Differential regulation of chemokine expression by estrogen in human periodontal ligament cells

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Background and Objective: Estrogen modulates inflammatory responses, but the mechanisms involved have not yet been identified. Periodontal ligament (PDL) cells produce chemokines (a group of chemoattractant molecules that recruit leukocytes) and it has been suggested that estrogen modulates periodontal inflammation by regulating the expression of chemokines by PDL cells. Therefore, the objectives of this study were to investigate the regulation of chemokine ligand 2 [CCL2/monocyte chemoattractant protein 1 (MCP-1)], chemokine ligand 3 [CCL3/macrophage inflammatory protein-1α (MIP-1α)] and chemokine ligand 5 (CCL5/RANTES) by estrogen in human PDL cells.

Material and Methods: PDL cells were obtained from the PDL of premolars, extracted for orthodontic reasons, from two boys and two girls (16 and 17 years of age). PDL cell *CCL2*, *CCL3* and *CCL5* mRNA transcripts were determined by quantitative real-time PCR. The concentrations of CCL2, CCL3 and CCL5 proteins were determined by ELISAs.

Results: Treatment with 0.5 μg/mL of lipopolysaccharide (LPS, from *Escherichia coli*) + 100 nM 17β-estradiol (E$_2$) for 24 h reduced the expression of *CCL3* mRNA by about 40% compared to PDL cells treated with LPS alone. Attenuation of *CCL3* mRNA was not associated with a decrease in CCL3 protein within 48 h, suggesting a slow turnover of the CCL3 protein. Interindividual differences in the effects of E$_2$ on *CCL5* mRNA expression were observed. E$_2$ (100 nM) increased the expression of *CCL5* by 40–60% in PDL cells derived from two subjects but reduced the expression of *CCL5* by about 30% in cells from another subject. *CCL2* mRNA and CCL2 protein were highly expressed, but not regulated by E$_2$. Similar data were observed in cells obtained from both boys and girls.

Conclusion: Regulation, by estrogen, of chemokine expression in PDL cells shows a complex pattern involving the down-regulation as well as the up-regulation of chemokines, suggesting that estrogen exerts both anti-inflammatory and proinflammatory effects through these mechanisms.

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The female sex hormone, estrogen, regulates gene transcription via estrogen receptor (ER)α and ERβ (1). Both ER subtypes are widely expressed in different cells and tissues and they show distinct and specific patterns of expression (1,2). Human periodontal ligament (PDL) cells have been shown to possess binding sites for radio-

labelled 17β-estradiol (E_2) and to express mRNA for ERs (3,4). We have shown that human PDL cells express preferentially ERβ immunoreactivity, while the signal for ERα is much lower, suggesting that PDL cells express predominantly ERβ protein (5,6). Taken together, these data show that PDL cells express ERs, but the functional importance of PDL cell ERs remains to be clarified. Stimulation of ERα and ERβ with the most important endogenous estrogen, E_2, has no effect on the functional properties of human PDL cells, such as collagen synthesis and cell proliferation (7). PDL cells are fibroblast-like cells that produce collagen, but data have been presented showing that these cells may be transformed into a more inflammatory-like cell phenotype that produces cytokines and chemokines (8–11), suggesting that PDL cells play a role as producers of cytokines and chemokines responsible for the recruitment of white blood cells in periodontal inflammation.

Estrogen has been suggested to exert both proinflammatory and anti-inflammatory effects (12–14). Stimulation of recruitment and adhesion of white blood cells to the vascular endothelium is an initial step in the inflammatory reaction, which is attenuated by estrogen (15–17). A possible mechanism behind estrogen-induced reduction of white blood cell recruitment to the endothelium is down-regulation of the vascular cell adhesion molecule-1, as shown previously by Caulin-Glaser *et al.* (18), Simoncini *et al.* (19) and Mukherjee *et al.* (20). Lowered chemokine production is another possible mechanism of action explaining estrogen-induced attenuation of white blood cell recruitment (21,22). Chemokine ligand 2 [CCL2/ monocyte chemoattractant protein 1 (MCP-1)], chemokine ligand 3 [CCL3/ macrophage inflammatory protein-1α (MIP-1α)] and chemokine ligand 5 (CCL5/RANTES) are three important chemokines produced by many different cell types stimulating the recruitment of white blood cells to the site of inflammation (23–25). The expression of CCL2 and CCL3 has been reported to be low in healthy periodontal tissue but to increase with severity of peri-odontal disease (26–28). Human PDL cells have been reported to express mRNA for *CCL2* and *CCL5* upon stimulation with viable *Porphyromonas gingivalis* (29).

Here, we investigated the effects of estrogen on the production of chemokines from PDL cells, and found that a physiological concentration of the endogenous estrogen, E_2, differentially regulates chemokine expression in human PDL cells.

Material and methods

Cells and cell culture

The PDL cells were collected from four subjects – two boys, 16 and 17 years of age, and two girls, 16 and 17 years of age – who were referred for extraction of premolars on orthodontic indications. The patients and their parents were informed orally, and in writing, of the purpose of the study and the parents gave written approval for the PDL cells to be used. The study design and the experiments were approved by the Human Ethical Committee at Lund University (Lund, Sweden). Immediately after extraction, the teeth were washed in phosphate-buffered saline (PBS) and the middle third of the periodontal ligament was scraped off using a sterile curette. The apical and gingival parts of the periodontal ligament were not used in order to avoid contamination with cell types other than PDL fibroblasts. PDL explants from each subject were seeded in cell-culture Petri dishes containing Dulbecco's modified Eagle's medium supplemented with antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), glutamine (1.16 g/L) and 10% fetal calf serum, and the dishes were then placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air. The cells migrating from the explants were trypsinized (0.25%) after reaching confluence and were then reseeded at a density of 600,000 cells/mL. Experiments were performed on cells reaching 80% confluence in passages three to five. At these passages the PDL cells show fibroblast morphology, with a spindle-like cell shape, which is characteristic of fibroblasts (6). Before the start of the experiments cell density was evaluated carefully using a phase-contrast microscope (Olympus CK40; Olympus Europa GmbH, Hamburg, Germany).

Experimental procedure

Twenty-four hours before starting the experiments, standard cell-culture medium was replaced with fetal calf serum-free and phenol red-free medium to achieve standardized conditions with quiescent cells and to remove the estrogen-like activity of phenol red. E_2 (Sigma Chemicals, St Louis, MO, USA) was included 2 h before lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4 LPS; Sigma) and was then present during the 24 or 48 h incubation with LPS. LPS was dissolved in PBS and E_2 was dissolved in ethanol. Controls received ethanol (< 0.1%) as vehicle. Each cell-culture dish (52 mm in diameter; Nunc, Roskilde, Denmark) containing PDL cells at 80% confluence represents one sample/ observation for either quantitative real-time PCR or ELISA. Each sample was analyzed in duplicate both for PCR assays and for ELISAs.

Quantitative real-time PCR

The PDL cells were washed carefully in PBS and then total RNA was extracted and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The concentration and purity of RNA was measured at 260/280 nm in a Nano-Drop ND-100 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The RNA concentration in each sample was about 75 ng/μL. The RNA samples were then subjected to one-step quantitative real-time PCR measurements using QuantiFast SYBR Green RT-PCR kits (Qiagen) and QuantiTect primer assays (Qiagen) on a Roche real-time thermal cycler (Roche, Basel, Switzerland). Each sample was analyzed in duplicate. The expression of *CCL2*, *CCL3* and *CCL5* genes was calculated using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene, as described by Pfaffl (30). The expression of *GAPDH*

mRNA was not affected by E_2 treatment. The PCR primers (QuantiTect Primer Assays) for CCL2 (HS_CCL2_1_SG), CCL3 (HS_CCL3_2_SG), CCL5 (HS_CCL5_1_SG) and GAPDH (HS_GAPDH_2_SG) were purchased from Qiagen. The CCL2, CCL3 and CCL5 primers showed similar efficiencies.

Measurement of chemokine proteins

The PDL cells were washed carefully in PBS and scraped off the culture dishes using cell scrapers (Sarstedt, Newton, NC, USA). Then the cells were sonicated 2×10 s on ice and centrifuged at 1700 g and $4°C$ for 5 min. The concentrations of CCL2 and CCL3 proteins were determined in the cell supernatant using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instructions supplied by the manufacturer. Each sample was analyzed in duplicate. The concentrations of CCL2 and CCL3 were normalized to the total protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Values are presented as means ± standard error of the mean. Statistical significance was calculated using the Student's two-tailed t-test, and p-values of < 0.05 were regarded as denoting statistical significance.

Results

Effects of E_2 on the *CCL3* mRNA level

Stimulation with E_2 (100 nM) in the presence of LPS (0.5 µg/mL) for 24 h reduced the *CCL3* mRNA level by about 35% vs. stimulation with LPS alone in PDL cells derived from a 16-year-old boy, suggesting that E_2 reduces *CCL3* expression (Fig. 1). Analysis of the *CCL3* mRNA level was repeated in PDL cells derived from another subject (a 17-year-old girl). In these cells, combined treatment with LPS (0.5 µg/mL) and E_2 (100 nM) also reduced (by about 40%) the *CCL3* mRNA level vs. treatment with LPS alone (1.00 in LPS-treated cells vs. 0.57 ± 0.12 in LPS + E_2-treated cells; $n = 6$ observations in each group,
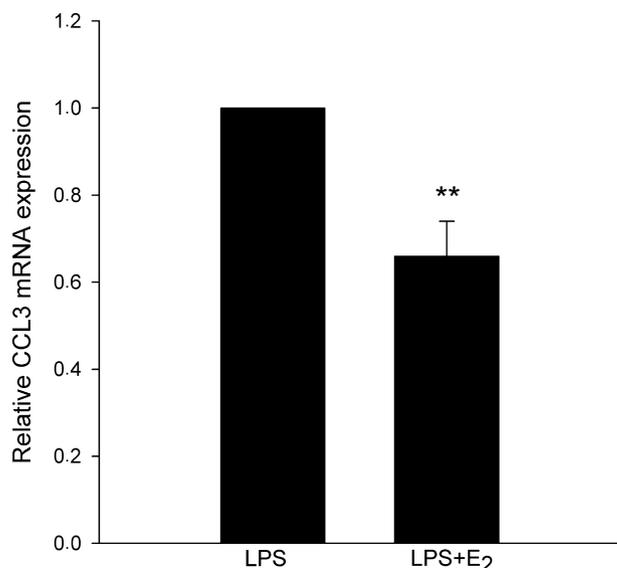
$p < 0.001$). Down-regulation of the *CCL3* mRNA transcript by E_2 was confirmed in PDL cells from a third subject, a 17-year-old boy (1.0 in cells treated with LPS alone vs. 0.55 ± 0.13 in cells treated with LPS + E_2; $n = 6$ observations in each group, $p < 0.001$). Down-regulation of *CCL3* mRNA by E_2 was thus observed in PDL cells originating from three different subjects.

Effects of E_2 on the PDL cell CCL3 protein concentration

The concentration of CCL3 protein in PDL cells was very low (at, or even below, the lowest standard). Treatment of PDL cells derived from three donors (the same donors analyzed for *CCL3* mRNA expression presented above) with LPS (0.5 µg/mL) + E_2 (100 nM) for 24 h tended, but not significantly, to decrease the CCL3 protein concentration vs. treatment with LPS alone (0.29 ± 0.11 pg/µg of protein in LPS-treated cells vs. 0.14 ± 0.03 pg/µg of protein in LPS + E_2-treated cells; $n = 8$ in each group). Treatment with LPS (0.5 µg/mL) + E_2 (100 nM) for a longer period of time (48 h) had no effect on the concentration of CCL3 protein vs. treatment with LPS alone (Fig. 2).

Effects of E_2 on the PDL cell *CCL2* mRNA level and the CCL2 protein concentration

The relative mRNA expression level for *CCL2*, normalized to that of the housekeeping gene *GAPDH*, was about 55% higher than the mRNA expression level of *CCL3* in LPS-stimulated (24 h of stimulation with 0.5 µg/mL of LPS) PDL cells derived from the 17-year-old girl (Fig. 3). Higher expression of *CCL2* mRNA vs. *CCL3* was observed also in cells derived from two other subjects. The PCR data showing high expression of *CCL2* was confirmed at the protein level. The CCL2 protein level was about three times higher than that of CCL3 in PDL cells treated with 0.5 µg/mL of LPS for 24 h (0.89 ± 0.07 pg/µg of protein for *CCL2* vs. 0.29 ± 0.11 pg/µg of protein for *CCL3*; $n = 3$ and 8 observations in each group, respectively, $p < 0.05$).

Fig. 1. Quantitative PCR shows that treatment with lipopolysaccharide (LPS) (0.5 µg/mL) + 17β-estradiol (E_2) (100 nM) reduces the level of *CCL3* mRNA transcript by about 35% compared to treatment with LPS alone in periodontal ligament (PDL) cells derived from a 16-year-old boy. Similar results were observed in cells derived from two other subjects included in the study. The cells were stimulated with LPS, with or without E_2, for 24 h. Values are presented as means ± standard error of the mean of six observations in each group. **$p < 0.01$ compared with LPS alone.
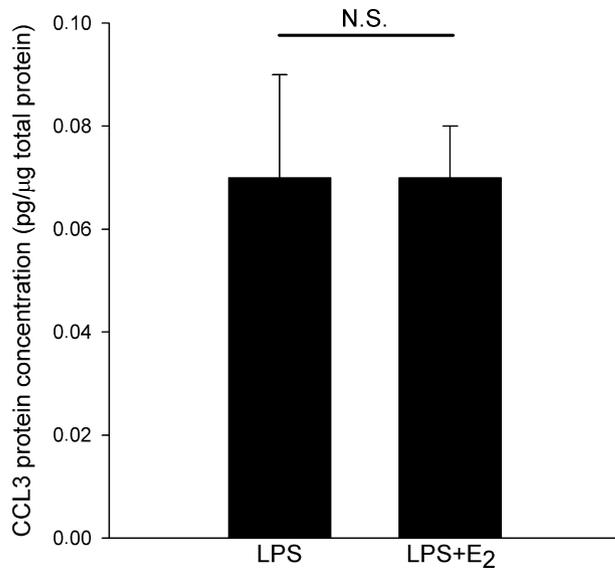
Fig. 2. 17β-Estradiol (E_2) (100 nM) has no effect on the CCL3 protein concentration, determined by ELISA, in periodontal ligament (PDL) cells derived from a 16-year-old boy. Cells derived from this subject were also used for real-time PCR and these data are presented in Fig. 1. The cells were stimulated with lipopolysaccharide (LPS) (0.5 μg/mL), with or without E_2, for 48 h. Values are presented as means ± standard error of the mean of four observations in each group. NS, not significant.
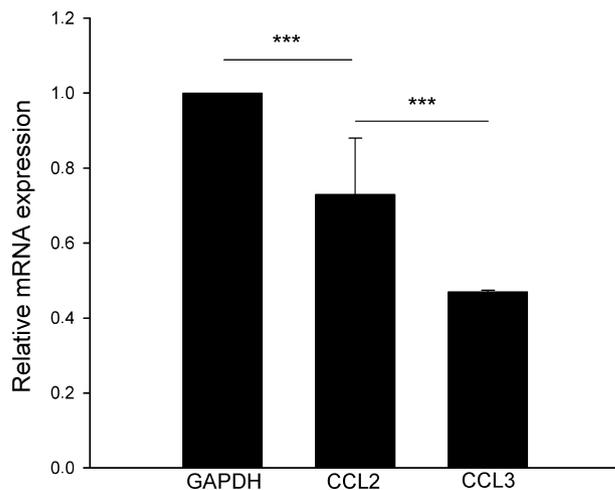

Fig. 3. Relative expression level of *CCL2* and *CCL3* mRNA transcripts normalized to that of *GAPDH* in lipopolysaccharide (LPS)-stimulated (0.5 μg/mL for 24 h) periodontal ligament (PDL) cells derived from a 17-year-old girl. The *CCL2* gene shows 55% higher expression compared with the *CCL3* gene. *CCL2* mRNA expression was higher than that of *CCL3* also in PDL cells derived from two other subjects included in this study. Values are means ± standard error of the mean of six observations in each group. ****p* < 0.001.

Treatment with LPS (0.5 μg/mL) + E_2 (100 nM) for 24 h had no effect on the *CCL2* mRNA transcript level and CCL2 protein concentration compared to stimulation with LPS alone in PDL cells derived from the 17-year-old girl (Fig. 4). Moreover, chronic treatment (21 d) with LPS (0.5 μg/mL) + E_2 (100 nM) had no effect on the concentration of CCL2 protein compared to treatment with LPS alone (data not shown). Similar data for CCL2 were observed in cells derived from the other three subjects included in this study.

Effects of E_2 on the mRNA level of *CCL5* in PDL cells

Treatment with 100 nM E_2 in the presence of LPS (0.5 μg/mL) for 24 h increased the *CCL5* mRNA level by about 60% compared to treatment with LPS alone in PDL cells derived from the 17-year-old boy (Fig. 5A). In PDL cells derived from the 16-year-old boy, costimulation with LPS and E_2 caused a 30% decrease in *CCL5* mRNA vs. stimulation with LPS alone (Fig. 5B). LPS + E_2 increased the *CCL5* transcript level by 40% vs. LPS alone in PDL cells obtained from the 17-year-old girl, whereas E_2 had no effect in cells derived from the 16-year-old girl (Fig. 5C,D). Taken together, the effects of E_2 on CCL5 vary between PDL cells originating from different subjects, suggesting that the response to E_2 is dependent on interindividual differences.

Discussion

In the present study we demonstrated a differential regulation of chemokine genes by E_2 in human PDL cells, suggesting that estrogen exerts both pro-inflammatory and anti-inflammatory effects through these mechanisms. We showed estrogen-induced down-regulation of *CCL3* mRNA, while the expression of *CCL2* mRNA was unaffected by estrogen in PDL cells derived from three individual subjects. Interindividual variations in E_2-induced effects on PDL cell *CCL5* expression were demonstrated, suggesting that the effects of estrogen on CCL5 depend on the genetic origin of the PDL cells. The E_2-evoked effects on chemokine expression were observed in cells derived from boys as well as girls, suggesting that these mechanisms are independent of gender. We used cells derived from subjects of similar age to minimize interindividual differences. PDL cells derived from male and female subjects express ERα and ERβ similarly (5), supporting the fact that PDL cells from male and female
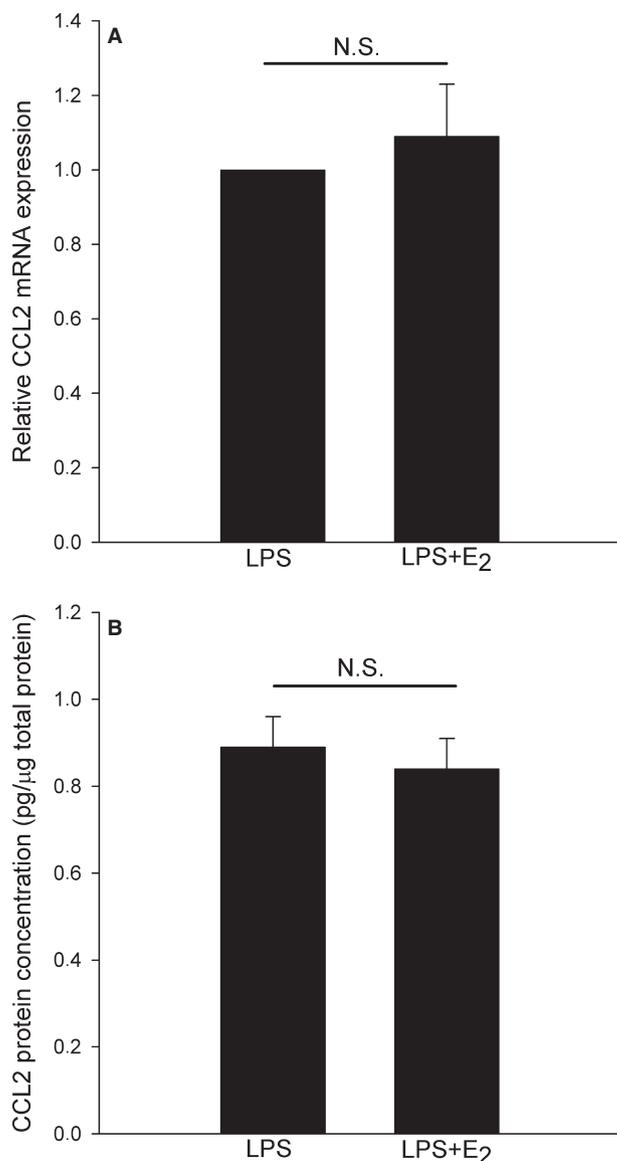
Fig. 4. Expression of (A) *CCL2* mRNA and (B) CCL2 protein in periodontal ligament (PDL) cells treated for 24 h with lipopolysaccharide (LPS) (0.5 μg/mL) in the absence or in the presence of 100 nM 17β-estradiol (E_2). The PDL cells were derived from a 17-year-old girl. Values are means ± standard error of the mean of three to six observations in each group. NS, not significant.

may be the combination of low CCL3 protein levels and a too low sensitivity of the CCL3 ELISA. We investigated the effects of estrogen on CCL3 protein at 24 h (i.e. the same time-point at which estrogen down-regulates the *CCL3* transcript) and at 48 h, but estrogen had no effect at either time-point, suggesting that the cellular CCL3 protein concentration is maintained for at least 48 h, although expression of the *CCL3* mRNA transcript is reduced by about 40%. These data suggest a slow turnover of the CCL3 protein.

In this study we identified the *CCL3* and the *CCL5* genes to be regulated by estrogen in human PDL cells subjected to stimulation with the *E. coli* promoter of inflammation, LPS. In human PDL cells, *E. coli* LPS and LPS from the well-known periodontal disease pathogen *P. gingivalis* have been shown to induce similar levels of cytokine expression (9), and thus it is reasonable to suggest that our data are representative for the *in vivo* situation. We used a concentration of LPS (0.5 μg/mL) that has been shown previously to induce cytokine and chemokine production without affecting collagen synthesis and cell proliferation in human PDL cells (11,32). *CCL3* mRNA has been reported to be expressed in human gingival epithelial cells, but not in human gingival fibroblasts (33). We demonstrated that human PDL cells express *CCL3* mRNA, suggesting cell-type-specific expression of this chemokine among different types of oral fibroblasts. Interestingly, the PDL cell expression level of the CCL2 chemokine was higher at both mRNA and protein levels than that of the CCL3 chemokine, suggesting that PDL cells are able to produce high amounts of CCL2. Thus, because the PDL cells show a high expression of CCL2, and this chemokine promotes recruitment of monocytes/macrophages (25), we suggest that PDL cells play an important role in attracting monocytes to the periodontal inflammation but that estrogen has no effect on this process.

We demonstrated that estrogen reduces *CCL3* gene expression in human PDL cells, suggesting that estrogen
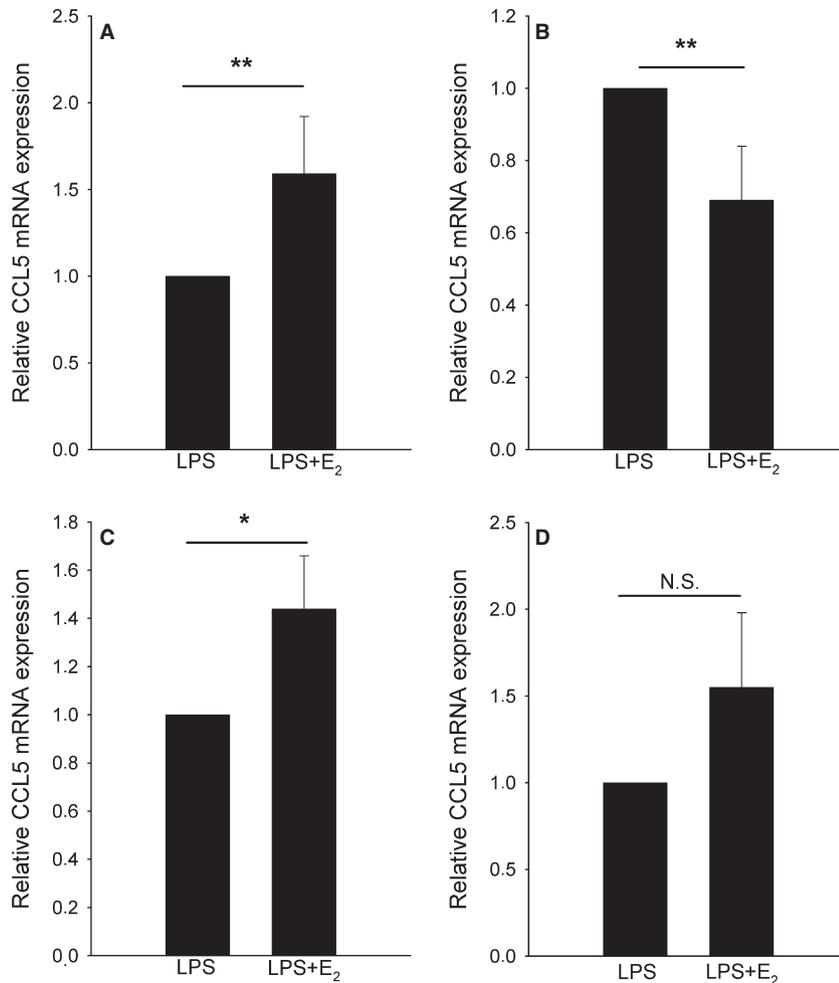
subjects respond similarly to estrogen. We used a high, but still physiological, concentration (100 nM) of E_2. This is about the same concentration of E_2 observed in plasma during pregnancy (31). Regulation of PDL cell chemokine expression by estrogen, as demonstrated here, is probably more important in situations with high plasma concentrations of estrogen (e.g. in premenopausal women and during pregnancy), than in situations with low

estrogen concentrations (such as after the menopause).

Treatment with estrogen decreased the expression of mRNA for *CCL3* but had no significant effect on the cellular concentration of CCL3 protein, suggesting that the reduction in *CCL3* mRNA induced by E_2 (−40%) was not sufficient to cause a reduction in the protein level. Another possible explanation for the lack of detectable E_2-induced reduction of CCL3 protein

Fig. 5. The effects of 17β-estradiol (E_2) on periodontal ligament (PDL) cell *CCL5* mRNA levels depend on interindividual variations. The PDL cells were treated for 24 h with lipopolysaccharide (LPS) (0.5 μg/mL) in the absence or in the presence of 100 nM E_2. Panels A and B show data from cells derived from the two boys, 17 and 16 years of age, respectively. Panels C and D show data from the two girls, 17 and 16 years of age, respectively. Values are means ± standard error of the mean of five to seven observations in each group. *$p < 0.05$; **$p < 0.01$. NS, not significant.

attenuates the recruitment of white blood cells to the inflammatory reaction via this mechanism. By contrast, estrogen up-regulated *CCL5* gene activity in PDL cells from two out of four subjects, suggesting that estrogen stimulates recruitment of T cells to the inflammatory reaction via this mechanism. Taken together, estrogen exerts both anti-inflammatory and pro-inflammatory effects via these mechanisms. We have previously shown that the chemokine GROα, which is another important chemoattractant for neutrophils, is also, like CCL2, not regulated by estrogen (34). In this study we showed that both CCL3 and

CCL5 are regulated by estrogen, while CCL2 is not. Thus, estrogen seems to differentially regulate chemokine expression in human PDL cells.

Acknowledgements

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