

The expression of interleukin-15 and interleukin-18 by human term placenta is not affected by lipopolysaccharide

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ABSTRACT. The aim of the study was to examine the stimulatory effect of the inflammatory agent lipopolysaccharide (LPS) on the capacity of human term placenta to secrete interleukin (IL)-15 and IL-18. Isolated placental cotyledons from normal human term deliveries were dually perfused for ten hours with perfusion medium alone (n = 5) or with perfusion medium containing LPS (1 µg/kg perfused placental tissue) (n = 5). Placental tissue was collected from three different placental compartments (amnion, chorion, and placenta) before and after perfusion. The placental tissues collected were homogenized and examined for IL-15 and IL-18 by ELISA. In addition, formalin-fixed and paraffin-embedded sections from term placentas before perfusion were stained by immunohistochemistry to characterize the cellular origin of placental IL-15 and IL-18. Statistical significance was determined using paired/unpaired t-test. p < 0.05 was considered significant. Our results show that IL-15 and IL-18 are produced more by chorionic tissue, as compared to the amnion and placental tissues. Moreover, we show that IL-15 and IL-18 are expressed by epithelial cells of the amnion, chorionic cells of the chorion and decidual cells of the decidua. However, IL-15, but not IL-18, was expressed also by syncytiotrophoblasts of the villi. Perfusion of LPS did not affect the capacity of amnion, chorion and placental tissues to secrete IL-15 and IL-18, as compared to control. The expression of IL-15 and IL-18 in the different compartments of the human placenta suggests a possible role for these two cytokines in normal placental development, pregnancy and labor. Moreover, our results indicate that IL-15 and IL-18 are not part of the mechanism of the response of human placenta to LPS.

Keywords: placenta, lipopolysaccharide, interleukin-15, interleukin-18

Cytokines are produced by various maternal and fetal cells within the uterus and the fetoplacental unit. The cells involved in cytokine production are not only immune cells, but also other cell types, such as maternal decidual cells and trophoblasts [1]. Many of the cytokines at the maternal-fetal interface thought to be involved not only in immune regulation, but also in physiological processes through pregnancy, are observed from implantation through to parturition [2, 3]. Therefore, imbalances and disruptions in cytokine production at the fetal-maternal interface have been implicated in different complications of pregnancy involving abnormalities of both placental growth and establishment and initiation of parturition [2, 4]. There is strong evidence that elevated cytokine production in gestational tissues is associated with spontaneous, preterm labor [4].

Interleukin (IL)-15 and IL-18 are part of the cytokine network that plays a critical physiological and immunological role at the maternal-fetal interface during preg-

nancy and parturition [5-9]. IL-15 is a novel cytokine that shares several functional properties with IL-2 including T-cell stimulation, activation of T-cell proliferation and B-cell proliferation and differentiation, in addition to promoting NK cell cytotoxicity [10-12]. IL-15 activity is mediated by a heterotrimeric receptor that consists of the β and γ subunits of IL-2R [13]. Moreover, proinflammatory cytokines production by macrophages has been reported to be regulated by IL-15 [14]. IL-15 is expressed in a wide variety of tissues and cells, suggesting at least partially distinct physiological functions [10].

IL-18, a cytokine belonging to the IL-1 family, was originally characterized as IFN- γ -inducing factor, and is present in the serum of animals with endotoxemia [15]. Like IL-1 β , IL-18 is a cytoplasmic protein synthesized as a biologically inactive 24-kDa precursor molecule lacking a signal peptide that requires cleavage into an active, mature 18-kDa molecule by the intracellular cysteine protease, IL-1 β -converting enzyme (caspase-1) [16]. IL-18 is produced by a wide variety of cells including monocytes, macrophages, intestinal epithelial cells, microglia, astro-

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cytes, osteoblasts, adrenal cortex and keratinocytes [17-21]. IL-18 is a multifunctional, proinflammatory cytokine that plays a key role in host defense against several infectious agents. It is known to participate in the regulation of both Th1 and Th2 responses, and it also up-regulates innate and acquired immunity [22].

The involvement of IL-15 and IL-18 in pregnancy, labor-onset and complications of pregnancy has been suggested [5-9]. IL-15 expression in human endometrium and decidua indicates a role for IL-15 in uterine functions during pregnancy [5]. Furthermore, Agarwal *et al.* 2001, showed that IL-15 is expressed by human placenta, and increases in its mRNA and protein levels have been shown to occur following the onset of labor at term [6]. A recent study has shown the presence of IL-15 in the amniotic fluid and also revealed that fetal membranes are a source of its production [23]. Moreover, increased levels of IL-15 in amniotic fluid during preterm labor [23] suggest a possible role for this cytokine in preterm labor. IL-18 is found to be present in the maternal-fetal interface and expressed by human chorion and decidua [8, 24]. Recently, it was reported that IL-18 levels are significantly elevated in serum sampled during the first trimester up until the onset of labor [7]. Moreover, Ida *et al.* 2000, showed that IL-18 levels are increased further once labor begins, and remain elevated until at least the third day post-partum [7]. High levels of IL-18 in serum were also observed in various complicated pregnancies, including premature rupture of membranes (PROM) [7]. Furthermore, Pacora *et al.* 2000, reported that microbial invasion of the amniotic cavity in either preterm or term parturition was associated with a significant increase in the levels of IL-18 in the amniotic fluid [8]. The current study was conducted to examine IL-15 and IL-18 expression in the different compartments of human term placenta, and to evaluate the effect of perfused LPS on the expression levels of these cytokines in placental compartments.

METHODS AND MATERIALS

Study design

Placentas from 10 uncomplicated, term pregnancies were collected immediately after vaginal delivery. The cord was properly clamped to maintain the dilatation of the fetal vascular system. The perfusion experiments were performed according to Holberg *et al.* [25]. After careful inspection of the chorionic and decidual surface, an intact cotyledon was selected, as defined by a single chorionic artery and vein with no anastomotic connection to vessels of a neighboring cotyledon, and with the corresponding region of decidual surface being non-traumatized.

A fetal artery and corresponding vein from a single cotyledon were cannulated within 15-20 minute of delivery. Following successful establishment of the fetal circulation, the placenta was mounted in a perfusion chamber, and the maternal circulation was simulated by placing four catheters into the intervillous space of the lobe, corresponding to the isolated perfused cotyledon. Maternal perfusate that returned from the intervillous space was continuously drained by a maternal venous catheter, placed at the lowest level on the maternal decidual surface, to avoid significant pooling of perfusate [25].

Perfusion medium consisted of M-199 cell culture medium [M199 media (Beit HaEmek, Israel)], enriched with bovine serum albumin (0.1 mg/mL), glucose (1.0 g/L) (Sigma Chemicals Co., St. Louis, USA), heparin (20 IU/mL) (Beit Kama, Israel) and Gentamycin (48 µg/mL) (Teva, Petah Tekva, Israel). The pH of the medium was adjusted to 7.4 with bicarbonate [25].

The two reservoirs, containing the perfusion medium for the maternal and the fetal circuit, were placed into heated water baths at 37°C, and were equilibrated with a prehumidified gas mixture of 95% O₂ and 5% CO₂ on the maternal side and 95% N₂ and 5% CO₂ on the fetal side. Perfusion pressure of 20-30 mmHg, giving a flow rate of 6-8 mL/min and 10-12 mL/min in the fetal and maternal circulation respectively, was established. The venous return could be recycled into the respective reservoir, giving a closed-circuit perfusion. Five term placentas were perfused with medium alone; another five term placentas were perfused with medium containing LPS in the maternal reservoir (1 µg/kg perfused placental tissue [Sigma]), for 10 hours. In order to validate the placental integrity, we performed histological examination of the cotyledon at the end of each experiment. They were found to be normal and no tissue damage was indicated [25].

Sample collection and homogenates preparation

Placental tissues before and after perfusion were separated into three different compartments; amnion, chorion and placenta (main placental tissue). Tissue samples were collected before and after perfusion, and stored at -70°C, or formalin-fixed and paraffin-embedded in order to characterize the cellular origin of these cytokines in each placental compartment by immunohistochemistry.

In order to evaluate the levels of IL-15 and IL-18 in each compartment, homogenates were prepared from amnion, chorion and placental compartments from each perfused placenta. Tissue samples were homogenized in 1 mL of cold PBS, centrifuged at 13 000 RPM for 15 minutes, and supernatant was collected and stored at -70°C. Total protein in each sample was determined using dye reagent (Biorad).

Evaluation of cytokines levels

IL-15 and IL-18 levels in placental homogenates were examined by enzyme-linked immunosorbent assay (ELISA). Specific paired antibodies and recombinant proteins were used for IL-15, mouse monoclonal anti-human IL-15 (first antibody) and biotinylated mouse anti-human IL-15 (second antibody); sensitivity was < 16 pg/mL, and standard curve range was 4-1 000 pg/mL, R&D Systems, Minneapolis, USA]; for IL-18, mouse monoclonal anti-human IL-18 (first antibody) and biotinylated goat anti-human IL-18 (second antibody); sensitivity was < 16 pg/mL, and standard curve range was 4-2 000 pg/mL, R&D Systems. The first antibodies were incubated in 96-well ELISA plates, overnight in room temperature, followed by washing and addition of blocking buffer for two hours at room temperature. Thereafter, the blocking buffer was washed and samples or recombinant cytokines (at different concentrations) were added. After one hour of incubation at room temperature, plates were washed and

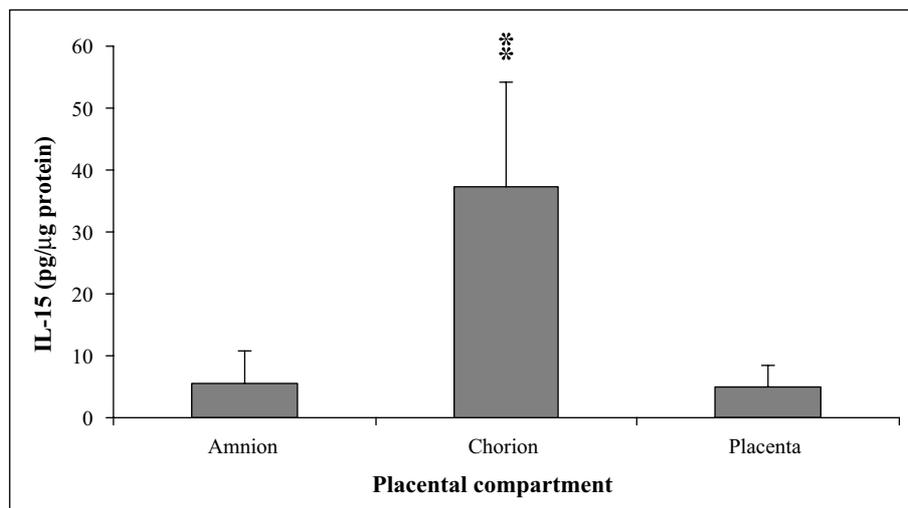


Figure 1

The levels of IL-15 in different compartments of normal human term placenta. IL-15 was detected in the homogenates of amnion, chorion and placenta (main placental tissue) of normal human term placenta after vaginal delivery, before perfusion, using specific ELISA antibody pairs. ELISA kit sensitivity was 16 pg/ml. Results are presented as mean \pm S.D. Statistical significance was determined using a paired and/or unpaired t-test and $p < 0.05$ was considered significant. ** $p < 0.01$.

Streptavidin HRP was added for 20 minutes room temperature. After washing, TMB was added for 15-20 minutes and the reaction was stopped by adding 2N H_2SO_4 . Absorbance was read using an ELISA reader at 450nm.

Immunohistochemical staining [26, 27]

Four micron-thick sections from formalin-fixed, paraffin-embedded tissue blocks were mounted on saline-coated slides, dried at 37°C for 48 hours and stored at room temperature. Antigen unmasking was carried out by boiling the sections in 6-M urea (Sigma) solution for 15 minute. Before the primary antibodies for IL-15 [mouse monoclonal anti-human IL-15 (2.5 μ g/mL) (R&D systems)] or IL-18 [rabbit polyclonal anti-human IL-18 (5 μ g/mL) (MBL, Naka-Ku, Nagoya, Japan)] were applied, blocking of the nonspecific background was achieved with PBS containing normal serum of the same origin as the secondary antibodies. This solution was also used to dilute the primary antibodies. After incubation for one hour at room temperature, the primary antibodies were removed and the sections were washed with PBS. The biotinylated antibodies (polyclonal goat anti-mouse and goat anti-rabbit [Zymed, San Francisco, CA, USA]) and the streptavidin-peroxidase conjugates (Zymed) were applied according to the supplier's directions. Blocking of the endogenous peroxidase was achieved with 3% H_2O_2 in 80% methanol for 15 minutes, before the streptavidin peroxidase conjugate (Frutarom, Ltd., Israel) was applied. Development was performed with 0.06% DAB (Sigma), and Mayer's hematoxylin (Sigma) was used for counterstaining. The sections were mounted in Eukitt. Negative controls were performed for each specimen using normal serum instead of the primary antibodies.

Statistical analyses

Statistical significance was determined using a paired and/or unpaired t-test; $p < 0.05$ was considered significant. Data are presented as mean \pm S.D. of cytokine/ μ g protein.

RESULTS

Expression and co-localization of IL-15 in fetal and maternal compartments of normal, human term placenta

The levels of IL-15 were detected in the homogenates of all three placental compartments examined (amnion, chorion and placenta [main placental tissue]) before perfusion (*figure 1*). IL-15 levels in the chorion were significantly higher (37 ± 17 pg/ μ g protein) as compared to those in the amnion (6 ± 5 pg/ μ g protein) ($p < 0.01$) and in the placenta (5 ± 4 pg/ μ g protein) ($p < 0.01$) (*figure 1*).

Immunohistochemical staining showed that IL-15 peptide is expressed by epithelial (Ep) and mesenchymal (Mes) cells of the amnion (*figure 2A*), cytotrophoblasts (Cyt), macrophages (M) and mesenchymal (Mes) cells in the chorion (*figure 2B*), syncytiotrophoblasts (Syn) of the villi (*figure 2C*) and decidual (Dec) cells in the maternal decidua (*figure 2D*).

Expression and co-localization of IL-18 in fetal and maternal compartments of normal, human term placenta

IL-18 levels were detected in the homogenates of all three placental compartments examined (amnion, chorion and placenta) before perfusion (*figure 3*). IL-18 levels in the chorion were significantly higher (29 ± 13 pg/ μ g protein) as compared to those in the amnion (13 ± 9 pg/ μ g protein) ($p < 0.01$) and in the placenta (8 ± 5 pg/ μ g protein) ($p < 0.01$) (*figure 3*).

IL-18 peptide was expressed in epithelial (Ep) cells of the amnion (*figure 4A*), cytotrophoblasts (Cyt), macrophages (M) and mesenchymal (Mes) cells in the chorion (*figure 4B*) and decidual (Dec) cells in the maternal decidua (*figure 4D*), but not in syncytiotrophoblasts (Syn) of the villi (*figure 4C*).

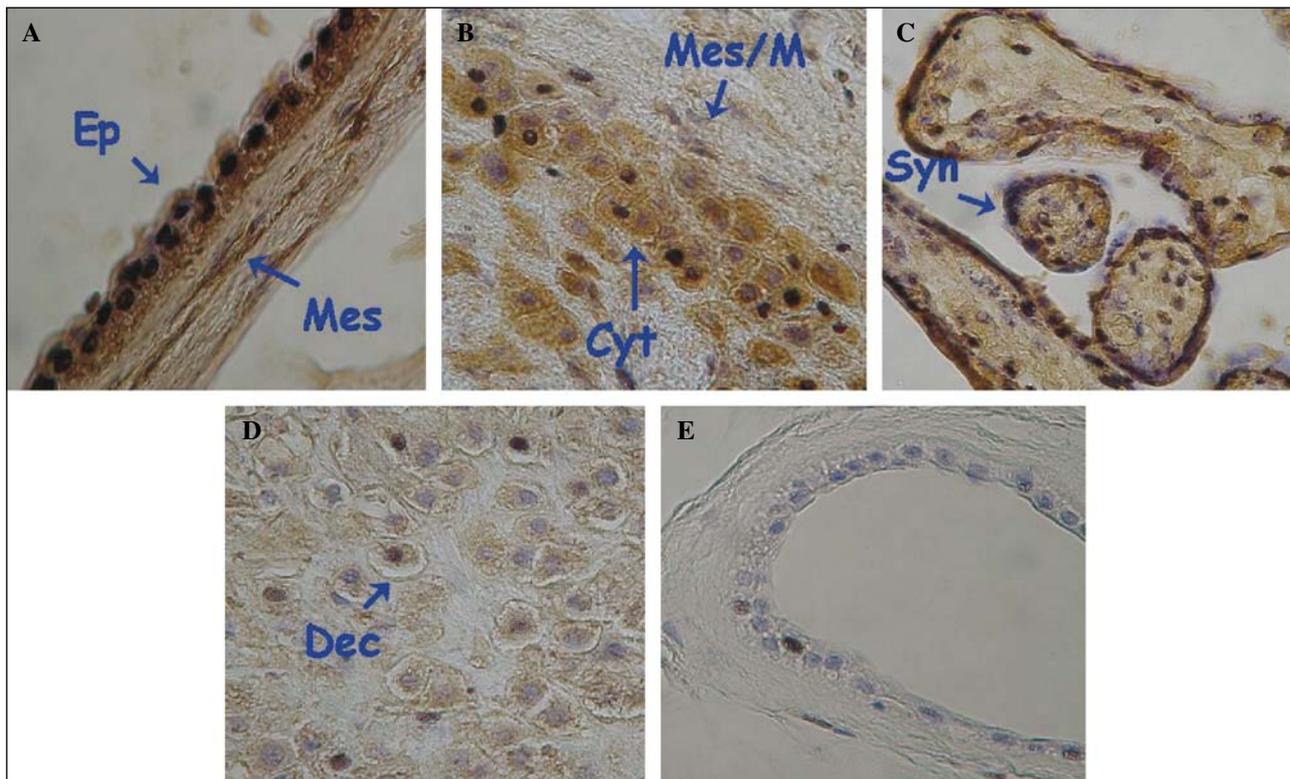


Figure 2

Localization of IL-15 in different placental compartments of normal human term placenta after vaginal delivery, before perfusion. Immunohistochemical staining showed the expression of IL-15 in epithelial (Ep) and mesenchymal (Mes) cells of the amnion (A), cytotrophoblasts (Cyt), macrophages (M) and mesenchymal (Mes) cells in the chorion (B), syncytiotrophoblasts (Syn) of the villi (C) and decidual cells (Dec) in the maternal decidua (D). Negative control (E). Magnification of the slides was x 200.

Stimulation of term placenta with LPS did not affect the capacity of the fetal and maternal compartments to produce IL-1

Perfusion of term placentas with medium containing LPS did not affect the capacity of the amnion, chorion or placenta to produce IL-15 (figure 5).

After perfusion in presence of LPS, the levels of IL-15 in the homogenates of amnion, chorion and placenta (11 ± 8 , 48 ± 20 , 4 ± 2 pg/ μ g protein, respectively) were similar to IL-15 levels after perfusion with medium alone (control) (15 ± 21 , 75 ± 40 , 7 ± 4 pg/ μ g protein, respectively) (figure 5).

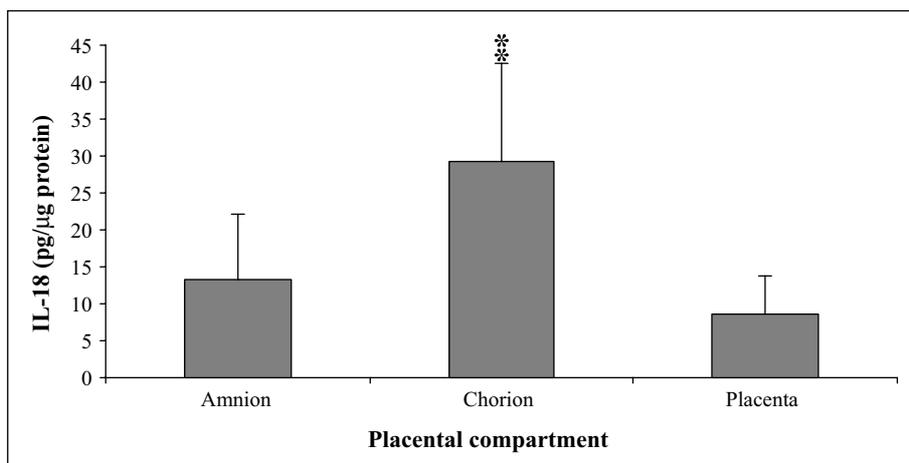


Figure 3

The levels of IL-18 in different compartments of normal human term placenta. IL-18 was detected in the homogenates of amnion, chorion and placenta (main placental tissue) of normal human term placenta after vaginal delivery, before perfusion, using specific ELISA antibody pairs. ELISA kit sensitivity was 16 pg/ml. Results are presented as mean \pm S.D. Statistical significance was determined using a paired and/or unpaired t-test and $p < 0.05$ was considered significant. $^{***} p < 0.01$.

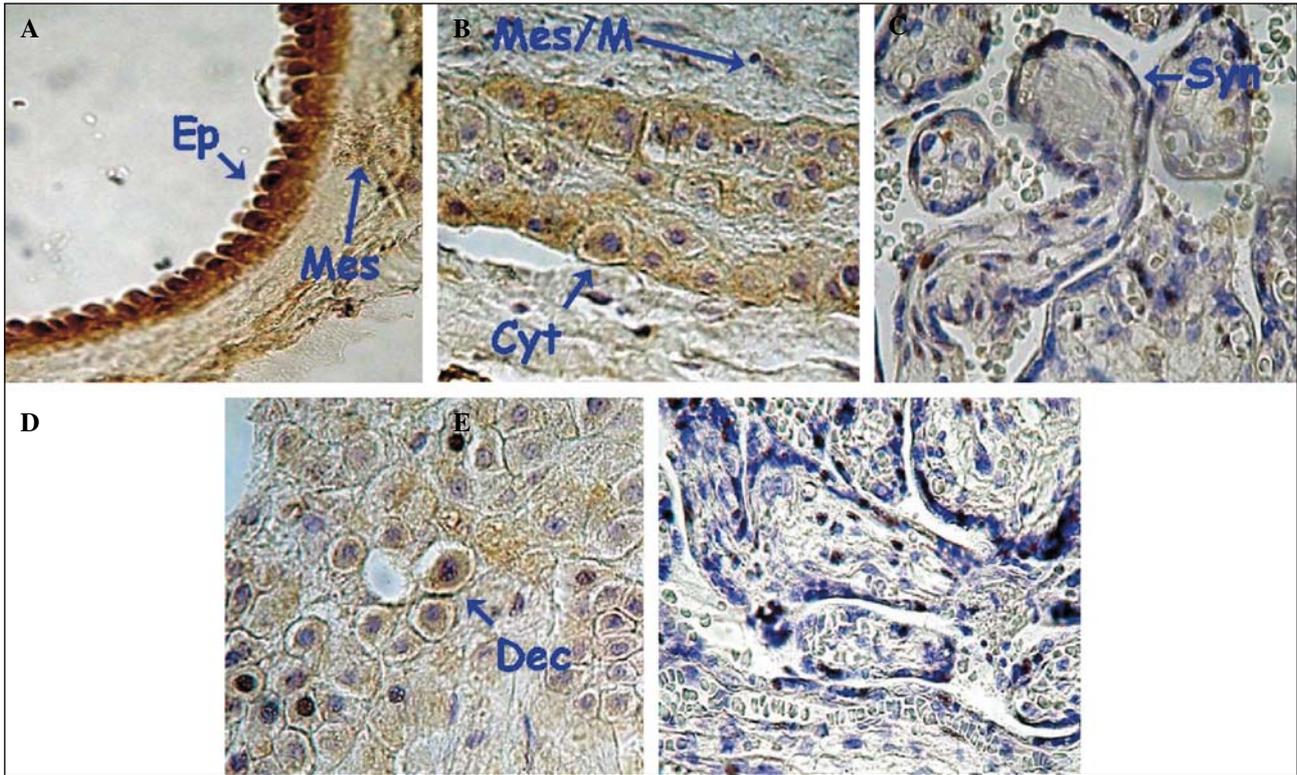


Figure 4

Localization of IL-18 in different placental compartments of normal human term placenta after vaginal delivery, before perfusion. Immunohistochemical staining showed the expression of IL-18 in epithelial (Ep) and mesenchymal (Mes) cells of the amnion (A), cytotrophoblasts (Cyt), macrophages (M) and mesenchymal (Mes) cells in the chorion (B) and decidual cells (Dec) in the maternal decidua (D), but not in syncytiotrophoblasts (Syn) of the villi (C). Negative control (E). Magnification of the slides was x 200.

Stimulation of term placenta with LPS did not affect the capacity of the fetal and maternal compartments to produce IL-18

Perfusion of term placentas with medium containing LPS did not affect the capacity of the amnion, chorion or placenta to produce IL-18 (figure 6). In the presence of LPS, the levels of IL-18 in the homogenates of amnion, chorion and placenta (19 ± 9 , 41 ± 19 , 21 ± 8 pg/ μ g protein, respectively), were similar to IL-18

levels after perfusion with medium alone (control) (22 ± 9 , 38 ± 14 , 18 ± 3 pg/ μ g protein, respectively) (figure 6).

DISCUSSION

IL-15 and IL-18 are two cytokines that play a key role in host defense against microbial infections [8, 28]. They have been suggested to be crucial cytokines that regulate cytokine production and the cytolytic potential of uterine

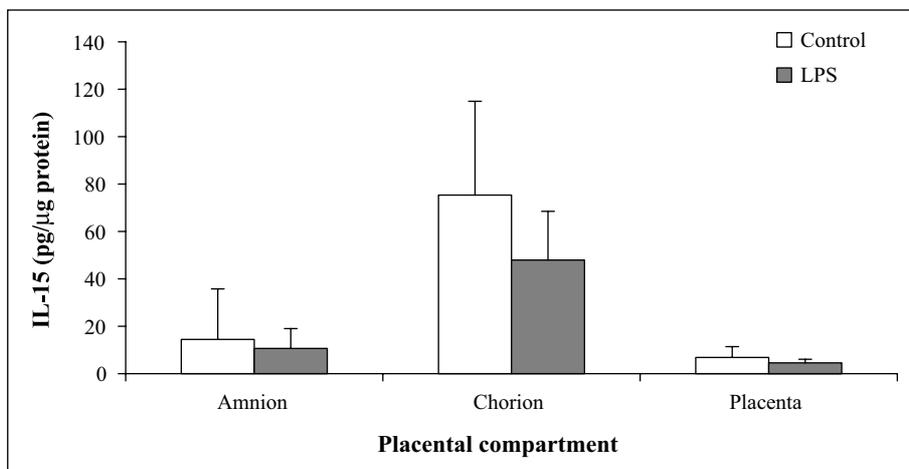


Figure 5

IL-15 levels in different compartments of perfused, normal human term placenta in the presence and absence of LPS. IL-15 levels were examined in the homogenates of the amnion, chorion and placenta (main placental tissue), following ten hours of perfusion with medium alone (control) or medium containing LPS (1 μ g/kg perfused tissue). Results are presented as mean \pm S.D. Statistical significance was determined using a paired and/or unpaired t-test and $p < 0.05$ was considered significant.

NK cells [9]. Moreover, IL-15 and IL-18 have been proposed to play a physiological role at the maternal-fetal interface during pregnancy and labor [9, 29]. In the current study, we examined the levels and the cellular origin of IL-15 and IL-18 in term placenta, and also evaluated the effect of the inflammatory agent LPS, on the capacity of different compartments of human, perfused term placenta to secrete these cytokines.

Our results show that IL-15 is produced by the fetal membranes (amnion and chorion) and the placenta. Moreover, we show that the chorionic tissue is the main source of IL-15 produced by human term placenta. Furthermore, LPS did not affect the capacity of human term placenta to produce IL-15.

Recently, Agarwal *et al.* [6] suggested that IL-15 is expressed by human placental tissue culture and that its levels correlated with the duration of the pregnancy. Moreover, Fortunato *et al.* [23] showed that IL-15 mRNA and peptide were present in the amnion, chorion, and decidual cells, and that stimulation with LPS did not affect IL-15 levels secreted by amnio-chorionic tissue cultures. Our current study, using a placental perfusion system, is in harmony with these *in vitro* data. We suggest that IL-15 may play an important role in normal human pregnancy, but may not be involved in the placental immune response to infection.

Our results also show that IL-18 is expressed by the amnion, chorion and decidual cells, but not by syncytiotrophoblasts of the placental villi, although it is detectable in all placental compartments of human term placenta. IL-18 expression in different placental compartments has been examined by number of groups. Recently, Menon *et al.* [24] showed that IL-18 is constitutively expressed by human chorion and decidua, but not by human amnion. Conversely, Splichalova *et al.*, [30], reported that IL-18 is expressed in the amnion tissue of pig placenta. However, our immunohistochemical results demonstrate constitutive expression of IL-18 in epithelial cells of the amnion. Moreover, Tokmadzic *et al.* [31] recently showed that IL-18 is not expressed by villus trophoblastic cells of first trimester, normal human placentas. Our results also show that syn-

cytrophoblasts of human term placenta do not express IL-18.

Furthermore, we show that the levels of IL-18 in the amnion, chorion and placenta were not affected by stimulation with LPS. Similar results for IL-18 expression were reported in rat liver [32]. However, Splichalova *et al.* [30] showed that IL-18 expression in amnion cells from pig pregnancies was increased in response to intra-amniotic infection with bacteria. These conflicting data may be explained by the different techniques and species used in our current work and the work conducted by Splichalova *et al.* Recently, it was reported that microbial invasion of the amniotic cavity in either preterm or term parturition was associated with a significant increase in the amniotic fluid concentration of IL-18 [8]. We suggest that, in the presence of intra-amniotic infection, the fetal membranes and the placenta are not the main source of these elevated levels of IL-18 in the amniotic fluids. The main source for these high levels of IL-18 could be the inflammatory cells that transmigrate through the fetal membranes into the amniotic fluid, resulting in the production of high levels of inflammatory cytokines [33].

IL-18 is a member of the IL-1 family and is structurally similar to IL-1 β [34]. Previously we have shown that stimulation with LPS induced the expression of IL-1a in the decidual tissue of perfused normal human term placenta [26]. Moreover, our unpublished data indicate that the secretion of IL-1b by perfused normal human term placenta is down-regulated after stimulation with LPS, while the secretion of IL-1 receptor antagonist (Ra) is up-regulated. These data, together with our current results, suggest that the IL-1 family may be differentially regulated in response to inflammation in human term placenta.

In conclusion, this study has demonstrated that IL-15 and IL-18 are expressed mainly by the chorionic tissue, and that LPS did not affect the levels of these two cytokine in the human placenta and fetal membranes. The expression of IL-15 and IL-18 in the different compartments of human placenta suggests a possible role for these two cytokines in normal placental development, pregnancy and labor. Moreover, it is possible to suggest that IL-15 and IL-18 are

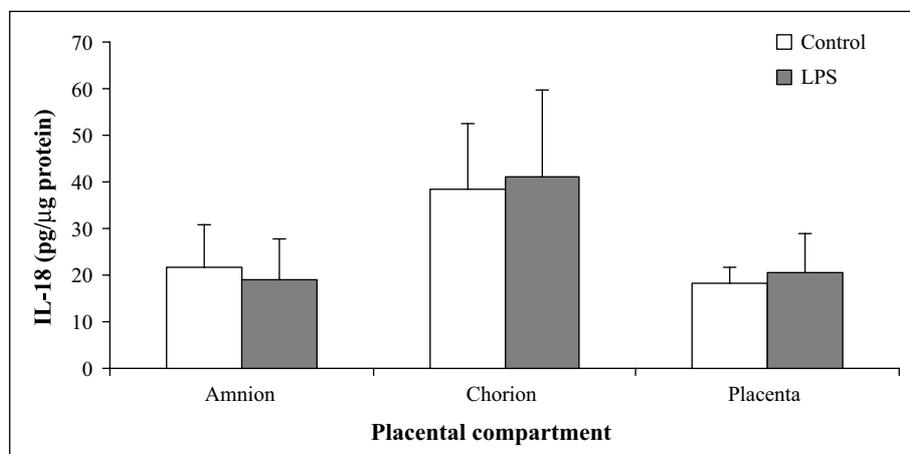


Figure 6

IL-18 levels in different compartments of perfused, normal human term placenta in the presence and absence of LPS. IL-18 levels were examined in the homogenates of the amnion, chorion and placenta (main placental tissue), following ten hours of perfusion with medium alone (control) or medium containing LPS (1 μ g/kg perfused tissue). Results are presented as mean \pm S.D. Statistical significance was determined using a paired and/or unpaired t-test and $p < 0.05$ was considered significant.

not involved in the inflammatory response mechanism in human placenta.

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