Pro-inflammatory and anti-inflammatory cytokines in human preterm and term cervical ripening

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Abstract

Cervical ripening is necessary for successful delivery. As cytokines are believed to be involved in this process, the aim of this study was to investigate possible changes in the mRNA and protein expression of pro-inflammatory (interleukin (IL)-1α, IL-1β, IL-12, IL-18) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines in the human cervix during pregnancy, term and preterm labor. Cervical biopsies were taken from 59 women: 21 at preterm labor, 24 at term labor, 10 at term not in labor and 4 from non-pregnant women. mRNA was analyzed with real-time RT-PCR and protein expression and/or secretion with immunohistochemistry and ELISA. There was an upregulation of mRNA for IL-10, IL-13, IL-1α and IL-1β in the laboring groups, while mRNA for IL-12 and IL-18 was downregulated ($p<0.05$). IL-4 mRNA was detected more frequently, while IL-12 mRNA expression was lower, in the preterm labor group than in the term labor group ($p<0.05$). The protein levels of IL-4 and IL-12 were lower and IL-18 tended to be higher in the labor groups, while IL-10 protein levels were unaffected by labor. IL-4 protein levels were significantly higher in the preterm subgroup with bacterial infection than in the non-infected group ($p<0.05$). IL-10 had higher expression in squamous epithelium at preterm labor than at term ($p<0.05$). In conclusion, the major changes in pro-inflammatory and anti-inflammatory cytokine mRNA and protein expression in cervix occur during the labor process irrespective of the length of gestation. However, our results indicate that dysregulation of anti-inflammatory cytokines in the human cervix could be involved in the pathogenesis of preterm labor.

Keywords: cervical ripening;cervix;cytokines; preterm birth

Abbreviations:

PPROM- preterm premature rupture of membranes, PTL-preterm labor, NP-non-pregnant, Th- T helper cells, TL- term labor, TnotL-term not in labor, TLR- Toll-like receptors

1. Introduction

The rate of preterm birth has not decreased in the past 30 years and the mechanisms underlying the initiation of both preterm and term labor remain largely unknown (reviewed in (Goldenberg et al. 2008)). At delivery, uterine contractions are coordinated with cervical ripening. This process is considered to be an inflammatory reaction associated with elevated levels of cytokines at the time of both preterm and term labor (Sennstrom et al. 2000; Tornblom et al. 2005). Furthermore, parturition itself can be regarded as an inflammatory process (reviewed in (Challis et al. 2009)).

For many years, a healthy pregnancy was described as a T helper (Th)-2 phenomenon while Th1 cytokines were believed to be harmful to pregnancy outcome (Wegmann et al. 1993; Raghupathy 1997). This concept has now developed further and nowadays a complex and dynamic cytokine balance is considered to prevail during gestation (Chaouat 2007). Although Th1 type of responses are associated with spontaneous abortions and reproductive failure, they also dominate early during the peri-implantation period as well as during labor (reviewed in (Raghupathy and Kalinka 2008)). IL-12 and IL-18 are important in regulating natural killer cell activities in early pregnancy, and are considered important for reproductive success. However, altered levels of these cytokines can be hazardous as higher circulating IL-12 levels in maternal blood have been described in preeclampsia and in preterm delivery (Bachmayer et al. 2006; Gargano et al. 2008), and IL-18 levels in maternal blood are lower in women giving birth before 34 weeks of gestation (Ekelund et al. 2008). Also, a pro-inflammatory cytokine bias is seen in placentas in women with preterm delivery and preterm premature rupture of membranes (PPROM) compared to term (El-Shazly et al. 2004).

IL-10 can be considered as a regulatory cytokine. In relation to pregnancy, it decreases production of pro-inflammatory cytokines such as IL-8, IL-6, TNF-α, IL-1β (Fortunato et al.

1996; Fortunato et al. 1997; Fortunato et al. 1998; Sato et al. 2003) and prostaglandin E_2 (Brown et al. 2000) in lipopolysaccharide-stimulated fetal membranes. IL-10 treatment significantly reduces IL-1β induced uterine contractility and amniotic fluid prostaglandins in pregnant rhesus monkeys (Sadowsky et al. 2003).

The cytokine balance in cervix is less well characterized. Several of the pro-inflammatory cytokines, like IL-6, IL-8, granulocyte colony stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1), and IL-1β, increase at parturition (Sennstrom et al. 2000; Osman et al. 2003; Tornblom et al. 2005). Non-infected preterm parturition is also associated with higher levels of IL-6, IL-8 and MCP-1 in human cervix (Tornblom et al. 2005). While, anti-inflammatory cytokines have been studied in the non-pregnant cervical tissue in association with human papilloma virus infection, human immunodeficiency virus infection, in premalignant and malignant lesions (de Gruijl et al. 1999; Nicol et al. 2005), no studies so far describe the balance of pro-inflammatory and anti-inflammatory cytokines in the cervical tissue during pregnancy and labor at term or preterm. Therefore, the aim of this study was to investigate possible changes in the mRNA and protein expression of pro-inflammatory (IL-1α, IL-1β, IL-12, IL-18) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines in the human cervix during pregnancy, term and preterm labor.

2. Materials and Methods

2.1 Patients

A total of 59 women were included in four study groups. The groups in labor included 21 women undergoing preterm labor (PTL) and 24 women undergoing normal term labor (TL). As controls, ten women who delivered at term by caesarean section prior to the onset of labor (TnotL) were recruited. Four non-pregnant women (NP) undergoing

hysterectomy for benign conditions such as myomas, were used as a reference group, since several new substances were investigated in cervical tissue.

Preterm delivery was defined as delivery before the 37th week of gestation. The labor groups (PTL and TL) were in active labor and demonstrated a ripe cervix, with dilatation more than 4 cm. All of these patients except three were delivered vaginally. One patient in the PTL group was delivered by emergency caesarean section due to breech presentation and two patients in the TL group due to protracted labor. Women in the TnotL group had unripe cervices (with a Bishop score of <5 points) and were delivered by caesarean section prior to the onset of labor. The indications were breech presentation, humanitarian, earlier caesarean section or disproportion.

None of the women included in the study suffered from pre-eclampsia, diabetes or other systemic or intercurrent disease.

Vaginal and urine cultures were taken from women in PTL group. Ten of 21 women in the PTL group had negative cultures. In six patients bacterial growth was demonstrated (three with ureaplasma urealyticum and three with group B streptococcus). In five patients the cultures were positive for candida. Ten of the PTL patients had preterm premature rupture of membranes (PPROM), defined as rupture of membranes at least one hour before contractions (Goldenberg et al. 2008). In the PPROM subgroup, there were four women with negative cultures, four with bacterial growth and two with candida growth in the cultures.

There were no significant differences between the groups of pregnant women with respect to maternal age, parity, previous preterm births and previous caesarean sections. Clinical data on the women is presented in Table I.

Before the study, the local Ethics Committee of Karolinska Institute gave its approval and the subjects gave their informed consent.

2.2 Sampling procedure

A cervical biopsy was taken as described previously (Dubicke et al. 2008) and divided into three pieces. The samples for mRNA analysis were immediately immersed in RNA*later*® (Ambion Inc, Austin, TX, USA), kept at 4° C for 24 hours and thereafter frozen and stored at -70°. The samples for protein analysis were immediately frozen and stored at –70° C. The biopsies for immunohistochemistry were rinsed in physiological saline solution and fixed in a 4% formaldehyde solution for a maximum of 24 hours, thereafter dehydrated in 70% ethanol solution and embedded in paraffin.

Not all subsequent analyses could be performed on all 59 women, due to the limited amount of tissue retrieved from some of the women.

2.3 Tissue homogenization and extraction of RNA

Tissue homogenization was carried out using a dismembranation apparatus (Retsch KG, Haan, Germany) and was followed by either RNA or protein extraction.

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4 Treatment with DNAse and reverse transcription (RT)

The concentration of total RNA obtained was determined employing NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). All the samples had a OD260/OD280 ratio higher than 1.8. One μg of total RNA, pre-treated with 1 μl RQ1 RNase-Free DNase (Promega, Madison, WI, USA), was used for RT reaction, which was performed using SuperScript™ RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) as described elsewhere (Tornblom et al. 2005). The cDNA was stored at -70° C prior to use.

2.5 Real-Time RT-PCR

mRNA levels encoding IL-4, IL-10, IL-13, IL-1α, IL-1β, IL-12a (p35), IL-12b (p40) and IL-18 were quantified with real-time RT-PCR employing the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (Dubicke et al. 2008). Appropriate primers and probes were purchased from commercially available Taqman® gene expression assays (Applied Biosystems) and are presented in Table IIA. 18S, β-actin and cyclophilin A were used as endogenous controls. The geometric mean of these three endogenous controls was used for normalizing of the mRNA levels for the gene of interest (Vandesompele et al. 2002). Relative gene expression was calculated using a $\Delta\Delta C_T$ method, with the non-pregnant group as control. The geometric mean of C_T of endogenous controls was subtracted from the C_T of the respective gene, followed by subtraction of the median control group ΔC_T value, giving the $\Delta\Delta C_T$. The amount of products doubles in each cycle, so the relative gene expression was calculated with the formula $2^{-\Delta\Delta C_T}$, given in the manufacturer's instructions. Serial dilutions of placental cDNA made from purchased total RNA (Ambion, Austin, TX, USA) were added to each plate for validation of the experiment.

2.6 Protein extraction

Following the tissue homogenization, 1 ml of phosphate-buffered saline (PBS), including 0.01% Triton X-100 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and protease inhibitor cocktail 25 Complete™ (Boehringer Mannheim GmbH, Ingelheim, Germany), was added. After centrifugation at 10000g, 4°C for 10 min, the supernatant was retrieved and stored in aliquots at -70 °C until analyzed.

Total protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions.

2.7 Determination of the protein levels of IL-4, IL-10, IL-12 and IL-18

The concentrations of IL-4, IL-10 and IL-12 in the supernatants were determined using Quantikine® HS high sensitivity human ELISA kits (Quantikine®, R&D systems, Minneapolis, MN, USA). IL-12 ELISA kit detects IL-12 heterodimer, p70. The concentration of IL-18 was measured with Human IL-18 ELISA kit (MBL, Nagoya, Japan). All the measurements were done according to the manufacturer's instructions. The results were interpolated from the standard reference curve provided with each kit. The sensitivity of the kits was 0.11 pg/ml for IL-4, 0.5 pg/ml for IL-10 and IL-12, 12.5 pg/ml for IL-18. The concentrations of the cytokines were normalized against the total protein concentration.

2.8 Immunohistochemical staining

The biopsies were sectioned, mounted on Menzel-gläzer SUPERFROST® PLUS glasses (Menzel GmbH&Co KG, Braunschweig, Germany) and stained using the MACH3™ Mouse–Probe HRP Polymer Kit (Biocare Medical, CA, USA). The slides were pre-heated for one hour at 60°C. Thereafter, deparaffinization and antigen retrieval was performed in a 2100 Retriever autoclave (Prestige Medical, Minworth, England) using Diva Decloaker™ (Biocare Medical) and Hot Rinse™ (Biocare Medical) according to the manufacturer's instructions. After washing in TBS-buffer (Biocare Medical), the activity of endogenous peroxidase was eliminated with Peroxidazed (Biocare Medical), and non-specific binding was blocked with Background Sniper (Biocare Medical). The sections were incubated for 60 min with the appropriate mouse monoclonal antibody. Dilutions and manufacturers are shown in Table IIB. Subsequently, the slides were incubated for 12 min with mouse-probe MACH3 (Biocare Medical), which was followed by incubation for 15 min with M-Polymer HRP (Biocare Medical). All stainings were developed using a Betazoid DAB (Biocare Medical). The glasses were washed with TBS-buffer between steps and rinsed with distilled water afterwards, followed by counterstaining with Mayer's hematoxylin. Finally, the slides were dehydrated in

increasing concentrations of ethanol and lastly Xylen. Stainings with primary isotype-matched immunoglobulin of irrelevant antigen-specificity IgG_{2B} (for IL-10) or IgG_1 (for IL-12) (R&D systems) were used as negative controls.

2.9 Evaluation of the immunohistochemical stainings

For all immunohistochemical examinations, the immunoreactivity was checked in the squamous epithelium, the glandular epithelium, the vascular endothelium and five fields in the stroma. A semiquantitative scale from 0 to + + + was used. The evaluation was performed blindly by three independent investigators (A.D., G.C. and E.A.) using conventional light microscopy. The mean was calculated from evaluations of all three observers and further used for statistical analysis.

2.10 Statistical analysis

Two independent groups were compared utilizing the Mann-Whitney *U* test. When more than two groups were compared, the Kruskal-Wallis test was applied, followed by multiple comparison with Dunns correction. Spearman's rho was utilized for analyzing non-parametric correlations. Fisher's exact test was used to test for non-random associations. In all cases a p-value of <0.05 was considered to be statistically significant. All calculations were performed with the STATISTICA 8.0 software (StatSoft Inc, Tulsa, OK, USA) and GraphPad Prism 5.01 (GraphPad Software Inc, CA, USA).

3. Results

3.1 mRNA expression

3.1.1 Anti-inflammatory cytokines

IL-4 mRNA expression was detected in 12 of 16 samples in the PTL, in 8 of 24 in the TL, in 4 of 10 in the TnotL and in 1 of 4 in the NP group. In all detected samples IL-4 mRNA expression was low, with C_T values around 40. IL-4 was detected more often in the PTL group, where 75% of the samples analyzed were positive for IL-4 mRNA, than in the TL group, where 33% of the samples were positive for IL-4 mRNA ($p<0.05$) (data not shown).

The mRNA expression of IL-10 was higher in the laboring groups, with 2-fold higher levels in the PTL group ($p<0.05$) and 3-fold higher levels in the TL group ($p<0.001$) than in the TnotL group (Figure 1A).

Similarly to IL-4, IL-13 mRNA expression was low or undetectable in all the samples. Nevertheless, IL-13 was detected more often in the laboring groups (9 of 16 in PTL, 10 of 24 in TL) and not at all in any of 10 samples in the TnotL group ($p<0.05$) (data not shown).

3.1.2 Pro-inflammatory cytokines

Both IL-1α and IL-1β mRNA expression was high in the laboring groups. Compared with the NP group, IL-1α mRNA expression was four times higher in the PTL group and seven times higher in the TL group ($p<0.05$) (Figure 1B). The mRNA expression of IL-1β showed a similar pattern with 22-fold higher expression in PTL ($p<0.01$) and 20-fold higher expression in TL ($p<0.001$) compared to the TnotL group (Figure 1C). IL-1β mRNA expression was also significantly higher in the laboring groups than in the NP group ($p<0.05$).

As against this, IL-12a mRNA expression was significantly lower in both the TL and the PTL group than in the TnotL ($p<0.001$ for PTL, $p<0.01$ for TL) and the NP group ($p<0.01$) (Figure 1D). Moreover, IL-12a mRNA expression was 2-fold lower in the PTL than in the TL group ($p<0.05$). IL-12b mRNA expression was generally low or undetectable in all the samples with no differences between the groups (data not shown).

Similarly to IL-12, IL-18 mRNA expression was lower in the PTL ($p<0.05$) and in the TL group ($p<0.001$) than in the TnotL (Figure 1E). The mRNA expression in the NP group was also significantly lower than in the TnotL ($p<0.05$).

Apart from IL-12a mRNA expression and IL-4 mRNA detection frequency, there were no differences in mRNA expression comparing PTL and TL groups. There was no correlation between cytokine levels and gestational age. Subgroup analysis revealed no differences associated with positive vaginal and/or urinary cultures or PPROM. We observed positive correlations between the mRNA levels of IL-10 and IL-1β (rho=0.6, $p<0.0001$), IL-18 and IL-12a (rho=0.38, $p<0.01$). There were negative correlations between mRNA expression of IL-10 and IL-18 (rho=-0.4, $p<0.01$), IL-18 and IL-1β (rho=-0.4, $p<0.01$), IL-12a and IL-1β (rho=-0.4, $p<0.01$).

3.2 Protein levels

3.2.1 Anti-inflammatory cytokines

The levels of IL-4 were generally low in all the samples, under 0.6 pg/mg protein. The laboring groups had lower IL-4 concentrations than the TnotL and the NP groups (Figure 2A), although the difference was statistically significant only for the PTL group ($p<0.05$). Also, IL-10 concentrations were low or very low and there were no differences between the groups in IL-10 concentration (Figure 2B).

3.2.2 Pro-inflammatory cytokines

IL-12 protein expression was generally low in all the samples, generally below 0.4 pg/mg protein, and it was undetectable in 3 out of 13 samples in the PTL, in 2 out of 13 samples in the TL and in all 4 samples in the NP group. There was a tendency towards lower IL-12 levels in the laboring groups than in the TnotL group, statistically significant only for the PTL group ($p<0.01$) (Figure 2C).

IL-18 concentrations were much higher than those of the other cytokines analyzed, ranging from 5.28 till 429.5 pg/mg protein. There was a tendency towards higher IL-18 concentrations in the laboring groups (PTL and TL) than in the TnotL group, while the highest IL-18 concentrations were seen in the NP group (Figure 2D).

We observed no differences between the PTL and TL groups in cytokine protein concentrations. There was no correlation between cytokine levels and gestational age. Analyzing the subgroups of the PTL, we observed a tendency towards lower levels of IL-4 and IL-18 in the PPROM group than in the rest of PTL group (Figure 3A-B). There were significantly higher IL-4 levels in the subgroup with bacterial infection than in the subgroup without infection ($p<0.05$) (Figure 3C).

There was a positive correlation between the levels of IL-18 and IL-4 (rho=0.35, p=0.04); and a negative correlation between concentrations of IL-18 and IL-12 (rho=-0.54, p=0.0008).

3.3 Immunohistochemical staining of IL-10 and IL-12

IL-10 and IL-12 were both readily identified with immunohistochemistry in the cervical tissue (Figure 4-5A-D). All these proteins stained positively in squamous epithelium, vascular endothelium, glandular epithelium and stroma. The corresponding negative control sections demonstrated no staining (Figure 4-5 E). There was a big variation between the samples in the same group. However, we observed higher IL-10 protein expression in the squamous epithelium close to basal membrane in the PTL than in the TL group ($p<0.05$) (Figure 4A-B, F). IL-12 expression was more pronounced in the pregnant groups (PTL, TL, TnotL) than in the NP group (Figure 5 A-D). This observation was statistically significant in the stroma and vascular endothelium in the PTL group ($p<0.05$) (Figure 5F-G). Subgroup analysis revealed no differences associated with positive vaginal or urinary cultures or PPROM.

4. Discussion

To our knowledge, this is the first study to investigate both pro-inflammatory and anti-inflammatory cytokines in the cervical tissue during pregnancy and preterm and term labor.

Here, we show major changes in pro- and anti-inflammatory cytokine mRNA and protein expression in labor irrespective of gestational age, which is in line with our earlier studies on preterm and term cervical ripening (Tornblom et al. 2004; Tornblom et al. 2005; Klimaviciute et al. 2006; Dubicke et al. 2008). We demonstrate that a cervix in labor expresses higher mRNA levels of IL-10, IL-13, IL-1α and IL-1β while IL-18 and IL-12 mRNA levels are decreased compared to a cervix unaffected by labor. In contrast, protein levels of IL-18 tended to be higher, IL-4 and IL-12 levels tended to be lower and IL-10 levels remained the same in labor. Considering previous findings from our group of increased IL-6, IL-8 and monocyte chemotactic protein-1 protein levels during labor (Sennstrom et al. 2000; Tornblom et al. 2005), the overall cytokine profile of the laboring cervix is indicative of a pro-inflammatory response.

Although the major changes in cytokine expression were seen between labor and non-labor groups, we observed some differences in the processes of cervical ripening at preterm and term. We found lower levels of IL-12 mRNA expression at preterm than at term. The same tendency was observed in the protein levels; however this was not statistically significant. The biological function of this decrease is unclear, but lower expression of placental IL-12 is also seen in other pregnancy complications such as pre-eclampsia (Bachmayer et al. 2006). In contrast, there was a higher protein expression of IL-10 in the squamous epithelium at preterm labor. This confirms our earlier hypothesis that cervical epithelium plays an important role in the process of cervical ripening (Klimaviciute et al. 2006), as we have identified several other important substances like fetal fibronectine (Sennstrom et al. 1998), interleukin-8 (Sennstrom et al. 2000), MMP-8 (Sennstrom et al. 2003), corticotropin-releasing hormone (Klimaviciute

et al. 2006) and syndican-1 (Sahlin et al. 2008) in the cervical epithelium. We also detected IL-4 mRNA more frequently in preterm labor group than term. Despite the small number of cases, we also saw significantly higher protein levels of IL-4 in the subgroup with bacterial infection. This could indicate higher levels of Th2-associated cytokines in the cervix at preterm labor, especially when infection is present. Women with a higher anti-inflammatory/pro-inflammatory cytokine ratio in cervical secretions during early pregnancy are at higher risk of subsequent spontaneous preterm birth (Simhan and Krohn 2009). Those authors speculated that this relative hyporesponsiveness can create a permissive environment for ascending infection. On the other hand, higher IL-10 levels in epithelium at preterm labor could be a protective mechanism against too early pro-inflammatory changes in the cervix, as pro-inflammatory cytokines such as IL-1β can upregulate IL-10 expression (Trautman et al. 1997). Further, IL-10 can inhibit cyclooxygenase-2 expression and reduce prostaglandin release in cultured placental explants from preterm labor deliveries (Hanna et al. 2006). IL-10 can also cause selective inhibition of NFκB activation in LPS-stimulated human monocytes, whereas IL-4 can enhance degradation of various cytokines mRNA (Wang et al. 1995). IL-4 can decrease mRNA and protein expression of Toll-like receptors (TLR), which in turn can protect from excessive TLR signaling (Mueller et al. 2006). In a recent study, we report lower mRNA expression of TLR2 and TLR4 in preterm labor, and even further reduction in the group with bacterial infection (unpublished data). All these findings support a possible protective role of IL-10 and IL-4 in the cervix during preterm labor. Moreover, higher levels of IL-10 were also detected in amniotic fluid of women with preterm labor who delivered at preterm than in those who delivered at term (Gotsch et al. 2008), which could reflect a mechanism to counter-regulate the pro-inflammatory cervical ripening and delivery process. However, there are also reports describing that IL-10 (Mitchell et al. 2004) and IL-4 (Dudley et al. 1996; Spaziani et al. 1996) can induce pro-inflammatory action in the amnion.

Interestingly, analyzing the subgroups of the PTL group we saw a tendency towards lower protein levels of IL-18 and IL-4 in the PPROM group than the rest of preterm labor group. Although there are too few cases to draw any firm conclusions, these findings are consistent with the hypothesis that PPROM and PTL could partly involve different mechanisms (Menon et al. 2001). However, Menon et al observed an increase in IL-18 in the amniotic fluid of women with PPROM compared to women with preterm or term labor (Menon et al. 2001). Elevated IL-18 in PPROM also correlated with a longer interval to delivery (Jacobsson et al. 2003). This could show different functions of IL-18 in maternal and fetal compartments, and is supported by the findings that high IL-18 in amniotic fluid, but not in cervical secretions, was associated with microbial invasion of the amniotic fluid, intra-amniotic inflammation and prompt delivery in preterm labor (Jacobsson et al. 2003).

In our study, there was a discrepancy between mRNA and protein expression of IL-10 and IL-18. No differences in IL-10 protein levels were shown between the groups, but an upregulation of mRNA of IL-10 in labor was seen. The mRNA of IL-10 could be upregulated due to elevated pro-inflammatory cytokines during labor (Trautman et al. 1997). We find a tendency towards higher levels of IL-18 protein in labor, but downregulation of mRNA. This could be a negative feedback mechanism - IL-18 mRNA could be downregulated due to higher IL-18 protein levels. It could also be explained by temporal differences between mRNA synthesis and protein expression and shorter half-life of mRNA than protein.

In conclusion, we demonstrate that the major changes in pro-inflammatory and anti-inflammatory cytokine mRNA and protein expression in the cervix occur during the labor process irrespective of gestational age. There seems to be a Th1 bias in the laboring cervix. However, higher IL-10 levels in cervical epithelium in preterm labor and higher levels of IL-4 in the group with bacterial infection suggest that dysregulation of anti-inflammatory cytokines in the human cervix could be involved in the process of preterm labor.

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Table I Clinical data on women included in the study

Parameter	Preterm labor (PTL)	Term labor (TL)	Term not in labor (TnotL)	Non-pregnant (NP)

n	21	24	10	4
Age	31 (24-38)	31 (23-40)	33 (26-42)	46 (37-49)
Parity	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-2)
Previuos preterm births in the group	2	0	0	0
Previuos caesarean sections in the group	0	0	3	0
Gestational age in fgw[a]	34 (25-36)	40 (38-41)	39 (37-39)	-
Gestational age in days	238 (175-256)	282 (266-292)	272 (264-278)	-
Treatment with corticosteroids	7	0	0	-

Note: Data is presented as median (range) if not otherwise stated.
[a] full gestational weeks

Table IIA. Assay IDs and Reference Sequence database accession numbers for gene expression assays used for Real-time RT-PCR.

Gene	Assay ID	Reference Sequence database accession number
IL-4	Hs00174122_m1	NM_172348.1, NM_000589.2
IL-10	Hs00961622_m1	NM_000572.2
IL-13	Hs00174379_m1	NM_002188.2
IL-1α	Hs00174092_m1	NM_000575.3
IL-1β	Hs00174097_m1	NM_000576.2
IL-12a (p35)	Hs00168405_m1	NM_000882.2
IL-12b (p40)	Hs00233688_m1	NM_002187.2
IL-18	Hs99999040_m1	NM_001562.2
18S[a]	4319413E	X03205.1
β-actin[a]	4352935E	NM_001101.2
Cyclophilin A[a]	4326316E	NM_021130.3

[a] 18s, β-actin and cyclophilin A were used as endogenous controls.

Table IIB. Monoclonal anti-human antibodies used for immunohistochemical staining

Protein	Manufacturer	Cat No	Type	conc. (μg/ml)
IL-10	R&D systems (Minneapolis, MN, USA)	MAB219	mouse	2.5
IL-12	R&D systems (Minneapolis, MN, USA)	MAB217	mouse	6.25

Figure legends

Figure 1. mRNA expression of IL-10 (A), IL-1α (B), IL-1β (C), IL-12a (p35) (D) and IL-18 (E) in cervical tissue

C_T - the threshold cycle at which an increase in reporter fluorescence above the baseline signal is first detected. mRNA levels are normalized using geometric mean of three endogenous controls (18s, β-actin and cyclophilin A). The calculations are done using a $\Delta\Delta C_T$ method, with the non-pregnant group as control. Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). The box represents median value with 25%-75% of all data falling within the box. The whiskers extend to the range. The number of samples analyzed in each group is shown under the group name. Statistically significant differences are indicated above the plot: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 2. Protein levels of IL-4 (A), IL-10 (B), IL-12 (C) and IL-18 (D) in cervical tissue

Groups studied: preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). The box represents median value with 25%-75% of all data falling within the box. The whiskers extend to the range. The number of samples analyzed in each group is shown under the group name. Statistically significant differences are indicated above the plot: * $p<0.05$, ** $p<0.01$.

Figure 3. Subgroup analysis of protein levels in the preterm labor group

Protein levels of IL-4 **(A)** and IL-18 **(B)** in women with preterm premature rupture of membranes (PPROM), in women with preterm labor (PTL) and in women delivered at term (TL). The levels of IL-4 protein **(C)** in the preterm group with bacterial infection (PTL b), with candida infection (PTL c), the preterm group with negative cultures (PTL n) and term in labor group (TL). Every point in the scatter plot represents one sample and the mark is median value. Statistically significant differences are indicated above the plot: * $p<0.05$.

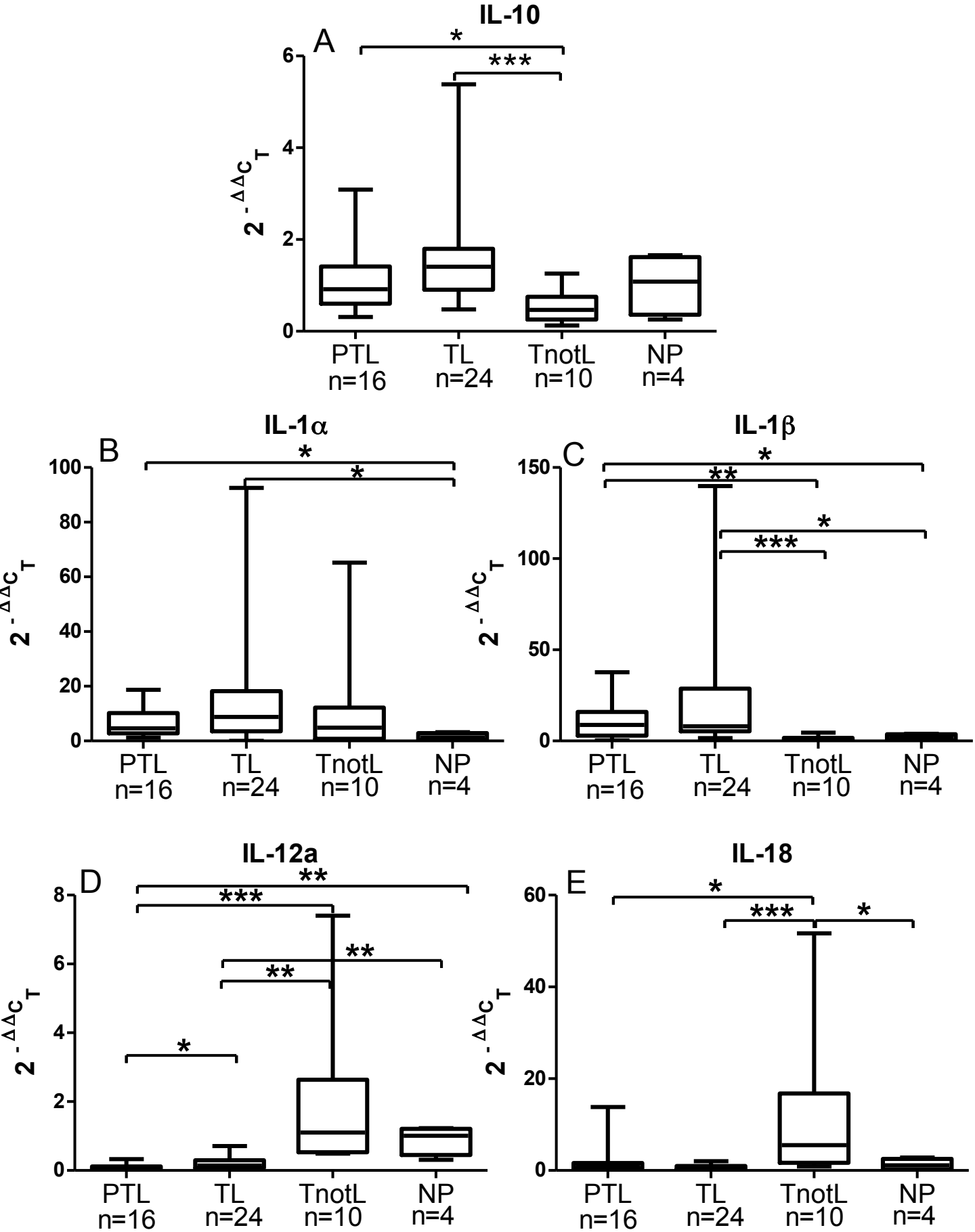
Figure 4. Immunohistochemical staining of IL-10 in cervical tissue

IL-10 in the cervical epithelium at preterm labor **(A),** term labor **(B),** term not in labor **(C)** and non-pregnant state **(D)**. Negative control **(E).** Magnification x400. The mark is 50 μm.

Box and whisker plots represent the staining of IL-10 in the squamous epithelium **(F).** Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). The box represents median value with 25%-75% of all data falling within the box. The whiskers extend to the range. The number of samples analyzed in each group is shown under the group name. A semiquantitative scale from 0 to + + + was applied. Statistically significant differences are indicated above the plot: * $p<0.05$.

Figure 5. Immunohistochemical staining of IL-12 in cervical tissue

IL-12 in the cervical epithelium at preterm labor **(A),** term labor **(B),** term not in labor **(C)** and non-pregnant state **(D)**. Negative control **(E).** Magnification x400. The mark is 50 μm. Box and whisker plots represent the staining of IL-12 in stroma **(F)** and vascular endothelium **(G).** Preterm labor (PTL), term labor (TL), term not in labor (TnotL) and non-pregnant (NP). The box represents median value with 25%-75% of all data falling within the box. The whiskers extend to the range. The number of samples analyzed in each group is shown under the group name. A semiquantitative scale from 0 to + + + was applied. Statistically significant differences are indicated above the plot: * $p<0.05$.


IL-10
A
*
***
6
4
2
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
IL-1α
B
*
*
100
80
60
40
20
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
IL-1β
C
*
**
*
***
150
100
50
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
IL-12a
D
**
***
**
**
*
8
6
4
2
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
IL-18
E
*
***
*
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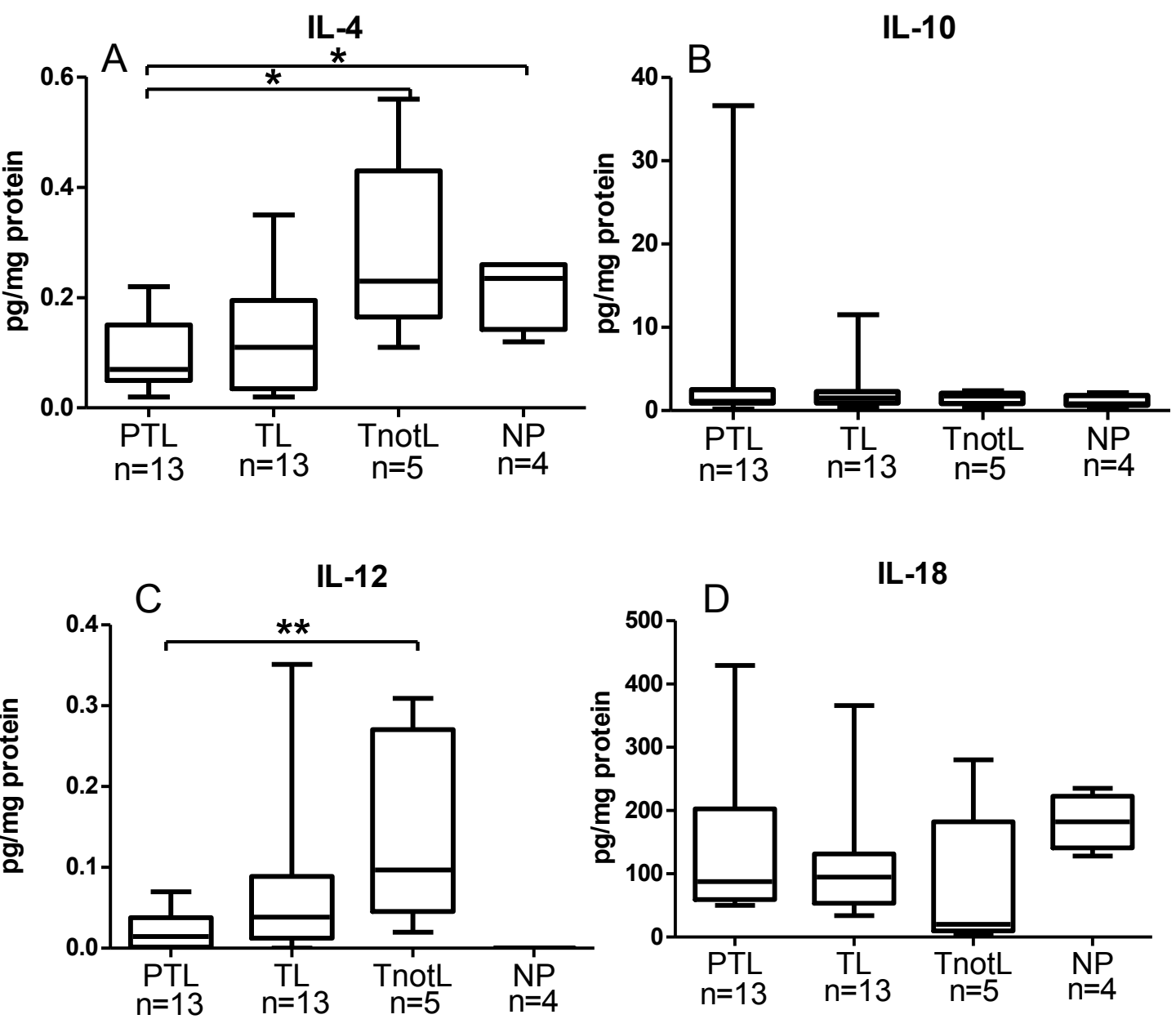
TL n=24
TnotL n=10
NP n=4

Figure 1


IL-4
A
*
*
0.6
0.4
0.2
0.0
pg/mg protein
PTL n=13
TL n=13
TnotL n=5
NP n=4
IL-10
B
40
30
20
10
0
pg/mg protein
PTL n=13
TL n=13
TnotL n=5
NP n=4
IL-12
C
**
0.4
0.3
0.2
0.1
0.0
pg/mg protein
PTL n=13
TL n=13
TnotL n=5
NP n=4
IL-18
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pg/mg protein
PTL n=13
TL n=13
TnotL n=5
NP n=4

Figure 2

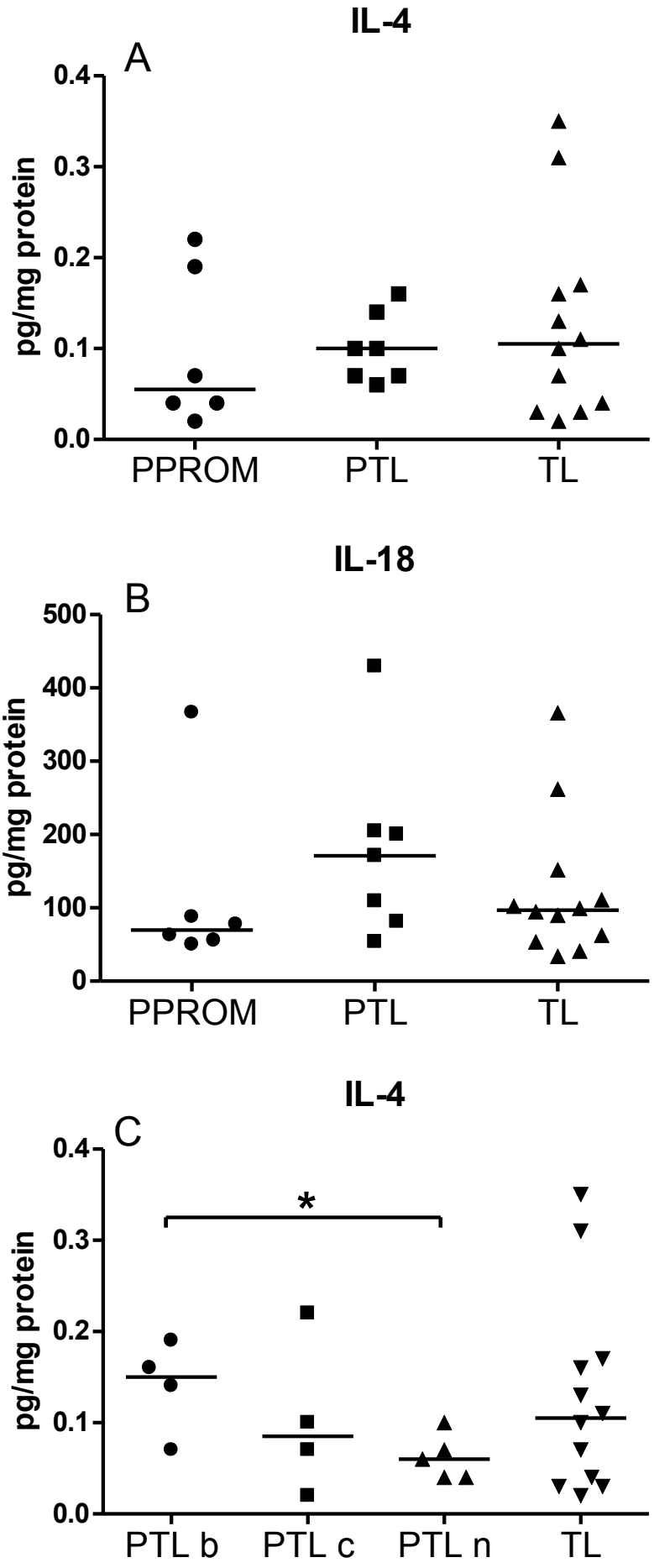
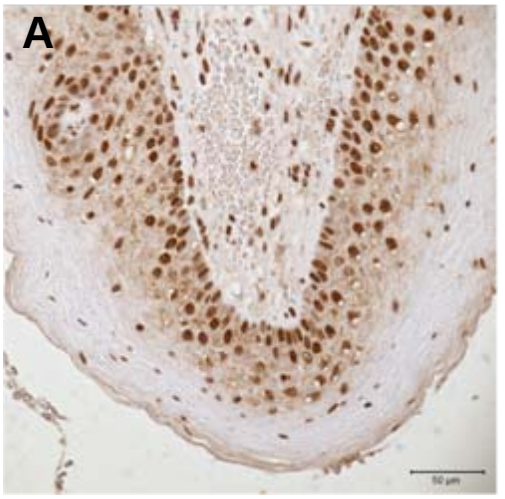

IL-4
A
pg/mg protein
0.4
0.3
0.2
0.1
0.0
PPROM
PTL
TL
IL-18
B
pg/mg protein
500
400
300
200
100
0
PPROM
PTL
TL
IL-4
C
pg/mg protein
0.4
0.3
0.2
0.1
0.0
*
PTL b
PTL c
PTL n
TL

Figure 3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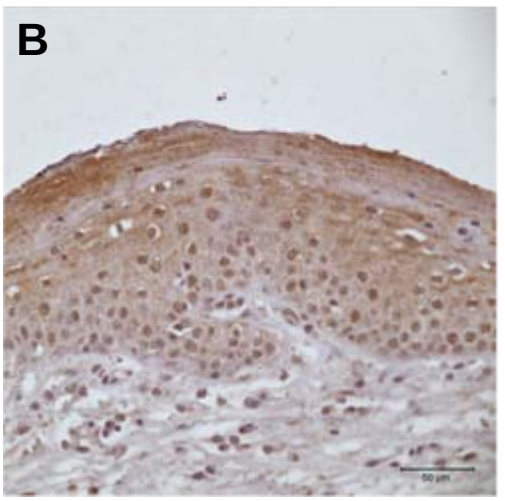
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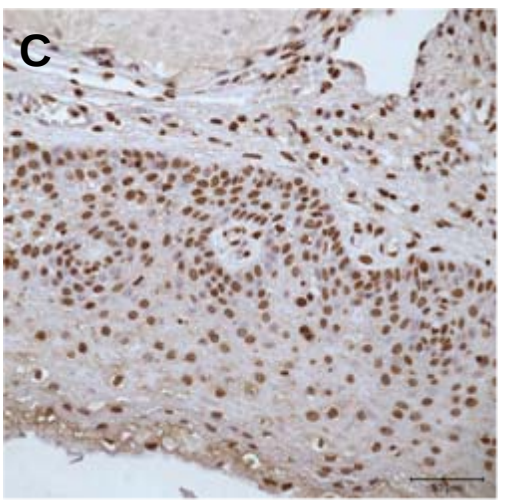
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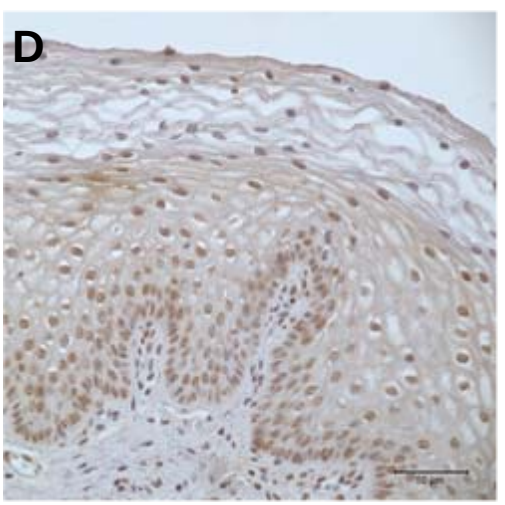
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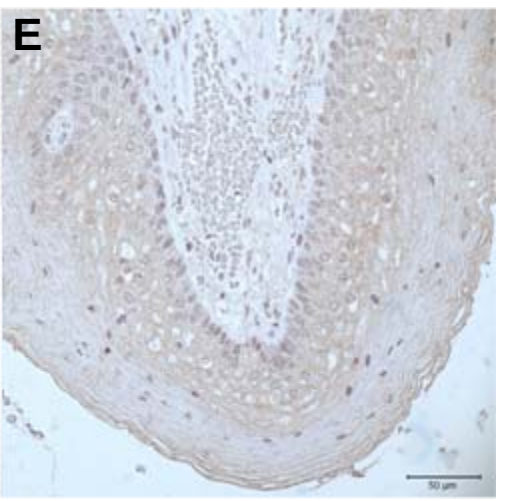
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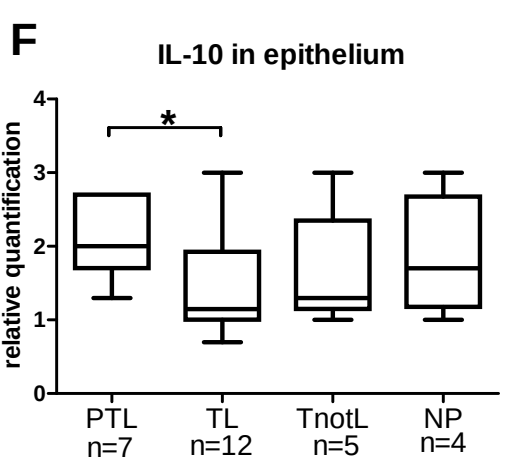
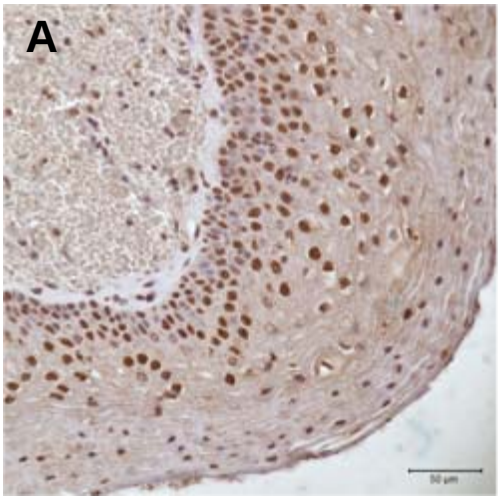

F
IL-10 in epithelium
relative quantification
4
3
2
1
0
*
PTL
n=7
TL
n=12
TnotL
n=5
NP
n=4

Figure 4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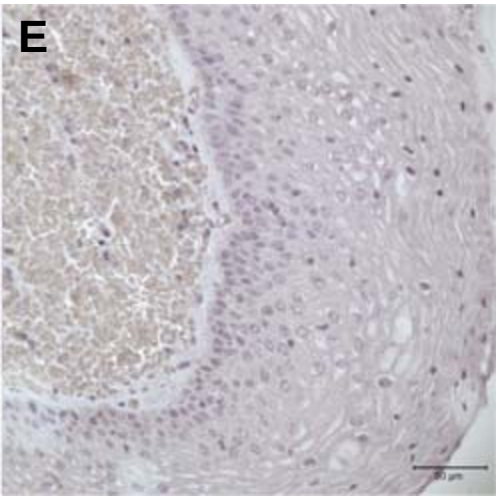
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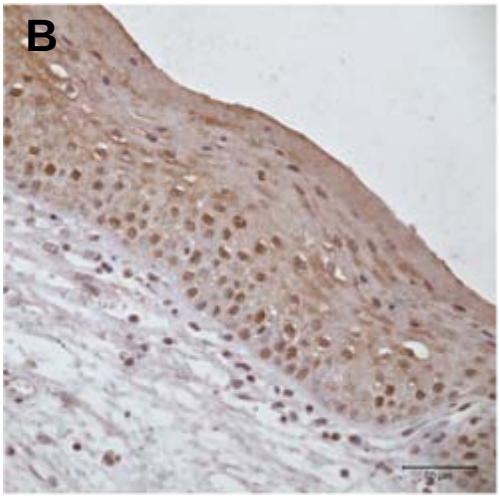
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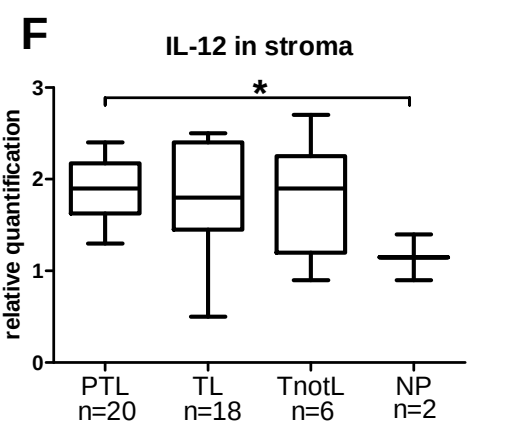
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IL-12 in stroma
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TL
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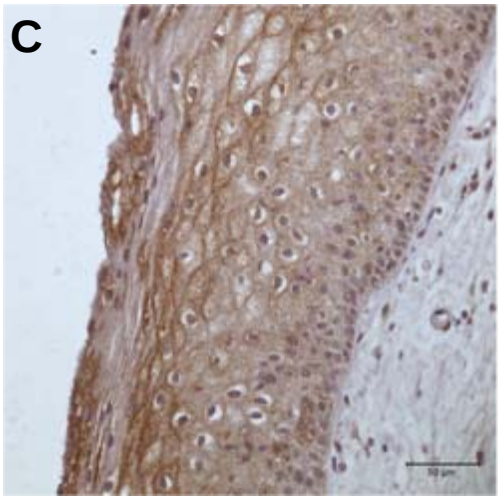
n=6
NP
n=2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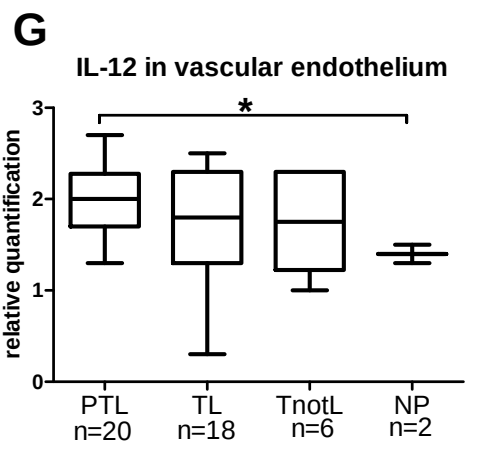
G
IL-12 in vascular endothelium
relative quantification
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PTL
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TL
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TnotL
n=6
NP
n=2

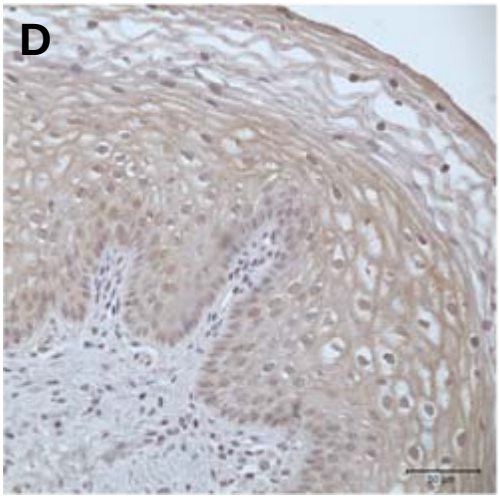
D

Figure 5