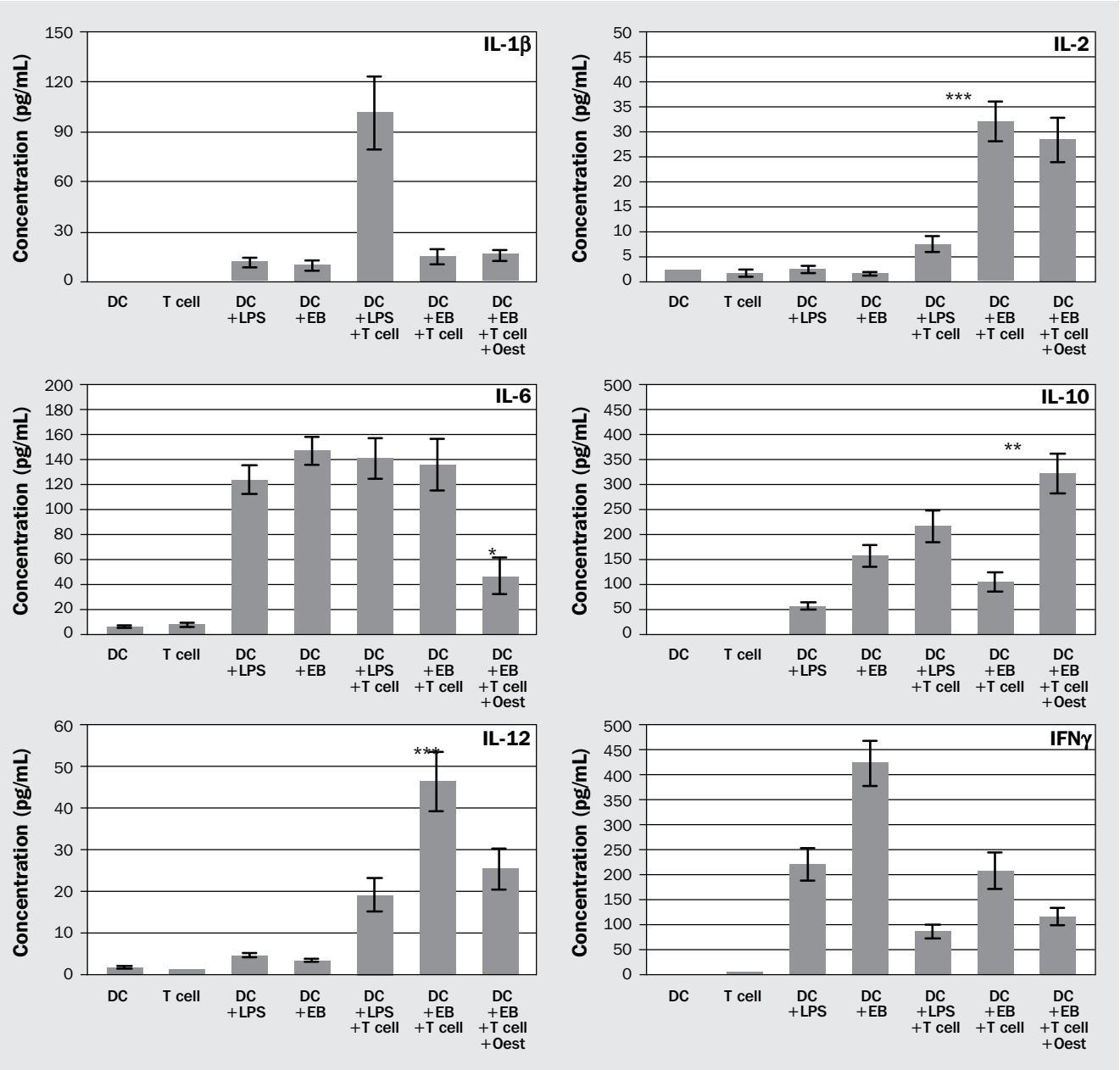

Fig. 4. Cytokine levels in supernatants of CD4⁺ T cells activated in the presence of dendritic cells pulsed with *C. trachomatis* EBs. IL-2 and IL-12 levels were significantly ($P<0.01$) higher in supernatants of EB-pulsed DC cultured with activated autologous CD4⁺ T cells. Treatment of dendritic cells with oestradiol significantly reduced secretion of IL-6 ($P<0.01$) and IL-12 ($P<0.05$) by CD4⁺ T cells; however, oestradiol increased secretion of IL-10 from activated CD4⁺ T cells. Statistical analysis used two-tailed Student's *t*-test. $P<0.05$ was considered significant.

activation of DCs, as they would give an insight into how DCs process and present chlamydial antigens to T cells. Pulsing DCs with live chlamydial EBs was characterised by the production of the cytokines IL-6, IL-10 and TNFα, together with the upregulation of the cell surface markers MHC-II, CD83 and B7-2 (CD86). Thus, protective immunity was achieved with the adoptive transfer of DCs pulsed with inactivated chlamydial organisms, and this protection correlates with the induction of a Th1 immune response, as found in other studies.[17–21]

The protective ability of the *Chlamydia*-pulsed DCs also correlates with their ability to secrete IL-12 and induce a Th1-type response when transferred *in vivo*.[19,20,22] However, adoptive transfer of DCs pulsed with recombinant chlamydial MOMP has induced a non-protective Th2 response.[23]

As the expression of DC co-stimulatory molecules is critical for effective DC-mediated T-cell activation,[24] EB-pulsed DCs should be more effective at stimulating a T-cell response due to the increased surface expression of MHC-II and CD86. Stimulated DCs secrete low levels of IL-10, which suppresses protective immunity against chlamydial infections;[18] however, this small amount may be necessary to curb an excessive pro-inflammatory response which may lead to inflammation and tissue scarring. IL-12 secretion is required to control chlamydial infections,[25] and TNFα is known to promote DC migration.[26] Thus, this cytokine profile and maturation phenotype is consistent with live EB-pulsed DCs being immunogenic and therefore effective in promoting an antichlamydial immune response.

When *Chlamydia*-pulsed DCs were co-cultured with autologous $CD4^+$ T cells it was found that dendritic cells efficiently presented chlamydial antigens to $CD4^+$ T cells, as shown by a significant proliferation of $CD4^+$ T cells (unpublished data). In these co-cultures, the cytokines IL-2, IL-12 and IFNγ were significantly increased compared to either cell type alone. IL-2 is an important cytokine secreted by T cells and acts in an autocrine manner, leading to proliferation of T cells into a large number of effector T cells. IL-12 induces Th1 differentiation with induction of IFNγ.[27] Thus, secretion of IL-12 following stimulation of $CD4^+$ T cells may help to provide a protective immune response to chlamydial infection.

Sex hormones such as oestradiol and progestin exert a powerful effect on various immune system functions. In the present study, the effect of oestradiol on chlamydial recognition and downstream signalling in DCs was examined. While there is no definitive information on the mechanism by which hormones affect the immune system, studies have demonstrated some degree of regulation of DC function by oestradiol.[28] Data from the present study show that oestradiol reduces TLR4 expression and its downstream signalling, which may lead to improper recognition of *Chlamydia* by DCs and increase susceptibility to chlamydial infection. Similar observations have been reported by Moeinpour *et al*.[13] of reduced TLR4 expression and thus inflammatory response in epidermal keratinocytes. In the present co-culture study where $CD4^+$ T cells and EB-pulsed DCs were used, it was found that oestradiol reduces the protective Th1 response and increases anti-inflammatory response.

In conclusion, it is suggested that pulsing DCs with live chlamydial EBs induces a Th1 immune response through secretion of IL-12 and IFNγ by dendritic cells and activated $CD4^+$ T cells. However, addition of oestradiol leads to poor recognition of *Chlamydia* by down-regulating TLR4 and its associated pathway, and also by upregulating secretion of IL-10 from $CD4^+$ T cells. As DCs are now being used as adjuvants in vaccine studies, these results enhance the knowledge of *Chlamydia* and DC interactions and will aid the design of a chlamydial vaccine that could induce protective responses even in women with high cytokine levels due to contraceptive use, pregnancy or other disorders. □

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