Investigation of ATP-mediated Interleukin-1β Secretion in Human Decidua

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Thesis submitted to the University of Nottingham in fulfilment of the requirements for the degree of Master of Research

September 2017

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Declaration

I confirm that the thesis is the result of my own work based on research that was undertaken at school of medicine, University of Nottingham during the period of October 2016 to September 2017, except where acknowledged. No material included in this work has been submitted for a previous degree/course, or any academic institution.

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25th, September, 2017

Acknowledgements

First of all, I need to express my sincere gratitude to my supervisor Prof. Raheela Khan for the continuous support of my study and research program. Her guidance helped my experiment development as well as thesis writing. She is the best advisor that always gave me useful comments and feedbacks.

Additionally, I wish to extend my greatest gratitude to Mrs Averil Warren who helped me in obtaining consent from patients and collecting samples. What's more, Mrs Averil Warren is always the one in the lab I would turn for help about the lab skills.

I also feel grateful for all the mothers who consented to participate in this research study. Besides, I would like to thank all the doctors, midwives and nurses those who made sample collection possible. Without all these people, it would have been impossible to complete the work.

Lastly, I would like to thank my family and boyfriend for all their understanding and support during my study.

Abstract

Introduction

The maternal immune system plays a significant role in pregnancy maintenance. Infections during pregnancy could stimulate inflammatory responses and lead to adverse pregnancy outcomes. Interleukin-1β (IL-1β), a vital pro-inflammatory cytokine, is implicated in pregnancy infection and infection-induced preterm labour. Meanwhile, human decidua, situated between the fetus and myometrium, has been hypothesized as the main source of cytokines in the presence of infection. Moreover, the most studied inflammasome – NLRP3 inflammasome, involved in the canonical IL-1β secretory pathway, has been found participating in normal pregnancy and pregnancy disorders. However, knowledge about how decidua is involved in the immune response is still limited. In this study, we investigated how IL-1β release with stimuli to TLR4 and the purinergic P2X7 receptor in the full-term decidua, especially on the key components of the inflammasome pathway.

Method

Full-term decidua explants, decidual leukocytes, decidual stromal cells and maternal peripheral blood mononuclear cells were isolated. Tissue explants and cells were stimulated with or without the TLR4 agonist-LPS and the $P2X_7$ receptor antagonist-A7 for 4 hours, and the last half hour stimulated with or without $P2X_7$ receptor agonist-BzATP. The secretion of IL-1β and TNF-α in culture media were detected by ELISA assay. The pro-IL-1β and NLRP3 inflammasome components proteins including NLRP3, caspase-1 and ASC in tissue lysates and culture media were evaluated by western blotting or ELISA.

Results

The levels of active IL-1β were higher under single stimulation with LPS in both of decidual tissue explants and maternal, and BzATP could significantly increase IL-1β release, while A7 could inhibit this increased effect. Besides, NLRP3, caspase-1, ASC are expressed in human decidual explants, and the expression of pro-caspase-1 increased after treatment with LPS. Also, NLRP3, p20 caspase-1, and ASC can be detected in culture media consistently, even without treatment. For the decidua cell types, decidual leukocytes, instead of stromal cells, can release IL-1β under costimulation with LPS and BzATP. Moreover, TNF-α secretion would be triggered with single stimulation with LPS in decidua explants, maternal PBMC, but these secretions were not affected by BzATP nor A7.

Conclusion

This study demonstrated that decidua explants can release IL-1β and TNF-α in response to LPS stimulation. Besides, exogenous BzATP could enhance the release of IL-1β, but not TNF-α, potentially through $P2X_7$ receptor. Furthermore, the expression of NLRP3 inflammasome components in decidua and the increased level of NLRP3 and caspase-1 after stimulation provided a clue that inflammasome may play a vital role in processing IL-1β in human decidua.

List of Abbreviations

β-ME	β -mercaptoethanol
A7	N-(1-((cyanoimino)(5-quinolinylamino) methyl amino)-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl) acetamide
AP	alkaline phosphatase
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BzATP	2’ (3’) -O- (4-Benzoylbenzoyl) adenosine’ 5-triphosphate triethylammonium salt
CARD	Caspase-associated recruitment domain
CD	Cluster of differentiation
DAMP	Damage Associated Molecular Pattern
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EI- LSCS	Elective caesarean section
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescence isothicyanate
HBSS	Hanks’ Balanced Salt Solution
HRP	Horseradish peroxidase

IFC	Immune flow cytometry
IL-1β	Interleukin-1β
IRF3	Interferon regulatory factor 3
K^+	Potassium ion
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MVB	Multivesicular bodies
MyD88	Myeloid differentiation primary response 88
NACHT	NOD-like nucleotide-binding domain
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NLR	NOD-like Receptor
NLRP3	NACHT, LRR and PYD domains-containing protein 3
$P2X_7$	Purinergic two receptor 7
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PRRs	Pattern recognition receptors
PTB	Preterm Birth
PYD	Pyrin domain
RA	Rheumatoid Arthritis
RIP1	Receptor-interacting serine/threonine-protein kinase 1
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute

SD	Standard deviation
SDS-PAGE	Sodium dodecyle sulphate polyacrylamide gel electrophoresis
SLE	Systemic Lupus Erythematosus
TAK1	Transforming growth factor beta-activated kinase 1
TBK1	serine/threonine-protein kinase 1
Th cell	T helper cell
TIRAP	TLR domain containing adaptor protein
TLR	Toll-like Receptors
TNF-α	Tumor necrosis factor TNF-β
TRAF	TNF receptor associated factors
TRAM	translocating chain-associating membrane protein
Treg	Regulatory T-Cell
TRIF	TIR domain-containing adaptor protein inducing interferon beta
WB	Western blotting

1. Introduction

The immune system protects the body from external pathogens and malignancy. Hence. a prerequisite of the immune system is the fundamental function of recognizing and eliminating "non-self" pathogens. Interestingly, this function raises an important issue in reproduction during both fertilization and pregnancy since the human fetus is semi-allogenic and also because of the intimate juxtaposition between maternal and fetal tissues (Manyonda, 2006).

Human decidua, the interface between fetal tissue and myometrium, consists of several cell types, including stromal cells, epithelial cells and a large population of leukocytes. Increasing evidence supports the fact that decidua is the key intrauterine source of bioactive molecules that are crucial in pregnancy maintenance and labour initiation. Consequently, the dysfunction of decidua would probably lead to maternal and fetal morbidity and mortality. Thus, studies regarding formation of the decidua and its functions are significant in further understanding the role of maternal side of placenta in pregnancy and providing scientific evidence for clinical treatment of placental disease.

Cytokines are soluble proteins synthesized mainly by immune cells that act on the receptor of their own cell (autocrine) or act on other cells (paracrine), leading to functional changes. The interactions of different cells, tissues and organs are controlled by the cytokine network. Among various cytokines, interleukin-1β (IL-1β) is a central mediator of the immune response, which is essential for host-defense responses to pathogens, but can also exacerbate the damage from inflammation (Garlanda et al., 2013). Additionally, IL-1β was found to be involved in the parturition and pathogenesis of preterm labour (Arcuri et al., 2009; Baergen et al., 1994; Døllner et al., 2002; Halgunset et al., 1994; Lockwood et al., 2006; Romero et al.,

1989a). Production of this cytokine is now known to be linked to inflammasomes that are innate immune system receptors and sensors, implicated in physiological and pathological inflammation during pregnancy (Khan and Hay, 2014). Among all the inflammasomes, NLRP3 type is the most studied inflammasome that is involved in the canonical IL-1β secretory pathway. However, how decidua mounts an inflammatory response is not fully understood. Thus, this study will focus on the decidua's role in immune response through IL-1β secretion, especially on the key factors of the inflammasome pathway.

1.1 Immune system

1.1.1 Innate and adaptive immune response

Inflammation, a protective immune response, serve to removal of harmful stimuli, such as microbial infection, tissue injury, and cardiac infarction, and to hasten the repair of damaged tissue (Medzhitov, 2008). The inflammatory process is rapidly activated and the damaged tissue is repaired. Insufficient inflammation can result in persistent infection, while excessive inflammation can cause auto inflammatory disease, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Apte et al., 2006; Burger et al., 2006).

The human body has three immune defense barriers against outside pathogens, including a physical barrier, the innate immune system as well as the adaptive immune system. Among them, the physical barrier is the first protection, which comprises the surface area such as skin and mucus membranes. The epithelial cells forming these barrier is the producer of cytokines and chemokines, and have the ability to actually recognize and process danger signals (Hato and Dagher, 2015). While, the innate immune system consists of the following

constituents: (1) soluble elements such as complement, interferon. (2) immune cells, such as macrophages, dendritic cells, neutrophils, and Natural Killer cells, etc (Manyonda, 2006). In addition, subsets of T and B cells have limited antigen receptor diversity and participate in innate immunity (e.g., B-1 B cells, natural killer T cells). The characteristic of the innate system is that it can induce non-specific responses to “non-self” pathogens, which are not affected by previous exposure (Janeway Jr and Medzhitov, 2002). Besides, the innate immune system can react fast to prevent and control the initial infection (Medzhitov, 2008). Many immune cells belonging to the innate immune system can identify pathogens through pattern-recognition receptor (PRRs) (Takeuchi and Akira, 2010). The activation of the PRRs leads to the secretion of cytokines and complement and then trigger inflammation, the details of which will be discussed in later section.

In most cases, innate immune protection is effective in defending from pathogens. However, sometimes, pathogens such as a virus can escape from innate immune response. In these cases, the adaptive immune system needs to respond to eliminate infection as the second line and to provide more rapid and vigorous protection through immunologic memory and specificity, which involves T cells, B cells, antibodies and receptors, etc (Manyonda, 2006). The adaptive immunity provides response to previously exposed pathogens which differ from innate immune system. The activation of adaptive immunity requires presentation of antigens by specialized antigen-presenting cells (APCs), production and secretion of cytokines and the amplification of lymphocyte clones (Gabbe et al., 2016). There are several other important differences in mechanism, including the pathogen recognitions and lymphocyte activation (Manyonda, 2006), that the innate immunity recognizes structures shares by

classes of microbes, whereas adaptive immunity recognizes the individual structures of microbes(antigens).

1.1.2 Immunology and pregnancy

The maternal immune system is a crucial system with the roles of protecting the maternal body against the pathogens and preventing damage to the fetus. It is characterized by a strong network of recognition, communication, and repair function during pregnancy (Sacks et al., 1999) and involves the placenta.

1.1.2.1 Structure and development of placenta

During pregnancy, the fetus develops in the amniotic cavity surrounding by amniotic fluid which is generated from maternal plasma (Underwood et al., 2005). This amniotic cavity is enclosed by the inner fetal membrane, the amnion and the outer membrane, the chorion. The interface of maternal and fetal connection is decidua, which is differentiated from the maternal uterine tissue-endometrium (Cartwright et al., 2010).

The placenta is composed of maternal decidua basalis and a fetal part of amnion and chorion frondosum, and the development of placenta starts at the time when blastocyst implanted into the endometrium, and the progress of implantation is regulated by a complex of interaction between the trophoblasts and the endometrium. Successful implantation after fertilization requires synchronization of endometrial maturation and embryonic development (Beier-Hellwig et al., 1995). To prepare for embryo implantation and pregnancy, the endometrium undergoes dynamic structural changes called decidulization, in response to the

changed estradiol and progesterone level, resulting in remorphological and biological reprogramming of endometrial stromal compartment involved increased gland secretion, vascular proliferation and production of a wide range of hormones, cytokines, growth factors and immune molecules regulating in recruitment of specific immune cell population and development of the placenta (Jones et al., 2006). These is a decidua part that the fetal membranes attached is decidua called decidua parietalis (Gabbe et al., 2016). Apart from that, for those attached by placenta it is called decidual basalis. As for embryo, the blastocyst sinks under the epithelium and be surrounded by the endometrium after implantation. After that, the invading trophoblast penetrate the endometrial blood vessels and form the intertrophoblastic blood filled sinuses. The maternal blood enters the placenta through uterine spiral arteries which contact with sinuses. At the same time, the fetal circulation develops and produces capillaries within chorionic villi, which is mainly formed by the syncytiotrophoblast layer, a core of cytotrophoblast in the early stage and a core of embryonic mesoderm in later stage； While the non- villous trophoblast constitutes the rest of the trophoblast tissue. (Benirschke and Kaufmann, 2000). In this way, the chorion leave, chorionic plate, the marginal zone and the basal plate of the placenta are formed. However, there is no vascular continuity and no direct blood connection between fetus and maternal blood (Manyonda, 2006). The connection between the fetus and placenta is via the umbilical cord consisting of two arteries, one vein and the outside Wharton's jelly (The white layer outside umbilical cord shown in Figure 2.2.1).

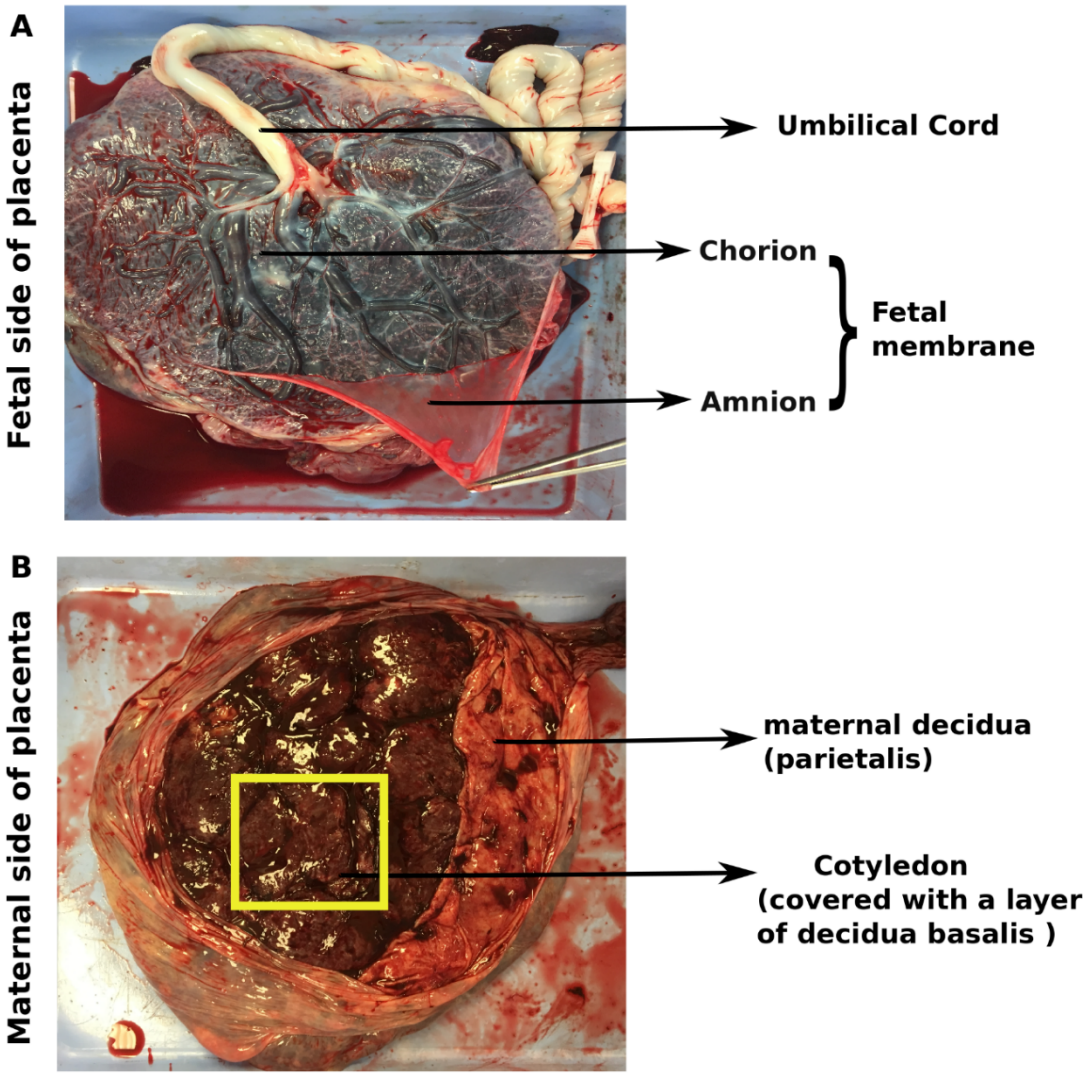

Figure 1.1.2 Structure of placenta

This figure shows the fetal side (A) and the maternal side (B) of placenta.

1.1.2.2 Immunology in normal pregnancy

1.1.2.2.1 Decidua leukocyte and pregnancy

In response to changed oestradiol and progesterone levels during menstruation (Irwin et al., 1989), the endometrium transforms into a specialized tissue, called decidua (maternal-fetal interface), which is situated between the fetus tissues and myometrium during pregnancy

(Cartwright et al., 2010). In normal pregnancy, there are a high number of immune cells in decidua, which is one of the most important changes of decidual tissue from non-pregnant endometrium. In early pregnancy, the immune cells form 40% of the total cells, 70% of them are uterine Natural Killer (uNK) cells, and 18% of them are macrophages, 10% T lymphocytes, 2% dendritic cells and several B lymphocytes (Wooding and Burton, 2008). However, how each of these cells participate in immunoregulatory during pregnancy is not yet fully defined.

The uNK cells is the most frequent cell type following the stromal cells in decidual tissue, and they are present before implantation and continue to proliferate in the decidua in the early pregnancy (Moffett-King, 2002). During the menstrual cycle, decidualising stromal cells can produce the signals (hormones, cytokines and chemokines) to recruit and guide uNK cells to differentiate to express a distinctive phenotype-the CD56+ uNK (Gabbe et al., 2016), and these uNK cells could produce a wide range of cytokine and chemokine under stimulation by trophoblast and stromal cells, which could be tolerogenic or cytotoxic to the embryo (Hanna et al., 2006; Moffett and Loke, 2006; Moffett-King, 2002; Shigeru et al., 2008). Successful pregnancy largely depends on the interaction between uNK cells and the invasive trophoblast to produce a suitable local micro-environment to support the immune tolerance throughout pregnancy. Besides, researches also suggested that uNK cells play an important role in the normal arterial remodeling (Anne Croy et al., 2006; Hanna et al., 2006; Pijnenborg et al., 2006; Shigeru et al., 2008).

Peripheral blood monocytes infiltrate the decidua and convert into macrophages, which comprise 20% of decidual immune cells (Bulmer and Johnson, 1984). Decidual macrophages (DMs) are considered to participate in protecting the fetus against intrauterine infections (Singh et al., 2005). Through secreting immunomodulatory molecules and inducing apoptosis

of unwanted or damaged cells, macrophages could inhibit anti-fetal activity by lymphocytes and participate in tissue remodeling to regulate trophoblast invasion and the expanding fetus(Renaud and Graham, 2008). Besides, the phenotype of $CD14^+$ decidual macrophage was shown to have an immune inhibitory function, probably in maintaining maternal tolerance to the developing fetus (Heikkinen et al., 2003; Renaud and Graham, 2008)). On the other hand, DMs can also secrete other cytokines such as TNF-α, IL-1, and IL-6 in response to an infection leading to an intrauterine inflammatory response which may trigger pre-term parturition (Mcgregor, 1988).

Apart from the innate system, the adaptive immune cells also involve in the pregnancy immunology. Previous study has shown that the maternal immune T cells could specifically recognize the paternal alloantigen but pregnancy could be maintained by inducing a state of transient T cells which is tolerance for paternal antigens (La Rocca et al., 2014). Researchers have found that the changed balances and correlation in Th1, Th2, Th17, and Treg cells in pregnancy complication, such as abortion, preeclampsia, preterm labour. (Saito et al., 2010; Saito and Sakai, 2003; Santner-Nanan et al., 2009)

1.1.2.2.2 Decidual stromal and immune cells

Decidualization starts with blood vessel pericytes and subepithelial stromal cells, followed by a comprehensive decidualization throughout the uterine stroma (Wooding and Burton, 2008). Apart from the supporting function, decidual stromal fibroblasts can secrete chemokines and cytokines participating in interactions of the immune system in the maternal decidua (Hess et al., 2007). Besides, through releasing cytokines and chemokines, stromal cells can allocate

and attract peripheral and resident leukocytes to support placental development and provide immune protection (Red-Horse et al., 2004; Red-Horse et al., 2001).

1.1.2.2.3 Cytokines in normal pregnancy

The release of cytokines by macrophage and other immune cells is a significant induced innate immune response (Gabbe et al., 2016). During normal pregnancy, cytokines network present as an anti-inflammatory condition. IL-2, IFN-γ and TNF-β, i.e. cytokines that have adverse effects on the pregnancy, are defined as Th1-cytokines, which would induce cytotoxic and inflammatory reactions being responsible for the induction of cell-mediated inflammatory reactions (Mosmann and Coffman, 1989; Mosmann and Sad, 1996). On the other hand, secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 from Th2 cells contributed in humoral immunity. It is supposed that normal pregnancy is characterized with a down-regulation of Th1-type activity and enhancement of Th2-type activity (Makhseed et al., 2001).

IL-1β, as a main proinflammatory cytokine, has aroused interest from reproductive immunologists. Studies have shown that during the late secretory phase, IL-1β mRNA is expressed by the endometrium and immune active IL-1β protein was also detectable. (Simón et al., 1993; Talbi et al., 2006). In proliferative phase, a weak IL-1β signal can be found in the endometrium (Tabibzadeh and Sun, 1992). As for implantation, animal and human experiments showed that IL-1β may be involved in the establishment of pregnancy (Arck et al., 1997; White et al., 2005) and control the invasion of trophoblast cell (Karmakar and Das, 2002). During pregnancy, human decidua was found to be a source of IL-1 in 1989 (Romero et al., 1989d). But most of the related researches focused on the first trimester placenta, that decidua could produce abundant IL-1β, and most thought it to be derived from leukocyte

rather than decidual cells (Jokhi et al., 1997; Lonsdale et al., 1996; Montes et al., 1995). Besides, IL-1β mRNA was expressed in chorion-decidua before the onset of labour (Osman et al., 2003).

1.1.3 Inflammation in pregnancy complications

Intrauterine infection and inflammation are potential pathologies in both preterm labour with or without PPROM, which may cause preterm parturition – a main cause of perinatal morbidity and mortality (Romero et al., 2007). Increasing evidence indicates that infection is a frequent and important mechanism of premature labour and delivery, such as microbial intrauterine injection or systemic administration of inflammatory agents in animal models leading to preterm labour (Elovitz and Mrinalini, 2004; Gravett et al., 1994). In addition, the relation of subclinical intrauterine infections or intrauterine inflammation patients and preterm delivery, as well as the effectiveness of using antibiotic to treat ascending intrauterine infections induced preterm delivery also indicate the correlation between infection and preterm labour (Romero et al., 2007).

There are solid studies showing that cytokines participate in inflammation-induced preterm parturition. IL-1β was the first pro-inflammatory cytokine to be implicated in pregnancy infection and infection induced preterm labour. Firstly, IL-1β was found at high levels in amniotic fluid, maternal blood, and vaginal fluid of women with intraamniotic infection (Romero et al., 1989a). Furthermore, the histologic chorioamnionitis is associated with the increased level of concentration of cytokines, such as IL-1β, IL-1α and TNF-α in amniotic fluid (Halgunset et al., 1994), umbilical cord blood (Døllner et al., 2002; Roberts et al., 2012), and decidual tissue (Arcuri et al., 2009; Baergen et al., 1994; Døllner et al., 2002; Lockwood et al.,

2006). IL-1β was also proved to stimulate myometrial contractions (Romero et al., 2007). Besides, the animal model has proved that intra-amniotic infusion of IL-1β could lead to preterm labour and was correlated with the most intense contraction pattern (Sadowsky et al., 2006). Furthermore, IL-1β is found to be with several aspects of the pathogenesis of preeclampsia (Lockwood et al., 2008; Rinehart et al., 1999). Although amnion, chorion, and decidua have all proved to contribute to the release of IL-1β in response to LPS in vitro experiments (Fortunato et al., 1996; Maneta et al., 2015; Paradowska et al., 1997; Romero et al., 1989d), it has been hypothesized that the main source of cytokines is decidua when infection is present (Steinborn et al., 1996), while fetal-origin cells may be the source of cytokines in normal labour at term (Steinborn et al., 1995; Taniguchi et al., 1991).

Similarly, TNF-α also proved to play important role in preterm parturition. For example, the concentration and bioactivity of TNF-α in amniotic fluid is ascended in women with preterm labor and intra-amniotic infection (Hillier et al., 1993). Besides, TNF-α could induce amnion, decidua, and myometrium stimulates to produce prostaglandins (Romero et al., 1989c); Furthermore, the systemically administration of TNF-α to pregnant animals can induce preterm parturition (Romero et al., 2007). Apart from IL-1β and TNF-α, there are other cytokines, such as IL-6, IL-18, colony stimulating factors (CSFs) and chemokines (IL-8), i.e. have also been implicated in the mechanisms of disease leading to preterm labour.

PGs (prostaglandins) play a central role in the process of term and preterm labour, involving in uterine contractility, membrane rupture and cervical ripening; the synthesis of which is regulated to ensure quiescence for the maintenance of pregnancy, and increased production in preparation for labour (Aoki and Narumiya, 2012; Sykes et al., 2014). When it comes to inflammation, it has been shown that chorioamnionitis could produce higher output of PGE

(prostaglandin E) and PGF (prostaglandin F) in feotal membranes and placentas, which may initiate preterm labor (Bernal et al., 1987; Van der Elst et al., 1992). On the other hands, the cytokines elevated in amniotic fluid and foetal membranes in preterm labour patients (Keelan et al., 2003) are likely to induce PGs production, leading to the premature initiation of foetal membrane remodeling manifested in the form of PPROM(Mitchell et al., 1991; Romero et al., 1989b).

1.2 Recognition of danger in pregnancy

Essentially, the germline-encoded pattern recognition receptors (PRRs) take the responsibility to sense the invading pathogens by recognizing structures present among microbial species, and these molecules structures are called pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira, 2010). In addition, recent evidence demonstrated that PRRs can also recognize endogenous molecules released from damaged cells, which is termed as damage-associated molecular patterns (DAMPs) (Gallucci and Matzinger, 2001).

Currently, four classes of PRRs families have been widely studied, including transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptor (CLRs); as well as cytoplasmic proteins like Retinoic acid-inducible gene-1-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

1.2.1 TLR family

The TLR family is one of the most-characterized among these four types PRRs, and it is responsible for sensing invading extracellular pathogens as well as the intracellular

endosomes and lysosomes (Akira et al., 2006). TLRs are divided into two subgroups according to their location and PAMP ligands. One group of TLRs is expressed on cell surface and can recognize microbial membranes components, such as TLR2, TLR4, and TLR6. These TLRs contains N-terminal leucine-rich repeats (LRRs) and a transmembrane region of cytoplasmic Toll/IL-1R homology domain (Kawai and Akira, 2010). The other group includes TLR3, TLR7, TLR8 and TLR9, which are located in intracellular vesicles. These TLRs can recognize nucleic acids (Kawai and Akira, 2010). **Table 1.2.1** shows that different TLRs recognize the different molecular pattern of microbial and self-components

Table 1.2.1 TLRs and their ligands

	Location	Ligand	pathogen
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	viruses
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR8	Endolysosome	ssRNA	Bacteria, viruses, self
TLR9	Endolysosome	CpG-DNA	Bacteria, viruses, self, protozoa
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	protozoa

For example, TLR4 can recognize the lipopolysaccharide (LPS) derived from membrane of Gram-negative bacteria, together with myeloid differentiation factor(MD2) on the cell surface(Barbalat et al., 2009). Furthermore, the TLR4 also participate in the recognition of viruses. To be specific, TLR4 can recognize the endogenous oxidized phospholipids produced by avian influenza virus (Imai et al., 2008). In contrast, a set of TLRs, consisting of TLR3, TLR7, TLR8 and TLR9 can recognize nucleic acids from bacteria, viruses, and endogenous nucleic acids (Akira et al., 2006).

1.2.1.1 TLR4 and signaling pathway

Recognition of PAMPs by TLR4 cause transcriptional upregulation of some genes, inducing a variety of inflammatory cytokines, chemokines, costimulatory molecules, and other effectors. According to the TIR domain containing adaptor molecules recruited to TLRs, the TLR signaling could be divided into two distinct pathways, MyD88-dependent and TRIF-dependent pathway (Takeuchi and Akira, 2010) (**Figure 1.2.1**). Briefly, the MyD88-dependent pathway is triggered by MyD88 interact with TRAF6 and IRAKs, resulting in activation of TAK1. TAK1 phosphorylates IKKβ, leading to IκBα and activation of NF-κB. NF-κB translocates to the nucleus to promote the transcription of proinflammatory genes (such as IL-1α/β, IL-18, IL-6, and TNFα). While, the TRIF-dependent pathways relies on TRIF interaction of RIP1 or TRAF3. TRAF3 activates IRF3 through TBK1, inducing transcription of type interferons (IFNs) and proinflammatory gene,

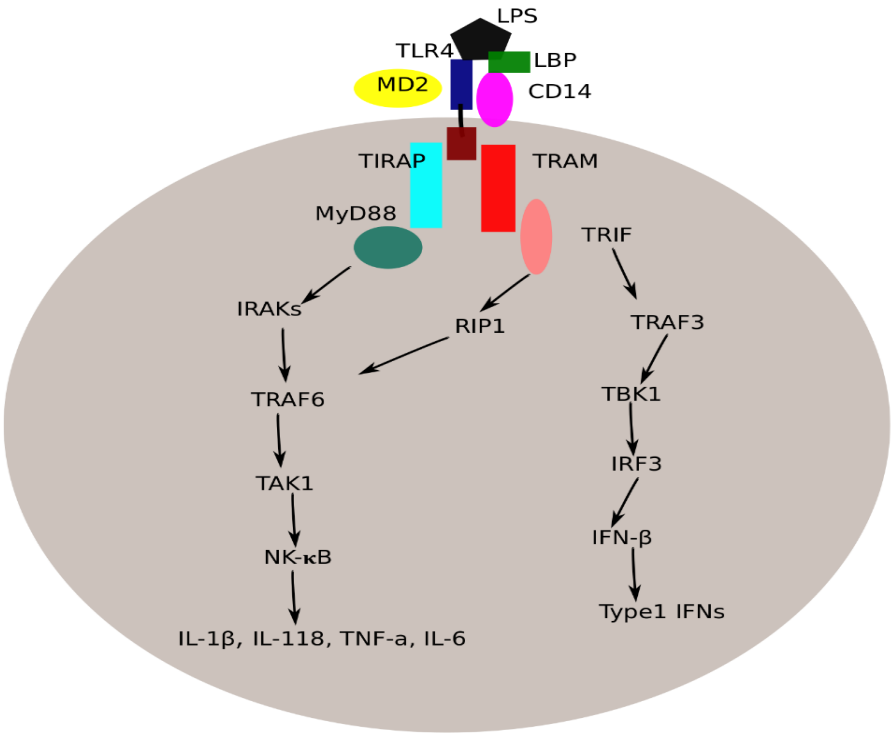

Figure 1.2.1 Schematic diagram of the TLR4 signaling pathway

This figure is modified from (Diamond et al., 2015). TIRAP:TLR domain containing adaptor protein； TRAM: translocating chain-associating membrane protein； MyD88: Myeloid differentiation primary response 88; TRIF: TIR domain-containing adaptor protein inducing interferon beta； TRAF: TNF receptor associated factors； RIP1: Receptor-interacting serine/threonine-protein kinase 1; TBK1: serine/threonine-protein kinase 1; TAK1: Transforming growth factor beta-activated kinase 1 ; IRF3: Interferon regulatory factor 3

1.2.1.2 TLR4 and preterm labour

Since TLRs are important for pathogens recognition, it could be anticipated that the activation of TLRs signaling will induce labour. The expression of TLR4 has been reported in cervical tissue, placenta, choriodecidua, amniotic epithelium and myometrium (Hassan et al., 2006; Maneta et al., 2015) after labour. Moreover, spontaneous labour at term and preterm labour with histologic choriamnionitis are associated with increased expression of TLR4 in choriamniotic membranes (Romero et al., 2007). Besides, animal models experiments

showed that the TLR4 mutation is less likely to deliver preterm after intrauterine infection with bacteria or LPS than wild type (Wang and Hirsch, 2003). All the information indicates that TLR4 plays an important role in preterm labour.

1.2.2 NLR family

When microbes cross the cell membrane barrier, the NOD-like receptors (NLRs) will work as the second line of defense. The NLR family consists of cytoplasmic pathogen sensors which are composed of a NOD-like nucleotide-binding domain (NACHT), a region of leucine-rich repeats at C-terminus (LRR) and the N-terminal effector domain. (Tschopp et al., 2003). The NACHT domains are key component for receptor activation through ATP-dependent dimerization and oligomerization mediated by AAA+ cassette (Neuwald et al., 1999). While the LRR domain plays a role in detection and ligand-binding and the N-terminus effector mediate the interaction with other protein domains. In addition, according to different N-terminus, NLRs families are classified as NLRA, NLRB, NLRC, and NLRP. (Stutz et al., 2009).

1.2.2.1 NLRP 3 and NLRP3 inflammasome

NLRP3, the best characterized NLR, contains the NLR typical elements: LRR, NACHT, and the N-terminal PYD which can recruit the adapter protein ASC by PYD interactions (Manji et al., 2002). In response to stimuli, the oligomerization of NLRP 3 and the subsequent recruitment of ASC could activate caspase-1 leading to IL-1β releasing, of which process is referred as inflammasome activation (Agostini et al., 2004). Notably, NLRP 3 is a unique NLR which can be triggered by many stimuli including diverse microbes (viruses, bacteria, and fungi), as well as various endogenous stress signals (Kawai and Akira, 2009). For example many microbe-

derived substances, such as MDP and LPS, can activate innate immune receptors and activate the NLRP inflammasome, with the administration with ATP (Kanneganti et al., 2007; Kanneganti et al., 2006). Additionally, a number of endogenous host stimuli, such as extracellular ATP, crystals, particulates and extracellular peptides, have been reported to activate the NLRP 3 inflammasome (Kawai and Akira, 2009). ATP, one of the reported danger-associated signal, the levels of which should be kept low in extracellular compartments (Mariathasan et al., 2006). However, the dying cells and some stimulated cell, such as lymphocytes, monocytes, have been shown to release ATP under certain stressful conditions (Filippini et al., 1990; Netea et al., 2009). The mechanism of NLRP3 inflammasome activation is introduced in next section.

1.3 IL-1β production and secretion

IL-1β is one the most studied and best characterized cytokines in pregnancy immunology for its roles in labour and preterm labour (mentioned in section 1.1.3). Intracellular pro-IL-1β, which is synthesized as an inactive 33 kDa precursor (Eder, 2009), is produced in response to molecular motif-PAMPs. However, the processing mechanism of IL-1β are different in different cells. For example, in neutrophils and macrophages, IL-1β transcription and secretion is carefully regulated and proceeds via a "double hit" mechanism, which means induction of pro-IL-1β expression is inefficient, and this primed cell must then encounter a further PAMPs or DAMPs to induce the processing and the secretion of an active IL-1β. One of the canonical mechanism of IL-1β activation is dependent on caspase-1 activation, that pro-IL-1β is enzymatically cleaved by the pro-inflammatory protease caspase-1 to generate a functional 17kDa product-IL-1β (Thornberry et al., 1992). Meanwhile, there some other

pathways for processing IL-1β For example, monocytes can release IL-1β following just a single stimulus (Netea et al., 2009; Ward et al., 2010).

1.3.1 Caspase-1 activation

Caspase-1 is synthesized as a 45 kDa cysteinyl aspartate protease with a CARD at the N-terminus (Martinon and Tschopp, 2007), which is an inactive pro-form in resting cells. The activation of caspase-1 occurs through the activation of inflammasome. To be specific, the PYD domain of NLRP3 can recruit the adaptor molecule ASC via PYD-PYD interactions, and then the pro-caspase-1 is recruited to ASC through CARD-CARD interactions leading to the activation of caspase-1 (Stutz et al., 2009) that pro-caspase-1 cleaves into two active subunit p20 (20 kDa) and a p10 (10 kDa). The active enzyme p20 and p10 assembles into two heterodimers of p20/p10 (Tabibzadeh and Sun, 1992). Following caspase-1 activation, the active p20/p10 can cleave the inactive pro-IL-1β generating the active IL-1β and then secreting IL-1β from cells (Guo et al., 2015).

1.3.2 Potential mechanism of NLRP3 inflammasome activation

Although the activation of caspase-1 through NLRP3 inflammasome assemble is well studied, the actual signal of its activation remains perplexing. The diversity of stimuli to NLRP3 inflammasome implicated that activation mechanism is likely to be very complex. Now there are four main mechanisms have been postulated. **Figure 1.3.2** shows the potential intracellular mechanism of NLRP3 activation, including intracellular potassium efflux out of the cell, the mitochondrial reactive oxygen species (ROS) generation, and the release of

cathepsins into cytosol. Each of these mechanism is briefly introduced below. However, it is necessary to note that not all of these events can be induced by all NLRP3 agonists, thus the precise mechanism of NLRP3 activation is still debated in different pathological conditions (Guo et al., 2015).

First of all, NLRP3 need be primed. For example, binding of LPS to TLR4, increasing the cellular expression of NLRP3 through NF-kB signaling (Bauernfeind et al., 2009). Additionally, recent research has shown that priming could induce the deubiquitinating of NLRP3 to licenses activation of mouse NLRP3 inflammasome (Bauernfeind et al., 2009; Py et al., 2013). Once be primed, NLRP3 can response to diverse stimuli and then assemble into NLRP3 inflammasome. During this process, ASC must be ubiquitinated linearly for the assembly of NLRP3 inflammasome (Guo et al., 2015).

One potential mechanism of NLRP3 activation is the lower intracellular potassium concentration, that intracellular K^+ efflux seems to be a need for NLRP3 activation in most of the cells (Stutz et al., 2009). Physiologically, the intracellular level of potassium tends to be high, with substantial K^+ exits warning the cell of danger. The K^+ efflux can happen due to the activity of some stimuli, and for example, pore formation or extracellular ATP reacts with $P2X_7$ receptor resulting in K^+ efflux (Colomar et al., 2003; Walev et al., 1995). Basically, low concentration of intracellular K^+ is required for full assembly of pyroptosome which is the supramolecular assembly of ASC dimers, the formation of which can activate caspase-1 leading to the secretion of pro-inflammatory cytokines (Fernandes-Alnemri et al., 2007).

The other theory is ROS act on a target on the upstream of the NLRP3 inflammasome and indirectly cause its activation (Stutz et al., 2009). Researchers found that ROD inhibitors could suppress the activation of the NALP3 inflammasome stimulated by asbestos, silica and alum,

which may involve with MAPK and PKB pathway (Kawai and Akira, 2009). Another potential inflammasome activation mechanism is the release of cathepsin B from the lysosome in sterile inflammation triggered by Crystalline or particulate matter (Guo et al., 2015). This theory is supported by the facts that the inhibition of lysosomal protease cathepsin B resulted in impaired inflammasome activation with decreased IL-1β (Hornung et al., 2009). However, a cathepsin B knockdown did not result in inhibition of caspase-1 activation (Newman et al., 2009).

1.3.3 $P2X_7$ receptor

$P2X_7$ receptor is a family member of an ion channel, which produces an initial cation current on the binding of ATP (Surprenant et al., 1996). $P2X_7$ receptor are expressed on immune cells, such as macrophage, and some antigen-presenting cells (Surprenant et al., 1996). Compared with other family member of P2X receptor, it is reported that $P2X_7$ receptor require higher concentration of ATP for activation, while BzATP is 300-fold more potent than ATP serving as the $P2X_7$ receptor agonists. (Bianchi et al., 1999; Donnelly-Roberts et al., 2009; Surprenant et al., 1996). As for $P2X_7$ receptor antagonist, A7 showed a greater potency in competitively blocking $P2X_7$ receptor activation compared with other antagonist, such as KN-62 and Brilliant Blue G(Donnelly-Roberts et al., 2009).

The activation of $P2X_7$ receptor could result in a variety of downstream events, such as cell proliferation, cell death, and proinflmmatory secretion(Bartlett et al., 2014), and it has been studied that they are associated with NLRP3 inflammasome (Duncan et al., 2007). Since the $P2X_7$ receptor antagonist showed the potential benefit in treatment of injury, inflammation, and neurologic disease, etc. For example, $P2X_7$ receptor antagonist was implicated that the

blocking of IL-1β release could inhibit the inflammatory pain in animal models as well we human clinical trials(Bartlett et al., 2014). As for the gestational tissue, previous studies shown that $P2X_7$ receptor was expressed in cord blood leukocyte, myometrium, and choriodecidua, which is involved in IL-1β release(Maneta et al., 2015; Warren et al., 2008). However, whether $P2X_7$ receptor expressed in human decidua is unclear. To investigate the role of $P2X_7$ receptor playing in IL-1β release, we primed tissue or cells with LPS as the PAMP via the TLR4 receptor, and used BzATP as a "second hit" to bind with $P2X_7$ receptor, to induce IL-1β production and release.

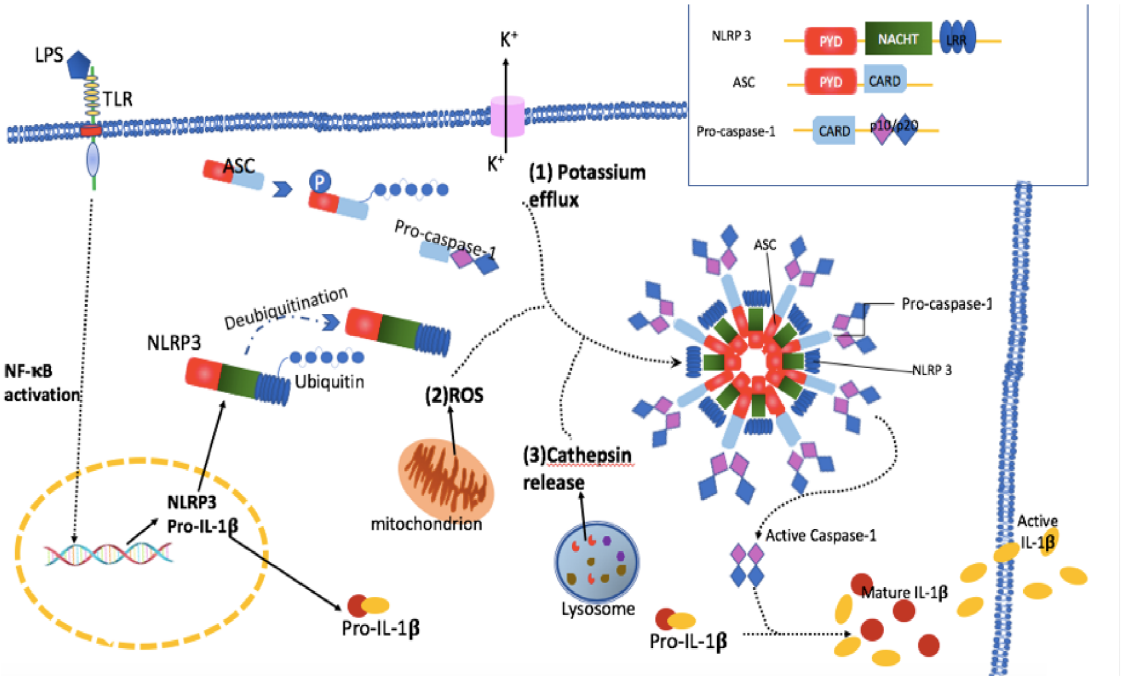

Figure 1.3.2 Potential mechanism of IL-1β production

The IL-1 production needs to "hit". The first hit is a NF-B-activating stimuli, such as LPS binding with TLR4, leading to the elevated expression of pro- IL-1 and NLRP3 or licensing NLRP3 with deubiquitination and licenses ASC with ubiquitination and phosphorylation. In canonical IL-1 production pathway, the activation of NLRP3 inflammasome needs a additional hit to activate the NLRP3 and format the NLRP3 inflammasome. The potential intracellular mechanism of NLRP3 activation, including intracellular potassium efflux out of the cell, the mitochondrial ROS generation, and the release of cathepsins into the cytosol. Then the NLRP3

interact with ASC through PYD-PYD, and the pro-caspase-1 filaments form off the ASC filaments through CARD-CARD interactions, leading to the auto-cleavage of pro-caspase-1 to caspase-1 p20/p10. The caspase-1 finally could cleave pro- IL-1β into its mature forms. Modified from (Guo et al., 2015)

1.3.4 IL-1β secretion

Although the previous researches have increased the knowledge of the potential mechanism involved in production and processing of IL-1β, the mechanism of IL-1β secretion has proven to be elusive and is poorly understood. The majority of secretory protein, such as cytokines IL-6 and TNF-α, are released through the conventional ER-Golgi route (Halban and Irminger, 1994). However, the release of IL-1β belongs to the non-classical secretory pathway. The schematic diagram (**Figure 1.3.4**) below shows five suggested different release mechanisms: (1) The first potential pathway is exocytosis of IL-1β containing secretory lysosomes (**Fig.1.3.4, pathway A**). which is supported by the evidence that intracellular pro-IL-1β and procaspase-1 were found co-localized with the lysosomal membrane protein Lamp-1 and the endolysosomal hydrolase cathepsin D by immunoelectron microscopy (Andrei et al., 1999); (2) Shedding of IL-1β-containing plasma membrane microvesicles from the cell surface (**Fig.1.3.4, pathway B**) has been suggested to be the main IL-1β release pathway of ATP-stimulated monocytes and dendritic cells (Bianco et al., 2005; MacKenzie et al., 2001; Pizzirani et al., 2007). And $P2X_7$ receptors expressed in the membrane of microvesicles appear to participate in the regulation of IL-1β release from shed microvesicles (Eder, 2009). (3) The fusion of multivesicular bodies (MVB) and subsequent release of IL-1β-containing exosomes was suggested as another main mechanism for IL-1β secretion (Qu et al., 2007). (**Fig.1.3.4, pathway C**). (4) Some studies have found that secretion of mature IL-1β occurs via the plasma membrane transporters (**Fig.1.3.4, pathway D**). The inhibition of ABC transporters has proved

to reduce IL-1β release (Ikeda et al., 2007; Marty et al., 2005), indicating that ABC transporter might participate in the IL-1β release. Apart from that, lysis cells containing IL-1β and consequent passive release IL-1β has been suggested as a possible mechanism (Laliberte et al., 1999). Especially under the pathological condition, that prolonged exposure to ATP or antimicrobial peptides results in the loss of membrane integrity and release of both pro-IL-1β and mature IL-1β (Brough and Rothwell, 2007; Verhoef et al., 2005).

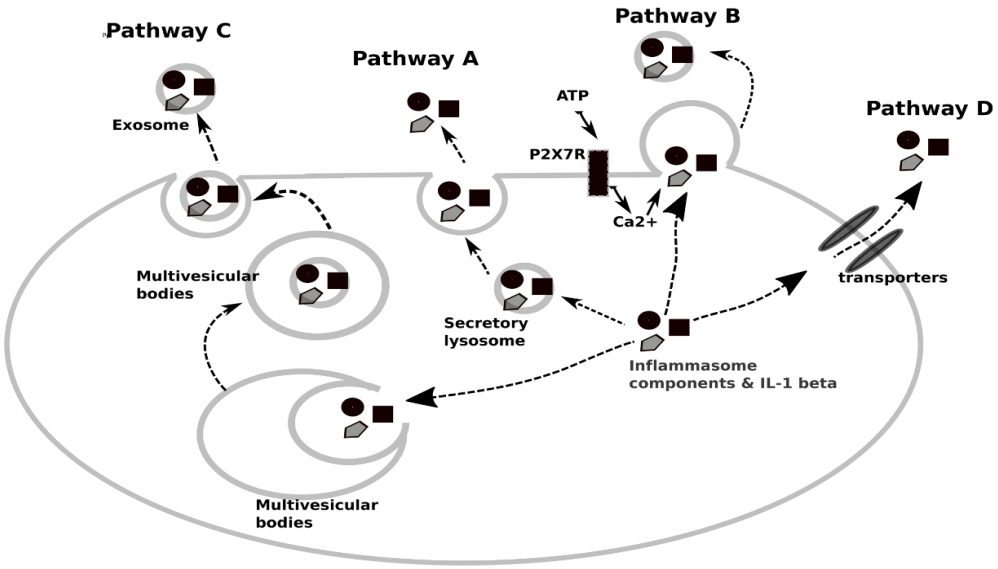

Figure 1.3.4 The schematic diagram of potential IL-1β release mechanism

This diagram shows four potential IL-1β release mechanism: pathway A presents the formation of multivesicular bodies and release the IL-1β contained exosomes. Pathway B indicates the release of IL-1β through secretory lysosome. Pathway C suggests the shedding of microvesicles and release of IL-1β from those shed vesicles. D shows the IL-1β release from cytosol to extracellular milieu via membrane transporters. Modified from (Eder, 2009)

1.3.5 NLRP3 inflammasome and pregnancy

NLRP3 Inflammasomes are implicated in physiological and pathological inflammations during pregnancy. The researcher found that the transcription of NLRP3 inflammasome components

was detected in chorion, amnion, decidual stromal cells as well as decidual endothelial cells (Hoang et al., 2014; Pontillo et al., 2013). Besides, the upregulation of inflammasome components in chorioamniotic membrane was characterized in spontaneous term-labour with acute histologic chorioamnionitis (Gomez-Lopez et al., 2017). Furthermore, a higher level of caspase-1 level in amniotic fluid indicated an activation of NLRP3 inflammasome in confirmed intrauterine infection (Gotsch et al., 2008). However, still further studies are needed for better understand how NLRP3 inflammasome involves in normal pregnancy and pathological conditions.

1.4 Hypothesis and aims

Starting from the current knowledge, increasing evidences support that decidua is the main source for IL-1β secretion participating in pregnancy complication. In addition, NLRP3 inflammasome components are found in gestational tissue with possible upregulation trend in intrauterine infection. However, how is IL-1β regulated in human decidua, and whether NLRP3 inflammasome pathway involves in its secretion mechanism are still unknown. The aim of this study is to investigate whether full-term decidua can stimulate IL-1β secretion and whether NLRP3 components play a role. The hypothesis to be tested is that IL-1β secretion occurs in maternal PBMC and decidua in response to pathogen or molecules. The specific aims of this study were:

(i) To detect IL-1β production and secretion in full term decidua explant, decidual stromal cells, decidual leukocytes and maternal PBMCs culture medium in response to

stimulation with the TLR4 receptor agonist and the $P2X_7$ receptor agonist and antagonist. This was done using ELISA.

(ii) To investigate the expression of NLRP3 inflammasome components and changes in decidual explant using western blotting after stimulation with the TLR4 receptor agonist and the $P2X_7$ receptor agonist and antagonist.

For these purpose, human full-term decidual explants, decidual stromal cells, decidual leukocytes and maternal PBMC were stimulated with bacterial lipopolysaccharide (LPS) and $P2X_7$ receptor agonist-BzATP and $P2X_7$ antagonist-A7. Then, the IL-1β secretion of tissue and cells and the expression of NLRP3 inflammasome components of decidua explants were evaluated.

2. Methods and materials

2.1 Sample Collection

2.1.1 Patient recruitment

Placentae were collected from patients attending the Department of Obstetrics and Gynaecology at Royal Derby Hospital. Ethical approval was obtained from the Derbyshire Local Research Ethics Committee (study number 09/h0401/90). All patients provided informed, written consent for the use of the placenta and maternal peripheral blood and then all the consent forms and sample information were documented, logged and kept in a secure file. Placenta from women who had pregnancy complications or twin pregnancy were not included in this study. Demographic data of the women who donated placentae for this study are shown in **Table 2.1.1**, including maternal age (years old), gestational age (weeks) and birth weight (g).

Table 2.1.1 Demographic date

	Age (years old)	Gestational age (weeks)	Birth weight (g)
Mean± SD or Median (95% CI)	33.25±5.24	39.00 (37.90, 39.21)	3474±408.6

2.1.2 Placenta collection

After checked by the midwife and with the cord clamped, the placentae were transported to the lab within 20 minutes after delivery. To acquire pure decidual tissue, the amnion of the placenta was peeled away before collecting decidua parietalis. Since the amnion is relatively firm and sturdy, it is easy to be peeled with forceps from fetal sides (**Figure 2.1.2 B**). After peeling away the amnion, vessels on the fetal aspect of the placenta could be seen more clearly (**Figure 2.1.2.A/C**) compared to the fetal side before and after peeling amnion. The parietal decidua tissues were then collected using ophthalmic forceps from the maternal side (**Figure 2.1.2 D/E**). Once the decidual sample was obtained, it was placed immediately in phosphate buffered saline (1X PBS) in a 50mL plastic tube, rinsed to remove blood, mucus and impurities **Figure 2.1.2.F**). Then decidual tissues were weighed and divided into several pieces. For decidual tissue explant assays, 100mg of tissue was needed for each treatment see below). For decidual mononuclear cell isolation, approximately 1000mg decidual tissue was obtained. Moreover, 500-1000mg of decidual tissue was needed for primary decidual cell culture. One placenta provided between 500-2000 mg of decidual tissue.

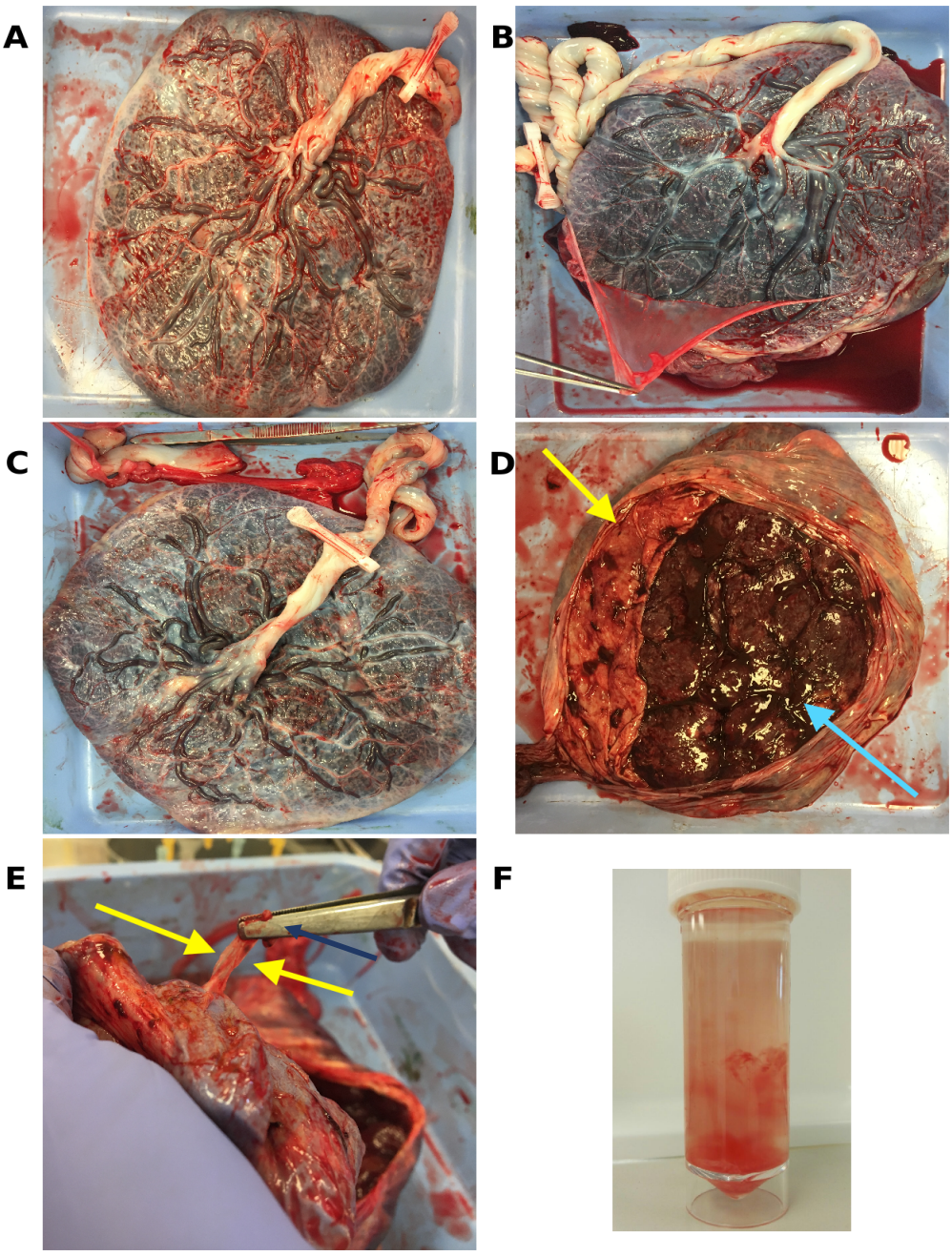

Figure 2.1.2 Collection of decidual tissue from placenta

A: the fetal side of placenta with amnion-covered the surface; B: peeled amnion membrane with forceps from fetal sides; C: fetal sides of the placenta after peeling amnion (vessels were clear); D: the maternal side of placenta: parietal decidua (yellow arrow); basal decidua (blue arrow); E: using ophthalmic forceps to collect parietal decidua tissues (yellow arrow indicate decidua tissue); F: collected decidual tissue in PBS buffer.

2.1.3 Maternal blood collection

Maternal blood was collected from women who were attending Pregnancy Day Care for a routine appointment from 20-28 weeks of pregnancy or immediately before EL LSCS. Approximately 10mL of venous blood was collected from the antecubital vein using a 21G needle into an EDTA vacutainer to prevent blood from clotting.

2.2 Sample preparation

2.2.1 Isolation of mononuclear cells

In this study, we used Histopaque®-1077 as the gradient medium containing poly sucrose and sodium diatrizoate with the density of 1.077g/mL, which facilitates the recovery of large numbers of viable mononuclear cells **(Figure 2.2.1 A).**

2.2.1.1 Maternal peripheral blood mononuclear cells (PBMC) isolation

The protocol of PBMC isolation was the same with the previous published research from our lab (Maneta et al., 2015), from which the flow cytometry of their harvested cells showed that the isolated cells mainly consisted of monocytes, lymphocytes and neutrophils. The CD45 positive cells accounted for 82.64%, CD14 positive cells accounted for 59.29%, while CD68 was 73.22%. The detailed method was: 10 ml of maternal blood was mixed 1:1 with 10 ml of RPMI (Gibco, Paisley, UK). The blood/RPMI mix was loaded onto the same volume of Histopaque 1077g (Sigma Aldrich, UK) and centrifuged 400g for 30 minutes at 20°C (Acceleration 1, Brake 1) (Falcon 6/300R, MSE, UK). After centrifugation, a visible lay of mononuclear cells formed between the mixture of plasma and media and Histopaque 1077g

interface (Figure 1.2.1 B-C. Then, the above solution was removed and the mononuclear layer was collected into a new 50 ml Falcon tube. The mononuclear cells were then washed with serum-free RPMI by centrifugation (250g, 10 minutes at 20°C). Then the pellet consisting mainly of PBMCs was suspended in 1mL of RPMI, cell counting performed using an automatic TC-10 cell counter (BioRad, Hercules, California, USA) and viability of cells preparations assessed using 0.4% solution of trypan blue (BioRad, Hercules, California, USA) (Described below). RPMI culture media was added up to make a cell concentration of 2×10^6 cells/ml. Cells were now ready for culture.

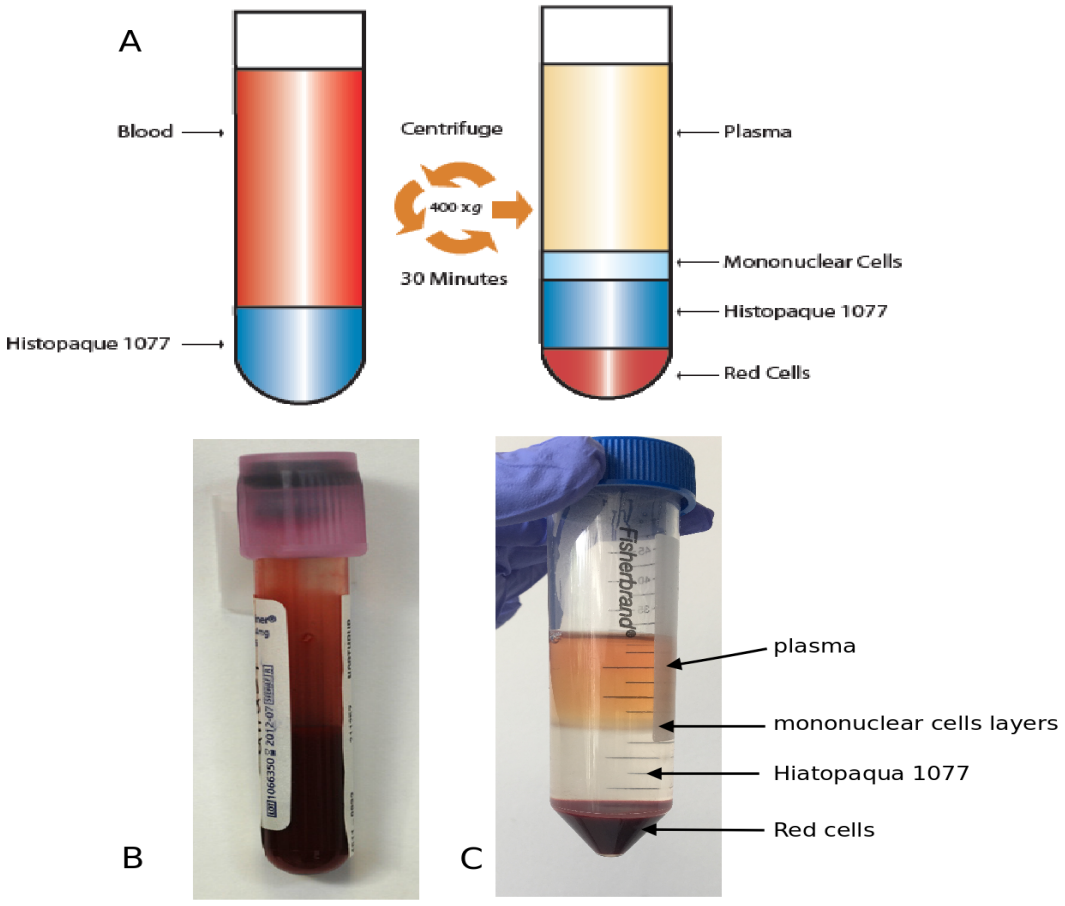

Figure 2.2.1 Demonstration of density gradient centrifugation using Histopaque-1077

The mixture of fresh blood and RPMI media was layered onto Histopaque-1077; after 30 minutes of centrifugation at 20^0C, a visible layer of mononuclear cells could be found at the Histopaque-1077/plasma interface.

2.2.1.2 Decidual mononuclear cell isolation

The method used in this study is similar with protocol of Yi Xu's lab (Xu et al., 2015). However, considering the high cost of cell detachment solution, we applied collagenase solution for cell detachment instead of Accutase used in their method.

Around 1g decidua tissue was rinsed in sterile 1X PBS to remove the excess blood from the membrane until the 1X PBS ran clear. The following step was to collect tissue pellets by centrifugation at 400 × g for 5 min at room temperature. Then, the supernatant was carefully aspirated without disturbing the pellet. The pellet was placed into a petri dish with 10 ml of cell detachment solution (0.2mg/mL collagenase, Sigma-Aldrich, UK) pre-warmed to 37 °C. Tissue was minced finely with a disposable Size 22 scalpel (Swann Morton, shefild, UK). After thorough mincing, the tissue mince and collagenase solution in the dish were transferred into a Universal and then enzymatically digested with collagenase at 37°C water bath for 45 to 60 minutes. Following incubation, 10 ml of 1X PBS was added to the digestion mixture, which was then filtered through a 100-µm cell strainer into a 50ml plastic tube. The tube was filled with 1X PBS to 50 mL and centrifuged at 400 × g for 5 min at room temperature. The supernatant was carefully removed and the pellet resuspended in 5 ml of ice-cold FACS buffer (1% BSA (Sigma-Aldrich, UK), 0.5% sodium azide (Sigma-Aldrich, UK) in PBS). The cell suspension was slowly overlain on top of the same volume of Histopaque®-1077 (Sigma-Aldrich, UK). The mixture was centrifuged with a swing-out rotor at 500 × g for 30 min at 4 °C without the brake (Acceleration 1, Brake 1). Leukocytes were present at the interface between Histopaque®-1077 and the FACS buffer. The upper layer was removed very carefully using a pipette. The interface layer contained the decidual mononuclear cells and a portion of the polymorphonuclear leukocytes. The cells were transferred from the interface

completely into a new Universal tube. Cells were washed with FACS buffer twice with centrifuging at 400 × g for 5 min at room temperature then re-suspended in 1 ml of RPMI. The viable cells were counted using an automatic TC-10 cell counter (Bio Rad, Hercules, California, USA) (detailed information were shown below) then diluted in RPMI to a a cell concentration of 2×10^6 cells/ml. Cells were now ready for culture (Xu et al., 2015).

2.2.2 Decidual stromal cell isolation

2.2.2.1 Primary decidual stromal cell isolation

0.5 to 1 g decidual tissue was used for decidual stromal cell isolation. The same steps described in section 1.2.1.1 was followed until digested tissue was filtered through a 100µm cell strainer into a 50ml plastic tube. Then, the purified cells were then washed twice with HBSS followed by overlaid cell suspension slowly on top of the same volume of 60% Percoll (Sigma-Aldrich, UK). The mixture was centrifuged with a swing-out rotor at 400 × g for 20 min at room temperature without the brake (Acceleration 1, Brake 1). Decidual stromal cells will be found in the interface between Percoll and the HBSS. The stromal cell layer was collected and washed with HBSS twice. Then, the cell pellet was suspended in culture medium (10% FBS (v/v) (Sigma-Aldrich, UK), 1% Penicillin(v/v) (Sigma-Aldrich, UK) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK)). These cells were now ready for primary cell culture.

2.2.2.2 Primary decidual stromal cell culture

Isolated cells were placed into a T25 flask and incubated at 37°C in air/5% CO_2 atmosphere. Cells were checked every day, and the cell culture medium was changed every other day. Once the adhered cells became 90%-100% confluent, cells were passaged following splitting. To split cells, appropriate volumes (according to the size of the flask) of 0.025% trypsin EDTA (Sigma-Aldrich, UK) was added to the flask and incubated at 37°C for 3 minutes until cells had detached from the base of the flask. Then the same amount of culture medium was added to stop trypsinisation. The cell suspension was centrifuged at 800 rpm for 5 minutes. The supernatant was then removed and the cell pellet washed with HBSS twice. Finally, the cell pellet was suspended with culture medium and transferred into a T75 flask and maintained with media changes every three days. Continued cell culture and passaged till Passage 3 cell which was ready for cell stimulation and fixation for fluorescence. After the third time of splitting, cells were counted and diluted to 5×10^5 cells/ml. Then 1 mL of stromal cell suspension were plated into 24 wells plate. After incubation at 37°C overnight, cells were checked. The decidual stromal cells were ready for stimulation when adhere cells became confluent.

2.2.3 Cell counting

TC-10 cell counter (Bio-Rad, Hercules, California, USA) was used in this experiment for primary decidual stromal cells, decidual mononuclear cell and maternal PBMC counting. 10uL of diluted cell (1/10-1/100) suspension was added to a TC-10 counting slide and then inserted into the machine. The number of cell/mL was shown, and the image of view was also displayed to assess whether the cells were in bulk on the slide. If the cells were in bulk, the

result of counting would be inaccurate. Further dilution would be needed and then repeated counting. Then, a 10µl aliquot of the cell suspension was mixed 1:1 in a 0.4% trypan blue solution. 10µl of diluted cell suspensions was added to the counting slide and read by machine. However, if the cells were still found in bulk in the view, haemocytometer counting was performed as an alternative assessment. The haemocytometer was then cleaned with 70% ethanol and allowed to air dry thoroughly. A coverslip was then placed on top of the haemocytometer and adhered using condensation as confirmed by Newton's Rings which could be observed as spherical patterns along the interface where the coverslip met the haemocytometer. 10µl of diluted cell suspensions was then added onto the haemocytometer which is drawn under the coverslip using capillary action. A light microscope (Olympus) at 100x magnification was used to visually count the viable and non-viable cells within the counting squares off each corner of the central square. Viable and non-viable cells could be easily distinguished from one another as trypan blue was not able to cross an intact cell membrane (Tennant, 1964). Therefore, the non-viable cells took up the trypan blue dye appearing a deep blue color, whereas the live cells did not take up the stain. The number of viable and non-viable cells counted was determined and the percentage of live cells calculated.

Decidual stromal cells or mononuclear cells suspensions were then diluted to obtain the appropriate cell concentration(s) for the assay requirements. All calculations were performed on total cell number, and samples with viabilities below 90% were not used any further.

2.3 in vitro stimulation experiments

2.3.1 Preparation of agonist and antagonist

Decidual tissue, isolated decidual mononuclear cells and stromal cells, as well as maternal PBMCs were treated with the TLR4 receptor agonist lipopolysaccharide from Escherichia coli 026:B6 (LPS, Sigma Aldrich, UK), $P2X_7$ receptor agonist BzATP (Sigma Aldrich, UK) and $P2X_7$ receptor antagonist A7 (A-740003) (Sigma Aldrich, UK). In this study, LPS was dissolved in RPMI at a concentration of 1mg/mL as stock concentration. The $P2X_7$ receptor agonist BzATP is a selective $P2X_7$ purinergic agonist (structure shown in the **Figure 2.3.1-A**), which is more potent than ATP at homodimeric $P2X_7$ receptors. BzATP was dissolved in H_2O at a concentration of 30mM. While A-740003 is a structurally novel and highly specific antagonist for mammalian $P2X_7$ receptors (structure shown in the **Figure 2.3.1-B**), the working concentration was 100µM dissolved in DMSO. The final assay concentration used in cell culture treatment and tissue culture treatment was shown in **Table 2.3.1** below. The stock concentration was 50-100 times higher than final assay concentration due to a 1:100/ 1:50 dilution onto the assay plate of cell stimulation or tissue stimulation respectively. (e.g. 5µL /10µL of LPS was added to 0.5mL RPMI medium in cells/ tissues stimulation).

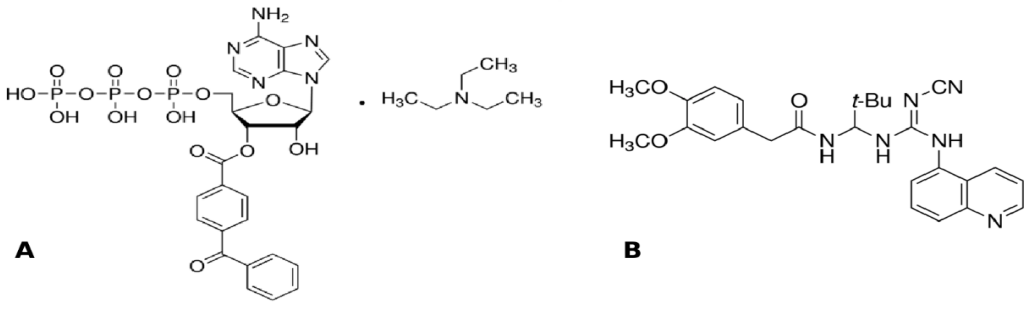

Figure 2.3.1 Structure of agonist and antagonist of $P2X_7$

A: $P2X_7$ receptor agonist (BzATP); B: $P2X_7$ receptor antagonist (A 740003)

Table 2.3.1 Concentration of treatments used in this study

		LPS	Bz ATP	A 740003
Stock concentration		1 mg/mL	30 mM	100μM
Final assay concentration	PBMC/DMC/DSC	10 μg/mL	300 μM	1 μM
	Decidual tissue	20 μg/mL	600μM	2 μM

2.3.2 Tissue and cell stimulation

100mg/well decidual tissue explants with 0.5mL RPMI 1640 medium (Gibco, Paisley, UK) or 0.5mL/well mononuclear cells suspensions (1×10^6 cells) were plated in 24 wells plates, respectively. In addition, for decidual stromal cells cultured in 24 wells plate overnight, previous media was changed and cells were washed with HBSS once before adding 0.5mL RPMI 1640 medium. Explants and cells were primed with LPS (10 μg/mL for cells, and 20 μg/mL for tissue explant) for 4 hours in air/5% CO_2 incubator at 37°C. The effects of the $P2X_7$ receptor antagonist was tested by pre-incubating cells with the A-740003 (1 μM for cells, and 2 μM for tissue explant) along with the LPS stimulation. After 3.5 hours incubation with LPS and with/without A-740003, BzATP (300 μM for cells and 600 μM) was added to wells for 30 mins to trigger IL-1β release. Negative controls consisted of unstimulated cells/tissue maintained in RPMI culture medium, which was without both LPS, A-74003 and BzATP. In contrast, positive controls were treated with both LPS and BzATP. Duplication of each drug and controls was conducted for each sample.

After incubation, for decidual tissue explants culture, supernatants were collected and labelled in EP tube, then frozen at -20℃ until required for quantitation by ELISA; the remaining tissue explants were collected in 2.0 ml cryo-tubes, snap frozen in liquid nitrogen and stored at -80℃. For mononuclear cell culture, the cell suspension was centrifuged at 800 × g for 10 min, and then the supernatant was frozen at -20℃ and the cell pellet stored at -80℃. For decidual stromal cells, supernatants were collected and cells disposed off.

2.4 Western blotting

2.4.1 Tissue homogenization

500-750 µL complete homogenization buffer was added to a 2.0 mL Lysing Matrix tube containing beads (Lysing Marix D, MP Biomedicals, USA). The frozen tissue was placed into the bead column tube directly just before homogenizing. The Lysing Matrix tube containing tissue and buffer was placed into the benchtop homogenizer machine (FastPrep-24, MP Biomedicals, USA), and the program run at 6M/S for 40 seconds. The tubes were checked for whether the tissue was well homogenized (no clumps could be seen); otherwise, another round was run until tissue was homogenized thoroughly. The tube was kept on ice while waiting for the homogenizer to cool down. After that, the homogenate was centrifuged (1000g, 10minutes, 4°C) (EBA 12R, Hettich, Germany). The supernatant (Supernatant 1) was then placed into an EP tube and centrifuged again (14000rpm, 60 minutes, 4°C). The resulting supernatant (Supernatant 2) was moved into a new EP tube again, which was saved at -80°C or used in another assay, such as BCA assay; The pellet was saved for further solubilization.

For solubilization, 30-50 µl of solubilization buffer was added to the pellet, which was also thoroughly vortexed and incubated at 4°C overnight. The pellet in buffer was sonicated the next day using an ultrasound cleansing bath (USC 100T, VWR collection, Vienna) for 15mins on ice before being centrifuged again at 1000g, 10mins at 4°C. After centrifugation, the supernatant (Supernatant 3) was then stored at -80°C.

2.4.2 Protein Determination

After homogenization, the concentration of total protein in supernatant was measured with BCA assay. The series of BSA concentrations was prepared at 1mg/ml, 0.8mg/ml, 0.6mg/ml, 0.4mg/ml, 0.2mg/ml, 0.1mg/ml, 0.05mg/ml consisting of stock BSA solution (10mg/mL) and 0.9% NaCl (w/v). Then samples were diluted 1 in 50 in saline for tissue homogenates. Then duplicates of 10ml aliquots of standard and diluted unknowns were similarly dispensed into wells of a plate. 200ml of BCA solution (0.2ml 4% copper sulphate (Sigma-Aldrich, UK) mixed with 9.8mls stock bicinchoninic acid reagent (Sigma-Aldrich, UK)) was added to each well. The plate was then incubated at 37°C for 30 minutes with shaking. The absorbance of all the standards and samples were read at 570nm using spectrophotometry (Thermo plate reader, MultiSkan ® Spectrum and Skanlt ® software version 2.2). A standard curve was constructed using Prism7.0 (Figure 3.2), and line equation was used to determine the protein concentration of the sample.

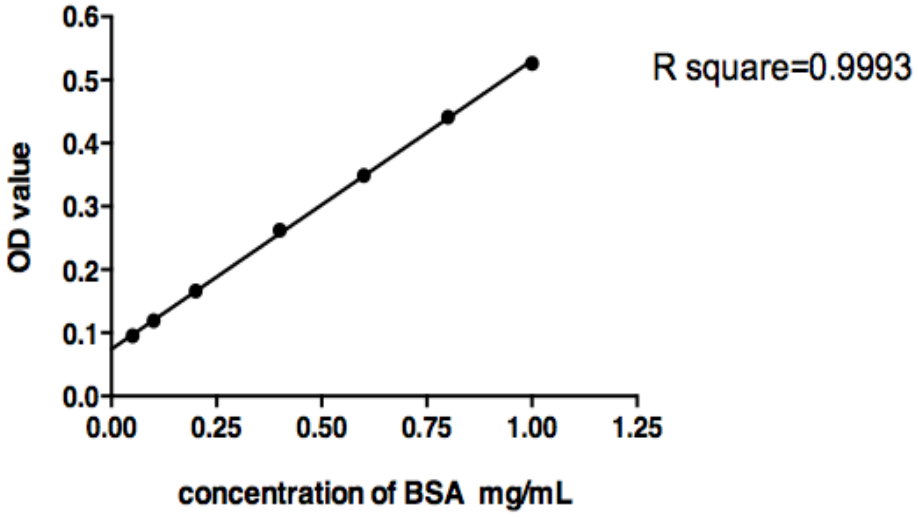

Figure 2.4.2 standard curve for BCA assay

Representative standard curve constructed for protein: the X axis was the concentration of BSA (mg/mL), while the Y axis was the OD value generated from plate reader. The adjusted R^2 of this standard curve was 0.999.

2.4.4 SDS-PAGE electrophoresis

Western blotting is a process involving three steps: separation of macromolecules, blotting and immune assay. A prevalent method for separating protein by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulphate (SDS) to denature the proteins. The method is called sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970; Laemmli, 1976).

SDS is an anionic detergent which has a net negative charge. This negative charges could destroy most of the complex structure of proteins, and the SDS binded proteins can be strongly attracted toward a positively charged electrode. Thus, the result of SDS incubation has two important feature: protein only retains their primary structure and have a large negative charge. In this way, proteins will migrate to the positives pole when placed in an

electric field. On the other aspect, polyacrylamide gels with different sized of tunnels throughout serve as an environment, in which proteins in different sized can move at different rates. (Roy and Kumar, 2014). When protein samples placed on the gel and the current was applied, the smaller size of the protein, the faster it can move. The final separation of proteins is almost entirely dependent on the its relative molecular mass, since charge-to-ratio is nearly the same among SDS-denatured polypeptides

2.4.5 Electroblotting

Electroblotting is a conventional method for transferring the protein from the acrylamide gel onto a nitrocellulose or PVDF which can be probed with antibody for target proteins. This approach accomplished by setting the gel flat upon a piece of the nitrocellulose membrane, and the electric field driveing the protein from the gel onto the membrane (**Figure 2.4.5.1**). The nitrocellulose can bind proteins tenaciously . When the protein moves from gel and bind to nitrocellulose membrane, the protein will preserve the original banding pattern. After that, the protein binding to nitrocellulose membrane could be stained with Ponceau Red (0.1% Ponceau (w/v) in 5% acetic acid (v/v), Sigma-Aldrich, UK) to red colour, by which the result of electrophoresis and protein transfer can be analyzed (**Figure 2.4.5.2**).

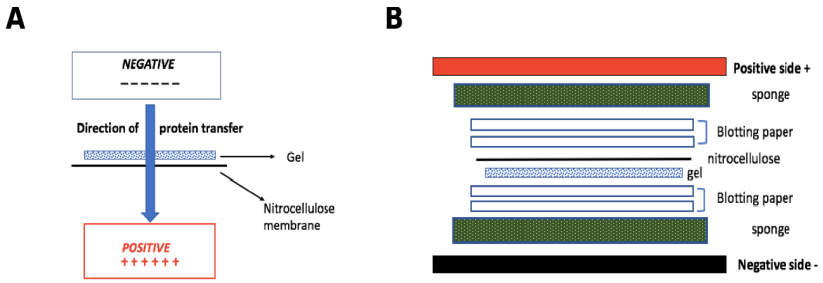

Figure 2.4.5.1 schematic of protein electrotransfering

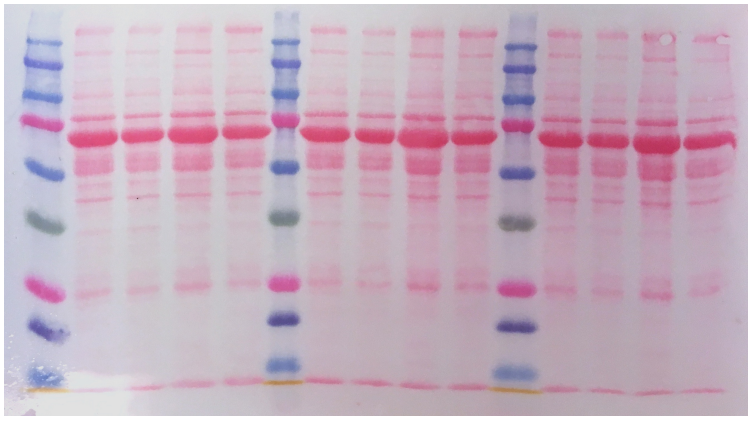

Figure 2.4.5.2 Visualization of proteins in membrane with Ponceau Red

2.4.6 Blocking and antibody incubation

After electroblotting, all proteins transferred onto the nitrocellulose membrane, including target protein and non-specific protein which would probably bind with target antibody. To minimise the non-specific binding, the blots need to be blocked with proteins, and we used 5% Marvel milk solution in TBS (w/v) (Marvel, premier b, Lincolnshire, UK).

After blocking, primary antibodies are utilized to detect the target protein. In this study, the primary antibodies included NLRP3 (Abcam, UK, 1:200 (v/v)), ASC (Enzo Science, US, 1:1000 (v/v)), Caspase-1(Bio-Vision, US, 1:200 (v/v)), and pro-IL-1β (Santa Cruz, US, 1:200 (v/v)), mature IL-1β (Cell signaling technology, The Netherlands, 1:1000 (v/v)) and β-actin (Sigma-Aldrich, UK, 1:5000 (v/v)). After incubation with primary antibodies, the second antibody serves as a conjugated antibody that when binding with the primary antibody, chemiluminescence will emit to allow the visualization of the target. As for the secondary antibody, there are two main types: Horseradish Peroxidase (HRP) -conjugated secondary antibodies and alkaline phosphatase (AP) antibody. The first one is conjugated with an enzyme from horseradish plant which could catalyze a reaction that emits chemiluminescence and can be detected (Kricka, 1991). However, The AP method is for the

detection of alkaline phosphatase activity using a colorimetric substrate (Bronstein et al., 1989). The secondary antibody used in this experiment was anti-Rabbit IgG-Alkaline Phosphatase antibody produced in goat from Sigma-Aldrich (UK) and Dako (Denmark).

2.4.7 Procedure for electrophoresis and electro blotting

For all western blotting experiment, SDS-PAGE gels were made of 12 or 15 % resolving gel and 4% stacking gel according to the molecular size of the target protein. 60-80 μg/ 20 μL of lysate samples or 20 μL of culture medium were loaded onto the gel. For electrophoresis, protein samples were diluted with same volume of loading buffer (5% 2β-mercaptoethanol (Sigma,UK) in Laemmli buffer (Biorad, Hercules, California, USA) (v/v)). The loading samples were heated at 88-95℃ for 5 minutes to further unfold the proteins followed by cooling on ice for 2 minutes to hold the proteins in that state before loading onto gel. Gels were run at 40mA for 50 minutes using Biorrad system for electrophoresis. Electroblotting onto the nitrocellulose membrane was conducted at 100mV with in cold transfer buffer for 1 hour, and the tank was settled on the ice. The protein transferred can be visualized by Ponceau red staining (Sigm,USA). All blots were blocked with 5% Marvel for 1.5 hours at room temperature, followed by incubation with 5-10 mL of the primary antibody at the required dilution in 3% Marvel and left overnight at 4℃ with gentle shaking. The AP method of immunodetection was used, and the AP-labeled secondary antibody in TBS was incubated on the blots for 1.5 hours at room temperature in the second day. Blots were washed with TBST and TBS after each step. Bio-rad detection system (Immun-Star™ AP Chemiluminescence Kits, Hercules, California, USA) was used for chemiluminescence detection that the enhancer and substrate were prepared and added onto the blots, then the blots were viewed using Chemi-

doc imaging system (Bio-Rad, California, USA). For normalisation of loading sample and quantification, blots were probed for a house-keeping protein. The β-actin antibody was used in this experiment. For reblotting blots, Reblot Plus solution (Chemicon Internationals) mild antibody stripping solution was used to strip off all antibodies. After stripping, blocked blots with 5% marvel in TBS for 1.5 hours. The incubated with β-actin primary antibody and follow with further steps described as above.

2.4.8 Data analysis of Western Blotting

Band intensities of proteins of interest were calculated using the Image J (National Institutes of Health, USA). Band intensities were calculated via comparison to the blot background. In addition, the intensities of the target protein and β-actin were both calculated, and the data were normalized with target protein intensity to β-actin intensity ratio. Comparison between different groups used the normalized data.

2.5 Enzyme-linked immunosorbent assay

2.5.1 ELISA procedure

IL-1β/ TNF-α ELISA detection reagent and Caspase-1 ELISA kits were purchased from R&D System (Human IL-1 beta/IL-1F2 ELISA DuoSet, and Human Caspase-1/ICE Quantikine ELISA Kit, R&D Systems, Abingdon, UK) and were used according to the manufacturers' recommendation. Samples were diluted using reagent diluent provided by Manufacturers, and the appropriate sample dilutions were decided upon by sample dilution series assay for different target proteins and samples. The brief protocol was shown as below: The first step

was blocking 96 wells plates with provided buffer. However, as for IL-1β/ TNF-α ELISA assay, coat antibodies were needed to be pre-incubated the night before doing the assay. After blocking, 100 µL of diluted sample, Standard and Control was added as duplicate into 96 pre-coated wells and incubated in room temperature for 1.5 to 2 hours according to protocol. Then, 100 µL of diluted detection antibodies were added to wells and incubated half to two hours, followed by adding diluted 100 µL of Streptavidin-HRP. 20 minutes incubation later, added Substrate solution to each wells. 96 wells plates were washed with washing buffer and completely removed liquid after each step. 50 µL of Stop solution was then added to Substrate solution. When the solution turned to be yellow, the plates were ready for reading.

2.5.2 Data analysis of ELISA

The optical density of standards and samples were determined from microplate reader. The standard curves were constructed using Prism7.0 and the Hyperbola equation within the program was used to determine the cytokines concentrations of sample.

2.6 Immunofluorescence

2.6.1 Cell fixation

For decidual stromal cells immunofluorescence, cell fixation was performed to Passage 3 decidual stromal cells. Culture medium was aspirated from wells, and cells were washed three times by 1×PBS, followed by cells fixation with 1mL of ice-cold acetone (Sigma, UK): methanol (Honeywell, USA) (1:1) (v/v). After 15-20 minutes' incubation at room temperature, aspirated the fixative and washed fixed cells with PBS three times. Then 1mL of PBS was added to wells,

and the plates were then covered by films and saved at 4 °C. After fixation, the plates were examined under microscope to confirm the presence of the cells.

2.6.2 Immunostaining

After fixation, cells were ready for immunostaining. PBS was aspirated, and 250 µL 20% (v/v) goat serum (Dako, Denmark) was added and incubated and slowly shook for 30 minutes at room for blocking. Meanwhile, the primary antibodies were made with 20% goat serum (vimentin: 1:20(v/v) and cytokeratin:1:50(v/v), (Dako, Denmark). After blocking aspirated the blocking solution and then add 200 µL of diluted corresponding primary antibody into wells. The plates were incubated overnight at 4 °C with shaking. In the following day, primary antibody was removed, then washed wells with PBS 3 times/10 minutes. The secondary FITC-labelled antibody was diluted in blocking solution (1:50 (v/v), (Sigma, UK) and then added 250 µL to each well. The plate was then covered with foil and incubated with shaking for 30 minutes at room temperature. Then, aspirated secondary antibody and washed with PBS 3 times/10 minutes under avoiding light exposure. Finally, 1mL of PBS was added to each plate and was examined in dark room. Zeiss Axiovert 25 fluorescence microscope was used to visualise the signal, and Cell F software was used for photography.

2.7 Data analysis and statistical methods

As described in each assay above, western blotting data was generated using Image J software, and the expression of target protein was shown as the ratio of target protein

intensity/ to β -actin intensity. As for the IL-1β/ TNF-α secretion analysis, the ELISA data was determined using Prism software according to standard curve.

Quantitative data presented as mean ± SD and statistical analysis was performed using GraphPad Prism 7 software. Shapiro-wilk test was conducted to test the normality. Data between groups were compared using paired or upaired one-way ANOVA with suitable post-hoc tests for multiple comparisons test (Dunn's multiple comparisons or Tukey's multiple comparisons according to the normality). The specific test used is indicated in figure legends. A probability (P) value of <0.05 was considered statistically significant.

3. Results

Decidua samples from women delivering at term gestation (N=17) and maternal blood samples (N=10) were used to assess the secretion of IL-1β , caspase-1 p20 and TNF-α in decidua tissue explant (N=7), decidual mononuclear cells (N=6), decidual stromal cells (N=4), and PBMC (N=10) under stimulation with LPS, BzATP and A740003. Additionally, the expression of NLRP3 inflammasome components, including NLRP3, pro-caspase-1 and ASC were investigated in decidual tissue explant (N=6).

The initial part of the project was to optimize and validate the antibodies and assays. After validation, the PBMCs were used as positive control to test the effect of treatment of LPS, BzATP, and A7 in the IL-1β secretion. Then, project went on to investigate the IL-1β process in decidua tissue explant, decidual stromal cells and mononuclear cells.

3.1 Assay validation

3.1.1 non-specific band in western blot

Western blotting experiments were conducted using decidual tissue lysates to detect the expression of specific proteins including pro-IL-1β caspase-1, NLRP3 and ASC. The reference protein β–actin was used in normalisation in this study. However, a consistent feature of the WB was the presence of a 50 kDa band on blots. Since the molecular size of some target protein were similar to this non-specific one, it is necessary to minimise/remove this 50 kDa band. In order to do this, a number of conditions and reagents were validated. Considering of the protein concentration yield from homogenization, 80 μg/lane of protein was used in subsequent experiment to test the effect of antibody and assay condition.

First of all, supernatant 2 and supernatant 3 from homogenized lysate were blotted with β–actin plus secondary antibody (**Figure. 3.1.1.1-A/C**) and with secondary antibody (anti-rabbit IgG, Sigma, Poole, UK) only (**Figure. 3.1.1.1-B/D**), to investigate whether this 50kDa band was due to non-specific binding of the primary antibody or secondary antibody. From **Figure. 3.1.1.1**, the 50kDa bands still existed in negative control assay with only secondary antibody applied. Thus, it was more likely that the secondary antibody was binding with the sample protein presenting in 50 kDa. As for the different part of tissue lysate, there were no differences in nonspecific bands when using Supernatant2 or Supernatant3. Since NLRP3, caspase-1, ASC pro-IL-1β are the cytoplasmic protein, supernatant 2 from homogenized lysate was applied in all the validation assays and later western blotting assays.

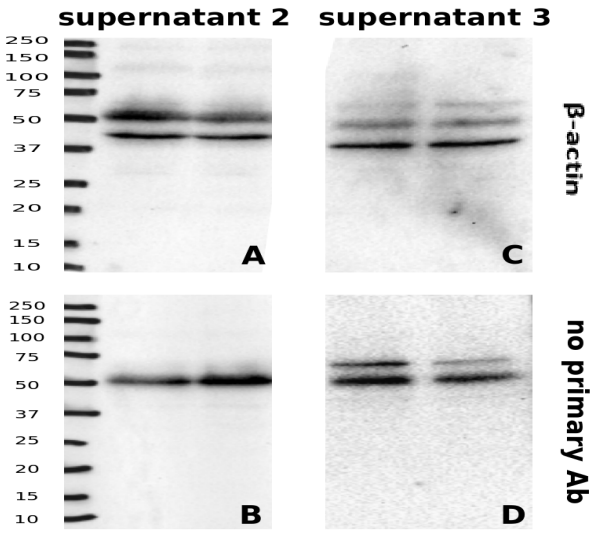

Figure 3.1.1.1 Non-specific band validation

Non-specific band around 50kDa appeared in both β-actin antibody incubated (A, C) and no primary antibody incubated (B, D) western blots. Supernatant 2 from homogenization was used for western blot in A/B, while Supernatant 3 from homogenization was used for western blot in C/D. But these non-specific bands appeared in all blots no matter using Supernatant 2(A/B) or Supernatant 3(C/D).

Consideration of the non-specific binding of the secondary antibody to sample, blocking with 5% BSA buffer and an alternative secondary antibody (anti-rabbit IgG, Dako, Denmark) were tried to eliminate the non-specific binding. Moreover, anti-human IgG agarose (Sigma, Poole, UK) was used to separate IgG from the sample by binding IgG with the agarose beads. We found that pre-clearing the sample with anti-human IgG agarose could decrease the intensity of the non-specific band while using the alternative Dako secondary antibody helped to reduce the 50 kDa band as well (**Figure. 3.1.1.2**). It may be because antibody's cross-reaction with human immunoglobulins and fetal calf serum has been removed by solid-phase absorption (cited from data sheet of this product). The antibody may cross-react with immunoglobulins from others species but not human. Considering the integrity of sample,

validation of alternative secondary antibody without pre-clearing was carried out in our western blotting assay.

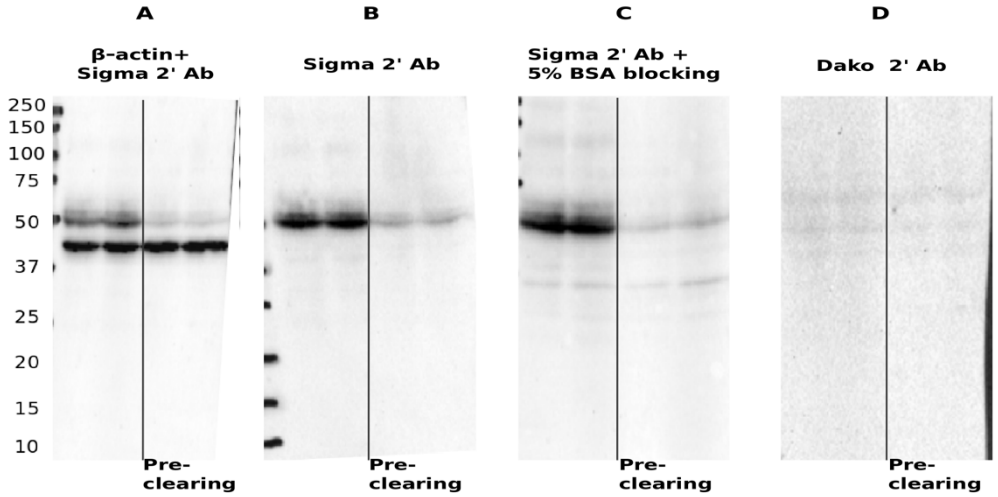

Figure 3.1.1.2 Validation of 50kDa non-specific band

Each blot shows the crude versus pre-cleared lysate (A): WB using supernatant 2 with β-actin antibody; (B): WB with secondary antibody (A3937, Sigma) only; (C): WB with secondary antibody (A3937, Sigma), but blocking with 5% BSA; (D): alternative secondary antibody (anti-Rabbit IgG, Dako). The exposure time in A-D was same. (Right sides of A-D): Tissue lysates were pre-incubated with anti-IgG agarose beads.

3.1.2 multiple bands in western blots

In western blotting assay, multiple bands were found in NLRP3, caspase-1, ASC and blots (**Figure 3.1.2**). As for the variants size of interested protein, we checked serval protein database, including UniProt (www.uniprot.org), genecard (www.genecards.org), Ensembl (www.ensembl.org) and pubmed (www.ncbi.nlm.nih.gov/pmc/) for all the possible isoforms.

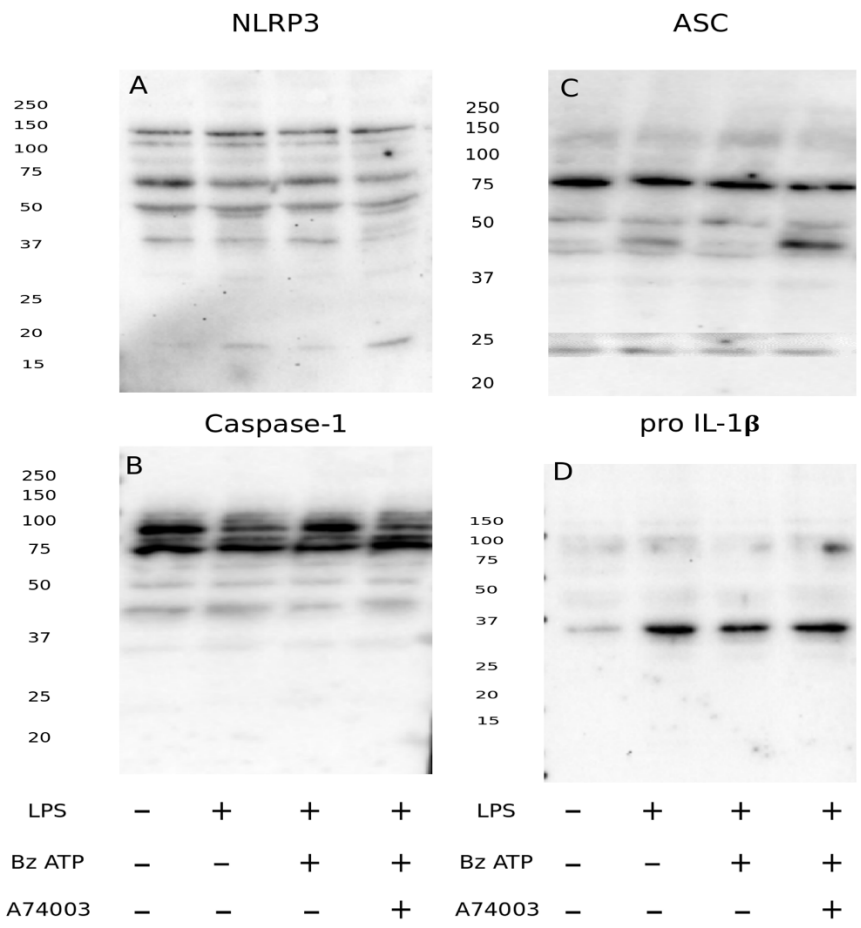

Figure 3.1.2 Multiple bands showing in Western Blotting

Western blotting shows multiple bands in NLRP3(A), Caspase-1(B), ASC(C) and Pro IL-1β (D) in all treatment groups.

For NLRP3, the protein sizes from top to bottom were 100kDa, 80kDa, 50kDa, 40kDa, 37kDa, 30kDa, 25kDa, and 15kDa (**Figure 3.1.2.A**). After searching the protein database (UniProt), there are 6 isoforms been identified in human. These isoforms are listed in **Table 3.1.2.1**. Comparing the molecule sizes with these isoform, the multiple bands are considered as NLRP3 isoform 1 (identifier: Q96P20-2) (around 100kDa), NLRP3 isoform 3 (around 80kDa) (identifier: Q96P20-3).

Table 3.1.2.1 The isoforms of NLRP3 in human

Isoform	Identifier	Length	Mass (Da)
Isoform 1	Q96P20-2	922	105,0975
Isoform 2	Q96P20-1	1,036	118,173
Isoform 3	Q96P20-3	719	83,533
Isoform 4	Q96P20-4	979	111,884
Isoform 5	Q96P20-5	979	112,263
Isoform 6	Q96P20-6	1,016	115,968

Human caspase-1 consists of PYD and p10/p20 domains. The protein sizes from top to bottom were 100kDa, 70kDa, 45kDa, 35 kDa (**Figure 3.1.2.B**). From Uniprot database, there were 5 caspase-1 isoforms. In this study, 45kDa protein was considered as isoform α (P29466-1), while 35kDa was considered as isoform γ P29466-3) (**Table 3.1.2.2**).

Table 3.1.2.2 The isoforms of caspase-1 in human

Isoform	Identifier	Length	Mass (Da)
Isoform α	P29466-1	404	45,159
Isoform β	P29466-2	383	42,888
Isoform γ	P29466-3	311	35,019
Isoform δ	P29466-4	263	29,821
Isoform ε	P29466-5	88	10.417

Human ASC protein (25 kDa) consists of PYD and CARD domains. The protein bands from top to bottom were 70kDa, 45kDa, 35 kDa, 25kDa (**Figure 3.1.2.3**). Among them,25 kDa band was considered as ASC isoform-1 (Q9ULZ3-1) (**Table 3.1.2.3**).

Table 3.1.2.3 The isoforms of ASC in human

Isoform	Identifier	Length	Mass (Da)
Isoform 1	Q9ULZ3-1	195	21,627
Isoform 2	Q9ULZ3-2	176	19,969
Isoform 3	Q9ULZ3-3	135	15,030

3.1.3 Lower molecular weight bands

The expected and most common seen molecular weight of proteolytically processed IL-1β is around 17kDa. However, in this study, the molecular size of active IL-1β of tissue culture medium is around 10-12kDa (**Figure 3.1.3-B**). After searching the database, an isoform of 12 kDa was reported before (**Table 3.1.3.1**), which is only an experimental evidence. To confirm this 10-12kDa band is our target protein, positive control and peptide competition assay were applied. From **Figure 3.1.3**, the positive control appears in the expected size (**Figure 3.1.3-A**). However, in the peptide competition assay, when blocking the primary antibody with IL-1β peptide, these bands disappeared (**Figure 3.1.3-C**). Thus, the 10-12kDa bands are considered as specifically recognized by the IL-1β antibody.

Table 3.1.3.1 Similar IL-1 IL-1β protein found in human

Protein	Sequence state	Identifier	Sequence Length	Protein size
Precursor IL-1β	Complete	P01584-1	269	30,748
Mature IL-1β	Fragment	B5BUQ8-1	153	17,377
Mature IL-1β	Fragment	C9JSC2-1	125	14,331
Mature IL-1β	Fragment	C9JWV2-1	143	16,393
Mature IL-1β	Fragment	C9JVK0-1	111	12,803

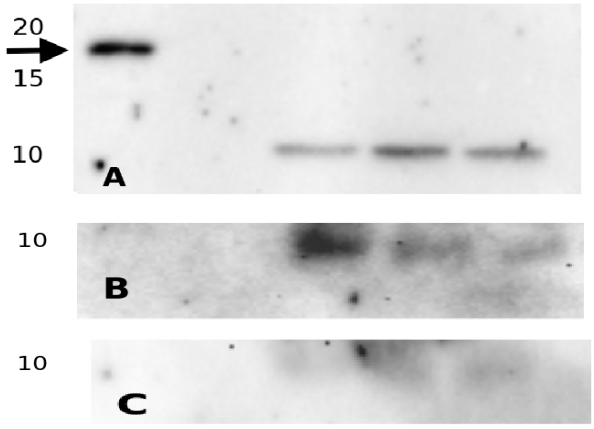

Figure 3.1.3 Confirmation of IL-1β in lower molecular weight

The positive control of IL-1β peptide appeared at around 17kDa (A with black arrow). IL-1β WB using tissue culture medium appeared at around 10-12 kDa (A and B). Moreover, peptide competition assay, in which five times weight of IL-1β peptide was incubated with one time weight of IL-1β primary antibody, presented no signal (C).

3.1.4 Determining the appropriate sample load for western blot

At beginning of the experiment, 80 μg/ lane of protein was loaded for validation assay for each antibody. However, we found some of the multiple bands were too dark. To find out the

appropriate sample loading amount, loading concentration serials assay was applied. 20 μg/ lane was set as a starting amount, followed by 40 μg, 60 μg, and 80 μg of protein sample. Considering of the target protein and reference protein signal and protein concentration, 40 μg/ lane was considered as suitable amount for the NLRP3, caspase-1, pro IL-1β. However, as for ASC protein, 80 μg/ lane was set as loading amount because of the low expression.

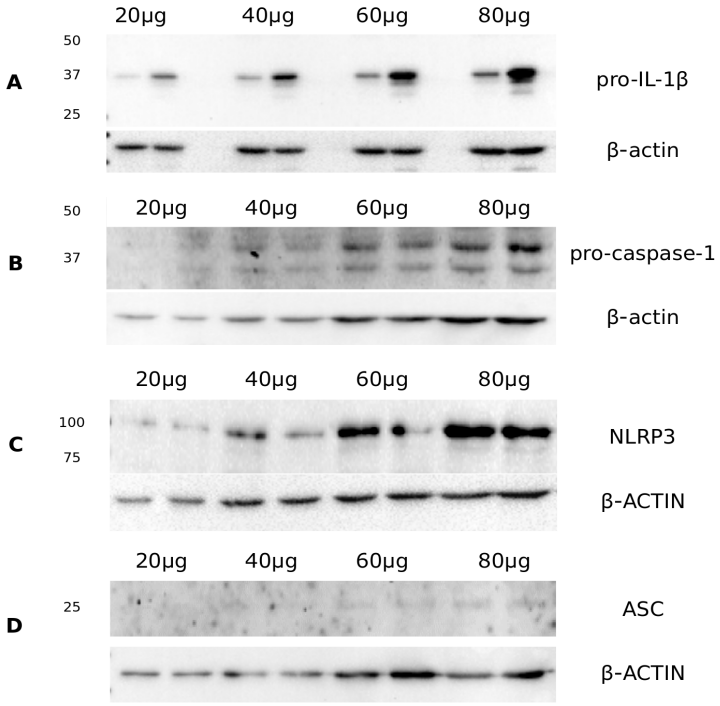

Figure 3.1.4 Western blotting sample loading test

Loading concentration serials from left to right were 20 μg, 40 μg, 60 μg, 80 μg, and antibodies included IL-1β (A), caspase-1(B), NLRP3 (C), and ASC(D).

Once validation was completed, the following conditions were employed: for all subsequent western blotting, using Dako's anti-Rabit IgG antibody as the secondary antibody, no pre-clearing, loading 40 μg/lane of protein, instead of 80 μg/lane, except for ASC.

3.2 The role of TLR4 and $P2X_7$ receptor in secretion of IL-1β in maternal PBMC

In order to determine whether decidua utilize the inflammasome pathway in the release of IL-1β, maternal PBMC were used as a positive control to confirm the effect of different treatments, including TLR4 activator (LPS 10μg/mL), $P2X_7$ receptor activator (BzATP 0.3mM) and $P2X_7$ receptor antagonist (A7 1μM).

Stimulation of TLR4 and $P2X_7$ receptor in maternal PBMC with LPS and BzATP triggered secretion of IL-1β (**Figure 3.2**). Compared with control group (15.06 ±6.59 pg/mL, N=10), the Level of IL-1β was higher in three treatment groups. Besides, the IL-1β level was highest in LPS + BzATP co-stimulation group (4497 ±1469 pg/mL, N=10), which is higher than LPS group (1072 ± 671.0 pg/mL, N=10, $p<0.0001$). To investigate the role of $P2X_7$ receptor in IL-1β production, $P2X_7$ receptor antagonist (A-74003) was added, with the secretion of IL-1β inhibited significantly comparing with BzATP group (LPS+A7+BzATP (2230±996.3 pg/mL, N=6, P=0.0005), while there is no difference between LPS and LPS+A7+BzATP groups.

Since the IL-1β ELISA kit could detect both pre-cursor and mature forms of the cytokine, western blot of IL-1β using PBMC culture medium (N=6 in each group) was applied to confirm the format of IL-1β. A similar effect was found in western blot (**Figure 3.2.1 B**) that the intensity was highest in LPS + BzATP group comparing with other groups.

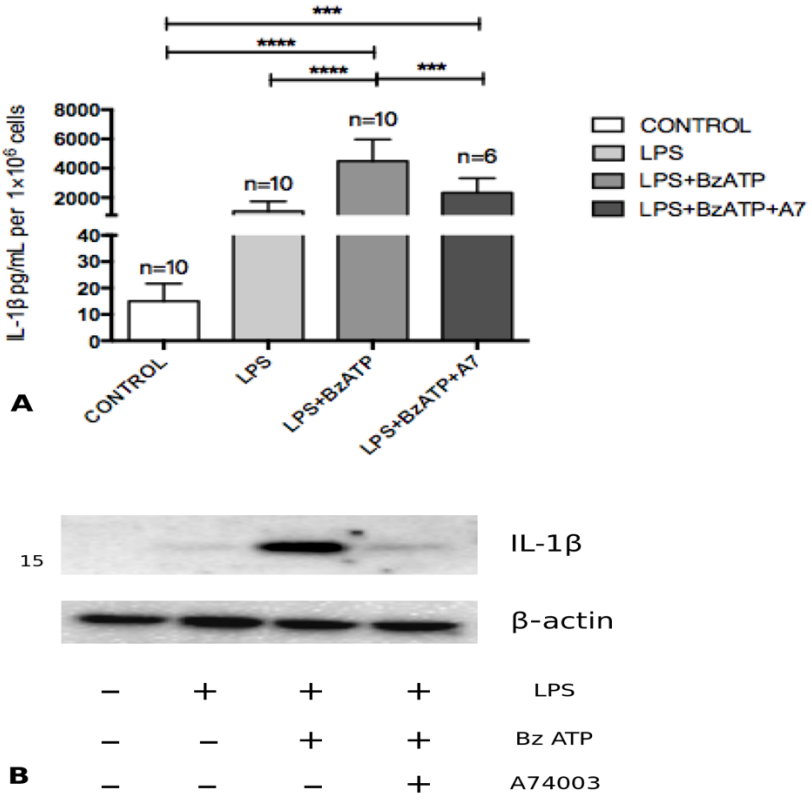

Figure 3.2 Changes of IL-1β secretion in maternal PBMC

1×10^6 PBMC cells were pre-treated with or without LPS (10 μg/mL) and A7 (1μM) for 3.5 hours before stimulating with Bz-ATP (0.2mM) for 30 minutes. Cell culture medium were collected for assessing IL-1β concentration using ELISA (A) and western blotting. A: Data presented as mean ± SD. One-way ANOVA with Tukey's post-multiple comparison test was used to show difference in different treatment groups. (P<0.001 (***) and P<0.0001(****)). B: Western blot analysis detected expected size protein band of mature IL-1β round 17 kDa in cell culture medium (IL-1β antibody diluted in 1:200) (N=6 in each group)

3.3 The process of IL-1β production in human decidua

3.3.1 The role of TLR4 and P2X7 receptor in IL-1β production in decidua

To investigate role of TLR4 and $P2X_7$ in production of IL-1β in decidual explants, we carried out western blot and ELISA assay of pro-IL-1β using tissue lysate. As for the quantification assay-ELISA, the basic pro-IL-1β amount in control group was 1241 ± 649.60 pg/mL (N=7), expression of pro-IL-1β elevated in three treatment groups comparing with control group (LPS group:3701 ± 861.0 pg/mL, N=7, p=0.002; LPS+BzATP group:2779±1053 pg/Ml, N=7, P=0.012;

LPS+A7+BzATP group: 2304 ± 724.9 pg/mL, N=7, p=0.048). However, there is no statistic differences in three treatment groups. In western blotting, the pro-IL-1β bands appeared at 30-37kDa, and the relative intensity of pro-IL-1β was lowest in control group. The expression of pro-IL-1β was at higher intensity after treatment with LPS (p<0.0001 versus control). In addition, pro-IL-1β was lower in co-treated with BzATP group comparing with LPS-treated group(p=0.036), but there was no statistical difference between LPS and A7 groups.

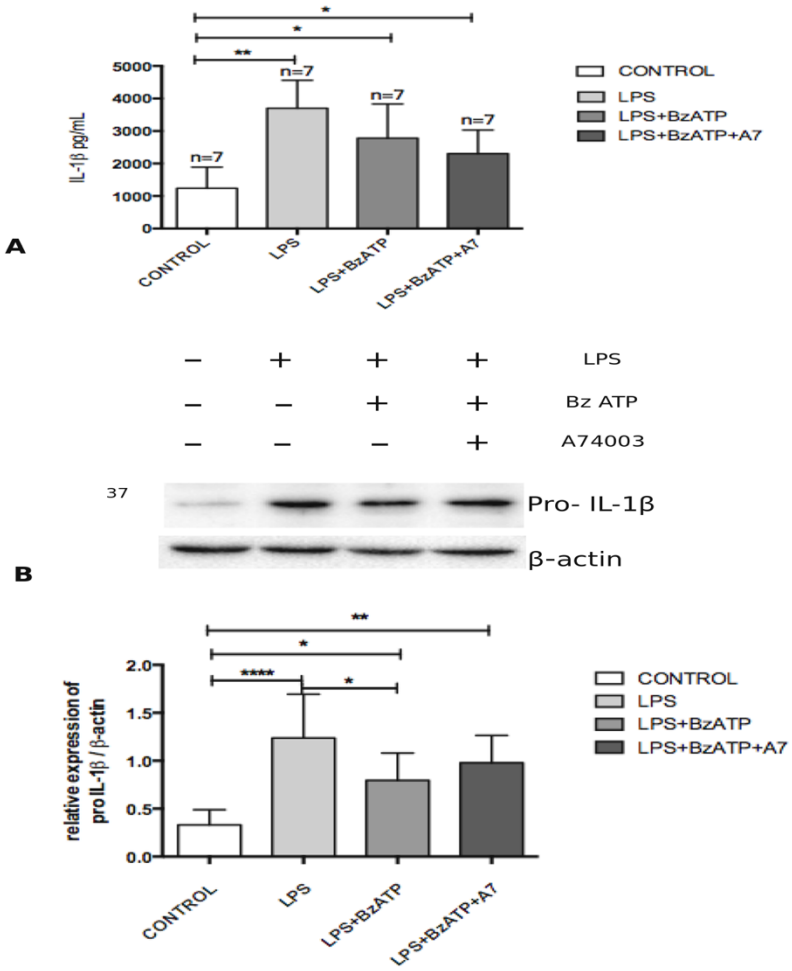

Figure 3.3.1.1 Changes of IL-1β level in decidual tissue lysate

100mg of decidual tissues were pre-treated with or without LPS(20μg/mL) and A7 (2μM) for 3.5 hours before stimulating with BzATP (0.6mM) for 30 minutes. Tissue lysates were collected for assessing IL-1β concentration using ELISA(A) and western blotting(B). Western blot analysis detected expected size protein band of pro- IL-1β round 35-37 kDa using tissue lysates (IL-1β antibody diluted in 1:1000). Data from ELISA presented as mean ± SD (A). Pro-IL-1β protein expression level from WB was presented as a ratio to that of corresponding β-actin (B). One-way ANOVA with Tukey's post-multiple comparison test was used to show difference in different treatment groups. P<0.05(*), p<0.001(**), p<0.0001(****).

3.3.2 The role of TLR4 and $P2X_7$ receptor in IL-1β secretion in decidua

The IL-1β secretion of decidua tissue was evaluated by detecting IL-1β in tissue culture medium, after treated with LPS (20μg/mL), BzATP(0.6mM), A7(2μM) separately. IL-1β ELISA results showed that the lower level of IL-1β was in control group (54.46±28.59 pg/ mL, N=7) and LPS group (82.12±44.88 pg/ mL, N=7), while LPS+ BzATP could trigger the IL-1β release (200.0±99.21 pg/mL, N=7, P=0.0147 versus control group, P=0.0191 versus LPS group). However, co-treated with $P2X_7$ receptor antagonist could inhibit the IL-1β release by BzATP (96.73 ±42.89 pg/mL, N=7, P=0.0284 versus LPS+ Bz-ATP group), but there was no difference in LPS groups and LPS+BzATP+A7 group (**Figure 3.3.2-A**). Western blotting assay with tissue culture medium further confirmed the ELISA result that the intensity of mature IL-1β was lowest in control group, but highest in LPS+ BzATP group (**Figure 3.3.2-B**).

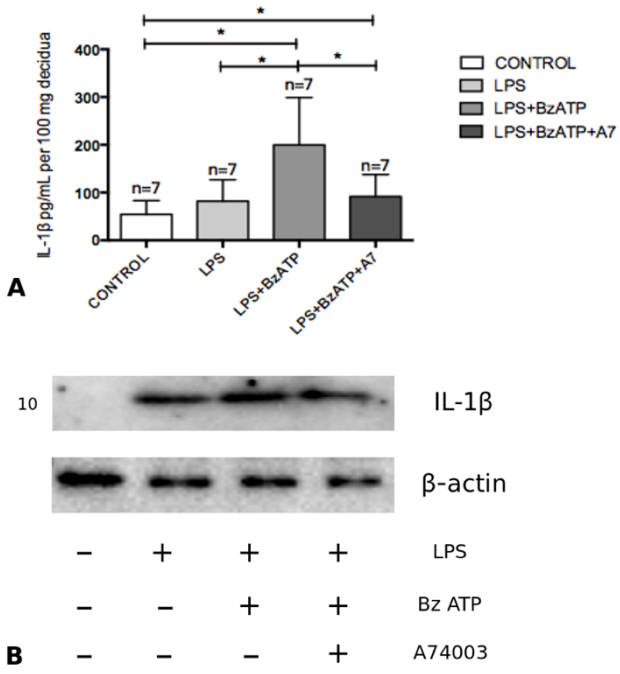

Figure 3.3.2 Changes of IL-1β secretion level in tissue culture medium detected by ELISA and western blotting

100mg of decidual tissues were pre-treated with or without LPS(20μg/mL) and A7 (2μM) for 3.5 hours before stimulating with BzATP (0.6mM) for 30 minutes. Tissue culture medium was collected for assessing IL-1β concentration using ELISA. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA with Tukey's post-multiple comparison test was used to show difference different treatments groups, P<0.05 (*) (A). Western blot analysis detected expected size protein band of mature-IL-1β round 10-12 kDa using tissue culture medium (IL-1β antibody diluted in 1:200) (B).

3.4 Decidual cell types involvement in IL-1β production

From above experiments, we found that IL-1β production could be triggered by TLR4 and $P2X_7$ activator in decidua tissue. Indeed, decidua tissue consists of several cell types, mainly including epithelial cells, stromal cells, immune cells. Further investigation of which cell types could secret IL-1β was carried out by isolating decidual leukocytes and decidual stromal cells from decidua tissue and then treating cells with TLR4 and $P2X_7$ receptor agonist.

3.4.1 Decidual stromal cell isolation and stimulation

Decidual tissue was dispersed and maintained in primary culture. Since cell sorting method was not performed, multiple cell types within decidua were harvested and cultured. From cells tested at different passages cells, it was found that the cell composition altered becoming more identical with increasing passage number (**Figure 3.4.1.1**). In passage 0, there were several morphological cell types: fibroblast-like cells, lymphoblast-like cells, and few epithelial-like cells (**Figure 3.4.1.1 A/C/D**). Since the most of lymphoblast-like immune cells grown in suspension without attaching to the base of the culture flask, the number of leukocyte-like cells reduced gradually as medium changing and culturing. As cell proliferating, fibroblast-like cells accounted for the main composition (**Figure 3.4.1.1 B/F**). After splitting, some of the epithelial-like cells and lymphoblast-like cells left on the surface of flask (**Figure**

3.4.1.1 E). Then, after splitting and passage, cells became more morphologically identical. After passage 2, most of the cells left were fibroblast-like cells (**Figure 3.4.1.2**). Considering the likely changes if cell functions in vitro, we used passage 2-3 cells to perform cell incubations and immunofluorescence. Immunofluorescence was conducted to detect vimentin and cytokeratin expression. Vimentin is an intermediate filament for mesenchymal tissue, which is found in various non-epithelial cells (Chang et al., 2002). Staining positive may confirm mesenchymal origin. Cytokeratin belongs to the family of intracytoplasmic structural proteins which are the dominant intermediate proteins of epithelial cells and hair forming cells. Cytokeratin (+) is used for diagnosis of epithelial versus non-epithelial cells or tumours (usually cytokeratin (+)). In this study, immunofluorescence staining of cultured P3 human decidual stromal cells revealed to be positive for vimentin but negative for cytokeratin (**Figure 3.4.1.3**). However, undetectable levels of IL-1 β were found in passage 3 stromal cell culture mediums after 4 hours stimulation with LPS and half hours with BzATP (**Figure 3.4.1.4**).

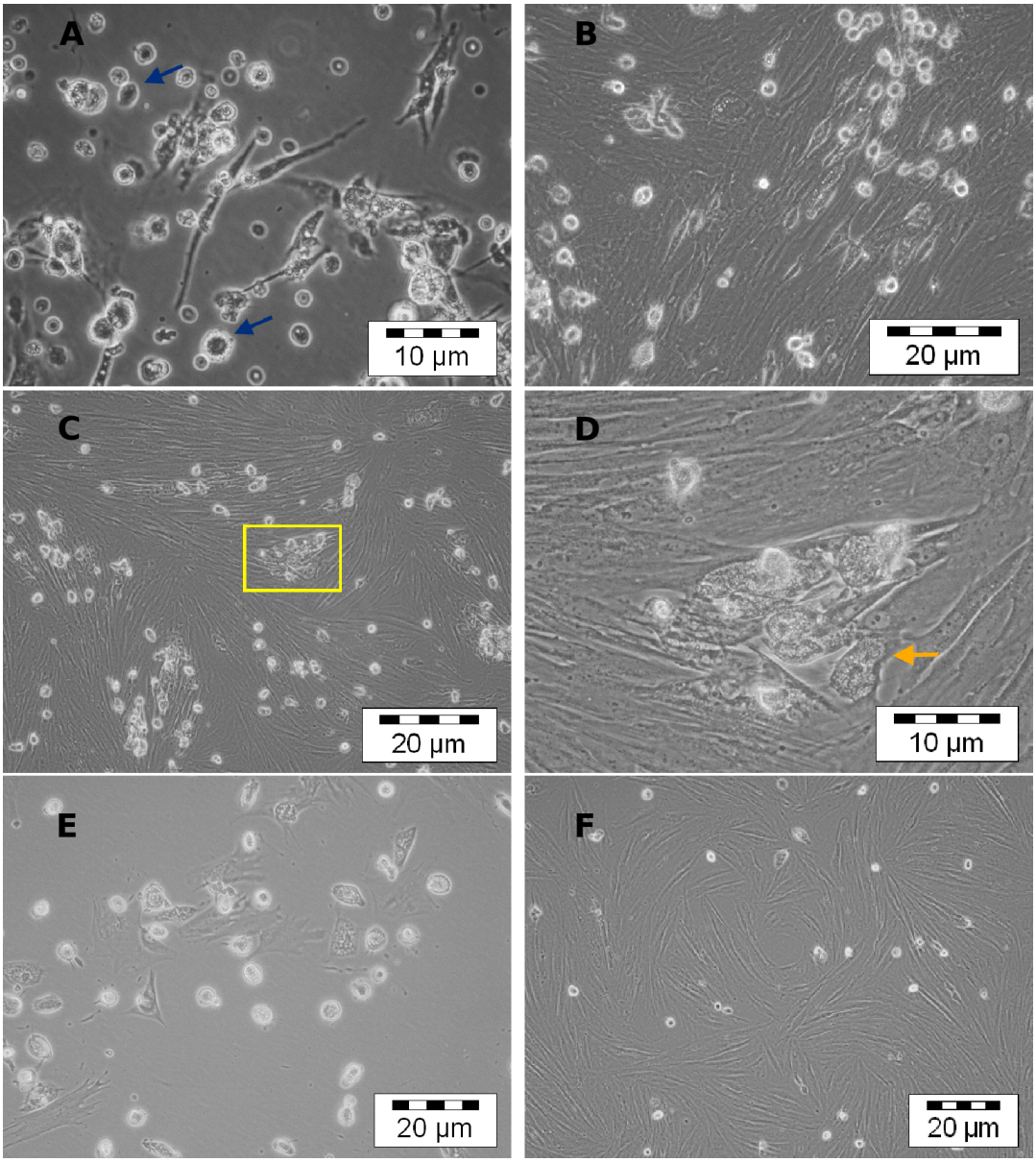

Figure 3.4.1.1 Morphological cell types in primary decidua cell from Passage 0 to 1.

Primary decidua cell isolated from fresh decidua, Passage 0 to 1 cells were visualized by light microscopy (200×magnification); (A): P0-D2 cells with different cell types, lymphoblast-like cells (blue arrow); (B): P0-D6 cells; (C) P0-D11 cells; (D) 20×magnification of image C, epithelial-like cells (yellow arrow); (E) Cells left after P0 cells splitting; (F) P1-D6 cells.

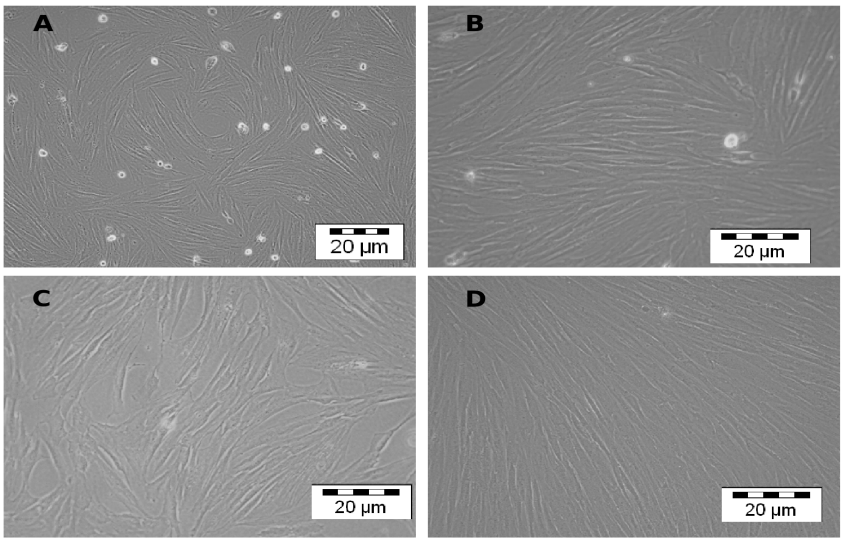

Figure 3.4.1.2 primary decidua cell culture

Primary decidua cell culture and passage were visualised by light microscopy (200 ×magnification). Figures from A to D were Passage 1, Passage 2, Passage 4 and Passage 5 cells from the same sample.

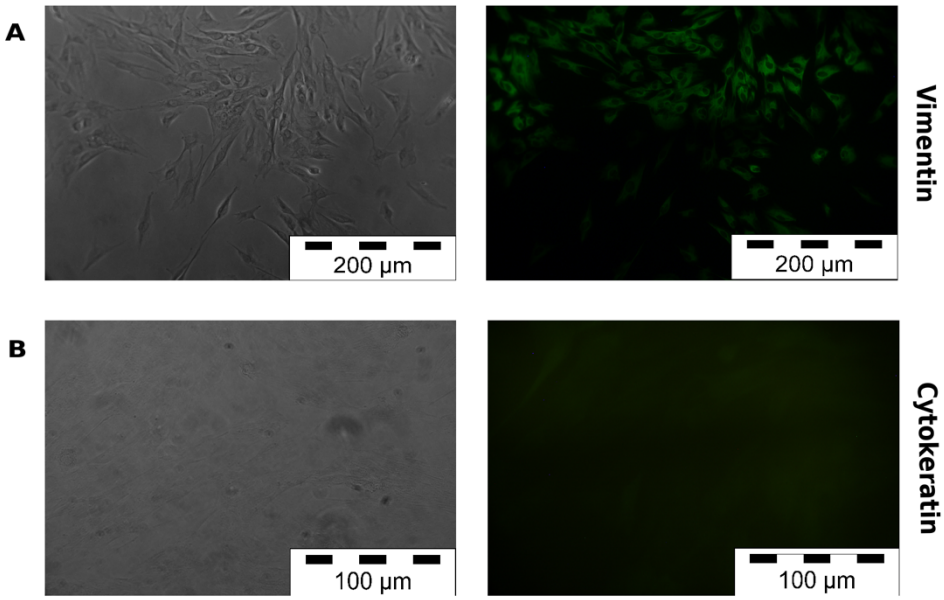

Figure 3.4.1.3 Expression of Vimentin and Cytokeratin in Passage 3 decidual stromal cells using Immunofluorescence

Immunofluorescence staining for Vimentin (antibody diluted in 1:20) (A right, 200×magnification) and Cytokeratin (antibody diluted in 1:50) (B right, 400×magnification). Left side images were visualised by light microscopy (A: 200×mag, B:400×mag).

3.4.2 Decidua leukocytes isolation and stimulation

Leukocytes were isolated from fresh decidua tissue using similar protocol from Yi Xu's lab (Xu et al., 2015). After harvesting and counting, different treatments were added to stimulate 1×10^6 leukocytes. We found that the increasing trend of IL-1β in LPS and BzATP group (122.6±78.52 pg/mL), while LPS+Bz-ATP+A7 group was significantly lower than LPS + BzATP group (36.01±15.23 pg/mL, *p*=0.0478) (**Figure 3.4.2**).

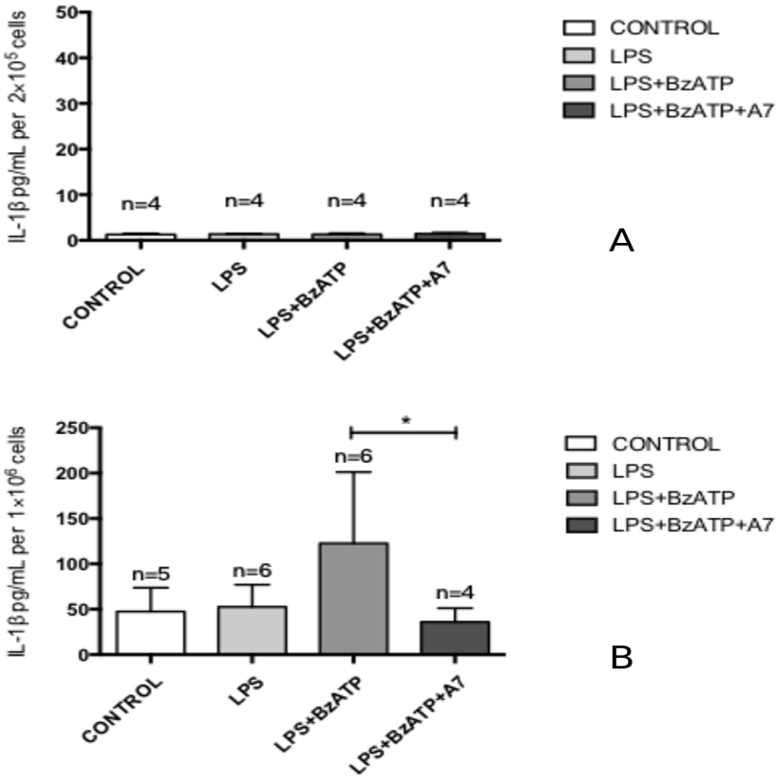

Figure 3.4.2 Changes of IL-1β secretion level in stromal cells and decidua leukocytes detected by ELISA

Passage 3 primary stromal cells (5×10^5) (A) and isolated decidual leukocytes (1×10^6) (B) were plated in 24 wells plates, and stromal cells were left overnight to grow confluent. Then cells were pre-treated with or without LPS(10μg/mL) and A7 (1μM) for 3.5 hours before stimulating with Bz-ATP (0.3mM) for 30 minutes. Cells culture medium was collected for assessing IL-1β concentration using ELISA. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA with Tukey's post-multiple comparison test was used to show difference different treatments groups，P<0.05 (*).

3.5 Regulation mechanism of TLR4 and $P2X_7$ receptor in IL-1β production.

To investigate the regulation mechanism of TLR4 and $P2X_7$ receptor in IL-1β production, we focused on the classic NLRP3 inflammasome activation mechanism. In addition, an inflammasome-independent pathway production-TNF-α was evaluated for at the same time.

3.5.1 classic NLRP3 inflammasome activation

To study the classic NLRP3 inflammasome activation pathway, the main components of NLRP3 inflammasome were observed, including NLRP3, caspase-1, and ASC. In this study, we used the western blotting to detect protein expression in tissue lysates as discussed and following the validation described.

The caspase-1 enzyme is responsible for the conversion of pro-IL-1β to biologically active IL-1β, which presents in an inactive format in the cytoplasm. NLRP3 inflammasome activation could lead to the cleavage of the inactive pro-caspase-1 into two active subunit p20 (20 kDa) and a p10(10 kDa). Then, p20 would be released extracellularly through. Thus, in this study, pro-caspase-1 in tissue lysate was detected by western blotting, while p20 in both tissue lysate and culture medium was observed using ELISA kit. As a positive control of caspase-1 release, p20 detection was performed in maternal PBMC culture medium. We found that p20 secretion was detected after treated maternal PBMC with LPS and BzATP, with the highest level (349.5±183.3 pg/mL, p=0.022 versus control group(139±29.42 pg/mL)) **(Figure 3.5.1.1.A).** Meanwhile, as for the PBMC cell lysate, the p20 level was higher in control group comparing with LPS + BzATP group (3298±1169 pg/mL versus 2401±1100 pg/mL, p=0.0392) **(Figure 3.5.1.1.B).**. However, as for the decidua tissue, there was no difference in caspaser-1

p20 levels in both culture media and tissue lysate between different groups **(Figure 3.5.1.1.C/D).**

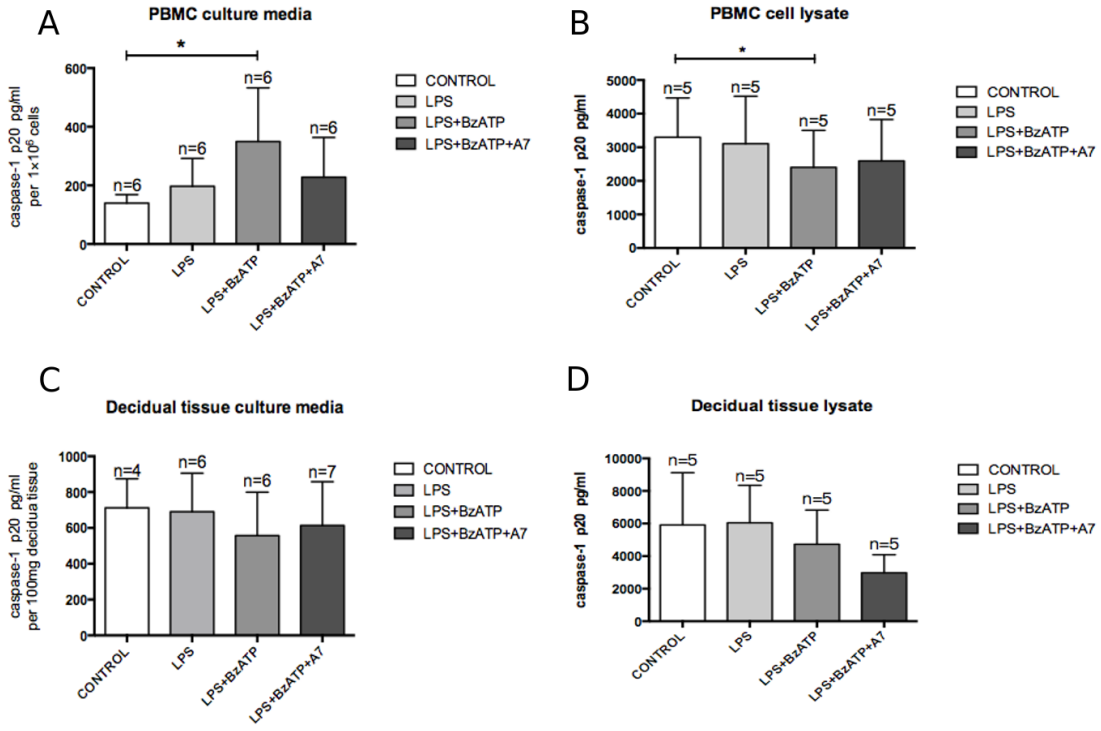

Figure 3.5.1.1 Changes of caspase-1 p20 secretion level in PBMC and decidua detected by ELISA kit

1×10^6 PBMC cells (A-D) or 100mg decidua tissue (E) were pre-treated with or without LPS and A7 for 3.5 hours before stimulating with BzATP for 30 minutes. Culture medium was collected for assessing caspase-1 p20 concentration using ELISA kit. Data presented as mean ± SD; Statistical analysis was carried out using one-way ANOVA with Tukey's post-multiple comparison test was used to show difference different treatments groups，P<0.05 (*).

As for the pro-caspase-1, western blotting results shows that capase-1 p45 intensity was higher after incubation with LPS, meanwhile there is significant difference when compared with control group (P=0.029**). (Figure 3.5.1.2.A).** NLRP3 is the main component of in the classic NLRP3 inflammasome activation mechanism. In this study, NLRP3 in tissue lysate was detected by western blotting. NLRP3 bands appeared in all groups, but LPS+BzATP group and A7 group had a relatively higher NLRP3 expression than control (**Figure 3.5.1.2.B**). As for the

ASC, we found ASC signal appeared in untreated tissue lysate; besides, the protein level did not change after stimulated with TLR4 and P2X7 receptor activator or antagonist (**Figure 3.5.1.2.C**). In addition, the NLRP3 and ASC were detected in explant culture medium, and the signals of NLRP3 and ASC were found in all four groups as well (**Figure 3.5.1.2.D**).

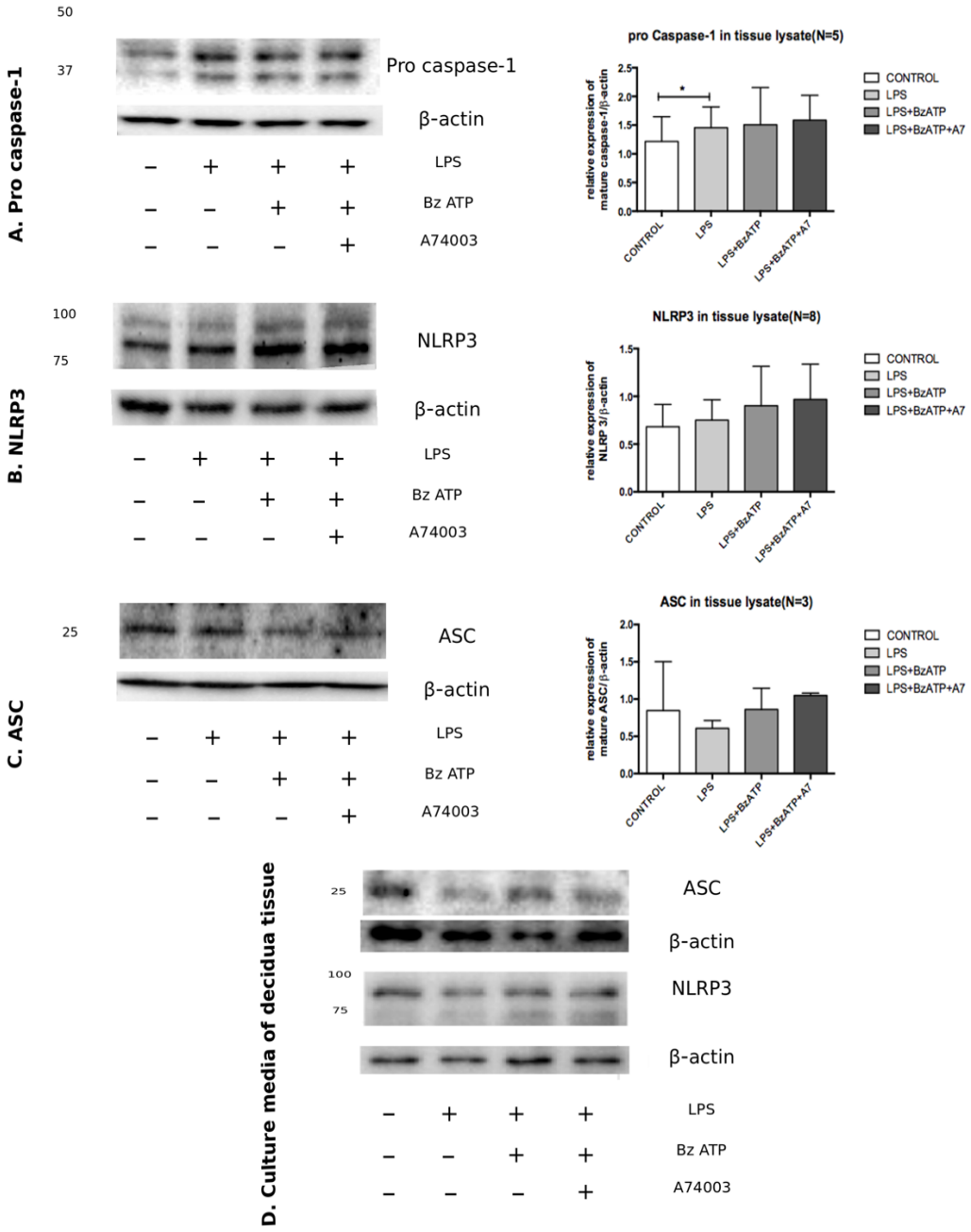

Figure 3.5.1.2 Changes of NLRP3 inflammasome components expression in tissue lysate detected by western blotting

100mg of decidual tissues were pre-treated with or without LPS(20μg/mL) and A7 (2μM) for 3.5 hours before stimulating with BzATP (0.6mM) for 30 minutes. Western blotting analysis detected expected size protein band of pro-caspase-1 35-45 kDa (caspase-1 antibody diluted in 1:400)(A); NLRP3 80-100 kDa (NLRP3 antibody diluted in 1:200)(B)； ASC 25 kDa (ASC antibody diluted in 1:1000)(C). Protein expression level in different groups were presented as a ratio to that of corresponding β-actin. Data presented as mean ± SD and statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison tests to test difference between control group and other three groups, P<0.05 (*). D: Western blotting of decidua explant culture medium shows positive expression of NLRP3 and ASC in all four groups (N=3)

3.5.2 Inflammasome-independent pathway.

As a parallel experiment, an inflammasome-independent LPS-induced production TNF-α was detected in the culture medium. In maternal PBMC culture medium, there was an increasing trend of TNF-α level in three treatment groups comparing with untreated control group, with significant statistic difference between LPS, LPS +BzATP+A7 groups and control group (Control group: 46.71±6.220 pg/mL; LPS group: 1109 ±723.5 pg/mL (*p*=0.0048 versus control); LPS +BzATP group: 707.7±412.1 pg/mL; LPS +BzATP+A7 group: 749.2±315.1 pg/mL (*p*=0.0448 versus control). However, there was no significant difference in three treatment groups. (**Figure 3.5.2.A**).

The same trend was found in decidua tissue culture medium, with the increased levels in TLR4 stimulated groups and lowest level in control group (Control group: 244.5 ±157.0 pg/mL; LPS group: 3665±2049 pg/mL (*p*=0.0105 versus control); LPS +Bz-ATP group: 2933 ± 943.8 pg/mL; and LPS +Bz-ATP+A7 group: 2975 ±1335 pg/mL) **(Figure 3.5.2.B)**. In addition, the changes between each group were similar in maternal PBMC and decidua tissue.

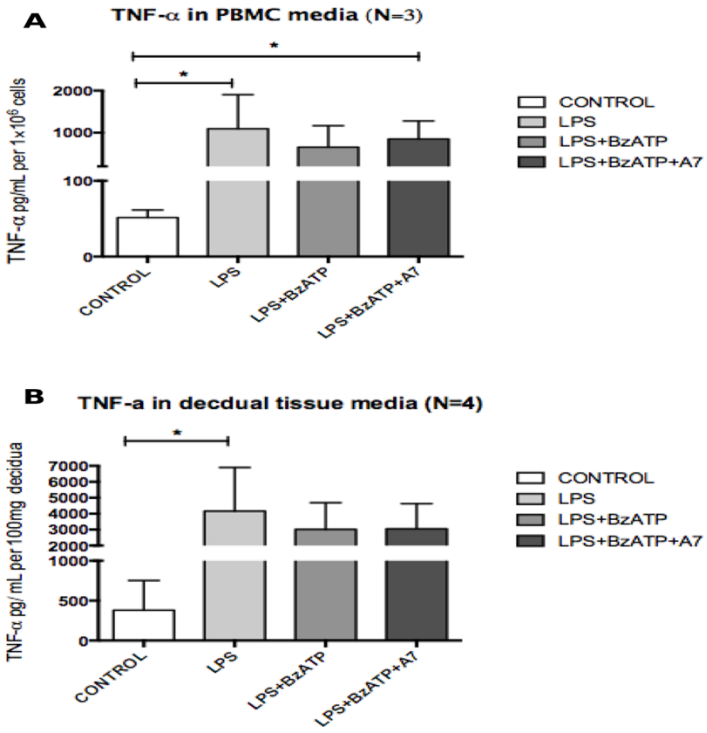

Figure 3.5.2 Changes of TNF-α secretion level in maternal PBMC and decidual tissue media detected by ELISA

1×10^6 PBMC cells(A) and 100mg of decidual tissues (B) were pre-treated with or without LPS (10μg/mL and 20μg/mL) and A7 (1μM and 2μM) for 3.5 hours before stimulating with BzATP (0.2mM and 0.4mM) for 30 minutes. Culture medium was collected for assessing TNF-α concentration using ELISA. Data presented as mean ± SD; and statistical analysis was carried out using one-way ANOVA with Dunnett's multiple comparison tests to test the difference between four different groups, P<0.05 (*).

4.Discussion

Infections during pregnancy could stimulate inflammatory responses at the maternal-fetal interface and lead to adverse pregnancy outcomes such as PTL based on the role of proinflammatory cytokines, such as IL-1β and TNF-a in the regulation of parturition and in the pathogenesis of infection-induced preterm labour (Arcuri et al., 2009; Baergen et al., 1994; Døllner et al., 2002; Halgunset et al., 1994; Lockwood et al., 2006; Romero et al., 1989a). Besides, human decidua, situated between the fetus and myometrium, is a key intrauterine source of bio-activate molecules, playing a crucial role in the cross-talk between maternal and fetal compartments (Liu et al., 2003). Although amnion, chorion, and decidua are all proven to contribute to the production of IL-1β in response to LPS in in vitro experiments (Fortunato et al., 1996; Paradowska et al., 1997; Romero et al., 1989d; Vince et al., 1992), it has been hypothesized that decidua is the main source of cytokines in the presence of infection (Steinborn et al., 1996)**,** while fetal origin cells may be the source of cytokines related with normal term labour (Steinborn et al., 1995; Taniguchi et al., 1991).However, the mechanism of IL-1β secretion in human decidua is not fully understand. It is well studied that NLRP3 inflammasome components, as innate immune system receptors and sensors are involved in the canonical IL-1β production and release pathway. Furthermore, previous evidence has indicated that inflammasomes are involved in normal pregnancy and pregnancy disorders, such as preterm labour (Khan and Hay, 2014), but no studies have reported whether NLRP3 inflammasome participates in the inflammatory response in full-term human decidua. Thus, the aim of this study was to determine whether decidual tissue plays a role in inflammation through IL-1β secretion in response to inflammatory stimulation and, especially whether key

factors of the inflammasome pathway are involved. Furthermore, the capacity of different decidual cell types to secrete IL-1β was also investigated.

The main findings from this research are that:

1) Decidual tissue explants could produce pro-IL-1β with single stimulation with LPS, while $P2X_7$ receptor agonist BzATP could stimulate the release of mature IL-1β, and $P2X_7$ receptor antagonist A7 could inhibit the BzATP triggered IL-1β secretion.

2) NLRP3 inflammasome components, include NLRP3, caspase-1, ASC are expressed in full term human decidual explants, and the expression of pro-caspase-1 increased after treatment with LPS. In addition, NLRP3, p20 caspase-1, and ASC can be detected in culture media consistently, even without treatment.

3) Decidual leukocytes can release IL-1β under co-stimulation with LPS and BzATP, and this secretion can be inhibited by a $P2X_7$ receptor antagonist.

4) Maternal PBMC could secrete IL-1β under single stimulation with LPS; $P2X_7$ receptor agonist BzATP could accelerate the release of IL-1β, and $P2X_7$ receptor antagonist could inhibit the secretion. Additionally, co-stimulated with both LPS and BzATP could trigger p20 caspase-1 release from maternal PBMC.

5) Decidual tissue explants, decidual leukocytes, and maternal PBMCs could secrete TNF-α under single stimulation with LPS, while $P2X_7$ receptor agonist/antagonist has no effect on the release of TNF-α.

6) The secretion of IL-1β nor TNF-α from cultured decidual stromal cells was undetectable under the same treatment condition.

4.1 IL-1β secretion from human decidua

First of all, we found that the LPS stimulate the expression of pro-IL-1β in all treatment group in decidual. Notably, no significant differences were found in IL- 1β level in three treatment groups by ELISA assay. Since the IL-1β ELISA kit used could detect both inactive pro-IL-1β and mature IL-1β, western blotting was carried out to distinguish the pro-IL-1β from IL-1β and the higher intensity of pro-IL-1β was found in LPS comparing with LPS+BzATP group. Elevated pro-IL-1β expression after LPS stimulation can be explained by the canonical IL-1β production mechanism that stimulation of cells with TLR ligands, such as LPS, or NLR agonists, can induce transcription of IL-1β mRNA and then translation into pro-IL-1β protein, in which the NF-kB pathway is involved (Dinarello, 1996; Watkins et al., 1999). Meanwhile, the results of higher level of secreted mature IL-1β and lower pro-IL-1β in lysate in LPS+BzATP group comparing with single LPS stimulation group were consisted with “double hit” theory: priming leads to transcription of pro IL-1β, but the second hits, such as potassium efflux triggered by BzATP bind to $P2X_7$ receptor, leading to NLRP3 inflammasome activation, is required for IL-1β release (Ferrari et al., 1997; Guo et al., 2015; Khan and Hay, 2014). Consistently, data from our lab demonstrated that extracellular ATP also promotes LPS induced-secretion of IL-1β from placenta blood, cord blood and choriodecidua (Maneta et al., 2015; Warren et al., 2008). The natural existing ATP could be a “dangerous” signal, when extracellular ATP exists in high concentration (Khan and Hay, 2014). In human pregnant uterus, elevated ATP could be released from endothelial cells in cord blood vessels (Yegutkin et al., 2000) and mechanical trauma from uterine stretch or contractions (Pedersen et al., 1999), which could explain the elevated cytokine in normal labour. Stimulating $P2X_7$ receptor with extracellular ATP only could not stimulate the secretion of IL-1β, which is indicated from our previous study (Maneta

et al., 2015; Warren et al., 2008), however, blocking $P2X_7$ receptor with antagonist (A7) could partially inhibit the LPS and BzATP stimulated IL-1β secretion in both PBMC and decidua explant, which was similar with other gestational tissue (Maneta et al., 2015; Warren et al., 2008) . However, the IL-1β secretion after blocking $P2X_7$ receptor still existed when comparing with control group. These phenomena can be explained by one of the IL-1β release mechanisms that $P2X_7$ receptors expressed in the membrane of microvesicles may participate in the regulation of IL-1β release from shed microvesicles (Eder, 2009). The inhibition of $P2X_7$ receptors proved to reduce IL-1β secretion substantially, indicating that $P2X_7$ receptors related pathway is the main IL-1β release mechanism in decidua explant, but IL-1β secretion can still be achieved through other pathways.

4.2 NLRP3 inflammasomes in decidua

The involvement of NLRP3 activation in the IL-1β processing pathway has been proved in cell lines, animal model and from human data. Besides, the potential role of inflammasomes in normal pregnancy and preterm birth was predicted from previous studies. Limited date in gestational tissues showed the expression of components of inflammasomes as well as the upstream signaling pathway leading to IL-1β release (Khan and Hay, 2014). Recently, Romero et al found that NLRP3 and caspase-1 were expressed in term chorioamniotic membranes, and the transcriptional level of them altered in women with or without term labour. (Romero et al., 2016). Besides, the release of endotoxin-induced IL-1β was found to be caspase-1 and $P2X_7$ dependent in chorioamniotic membrane, mononuclear cells from cord blood, and placental blood with a reduced level of IL-1β when treated with caspase-1 inhibitor (Gomez-Lopez et al., 2017). As for the decidua, Pontillo et al found that mRNA of caspase-1, IL-1β and

NLRP3 increased in early pregnancy decidua stromal cells and cytotrophoblasts (Pontillo et al., 2013). This study, for the first time, investigated the NLRP3 inflammasome components protein expression in human full term decidua. In this study, the western blotting assay demonstrated that NLRP3, pro-caspase-1, and ASC were expressed in decidua tissue without treatment. Besides, LPS stimulation increased pro-caspase-1 protein levels. Meanwhile, a slightly higher NLRP3 expression in LPS+BzATP are consistent with unpublished data from our lab where we found that the NLRP3 mRNA level increased after treated with LPS+ATP. On the other hand, p20 caspase-1, as the result of activation of inflammasome, was assumed to release along with IL-1β through different pathways (Eder, 2009). However, no difference was found in p20 caspase-1 from decidua explant culture media with or without treatment. This phenomenon indicated that p20 caspase-1 may participate in other biology activity or can be release by another secretion pathway. In this study, we only detected the activation of caspase-1 by measuring the level of p20. But there are other methods to detect the activity of caspaser-1, such as detection of caspaser-1 cleavage of substrate YVAD-AFC or Z-WEHD, and these methods can provide further evidence of the degree of caspase activity.

Interestingly, NLRP3 and ASC protein can also be detected in media, even without treatment. Extracellular NLRP3 and ASC were reported with the role of amplifying inflammatory response (Baroja-Mazo et al., 2014); and the extracellular NLRP3 and ASC could be detected after 15 minutes from macrophages treated with ATP (Baroja-Mazo et al., 2014). However, in this study, NLRP3 and ASC protein presented in untreated media as well. A possible explanation for these observations may be that the NLRP3 inflammasome was activated in full term decidua explant, and this activation is possible due to normal labour process. This hypothesis is based on Romero's finding that women who underwent labour had a higher concentration

of NLRP3, a greater immunoreactivity for caspase-1, and a higher quantity of p20 capase-1 and pro- and mature-IL-1β in their chorioamniotic membranes (Romero et al., 2016). Additionally, the already functional NLRP3 inflammasome can explain the release of IL-1β and p20 capase-1 from untreated decidua tissue. Their appearance in media could also be due to cell/tissue death releasing their contents into the culture media. However, how NLRP3 inflammasome is activated remains unclear as labour process therefore further questions, such as how inflammasome component expression differ between decidua and chorioamniotic membranes or how inflammasome changes in decidua under different pathological conditions, need to be addressed in the future.

4.3 Different role of IL-1β secretion in decidual stromal cells and decidual leukocytes

To investigate IL-1β processing in maternal decidua, the decidua explant was used in this study. The major advantage of explant culture is the maintenance of near *in vivo* environment *in vitro* for a short duration of time. However, decidua tissue consists of a heterogeneous cell population. Flow cytometric analysis of human decidual cell suspension has demonstrated that more than half of the cells are of stromal origin, with the remaining cells expressing the leukocyte common antigen (CD45) of hematopoietic linage (Vince et al., 1990). Apart from immune cells themselves, decidual stromal cells are reported as contributing to the immune response in placental immune defence (Hess et al., 2007; Pontillo et al., 2013; Wu et al., 2014). To investigate the role of different decidual cells in the immune response, decidual stromal cells and decidual leukocytes were isolated and IL-1β secretion determined with different treatments.

The method of isolation of decidual leukocytes cells was modified from Xu Yi's method (Xu et al., 2015). They found that using the method to isolate infiltrated leukocytes resulted in a high yield of viability (more than 90%) and the isolated cells mainly consisted of neutrophils ($CD25^+$), macrophages ($CD14^+$), B cells ($CD19^+$), T cells ($CD3^+$) and NK cells (CD56+). They thought that this method is optimal for immunophenotyping of leukocytes, cell culture, or leukocytes functional studies at the human maternal-fetal interface. However, considering the high cost of cell detachment solution, we applied collagenase solution for cell detachment instead of Accutase used in their method, which may probably cause a difference in the result. Thus, a further flow cytometry is required to confirm the population of isolated decidual leukocytes in future.

As for the decidual stromal cells, we cultured the isolated cells *in vitro* till passage 2, and used the cells in passage 3 for stimulation. The yield resulted in a mixed cell population of leukocytes and stromal cells distinguishable by size and morphology. Using cell sorting flow cytometry, it was confirmed that 97% of leukocytes can be removed (Liu et al., 2005).Using this method, , After cell culture and passage, the cells used for stimulation were large size, spindle appearance, and staining for vimentin identifying them as stromal cells.

The secretion of IL-1β increased significantly by decidual leukocytes when co-treated with both LPS and BzATP. Macrophage and uNK cells are the two major decidual immune cell population, and a previous study showed that only macrophages secrete IL-1β after being treated with TLR agonists while NK cells cannot (Duriez et al., 2014). Besides, the canonical IL-1β processing mechanism is based on experiment in macrophages (Bauernfeind et al., 2009; Colomar et al., 2003). Since this observation coincides with the canonical IL-1β release mechanism, which depends on two stimulations (Eder, 2009), we assume that it is

macrophages which plays a dominant role in IL-1β secretion in decidual immune cells. However, further experiments using flow cytometry to sort immune cells are required to understand the individual roles of each immune cell type. Besides, the secretion of p20 caspase-1 was found in similar levels in each group. As for decidual explants, p20 caspase-1 and IL-1β were secreted from untreated decidual leukocytes, which may suggest that the activated caspase-1 in decidua is from decidual leukocytes. To better understand it, a further study on NLRP3 inflammasome in decidua leukocyte may needed.

On the other hand, no IL-1β secretion was detected from stromal cells, and it seemed stromal cells cannot respond to LPS and BzATP. However, Pontillo et al isolated decidual stromal cells from early pregnancy placenta, and cells used were passage 2 or passage 6. They found that LPS could stimulate decidual stromal cell to secrete IL-1β with elevated transcription of NLRP1,NLRP3, NLRC4, ASC, and caspase-1 (Pontillo et al., 2013). However, they set 4 hours and 24 hours as time courses for LPS stimulation, and observed that NLRP3 and IL-1β expression was augmented after 4 hours stimulation, and IL-1β secretion was only found after 24 hours stimulation but undetectable after 4 hours' stimulation. According to this study, we can tell that the time course is important for stromal cells to secrete IL-1β. The undetectable IL-1β in decidual stromal cells may due to insufficient stimulating time. Thus, further experiments using different time course and mRNA level tests are needed to understand whether decidual stromal cells from full term decidua are involve in IL-1b production.

4.4 IL-1β secretion from PBMC

In this study, maternal PBMCs were used as a positive control to test the effect of treatment of LPS, BzATP, and A7 on IL-1β secretion instead of cord blood, since blood mononuclear cells

are a rich source of IL-1β production (Fenton et al., 1987; Netea et al., 2009), and NLRP3 inflammasome is well studied in peripheral blood. As for cord blood, previous studies showed that term cord blood produces a lower level of IL-1β in response to endotoxin (LPS) in (Dinarello et al., 1981; Strunk et al., 2012). This damaging inflammatory response was considered due to the lower CD14 expressing/ $CD16^{pos}$ monocytes and a downregulation of TLR-mediated NLRP3 expression in cord blood (Sharma et al., 2015). Thus, the maternal blood was used to present the relatively normal immune response from maternal aspect. Our finding indicated that a single stimulation of TLR4 with LPS could lead to the trend of IL-1β release in human PBMC (**Figure 3.2**). This could be explained by Netea's research in 2009, they found that LPS alone could induce the release of mature IL-1β alone in human monocytes (Netea et al., 2009). Meanwhile, monocytes are the primarily source for the production of IL-1β in circulating blood (Allen et al., 1992; HSI and REMICK, 1995). Subsequent research found that exocytosis of ATP may explain the capacity of TLR ligands to activate IL-1β production and secretion (Pelegrin and Surprenant, 2006, 2007; Piccini et al., 2008). When treated the $P2X_7$ with antagonist A7 at the same time with LPS, a significant inhibition to external ATP could be achieved by $P2X_7$ antagonist, but there was no difference on comparing single LPS stimulation with A7 stimulation, which is similar with decidua explant. Thus, we suggested that PBMC can be stimulated through TLR4 with LPS, which is independent of the $P2X_7$ receptor; but the release of IL-1β was enhanced by exogenous ATP binding with $P2X_7$. In addition, increasing level of caspaser-1 p20 after co-stimulated with LPS and BzATP was found in PBMC media, which indicated that the enhancement of ATP induced IL-1β release was possibly related with capase-1 activation. In contrast, p20 caspase-1 release might not correlate with LPS stimulated IL-1β secretion. Since this study focused on decidua and the maternal PBMC was used as positive control for different treatment, we did not categorise

the population of isolated PBMCs by flow cytometry however previous researchers in our team found that the isolated cells mainly consisted of monocytes, lymphocytes and neutrophils. Among them, the CD45 positive cells accounted for 82.64%, CD14 positive cells accounted for 59.29%, while CD68 was 73.22% (Maneta et al., 2015).

4.5 TNF-a secretion with the LPS stimulation

In this study, TNF-α, the another LPS-induced pro-inflammatory cytokine, was detected as a parallel indicator. The mechanism of TNF-α production was found to involve the Raf-1/ MEK1-MEK2/ ERK1-ERK2 pathway (Tjomme van der Bruggen,1999), instead of caspase-1-independent pathway. Here in, we found that the LPS can stimulate decidua explant, decidua leukocyte, and maternal PBMC to secrete TNF-α, while decidual stromal cells cannot secrete TNF-α in the same condition. This result is consistent with previous reports, that the decidua explants could produce TNF-α (Casey et al., 1989; Romero et al., 1991) while the endometrial stromal cells did not produce TNF-a in detectable amounts (Casey et al., 1989). However, the stromal cells they used were isolated from the uteri of premenopausal women instead of decidua. In the present study, we found that it is only decidual leukocytes instead of stromal cells could produce TNF-a, which may depend on the stimulation drugs and time we used.

4.6 Assay validation

In this study, the assay validation was the focus on the problem of non-specific band and multiple bands in western blotting. As for the non-specific band appearing around 50kDa, this was assumed to be human IgG reacting with Sigma secondary antibody used. Human IgG is

large molecule made of four peptides chains, including two class heavy chains of 50kDa and two light chains of 25kDa. IgG antibodies are important for neonate immune system since it is the only immunoglobulin isotype that could passage through the human placenta and protect the fetus in the uterus (Morell et al., 1971). And the transformation of IgG relies on specific receptors on the trophoblast for the Fc (crystallizable fragment) region of IgG (Leach et al., 1996). Thus, the abundant heavy chain of human IgG (50kDa) in placenta tissue was considered as the protein that reacted with secondary antibody (Sigma, UK).

Another problem with western blotting was the multiple bands for NLRP3, caspase-1, and ASC blots. The variant isoforms of target protein were checked on the accessible protein database, however, there some bands remain unknown. Multiple bands appearing on the blots could be caused by several factors. To understand and confirm whether these multiple bands are specifically recognized by the antibody, control sample assay and blocking peptides could be considered as effective methods. For control sample assay, both positive sample (contains the protein of interest) and negative protein (lack of interested protein) are needed to identify those bands due to non-specific interactions. At the same time, blocking antibodies with higher concentrations of according peptides could help to determine which bands are recognized by the antibodies. Besides, (Liu et al., 2014). However, because of the limited resource and time, these control assay and blocking peptide assay for NLRP3, caspase-1 and ASC antibodies were not applied in this study, except for the conformation of the lower molecular size of IL-1β. Apart from these assays, alternative monoclonal antibodies and lower antibody concentration were also worth validating. As for the antibody concentration, several dilutions have been tried. Dilutions of NLRP3 antibody was tried in 1:1000 and 1：500 and the dilution of caspase-1 was tried in 1:1000, but the target signals were too faint to be

recognized in these dilution. Thus, the dilution of caspase-1 in 1:400, and dilution of ASC in 1:1000 was used. In addition, antibodies used were polyclonal in this study, which means these antibodies can bind to multiple epitopes leading to inappropriate binding, while the monoclonal antibody would bind to the same epitopes. Thus, alternative monoclonal antibodies can be further validated.

4.7 Limitation of the study

As mentioned above, there are some limitation in this program. First of all, the results presented in this study reveal purely the protein level modulation of IL-1β and NLRP3 inflammsome components in response to inflammatory stimuli. There are, however, other measurements, such as using qPCR to detect mRNA level changes, that may reflect the changes under stimulation during IL-1β production process, and caspase assay to test the caspase activity. In addition, although the mononuclear cell isolation protocol has proved effective in previous studies, still the isolated cell population is consisted of various types cells, e.g. macrophages, monocytes, and NK cells, which could potentially account for the high variation of IL-1β concentration within PBMC culture media. Furthermore, the The concentration and time course of stimulation can be further validated. Due to the lack of sample source, the stimulation group setting, drug concentration and time course of each stimulus was based on previous studies rather than titrated probably. This could be possibly responsible for the undetectable level of IL-1β from decidual stromal cells. Besides, for western blotting assay validation, the positive sample (contains the protein of interest), negative protein (lack of protein of interest), and monoclonal antibodies were needed to confirm those multiple bands.

4.8 Further work

1. To validate the Western blotting of NLRP3 inflammasome components, trying alternative monoclonal antibodies and carrying out positive and negative control assays in each antibody;
2. To develop the immune stimulation experiment, time course experiment and drugs concentration serials assays are need.
3. To better understand the role of NLRP3 inflammasome pathway role in IL-1β secretion, other drugs stimulate/ inhibit components are required, such as caspase-1 inhibitor, NLRP3 inhibitor, and other PAMPs or DAMPs.
4. To understand the change of NLRP3 inflammasome components under stimulation, the mRNA level experiments are needed.
5. To better understand the role of TLR4 and $P2X_7$ receptor in the decidua, their expressions mRNA and protein level should be conducted.
6. To study in more details, using flow cytometry to observe the cells population of maternal PBMC and decidual leukocytes; and using immune staining to investigate the expression of NLRP3 inflammasome components in PBMC and decidua leukocyte.
7. To investigate the mechanism that how decidua immune response affect pregnancy, the similar experiments are needed be conducted in different pathological conditions.

4.9 Conclusion

IL-1β is an important proinflammatory cytokine. Increased level of IL-1β was found in normal labour as well as infection correlated preterm labour. Data obtained in this study has proved the hypothesis that decidua, when stimulated with LPS, decidua explants can produce pro-IL-1β. But the release of IL-1β can be stimulated with the addition of exogenous BzATP, potentially through the $P2X_7$ receptor associated pathway. This effect was also accompanied by an increased level of NLRP3, caspase-1, two major components of NLRP3 inflammasome components, which provided a clue that inflammasome may play an important role in processing IL-1β in human decidua. In addition, decidual leukocytes were proved to be the main cell type responsible for producing IL-1β.

This study for the first time investigate the IL-1β processing and NLRP3 inflammasome components in full term decidua explant, which provides an insight into NLRP3 inflammasomes roles in immune response and inflammation in decidua. Further studies on NLRP3 inflammasomes in different pregnancy disorders, with specific NLRP3 inflammasome components stimulation, such as caspase-1 inhibitor and NLRP3 inhibitor; as well as other DAMPs and PAMPs, and the expression on different level are required to unravel the how do maternal immune system, especially NLRP3 inflammasome participate in pregnancy process and effect the pregnancy outcomes.

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