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Differential effect of LPS and IL-1β in term placental explants

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ABSTRACT

Introduction: Inflammation is an important cause of placental dysfunction often associated with pregnancy complications. One well-known cause of inflammation is infection, through conserved "pathogen-associated molecular patterns" (PAMPs). Endogenous inducers of inflammation, known as "damage-associated molecular patterns" (DAMPs), have also been associated with pathological pregnancies and could contribute to the observed placental inflammation. Although both stimuli (i.e. PAMPs/DAMPs) can induce inflammation, they have yet to be studied together to compare their inflammatory effects on the placenta.
Methods: We used a model of term placental explants to compare the effects of a classical PAMP, bacterial lipopolysaccharide (LPS), and a DAMP, the pro-inflammatory cytokine interleukin (IL)-1. Gene and protein expression of several cytokines were analysed by qPCR and ELISAs and immunohistochemistry performed to study placental resident immune cells and apoptosis.
Results: LPS induced pro-inflammatory mediators (IL-6, IL-1β/α, TNF-α) whereas IL-1β induced only IL-6. Furthermore, LPS but not IL-1 exposure, led to elevated IL-10 and IL-1Ra secretion. Blocking the IL-1 signalling pathway abrogated the pro-inflammatory actions of LPS, whilst anti-inflammatory effects were preserved. The number of $CD45^+$ immune cells was elevated in explants treated with LPS only. A subpopulation of $CD45^+$ cells were positive for PCNA indicating proliferation of tissue resident macrophages.
Discussion: We conclude that LPS, a classical PAMP, and IL-1, a DAMP, have shared and distinct actions with pro-inflammatory effects mediated through IL-1 but anti-inflammatory actions having a distinct pathway. Identification of an inflammatory mediator (i.e. IL-1) common to multiple stimuli could be a therapeutic target to preserve the placenta.

1. Introduction

Inflammation during pregnancy is important for proper embryo implantation and timely labour initiation. Aside from these two specific events, pregnancy is a state of immune tolerance, with a tightly maintained balance of pro- and anti-inflammatory pathways. However, when this balance shifts to a pro-inflammatory state, it can have devastating consequences on the placenta and subsequently, the fetus. Studies have shown evidence linking inflammation and pregnancy complications, especially in cases of spontaneous preterm labour and preeclampsia [1,2].

Infections are the best-known cause of inflammation which they induce through their conserved "Pathogen-Associated Molecular Pattern" (PAMPs). Infections are associated with approximately 20–30% of cases of preterm birth. However, in the remaining 70% of cases, infections are not detectable, despite heightened inflammation [3]. Endogenous inducers of inflammation, known as "Damage-Associated Molecular Patterns" (DAMPs) or alarmins, have been shown to induce inflammation through the same receptors as PAMPs and have been increasingly associated with pregnancy complications like preeclampsia and reduced fetal movements [3–7]. However, more investigation is needed to understand the role of DAMPs in pregnancy pathologies.

Both PAMPs and DAMPs act through Toll-like Receptors (TLRs) and

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Nod-like Receptors (NLRs) leading to the activation of similar downstream pathways [8]. These receptors are expressed by trophoblast cells and there is evidence for elevated expression in pregnancy complications especially in preeclampsia [9]. No studies have directly compared the actions of PAMP and DAMP in the placenta. Such knowledge would greatly improve our understanding of these potentially overlapping inflammatory mediators and facilitate the development of efficient therapeutic strategies. Furthermore, DAMPs can be liberated in response to PAMPs and a better understanding of their effects is important even when targeting PAMPs. In this study, we profiled inflammatory responses of term placental explants to a classical PAMP, bacterial lipopolysaccharide (LPS) compared to a typical DAMP, IL-1.

2. Methods

2.1. Ethical approval

Approval was obtained from North West Research Ethics Committee in Manchester, UK (Ref: 08/H1010/55) and the Sainte-Justine Hospital Ethic Board (Ref: 3988) to obtain placentas from uncomplicated term pregnancies, delivered by caesarean-section without labor. All women gave informed written consent.

2.2. Placental explants

Placenta were obtained within 30 min after delivery and villous tissue was randomly sampled for explant preparation. Biopsies from villi samples were selected from within the thickest portion of at least three different cotyledons for each placenta. Biopsies were washed with PBS to remove maternal blood and three explants (2–3 mm each) were randomly selected and placed into netwells in a 12well/plate (74 μm mesh; Corning, Sigma-Aldrich, ON, Canada) with 1.5 mL of culture media as previously described [6]. They were maintained at 37 °C, 5% CO_2 and media changed daily until the fourth day when they were treated with LPS (1 μg/mL; Sigma-Aldrich, MO, USA), recombinant human (rh) IL-1β or IL-1α (10 ng/ml; Peprotech, Qc, Canada), IL-1 receptor antagonist (IL-1Ra) (1 μg/ml; Biovitrum, ON, Canada), all in PBS which was used as vehicle, for 4 h, 24 or 48 h. Multiple doses were initially tested for both IL-1α and IL-1β, namely 0.1, 1 and 10 ng/ml; the highest dose had the most potent effects on cytokine expression and was therefore selected for the remaining of the study. Explants and supernatants were then collected and explants were either fixed in 10% formalin and paraffin embedded for histology or frozen at −80 °C until RNA/protein extraction. The placental explant model has been well characterized, as previously described [10] and it is important to maintain the explants in culture and perform the experiments on the 4th day since the external layer (i.e. syncytiotrophoblast layer) has time to shed and regenerate morphologically and functionally by day 4. This way, the explants are as close as possible to “normal” villi.

2.3. Gene expression by qPCR

Total RNA was extracted from placental villi (Trizol, Life Sciences, Paisley, UK) and concentration and purity were determined using a Nanodrop Spectrophotometer (Thermo Scientific, UK). Two hundred fifty ng of RNA were reverse transcribed using M-MLV RT, oligo-dT, RNase out, and dNTPs (Life Sciences, Paisley, UK). qPCR was performed as previously described [11], using primers for the following inflammatory mediators: IL-1β, IL-1α, IL-6, TNFα, IL-1Ra, and IL-10 (QuantiTect Primer, Qiagen, UK). Quantitative PCR system (7900 HT; Applied Biosystem, Warrington, UK) was used, mRNA expression quantified using SYBR green I with a dissociation curve analysis performed to ensure amplification specificity. Relative gene expression in villous tissue samples was normalized to the housekeeping gene YWHAZ (forward: CCTGCATGAAGTCTGTAACTGAG, reverse: TTGAG ACGACCCTCCAAGATG, Invitrogen, UK), and the data presented as relative expression (ΔΔCt).

2.4. Protein analysis

Explants were homogenized in lysis buffer consisting of PBS with (1% Triton X-100, Sigma-Aldrich, ON, Canada) containing protease inhibitor cocktail (Calbiochem, Millipore, ON, Canada), 1 mM sodium fluoride (NaF, Anachemia Canada, Qc, Canada) and 1 mM sodium orthovanadate (Na_3VO_4, Sigma-Aldrich, ON, Canada) and centrifuged at 13000 rpm for 10 min at 4 °C. Protein concentration, used to normalize cytokine data, was determined by Bradford assay (BioRad, ON, Canada) and levels of IL-1β, IL-1α, IL-6, TNFα, IL-1Ra, and IL-10 were determined by DuoSet ELISAs (R&D Systems, MN, USA) following manufacturer's instructions, with a mean inter-assay CV of 7.53% and a mean intra-assay CV of 4.36%.

2.5. Histology

Immunohistochemistry (IHC) was performed on 5 μm paraffin sections of placental explants as previously described [4]. Antigens were retrieved by incubating the sections 10 min in 0.01 M sodium citrate. Endogenous peroxidase was quenched with H_2O_2 before blocking solution (10% bovine serum albumin, 2% calf serum, in PBS-Tween 0.1% (Sigma-Aldrich, ON, Canada)) was applied for 30 min at room temperature. Primary antibody (M30 caspase-cleaved cytokeratin 8, 66 pg/ml, Roche, USA; cleaved caspase-3 (D175), 1:50, Cell Signalling Technology, Ontario, Canada; CD45, 2 μg/ml, Santa Cruz Biotechnology, USA; and PCNA, 4 μg/ml Santa Cruz Biotechnology, USA; Ki67, 46 μg/ml, Dako, CA, USA) were diluted in blocking solution and was incubated overnight at 4 °C as previously described [6,12–14]. Sections were then exposed to the HRP-conjugated-goat anti-mouse or rabbit IgG (20 μg/ml) for 45 min at room temperature (Thermo Fisher, CA, USA). Positive antibody binding was revealed by application of 3,3’-diaminobenzidine. Omission of primary antibodies were performed alongside each experiment as negative controls. Slides were counterstained with hematoxylin, dehydrated and cleared before coverslips were applied with Cytoseal™ 60 (Thermo Scientific, Cheshire, UK). Immunofluorescence (IF) was performed the same way with addition of a step to quench autofluorescence (30 min incubation in 1% sodium borohydride (Sigma-Aldrich, ON, Canada)). Fluor-conjugated secondary antibodies were used (AlexaFluor488 and AlexaFluor594, Life Technologies, OR, USA) and the slides were assemble using DAPI containing media (Fluoromount-G, SouthernBiotech, AL, USA). Slides were scanned (Axioscan, Zeiss, ON, Canada) and images (10/experimental condition/placenta) randomly taken with the Zen software 2.3. Positive cells were manually counted by an observer blinded to the experimental conditions using Image J (NIH Image) and results presented as percentage of positive cells vs total number of cells.

2.6. Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical comparison between multiple groups was assessed using one-way ANOVA with Tukey's multiple comparison test. Statistical analysis was performed with GraphPad Prism 7.02 (GraphPad Software, CA, USA) and $p < 0.05$ was considered significant.

3. Results

3.1. Cytokine mRNA expression in term placental explants exposed to PAMPs or DAMPs

For all parameters studied, the effects of IL-1α were the same as IL-1β and therefore only the data for IL-1β are shown. Exposure of term placental explants to a PAMP, LPS, induced significantly higher expression of IL-6 and IL-1β at every time point studied (4 h, 24 h and

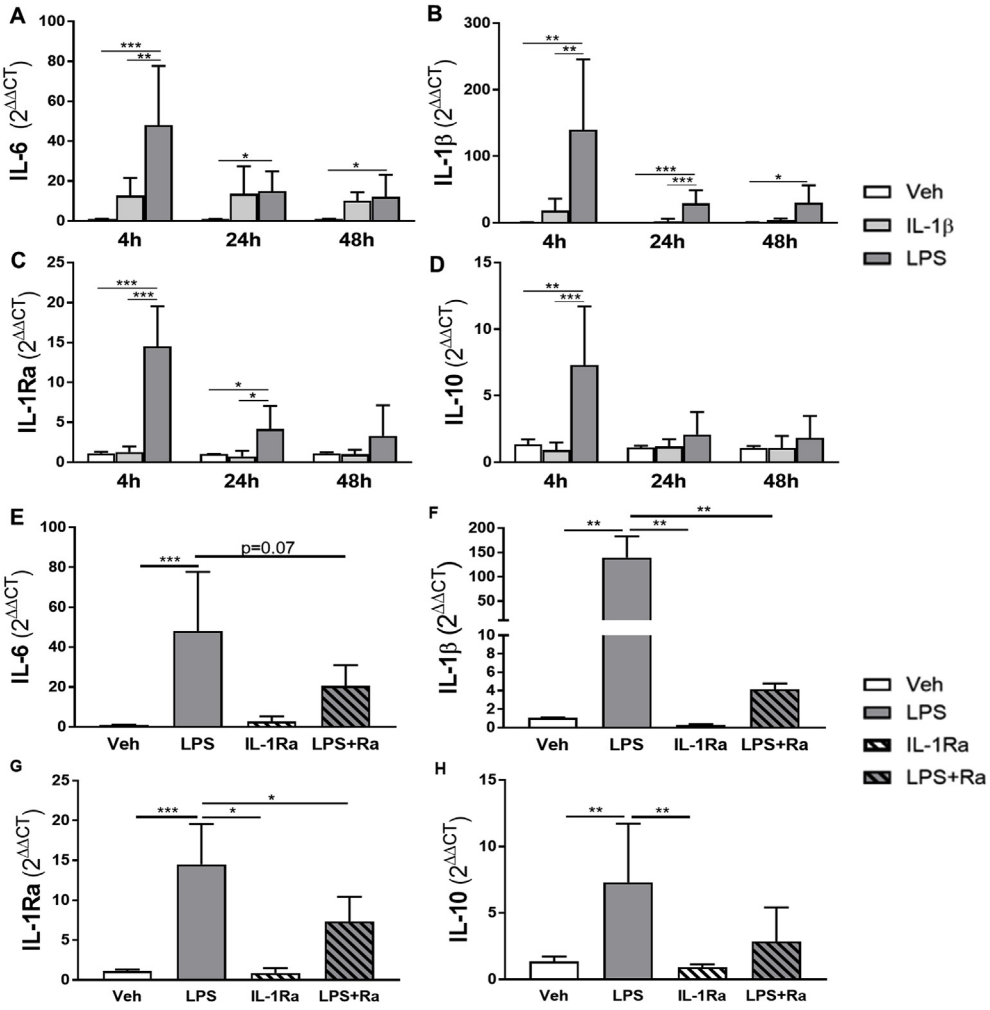

Fig. 1. Placental explants mRNA expression of cytokines following IL-1β or LPS exposure. (A) IL-6 and (B) IL-1β mRNA expression were elevated by LPS as were anti-inflammatory cytokines (C) IL-1Ra and (D) IL-10 and with LPS and IL-1Ra. (E) IL-6, (F) IL-1β and (G) IL-1Ra mRNA expression were decreased by the addition of IL-1Ra for 4 h whilst (H) IL-10 expression was not affected. Concomitant LPS + IL-1Ra decreased pro-inflammatory cytokines expression whilst the level of expression of anti-inflammatory cytokines was preserved. Data presented as $2^{\Delta\Delta CT}$, normalization to YWHAZ and vehicle control and presented as mean ± SD (A–D: n = 6 at 4 h and n = 7 for 24 and 48 h; E–H: n = 7 for Veh and LPS and n = 4 for IL-1Ra and LPS + IL-1Ra); One-way ANOVA, Tukey's multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001.

48 h) but with highest expression after 4 h (IL-6: 45.9-fold increased, p < 0.001 vs vehicle; IL-1β: 132.2-fold increased, p < 0.01 vs vehicle) (Fig. 1A and B) whilst exposure to IL-1β induced IL-6 expression (12.1-fold increased, p < 0.01 vs vehicle). Aside from pro-inflammatory cytokines, LPS also induced the expression of the anti-inflammatory cytokines IL-1Ra and IL-10 after 4 h (14.5-fold and 7.3-fold increased respectively vs vehicle, p < 0.001) (Fig. 1C and D). Expression of pro-inflammatory cytokines by LPS was reduced by co-treatment with IL-1Ra as seen by the tendency decreased expression (IL-6, p = 0.07) and significantly decreased IL-1β expression at 4 h (p < 0.01, Fig E–F). Administration of IL-1Ra did not alter its own expression or the expression of IL-10 following LPS treatment, at 4 h (Fig. 1G and H) or any of the other time point studied (24 h, 48 h, data not shown).

3.2. Production of cytokines by term placental explants following LPS or IL-1β exposures

LPS treatment led to elevated secretion of IL-1α (24.1-fold, p < 0.001), IL-1β (83-fold, p < 0.05), TNF-α (699.2-fold, p < 0.001), IL-6 (3.2-fold, p < 0.05) as well as anti-inflammatory IL-10 (39.7-fold, p < 0.001) and IL-1Ra (5.1-fold, p < 0.001) compared to vehicle at 24 and 48 h (Fig. 2A–F). These were significantly higher than following treatment with IL-1β (Fig. 2A–E). When explants were treated with LPS + IL-1Ra, a significant decrease in the secretion of pro-inflammatory cytokines was observed at 48 h (namely IL-1α (4.5-fold, p < 0.001), IL-1β (4.8-fold, p < 0.05) and TNF-α (25.4-fold, p < 0.001); Fig. 2G–J). However, secretion of anti-inflammatory IL-10 was preserved (Fig. 2K). In regards to the intracellular levels of cytokines, LPS increased IL-1α (40-fold, p < 0.01), IL-1β (26.9-fold, p < 0.001), IL-6 (5.2-fold, p < 0.01), TNF-α (129.3-fold, p < 0.01) as pro-inflammatory cytokine levels and IL-1Ra (2.7-fold, p < 0.01) and IL-10 (6.6-fold, p < 0.05) as anti-inflammatory cytokine all as compared to vehicle and all after 48 h (Fig. 3A–F). No significant changes were observed in the intracellular levels of cytokines following IL-1Ra treatment for 48 h (Fig. 3G–K) except for significantly decreased production of IL-6 (2.9-fold decreased, p < 0.05) (Fig. 3J).

3.3. LPS induced inflammation led to cell death and immune cell proliferation in term human placental explants

LPS treatment significantly elevated the percentage of M30 + apoptotic cells compared to vehicle at 48 h (12.6% vs. 5.2%, p < 0.01, Fig. 4). Although on the bar graph there is seemingly a tendency to increased cell death with IL-1β treatment, this was not statistically significant (7.4% vs 5.2%, p = 0.78). Co-treatment of IL-1Ra with LPS did not significantly reduce the % of M30 + cells (12.6% vs 7.6%, p = 0.11, Fig. 4). These results were confirmed with another marker of apoptosis, cleaved caspase-3 staining (Fig. 4C and data not shown).

The number of $CD45^+$ immune cells was significantly elevated in explants treated with LPS for 48 h compared to IL-1β or veh-treated explants (24.0% vs 10.7% and 15.5%, respectively, p < 0.05; Fig. 5A). Addition of IL-1Ra did not significantly reduce the number of $CD45^+$ cells (Fig. 5A and B). We then addressed whether resident immune cells proliferation was responsible for the increased number of

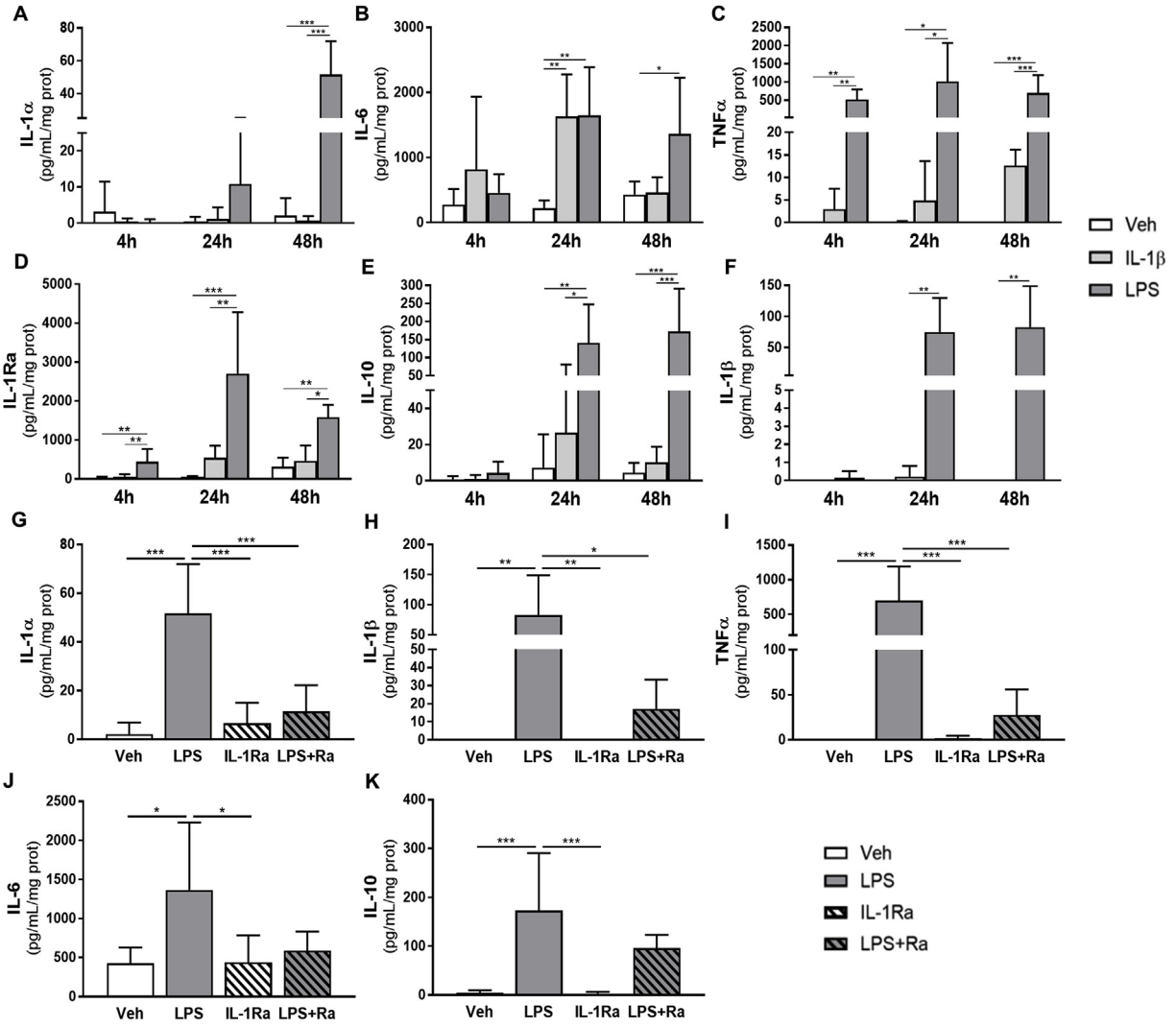

Fig. 2. Secretion of cytokines by placental explants induced by LPS and IL-1β with concomitant treatment of IL-1Ra. (A) IL-1α, (B) IL-6, (C) TNFα, (D) IL-Ra, (E) IL-10 and (F) IL-1β secretion was induced when treated with LPS and (G) IL-1α, (H) IL-1β and (I) TNFα secretion was abrogated by concomitant treatment with IL-1Ra and (J) IL-6 and (K) IL-10 were not significantly changed. Data presented as mean ± SD; one-way ANOVA, Tukey's multiple comparison test (A: n = 7; B-C: n = 6; D–F: n = 7; G: n = 4 for LPS and n = 6 for the others; H-K: n = 6), *p < 0.05, **p < 0.01, ***p < 0.001.

$CD45^+$ cells in LPS-treated explants. We observed a non-significant elevation of PCNA + cells, a proliferation marker, primarily in cytotrophoblasts (Fig. 5C and D), and there was also a trend for increased percentage of Ki67 + cells (p = 0.07; Supplementary Fig 1), but double positive cells (PCNA + $CD45^+$) were observed only in LPS-treated explants (Fig. 5E).

4. Discussion

In this study, we investigated the differences in the inflammatory actions between a classical PAMP, LPS, and a proto-typical DAMP, IL-1, in the term placenta. Although both PAMPs and DAMPs are known inducers of inflammation, it is of high importance to determine the differences in their actions to develop more appropriate therapeutic interventions in the future. We observed differences between LPS and IL-1 with both inducing pro-inflammatory cytokines, although to a lower extent for IL-1, and divergence was observed in terms of anti-inflammatory effects only for LPS treatment. Furthermore, blocking the IL-1 pathway in LPS-treated explants led to decreased expression, secretion and production of inflammatory cytokines, whilst anti-inflammatory cytokines were unaffected, suggesting that only pro-inflammatory pathways are mediated by IL-1 action. Of interest, we noted proliferation of placental resident immune cells (Hofbauer cells) in response to LPS, but not to IL-1. Globally this shows that the inflammatory actions of LPS are mediated, at least in part, through IL-1 and that anti-inflammatory cytokines are induced in an IL-1-independent manner.

LPS induced pro-inflammatory cytokine mRNA expression by placental explants. This is likely due to the downstream pathways induced and the retro activation that also occurs in response to the expression and release of cytokines, including IL-1, through NFκB gene activation [9]. In particular, the inflammation is likely due to the action of IL-1 secreted following LPS exposure since concomitant treatment with IL-1 receptor antagonist (IL-1Ra), significantly decreased the secretion of pro-inflammatory cytokines. LPS induced elevated anti-inflammatory cytokine expression, consistent with previously published work ([15–17]). However, this did not occur in response to IL-1, suggesting that different downstream pathways are implicated to resolve inflammation. This was confirmed by the failure of IL-1Ra to block LPS-induced IL-10 secretion, an anti-inflammatory response essential to clear the inflammation.

When we blocked the IL-1 receptor, a decrease in cytokine production and secretion was only detected after 24 h and 48 h, with no effect at 4 h (data not shown). This suggests that LPS has rapid effects

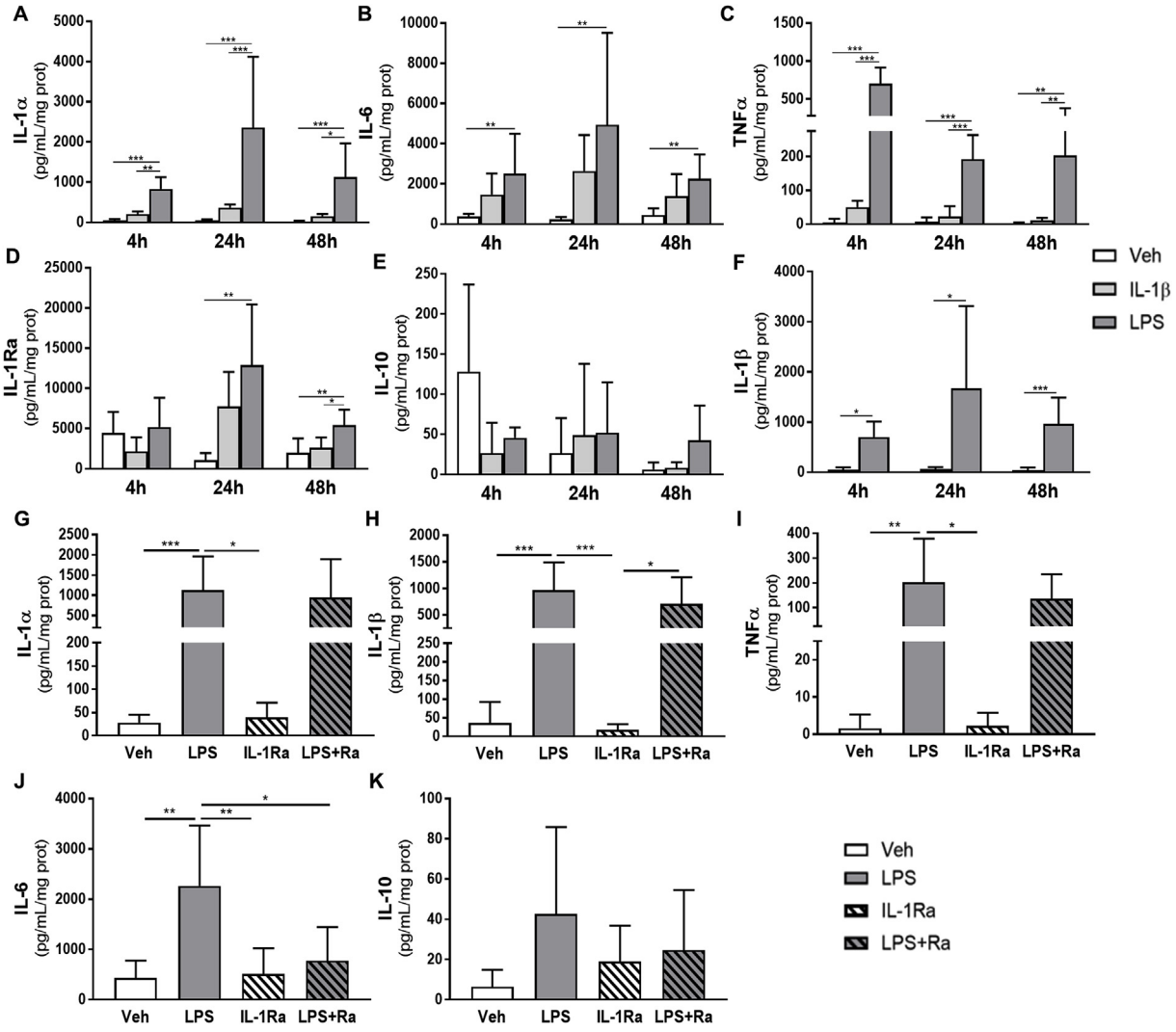

Fig. 3. Placental explants production of cytokines following inflammatory stimuli and IL-1Ra treatment. (A)(G) IL-1α, (B)(J) IL-6, (C)(I) TNFα, (D) IL-1Ra, (E)(K) IL-10 and (F)(H) IL-1β were induced by LPS and only (J) IL-6 expression was reduced by IL-1Ra treatment. Data are presented as mean ± SD; one-way ANOVA, Tukey's multiple comparison test, (A–E: n = 7; F: n = 6; G–K: n = 7 for Veh and LPS and n = 5 for IL-1Ra and LPS + IL-1Ra), *p < 0.05, **p < 0.01, ***p < 0.001.

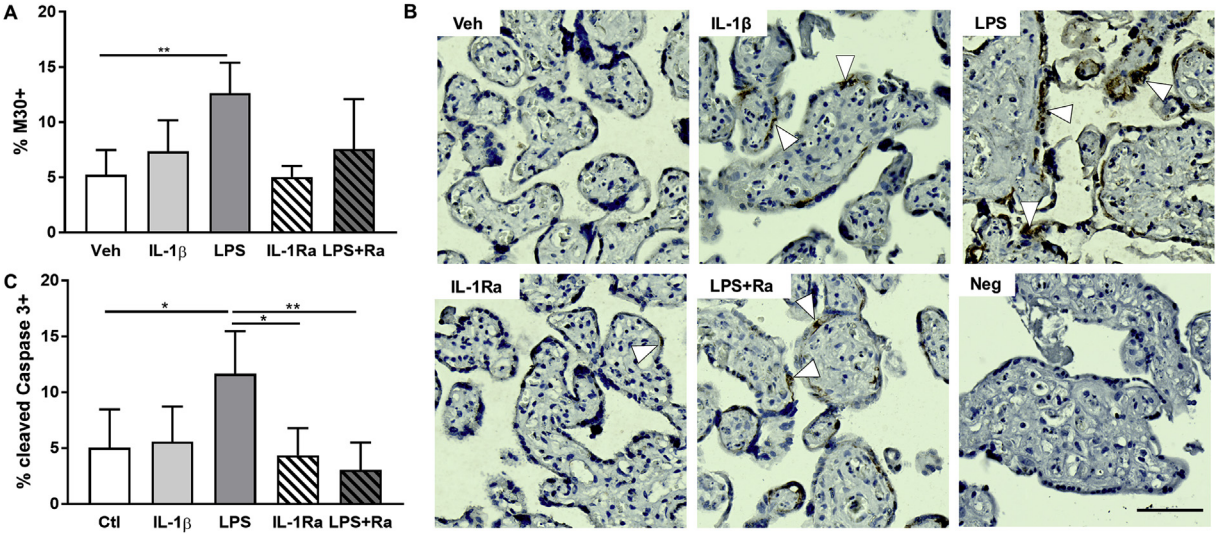

Fig. 4. Apoptosis (M30 + cells), observed at 48 h, in placental explants was induced by LPS (A) and was mainly observed in syncytiotrophoblast (arrowheads in representative example in B). Data are presented as mean ± SD, one-way ANOVA, Tukey's multiple comparison test (A: n = 4 for LPS and n = 5 for the others); **p < 0.01. Scale bar: 50 μm.

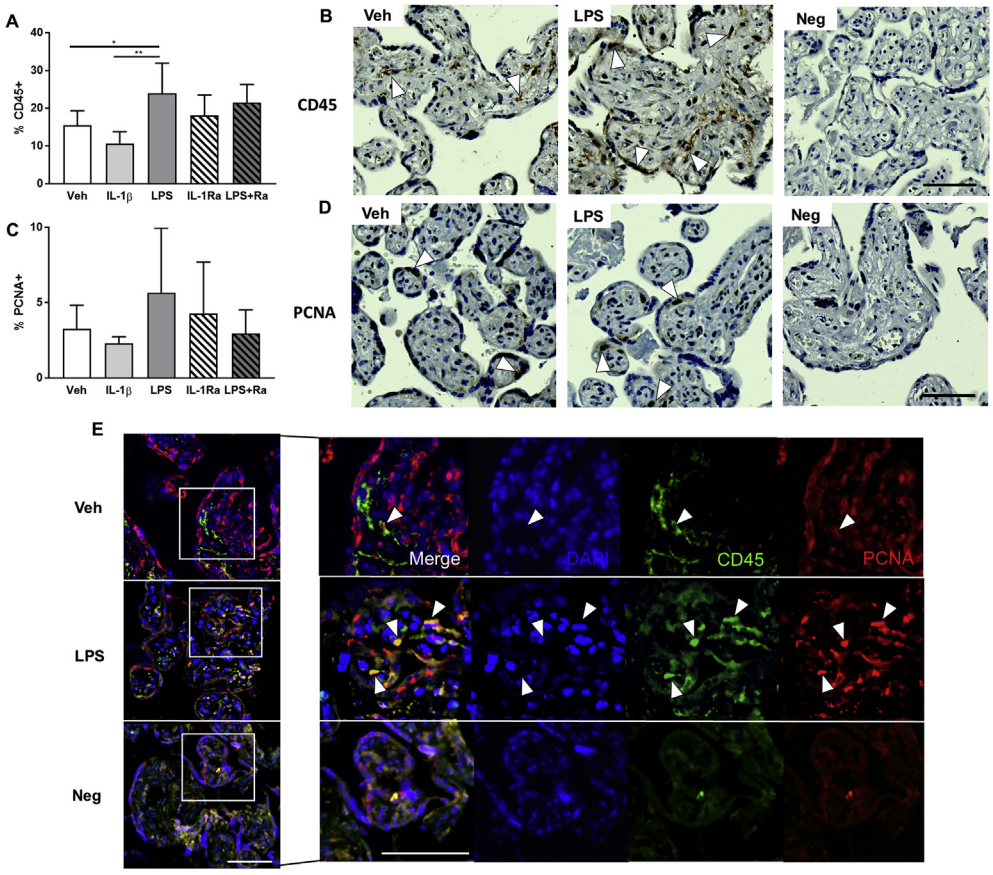

Fig. 5. Immunochemical analysis of immune cells in placental explants. (A) The percentage of immune cells ($CD45^+$) was elevated following LPS treatment. (B) Representative example of CD45 staining. (C) Non-significant elevation in the percentage of PCNA + cells in LPS treated explants. (D) Representative example of PCNA staining showing that cytotrophoblast as well as other cells are PCNA positive. (D) Colocalization studies revealed that double CD45 + /PCNA + cells (arrowheads) were observed only in LPS treated explants. Data are presented as mean ± SD, one-way ANOVA, Tukey's multiple comparison test, *$p < 0.05$, **$p < 0.01$. (A, C: $n = 5$) Scale bar: 50 μm.

on pro-inflammatory cytokine production and that the inhibition of IL-1 pathway is secondary potentially leading to the sustained effects which are abrogated with IL-1Ra. The activation of the IL-1 pathway has been shown to be implicated in multiple inflammatory responses following different inflammatory stimuli like LPS [18], a bacterial-derived PAMP, as well as in a non-infectious model of inflammation with uric acid [6].

We observed immune cell proliferation in explants treated with LPS. The main immune cells of the placenta are Hofbauer cells, fetal resident macrophages with a classic anti-inflammatory (M2) phenotype [19]. Other groups have shown that Hofbauer cells have a stem-cell like phenotype and they have the capacity to proliferate following stimulation [19]. This was also observed recently in second trimester placentas infected with the Zika virus [20] and a CD45 positive population have been shown to be elevated in placenta from pregnancy complications such as reduced fetal movements [4] and stillbirth [21]. This is in line with our results showing that LPS-induced inflammation led to increased number of $CD45^+$ cells in an ex vivo explant model and proliferation is a potential mechanism through which this could be occurring. Some viruses have been shown to have the ability to cross the syncytiotrophoblast layer [22] which is not the case for LPS or inflammatory cytokines [23]. Our study suggests that LPS could have an indirect effect through the action on syncytiotrophoblast cells, leading to the proliferation of third trimester Hofbauer cells but we cannot rule out the possibility that the cell culture system facilitated the entry of LPS into the villi facilitating direct interaction with Hofbauer cells. Thus, we hypothesized that the observed proliferation in our study occurs in resident macrophages of the placenta because there cannot be any hematopoietic progenitors present in the explant culture system.

We also observed an elevation of M30 positive cells as well as elevated active caspase-3 positive cells primarily in the syncytiotrophoblast layer when explants were treated with LPS, indicating increased apoptosis. Although it is possible that other types of cell death are also observed in the explants, our goal was not to distinguish between subtypes of cell death, but to investigate whether LPS or IL-1-induced inflammation altered the physiological apoptotic processes observed in the placenta. It has been demonstrated that trophoblast apoptosis can be induced through TLR2 and TLR4 activation, leading to cytokine production (IL-6 and IL-8) in the human placenta. Together these PRRs act in cooperation to activate the innate immune response to a "non-self" agent [24,25] and a similar process could be occurring in our explant model. Inflammatory processes have been shown to induce apoptosis [24,25] and, uncontrolled cell death within the placenta could have important detrimental effects on function, which could then lead to a positive feedback loop on inflammation, hence why studying apoptosis, alongside inflammation, is so important.

A limitation of our study is the use of a supra-physiological dose of LPS (1 μg/ml). For IL-1, we performed a dose response study and selected the highest dose (10 ng/ml) which had similar effects to the 1 ng/ml dose on many parameters and therefore we believe that the effect has plateaued at this dose. Furthermore, even though the fold changes are different between LPS and IL-1, the most important differences (induction of anti-inflammatory cytokines and proliferation of Hofbauer cells by LPS) are totally lacking in the IL-1 treated explants even though inflammation is observed. Moreover, we were still able to address the contribution of IL-1 to the effects of LPS using the specific IL-1R antagonist (IL-1Ra).

5. Conclusion

Our work showed important differences in the effects of a classical PAMP, LPS, compared to the typical DAMP, IL-1. The contribution of IL-1 to the inflammatory reaction induced by LPS, without affecting the production of anti-inflammatory cytokines, implicated in the resolution of inflammation, strongly supports the use of blockers of the IL-1 pathway in both PAMPs or DAMPs induced inflammation in the placenta. Moreover, explants treated with LPS induced elevated

syncytiotrophoblast cell death compared to IL-1 treatment. We also demonstrated Hofbauer cell proliferation *in vitro* following LPS exposure. Finally, this study supports the differential inflammatory effect induced by PAMPs and DAMPs at the maternal-fetal interface.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2018.11.006.

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