Characterization of the mechanism of action of an anti-IL-6R small peptide in an infection- and inflammation-induced preterm labor model

Estefania Marin Sierra
Department of Pharmacology and Therapeutics McGill University, Montreal

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ABSTRACT

Preterm birth (PTB; delivery before 37 weeks of gestation), representing approximately 8% of the births, is an increasing health concern worldwide. The reason why PTB numbers are in a constant increase is because even 50% of the cases present unknown causes. The only confirmed causal link is the existence of an infection and thus its related inflammation. The most important molecules related to both, inflammation and PTB are interleukin- (IL-) 6, IL-1β and tumor necrosis factor alpha (TNFα). Current reports show how the levels of IL-6 significantly increase in amniotic fluid in the presence of an infection that will later become PTB. Therefore, it will be important to study the possibility of targeting IL-6 in order to prevent PTB and its co-morbidities. IL-6 acts by binding to the IL-6R and this complex will later bind to the intracellular receptor gp130 generating an intracellular response. The action of IL-6 will translate into the activation of three main signaling pathways whose start-up point is characterized by the phosphorylation of the proteins STAT3, Erk1/2 and Akt. Our group has developed a small peptide, called 633, by analyzing the extracellular flexible motifs of the IL-6R. Some reports indicate that the generation of antagonists based on this flexible motifs tends to produce molecules with allosteric characteristics. In this work, we characterized the effect of 633 in the IL-6 signaling and in its pro-inflammatory function. We analyzed the possibility of 633 of presenting allosteric features. Then, we tested the potential of 633 in an IL-6-induced PTB mice model, in order to validate IL-6 as a valuable target and to study 633 as a potential therapeutic agent to prevent PTB. Lastly, we confirmed 633 efficacy in a lipopolysaccharide (LPS)-induced PTB mice model. The results showed the presence of the first allosteric characteristic of 633, biased agonism, by the differential modulation of IL-6 signaling. The effect of 633 produced a significant reduction of the STAT3 pathway without inhibiting the Akt or Erk1/2 pathway. We demonstrate a dose-response behavior of 633 against the IL-6-driven induction of pro-inflammatory cytokines (IL-6, IL-1β and TNFα) *in vitro*. We displayed the second allosteric characteristic by showing a differential 633 modulation of IL-6 efficacy and affinity. This result demonstrate the ability of 633 to reduce IL-6 efficacy and in the highest 633 doses, to even increase IL-6 affinity for its receptor. We validate IL-6 as an important target in the prevention of PTB by showing that the sole action of IL-6

is sufficient to generate the onset of PTB. We also prove how 633 was able to prevent the IL-6 prematurity by selectively modulating gene upregulation of proinflammatory cytokines and uterine activation proteins in maternal and gestational tissues. Lastly, we confirmed 633 efficacy by presenting evidence how 633 can prevent LPS-induced PTB by selectively modulating pro-inflammatory gene and protein upregulation in maternal and gestational tissues and fluids. We also demonstrate the ability of 633 to rescue the neonate survival and weight. For all the aforementioned, we conclude that 633 is a valuable therapeutic molecule for the treatment of PTB and that this work set the basis for the potential clinical development and later use of 633 in humans.

RESUMÉ

La naissance prématurée (PTB; accouchement avant 37 semaines de gestation), laquelle représente approximativement 8% des naissances, est une problématique de santé en hausse dans le monde. La raison pour laquelle le nombre de PTB augmente constamment est que les causes demeurent inconnues. La seule cause de PTB qui a été confirmée est la présence d'une infection et donc, de l'inflammation qui s'ensuit. Les molécules d'importance majeure liées à la fois à l'inflammation et à la PTB sont l'interleukine (IL)-6, l'IL-1 et le facteur de nécrose tumoral alpha (TNFα). Des études récentes ont montré que les concentrations d'IL-6 augmentent significativement dans le liquide amniotique, en présence d'infection, ce qui conduira à la PTB. Par conséquent, il est important d'étudier la possibilité de cibler IL-6 dans le but de prévenir la PTB et ses comorbidités. IL-6 agit en se liant au récepteur de l'IL-6 (IL-6R), ce qui forme un complexe qui se lie par la suite à la protéine accessoire gp130, générant une réponse intracellulaire. L'action d'IL-6 se traduit par l'activation de trois principales voies de signalisation, lesquelles débutent par la phosphorylation des protéines STAT3, Erk1/2 et Akt. Notre groupe a développé un petit peptide, nommé 633, en analysant les motifs flexibles de la portion extracellulaire du IL-6R. Des études ont montré que la génération d'antagonistes à partir de ces motifs flexibles tend à produire des molécules possédant des caractéristiques allostériques. Dans ce projet, nous avons caractérisé l'effet du 633 sur la signalisation et les fonctions proinflammatoires de l'IL-6. Nous avons également étudié la possibilité que 633 possède des propriétés allostériques. Ensuite, nous avons testé le potentiel du 633 dans un modèle murin de travail préterme induit par IL-6, dans le but de valider que l'IL-6 constitue une cible valable et pour étudier la possibilité que 633 constitue un agent thérapeutique potentiel pour prévenir la PTB. Finalement, nous avons confirmé l'efficacité du 633 dans un modèle murin de travail préterme induit par l'administration de lipopolysaccharide (LPS). Les résultats ont montré la présence d'une première caractéristique allostérique du 633, soit l'agonisme biaisé, via la modulation différentielle de la signalisation de l'IL-6. Le 633 engendre une réduction significative de la voie de signalisation de STAT3 sans inhiber les voies de Akt ou

Erk1/2. Nous avons démontré, *in vitro*, que le 633 modulait l'induction des cytokines pro-inflammatoires (IL-6, IL-1β et TNFα) en réponse à IL-6 de façon dosedépendante. Nous avons prouvé que le 633 possédait une seconde caractéristique allostérique en montrant que le 633 modulait de manière différentielle l'efficacité et l'affinité de l'IL-6. Ce résultat montre la capacité du 633 à réduire l'efficacité de l'IL6 et, aux doses les plus élevées, à augmenter l'affinité de l'IL-6 pour son récepteur.

Nous avons validé que l'IL-6 constitue une cible importante dans la prévention de la PTB en montrant que IL-6 seule est capable de déclencher une PTB. Nous avons aussi prouvé que 633 était capable de prévenir la prématurité due à l'IL-6 en modulant sélectivement l'augmentation de l'expression des gènes de cytokines proinflammatoires et de protéines activatrices de l'utérus dans les tissus maternels et gestationnels. Finalement, nous avons confirmé que 633 était efficace en prouvant que cette molécule pouvait prévenir la naissance prématurée induite par le LPS via la modulation sélective de l'augmentation de l'expression des gènes et des protéines pro-inflammatoires dans les tissus et fluides maternels et gestationnels. Nous avons aussi démontré la capacité du 633 à rétablir la survie néonatale et le poids. À la lumière des résultats obtenus, nous pouvons conclure que le 633 est une molécule thérapeutique à fort potentiel dans le traitement des PTB et que ce projet établit la base d'un développement clinique ultérieur favorisant l'usage du 633 chez l'humain.

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LIST OF ABBREVIATIONS

633	peptide designated name
Ab	antibody
AKT	protein kinase B
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
CAP	contraction associated proteins
CASP1	caspase 1
CCL2	c-c motif chemokine ligand 2
cDNA	compermentary deoxyribonucleic acid
cff-DNA	cell free fetal DNA
CRP	c reactive protein
CX43	connexin 43
CXCL15	c-x-c motif chemokine ligand 15
DMEM	dubelcco´s modified eagle medium
ECL	electrogenerated chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase p42 and p44 subunit
FIRS	fetal inflammatory response syndrome
gp130	glycoprotein 130
HEK	human embryonic kidney cells
hIL-6	human interleukin-6
hkb-hil6	human embryonic kidney cells transfected to express the IL-6 receptor
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal axis
HRP	horseradish peroxidase
IC50	half maximal inhibitory concentration
IFNϒ	interferon gamma
IL-	interleukin-
IL-6R	interleukin-6 receptor
LPS	lipopolysaccharides
LTA	lipoteichoic acid
mIL-6	recombinant murine interleukin-6
MLCK	myosine light-chain kinase
MMP	metalloproteinase
NFkB	nuclear factor kappa B
NFKB	nuclear factor kappa B subunit p50 subunit
OXTR	oxytocin receptor

p-AKT	phospho-protein kinase B
p-ERK1/2	phospho-extracellular signal-regulated kinase p42 and p44 subunit
p-STAT3	phospho- signal transducer and activator of transcription 3
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGDH	prostaglandin dehydrogenase
PGE2	prostaglandin E2
PGEP1	prostaglandin receptor EP1 isoform
PGEP3	prostaglandin receptor EP3 isoform
PGES1	prostaglandin E synthase
PGF2a	prostaglandin F2α
PGSH	prostaglandin H2 synthase
PLV	periventricular leukomalacia
pPROM	premature rupture of membranes
PR-A	progesterone receptor isoform A
PRR	pattern recognition receptors
PTB	preterm birth
PTGER3	prostanglandin E receptor 3
PTGHS2	prostagandin F2 alpha receptor
PTGFR	prostaglandin F receptor
RBC	red blood cells
RELA	nuclear factor kappa B p65 subunit
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SHP2	protein tyrosine phosphatase 2
sIL-6R	soluble form of the IL-6 receptor
SOCS	suppressor-of-cytokine-signalling
STAT3	signal transducer and activator of transcription 3
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TOC	tocilizumab
UAP	uterine activation proteins
WBC	White blood cells

1. INTRODUCTION

Preterm birth (PTB) is a major concern worldwide and despite of all the current efforts to generate a treatment or a better diagnosis, there is an increasing incidence of cases in both in-development and developed countries. This increase may be due to the lack of understanding in all the aspects of the pathology, going from causes, onset, immunological activation pathway and later short and long term effects on the neonate. The first strategy to target PTB, which is still currently used, is the administration of tocolytics, which are medications that can arrest the uterine contractility and donate some extra time to achieve fetal lung maturation. The problem with the use of tocolytics falls in the fact that these pharmacological agents are just effective into delaying the birth by 48 hours and they are unable to counter for the associated morbidities in the infant. One of the major linked causes to all fetal comorbidities is the presence of an inflammatory process. Inflammation is marked by the upregulation of different molecules like cytokines, chemokines, eicosanoids and hormones.

The objective of this work is to introduce a new therapeutic approach by targeting one of the major inflammation regulators, interleukin-6 (IL-6). In the course of this work we characterized the activity of this therapeutic small peptide in different features of PTB; mainly by analyzing its activity on the IL-6 signalling pathway, *in vitro, ex vivo* and *in vivo*.

1.1. Preterm birth

PTB is defined as the delivery before 37 weeks of gestation. It is thought that term and preterm birth are driven by the same physiological process just differing in the onset time and causes (being pathological signals in the case of PTB) (R Romero et al., 2006). The onset of birth involves the activation of different elements either in the fetal or maternal side which, by the presence of continue communication, will finalize with the recruitment of all the different required physiological adaptations that will later lead to uterine contractility and deliver of the neonate (Roberto Romero, Dey, & Fisher, 2014). The presence of PTB generates a high risk of mortality and later

health and developmental complications on the infant. All this detrimental effects may be due to biological immaturity of the fetus at the beginning of the pathology (Behrman & Stith Butler, 2007).

1.1.1. *Epidemiology and statistics*

The incidence of PTB is increasing despite the current efforts in the implementation of treatments and clinical interventions. The most current reports on PTB stated that 15 million babies are born prematurely; from them, 1 million babies die at parturition and 2.1 million babies die due to later complications (Blencowe et al., 2013; Cappelletti, Bella, Ferrazzi, Mavilio, & Divanovic, 2016). The number of complications related to PTB has increased in such manner that PTB is ranked since 2015 as the leading cause of mortality on children under 5 years old, surpassing pneumonia which was leading this category in 2013. Prognostics of PTB incidence have ranked it to remain the leading cause of mortality of children under 5 years old by 2030 (Liu et al., 2015). The population statistics reported that the incidence is higher in males, covering 55% of the total preterm births. The incidence of PTB increases with the presence of multiple births. This characteristic can explain the presence of high frequency of PTB in places with high rates of natural multiple births like West Africa or Japan and hormone-induced multiple births like England, France and United States (Blencowe et al., 2013). Canada presented a rate of PTB that fluctuated between 7.5 and 8.2% of total births in between 2001 and 2010 (Health Agency of Canada 2013).

PTB is also an economical concern since the hospitalization costs related to the preterm infants account for 47% of all the birth costs even though it just represents 8% of the total births (Barradas et al., 2016).

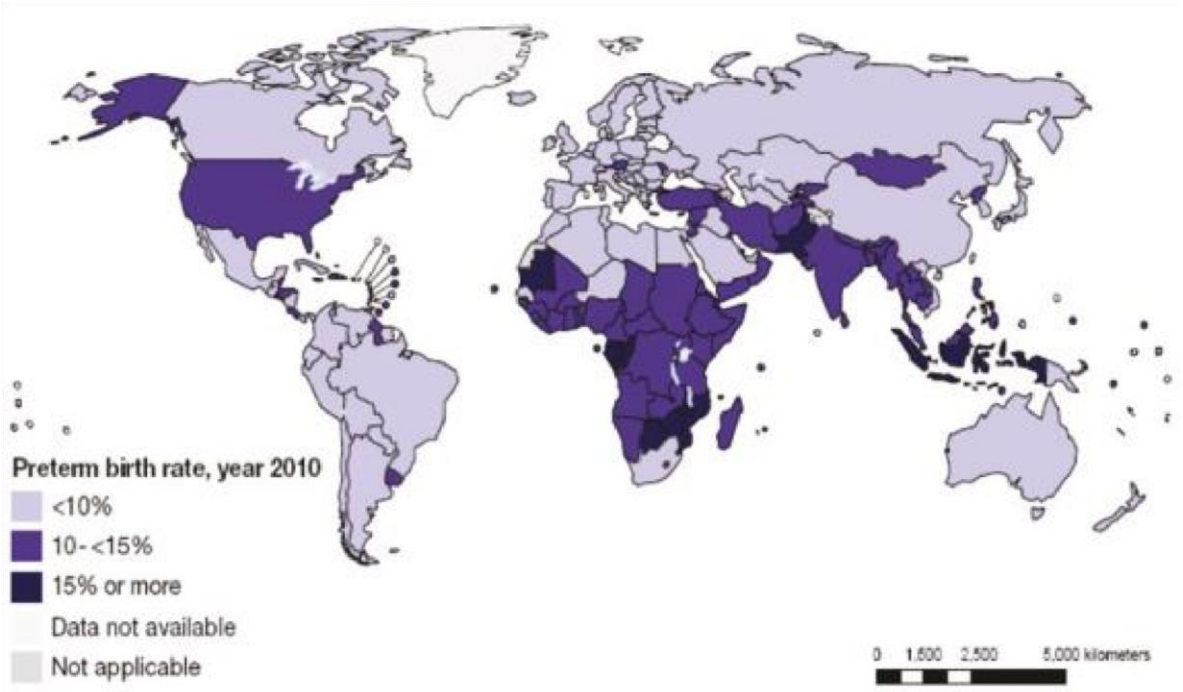


Figure 1.1 | Epidemiology of PTB. Percentage of cases of PTB by country in 2010 (WHO World Health Organization 2012)

1.1.2. *Parturition pathway*

Parturition is defined by the presence of changes in different organ systems in the fetus as well as in the mother. In the uterus there is the presence of three major modifications called 1) increased myometrial contractility, 2) cervical ripening and 3) decidual activation. Relevant extrauterine features are expressed as changes in concentration of different hormones and maternal metabolism (R Romero et al., 2006).

The activation of uterine components can be synchronous and asynchronous. An asynchronous activation may present premature activation of one of the uterine features but the birth can still happen at term (Roberto Romero, Yeo, Chaemsaithong, Chaiworapongsa, & Hassan, 2014).

The process of parturition is divided in four phases, each of them characterized by unique features.

- Phase 0 “Quiescence”: myometrial activity is inhibited by the action of progesterone. This molecule increases the levels of cyclic nucleotides (cAMP) which will later repress the central activators of uterine contractility: 1) release of calcium or 2) myosin-phosphorylation activity of myosin lightchain kinase (MLCK) (Behrman & Stith Butler, 2007).
- Phase 1 “Activation”: fetal growth and/or hypothalamic-pituitary-adrenal axis (HPA) maturation produce increased expression of contraction-associated proteins (CAP) like connexin 43 (CX43), oxytocin and prostaglandin receptors. Fetal hypothalamus and placenta will favor the secretion of estrogens which will lead to the progression to the phase 2 (Behrman & Stith Butler, 2007).
- Phase 2 “Stimulation”: increased levels of estrogen and progesterone receptor A (PR-A) will generate the secretion of CAPs like CX43, oxytocin receptor (OXTR), cyclooxygenase-2 (COX-2), MLCK and prostaglandins. Increased generation of corticotropin-releasing hormone (CHR), IL-1β and TNFα from placenta and fetal membranes will promote expression of prostaglandin H2 synthase (PGSH) and inhibit prostaglandin dehydrogenase

  (PGDH) in fetal membranes (Roberto Romero, Dey, et al., 2014). Prostaglandin E2 (PGE2) will bind to the prostaglandin receptor EP1 and EP3 and induce myometrial contraction and production of matrix metalloproteinases (MMP) in cervix and decidua to generate later cervical ripening and membrane activation (Challis et al., 2009). Prostaglandin F2α (PGF2a) will bind to FP receptor (PTGHS2) and induce myometrial contraction (Behrman & Stith Butler, 2007).
- Phase 3 “Involution”: oxytocin secretion enhances uterine contraction to prevent bleeding after placental separation by the cleavage along the decidua basalis (Behrman & Stith Butler, 2007).

1.1.3. *Aetiology*

PTB is a multifactorial syndrome, in which 50% of the cases are unknown and only infection has been established as a causal link to the onset of PTB (NadeauVallée et al., 2015).

Some of the potential causes of PTB are:

- *Microbial-induced inflammation*: normally presented as subclinical inflammation marked by the presence of chemokines, cytokines prostaglandins and proteases that will lead to the activation of the parturition pathway. (Roberto Romero, Dey, et al., 2014).
- *Decidual hemorrhage and vascular disease*: bleeding can generate thrombin which will later cause myometrial contractility and degradation of extracellular matrix causing premature rupture of membranes (pPROM) (Zhou et al., 2007). Some set of patients that present bleeding will also present preeclampsia features (Roberto Romero, Dey, et al., 2014).
- *Membrane senescence*: it has been shown that PTB patients present senescent membranes (Menon, 2016), whether this is a cause or a consequence of other mechanism should still be determined (Roberto Romero, Dey, et al., 2014).
- *Disruption of maternal-fetal tolerance*: some patients with chronic chorioamnionitis present maternal sensitization to fetal human leukocyte antigen (HLA) accompanied by complement deposition in umbilical vein (Richani et al., 2005). The linking mechanisms between loss of tolerance and PTB is still unclear (Roberto Romero, Dey, et al., 2014).
- *Decline in progesterone action*: administration of progesterone receptor antagonists induces cervical ripening and PTB (Roberto Romero, 2011). The possible use of progesterone to prevent PTB has been much more studied than the possibility of progesterone drop as a cause of PTB (Roberto Romero, Dey, et al., 2014).

- *Uterine overdistension*: formation of intra-amniotic balloons due to stretching of human myometrium can stimulate uterine contractility and secretion of inflammatory cytokines (Roberto Romero, Dey, et al., 2014).
- *Maternal stress*: presence of stress situations can lead to secretion of maternal and fetal cortisol which will induce production of CRH and later the onset of PTB (Roberto Romero, Dey, et al., 2014).
- *Cell-free fetal DNA (cff-DNA)*: levels of cff-DNA peak just prior onset of parturition. Patients with high levels of cff-DNA in midtrimester present higher risk of PTB (Farina et al., 2005). Cff-DNA can bind to TLR-9 and induce an inflammatory response that will activate the common pathway of parturition (Roberto Romero, Dey, et al., 2014).

a) Infection

Infection is strongly evidenced to play an important role in the onset of PTB and neonate complications (Roberto Romero et al., 2001). It is confirmed that systemic maternal infection can provoke PTB. Intrauterine infection, which is usually subclinical, is the most important infection associated to PTB. In intrauterine infection, bacteria will ascend from the vagina into choriodecidual space, transverse maternal-fetal membranes, replicate in amniotic fluid space and subsequently generate an infection that will present direct contact with the neonate (Grigsby, Novy, Adams Waldorf, Sadowsky, & Gravett, 2010). There are already cases of reversible PTB, where a woman with a chronic infection can be treated with antibiotics and therefore delay the beginning of uterine contractility (Roberto Romero, Espinoza, Chaiworapongsa, & Kalache, 2002). The existence of reversible PTB patients opens the perspective for the search of therapeutic agents that can delay the parturition and reduce the co-morbidities on the neonate.

Frequency

Infection is found in 25% of the PTB cases and accounts 79% of the extreme PTB circumstances (Cappelletti et al., 2016). PTB early onset infection includes bacteremia, meningitis, pneumonia and urinary tract infection. Even though less than 1% of women will present bacteria in the amniotic fluid, the incidence of bacteremia in North America is of 1.5% of live births and it generates a mortality rate of 40%. This type of infection nowadays is mainly caused by gram negative coliforms (Mcguire, Clerihew, & Fowlie, 2004). Additionally to bacterial infections, the presence of viruses are associated to PTB, by the presence of adenoviral genome in 40% of PTB cases (Cappelletti et al., 2016).

Evidence of causality

There are different proofs that link infection to the onset of PTB: a) as seen by several groups, administration of bacteria or bacterial products to animals results in abortion or labor; b) several reports indicate that presence of an extrauterine infection can develop later into PTB; c) presence of histological chorioamnionitis in PTB patients (Roberto Romero et al., 2001); d) amniotic fluid of PTB patients present high levels of microbial colonization and inflammatory cytokines and e) signals of infection in amniotic fluid in mid-trimester is a biomarker of high risk of PTB (Agrawal & Hirsch, 2012). It is important to remark that the sole presence of bacteria is not enough to produce PTB, therefore the process of infection-induced inflammation is vital for the onset of PTB (R. Romero et al., 2007).

Pathways of infection

It is well known that the presence of bacteria or microbial products in the amniotic fluid can trigger a massive proinflammatory response in the fetal compartment, having deleterious effects in the pregnancy and/or the neonate (Witkin, Linhares, Bongiovanni, Herway, & Skupski, 2011). Microorganisms can get into amniotic cavity ascending from the vagina and cervix, disseminating trough the

placenta, retrograde seeding from the peritoneal cavity and accidental introduction on invasive procedures. There are different sources of bacteria that will lead to PTB. Some of these registered sources are pyelonephritis, pneumonia, appendicitis, periodontitis and ascending route (Espinoza, Erez, & Romero, 2006; Fidel et al., 1994; Saini, Saini, & Saini, 2010). Diverse evidence places ascending route as the most important, because histological chorioamnionitis is more severe at the site of membrane rupture, bacteria found in congenital infection are similar to the ones found in lower genital tract and in twin patients the histological chorioamnionitis is more severe in the firstborn twin (Roberto Romero et al., 2001).

Toll-like receptors

In the presence of infection, the innate immune system will recognize pathogen-associated molecular patterns (PAMP) with pattern recognition receptors (PRR). One of the most important families of PRR is the family of toll-like receptors (TLR), which presents 11 members, each with different specificity. The activation of TLR produces the activation of the NFκB pathway characterized by the activation of cytokines and co-stimulatory molecules. Studies in gestational tissues have demonstrated the expression mainly of TLR-2 and TLR-4 and the presence of synergy of these two with TLR-3. TLR-2 recognizes gram positive bacterial lipoproteins, peptidoglycan and lipoteichoic acid (LTA), TLR-4 distinguishes gram negative bacterial LPS and TLR-3 is involved in response to viral infections (Agrawal & Hirsch, 2012). TLR-2 has been associated with the generation of an apoptotic response while the activation of TLR-4 has been linked mostly with the generation of cytokines. Studies have shown increased expression of TLR-2 and TLR-4 in amniotic epithelium, mainly on the basal surface, during spontaneous labor (Kim et al., 2004). Activation of any of these TLR in placenta has been demonstrated to induce the secretion of interleukin- (IL-) 6 and 8 (Abrahams et al., 2004). Placental TLRs are also expressed in decidua and myometrium as result of normal hemochorial placentation and present an overexpression in presence of chorioamnionitis (Yeon et al., 2005). These discoveries have suggested that infection-induced PTB must be mainly driven by the upstream activation of these TLR.

b) Inflammation

Inflammation is a non-specific immediate immune response that can help maintain homeostasis but can also become exaggerated and generate a pathogenic response. Clinical inflammation is defined as heat, pain, redness, swelling and impaired function. Vasodilatation and increased permeability account for the changes in temperature, redness and swelling while the presence of effector cells and mediators can account for the pain and loss of function (R. Romero et al., 2007). Histological inflammation is related to the infiltration of neutrophils, macrophages and lymphocytes into the inflamed tissue. Inflammation has been widely involved as one of the responsible mechanisms for PTB, parturition and fetal injury (Roberto Romero et al., 2006).

Benefits of Inflammatory response

The inflammatory process is brought in the presence of infection to deliver cells and molecules to supress infection. The cells recruited to the injured tissue will secrete molecules like cytokines, antimicrobial peptides and mediators. This molecules will enhance the killing response of effector cells towards the pathological insult. Another functions of inflammation are forming a physical barrier to halt the spread of infection by activating the coagulation system and forming thrombi; and promote the reparation of the injured tissue (Roberto Romero et al., 2006).

Role of inflammation in Pathology

Inflammation is a normal and fundamental response of the innate immune system to counter for insults but also it plays important roles in reproductive physiology. Inflammation can drive the generation of an adequate and specific adaptive response to counter for the presence of an insult, in the case of PTB, an infection (R. Romero et al., 2007). In chorioamnionitis most of the normal symptoms and signs of inflammation are not expressed, as the presented inflammation is subclinical. Absence of an inflammatory signature in maternal circulation has suggest

that PTB-related inflammation is localized specifically in gestational membranes (Gotsch et al., 2009; Roberto Romero et al., 2006).

An inflammatory environment can be detected in gestational tissues in patients of PTB even in absence of infection. Some studies have confirmed the involvement of TLRs expressed in human decidua in this non-infectious inflammatory process by an unidentified mechanism (Challis et al., 2009). One of the theories to explain this non-infectious inflammation is the presence of inflammasomes. This inflammasomes are complexes formed by a sensor molecule, an adaptor protein and pro-caspase 1. These complexes are found in the cytoplasm and capable of inducing an inflammatory response trough the activation of caspase 1 (CASP1) which will help in the maturation of IL-1β and IL-18, inducing an acute inflammatory response (Roberto Romero et al., 2016).

Cellular network

The type of infiltrating cells can be used to classify the inflammation as acute or chronic. In PTB this classification presents difficulties due to the fact that gestational tissues present physiologic infiltration of inflammatory cells, therefore, in order to generate the classification, we have to look at the magnitude of the infiltration. The cells normally recruited are macrophages, neutrophils and lymphocytes (R. Romero et al., 2007). Data showed that inflammation related to parturition is driven by the excessive influx of macrophages and neutrophils in the upper and lower segment of the myometrium (Challis et al., 2009).

The most prominent feature of histological inflammation is the infiltration of neutrophils in maternal and fetal membranes (Chau, Mcfadden, Poskitt, & Miller, 2014). CXCL8, a neutrophil chemoattractant, is upregulated in myometrium at birth, suggesting the requirement of neutrophils during parturition. Neutrophils participate in the process of PTB by secreting proinflammatory cytokines and MMP. Even though neutrophil depletion reduces the level of IL-1β, it does not prevent PTB (Gomez-Lopez, StLouis, Lehr, Sanchez-Rodriguez, & Arenas-Hernandez, 2014).

Macrophages are important in late gestation by secreting IL-1β, TNFα, IL-6, MMPs and nitric oxide. CCL2, a chemoattractant for neutrophils and macrophages, gets increased in the myometrium and amniotic fluid near delivery. The number of uterine macrophages increases previous parturition and dropped to non-pregnant levels just after birth. Macrophages are so significant for labor that its depletion protected LPS-induced PTB (Gomez-Lopez et al., 2014).

Mast cells are also important for cytokine secretion but its main function is the expression of endothelial adhesion molecules. Mast cell degranulation has been also associated with the onset of contractility (Gomez-Lopez et al., 2014). The profile of adaptive immune cells is marked by the upregulation of T $CD4^+$ effector cells mainly in the rupture zone of the fetal membranes and downregulation of T $CD4^+$ regulatory cells (Gomez-Lopez et al., 2014).

Cytokine network

The presence of a molecular signature of inflammation usually precedes the histological inflammation and can be present even in subclinical inflammation like the one presented in PTB. This signature is described as the presence of specific proinflammatory molecular markers like chemokines, cytokines, antimicrobial peptides and mediators (R. Romero et al., 2007). Cytokines play a central role in infection-induced PTB. This role is confirmed by the bacterial-driven production of cytokines in gestational tissues, concentration of bioactive cytokines in amniotic fluid and the confirmation of cytokine-induced PTB and blockade of PTB by the administration of cytokine antagonists. Spontaneous labor and PTB are associated with upregulation of pro-inflammatory cytokines like IL-1β, IL-6 and TNFα and downregulation of anti-inflammatory cytokines like IL-10 mainly in chorioamniotic membranes (Roberto Romero et al., 2006). The cytokines presented in PTB seemed to have certain functional redundancy but it has been shown that the specific blockade of one of this cytokines can generate enough response to reduce PTB incidence (Nadeau-Vallee et al., 2015).

The cytokine network can be subdivided into two main groups depending on the activation of a Th1 or a Th2 reaction. Th1 will produce cytokines like IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, interferon gamma (IFNY) and TNFα which will produce a strong cell-mediated response. Th2 response will be mediated by the production of IL-4, IL-5, IL-10 and IL-13 generating a humoral response. During pregnancy the Th1/Th2 balance is toward Th2 response creating a protective role in the fetomaternal relation. Inflammation/infection alters this balance creating a shift to the Th1 response, having as a result PTB. In inflammation, cytokines like IL-1β and IL6 will activate transcription factors that promote expression of molecules and receptors that play a key role in uterine contractility and parturition (Challis et al., 2009).

Uterine Activation Proteins

PTB arise from the secretion of different proinflammatory factors that occur in cervix, fetus, placenta, myometrium and fetal membranes. Generation of these factors will lead to activation of one or more of the components required for parturition; this components are called uterine activation proteins (UAP). One of the main components leading to PTB is the inflammatory environment, commonly expressed as upregulation of COX2 and IL-6 in the myometrium, and the secretion of MMP, prostaglandins and its receptors. The secretion of these molecules will produce ultimately a rupture of gestational membranes. The rupture of membranes is mainly due to neutrophil secretion of MMP which are enzymes that can break the collagen found in extracellular matrix of amnion and choriodecidua. As abovementioned, prostaglandins play a major role in the onset of PTB. Prostaglandins, mostly PGE2, get synthesised mainly in amnion fibroblasts by the action of phospholipases, COX and synthase enzymes. The action of PGE2 and other prostaglandins will be generated by its binding to the different prostaglandin receptors in myometrium, amnion and choriodecidual membranes producing a dilatation in the cervix. The beginning of uterine contractility will be driven firstly by the upregulation of oxytocin and its receptors in the myometrium due to the effect of proinflammatory cytokines like IL-1β and IL-6 (Challis et al., 2009).

1.1.4. *Clinical classification*

All the different causes of PTB exposed before have been tried to be classified by making a distinction with time or clinical presentation but these classifications are really general to address the whole problematic. In 2012, there was the presentation of a classification that incorporate five different components. This classification tends to rely on the delivery hindering the underlying mechanism of action. This has been a highly debatable subject because some of the known mechanisms of action are not directly measurable or the effect of them is unknown, giving to this classification, a big merit. The classification is based on one or more characteristics of the mother, the fetus or the delivery. The minimum requirements to create a classification system are (Esplin, 2016):

- *Obstetric estimate*. This includes the accuracy of gestational date which will allow to make meaningful comparisons (Esplin, 2016).
- *Spontaneous versus indicated PTB*. The main problem with this characteristic relies on the women with subtle evidence of parturition given that parturition goes beyond the scope of simple uterine contraction (Esplin, 2016).
- *Antepartum and intrapartum factors*. The best classification should differentiate between fetal and maternal stress and should include information on cervical insufficiency, maternal comorbidities and personal history (Esplin, 2016).
- *Fetal factors*. It is important to consider fetal characteristics like multiple gestations and congenital abnormalities (Esplin, 2016).
- *Placental pathology*. The state of the placenta can give a lot of information to the pathophysiology of the PTB (Esplin, 2016).

1.1.5. *Preterm birth outcomes*

Even though survival rates in PTB patients have improved and there is an increased use of antenatal steroids, assisted ventilation at delivery and surfactant therapy; there is an increased concern about the risk on PTB patients of developing afterward complications (Iacovidou, Varsami, & Syggellou, 2010). Although most

organs are implicated, the brain and the lung are especially susceptible. The aftereffect range will vary between late preterm neonates (32-36 weeks) that, beyond the risk of neonate mortality, present higher risk of developing temperature instability, respiratory distress, apnea, hypoglycemia, seizures, jaundice, kernicterus, feeding difficulties and periventricular leukomalacia (PLV) (Saigal & Doyle, 2008); to extreme preterm neonates (<28 weeks) which will present the highest risks for neonatal mortality, development of bronchopulmonary dysplasia, severe visual impairments and cerebral palsy (Platt, 2014).

Some presented neurodevelopmental impairments are cerebral palsy, mental retardation and sensory deficits. Many PTB patients also present developmental lags that are not classified as impairments (Saigal & Doyle, 2008). Ultrasound findings on PTB neonates showed brain injury and it is known that the presence of a normal ultrasound does not discard the presence of small cysts or diffuse cerebral white matter lesions (Iacovidou et al., 2010).

A set of PTB infants present dysfunctions in behaviour and cognition. This infants present different syndromes like attention deficit hyperactive disorder or behavioural highlights like shyness, unassertiveness and social maladaptation that follow the infant through adolescence and early adulthood (Saigal & Doyle, 2008). Other consequences presented as a result of PTB are higher risk of respiratory illness due to lower respiratory tract infections and bronchopulmonary dysplasia. Some late onset diseases which present a higher risk in PTB patients are retinopathy, myopia, hypermetropia, hearing difficulties and necrotizing enterocolitis (Saigal & Doyle, 2008).

One major outcome on the whole PTB picture is the generation of psychosocial and emotional effect on the families. This type of event generates a parental distress that persists through the years (Saigal & Doyle, 2008).

a) FIRS

The fetal inflammatory response syndrome (FIRS) is a condition characterized by the presence of systemic inflammation, due to systemic activation of the fetal

innate immune system, and elevation of fetal plasma IL-6. FIRS is mainly presented in neonates who undergo PTB. This syndrome is currently considered as a potential cause of short-term mortality and morbidity because of the presence of a multiorgan involvement (Gotsch et al., 2007). There are reports on the incidence of FIRS in the absence of proven intraamniotic infection. It is thought that occurrence of extraamniotic infection (between amnion and chorion) can produce inflammation in amniotic fluid. The presence of elevated IL-6 in fetal plasma due to alloimmune hemolytic anemia, because of Rh diseases, have also open the possibility of existance of non-infectious FIRS (Lee et al., 2007).

		n	Procedure-to-Delivery Interval (median, range, days)
I	AF IL-6 ≤ 7.9 ng/ml FP IL-6 ≤ 11 pg/ml	14	5 (0.2-33.6)
II	AF IL-6 > 7.9 ng/ml FP IL-6 ≤ 11 pg/ml	5	7 (1.5-32)
III	AF IL-6 > 7.9 ng/ml FP IL-6 > 11 pg/ml	6	1.2 (0.25-2)
IV	AF IL-6 ≤ 7.9 ng/ml FP IL-6 > 11 pg/ml	5	0.75 (0.13-10)

Figure 1.2 | FIRS scale. Measurement to determine stages in FIRS and average time of delivery after detection (R. Romero et al., 2007)

Role of fetus in spontaneous preterm birth

Some mechanisms help the establishment of an intrauterine immune response like clonal deletion of T lymphocytes, presence of T regulatory cells and T cell activity in the thymus and lymphoid organs. These regulatory mechanisms allow the set of a fetus innate and adaptive immune response. This response is revealed by the presence of neutrophil and monocyte activation in presence of PTB, elevation of IL-6 and C reactive protein (CRP) and activation of Th1 and Th2 response in umbilical cord in the presence of infection. The existence of an adaptive immune response in fetus has been demonstrated by the detection of IgE in cord blood. The fetal option of generating an immune response gives the risk of triggering FIRS, defined clinically as IL-6 plasma concentration higher than 11 pg/ml or histologically

as funisitis and chorionic vasculitis. Funisitis is associated with endothelial activation which is a key mechanism in neutrophil infiltration and organ damage (Lee et al., 2007; Roberto Romero, Gotsch, Pineles, & Kusanovic, 2007). There is an assumption that in context of infection and inflammation, there is a response that will present a survival value for both mother and fetus. In this response, the fetus presents an active role by secreting proinflammatory cytokines to signal the onset of labor and leave the inadequate environment. Therefore, FIRS is associated to the onset of labor by signalling of the fetus but also, by the involvement of chorioamniotic membranes and decidua (Gotsch et al., 2007).

Causes of FIRS

Fetal microbial invasion, intraamniotic inflammation or viral insults can result in the development of FIRS (Lee et al., 2007). As reviewed before, fetal invasion is also the main cause of the onset of PTB. In the presence of an insult, the fetus will produce an upregulation of fetal plasma MMP9 which is involve in the degradation of matrix collagen type IV (Roberto Romero et al., 2007). One of the major effector molecules in FIRS is IL-6, which even defines the whole syndrome. Some observations that compared the concentration of IL-6 in the umbilical artery and vein, suggested a fetal origin of the IL-6 upregulation in presence of FIRS (Gotsch et al., 2007).

Multiorgan involvement

FIRS can progress toward multiple organ involvement, causing organ dysfunction, septic shock and it can even produce death. The presence of FIRS can be differentially expressed in each fetal biological system. In the hematopoietic system, FIRS is expressed as major changes in granulocytes and red blood cells, presenting neutrophilia and activation of monocytes and neutrophils. The thymus of the FIRS patients seemed to be smaller when the syndrome comes from an infection/inflammation PTB. In the skin, FIRS patients present fetal dermatitis due to the constant recognition of microorganisms. Fetal plasma IL-6 concentration is

directly proportional to the presence of oligohydramnios, which leads to a reduced immunity for the fetus. Occurrence of FIRS is associated with changes in diastolic functions, hypotension and brain ischaemia; this decreased function has been presented as a correlation with IL-6 concentration in umbilical concentration. Existence of bacteria and inflammatory molecules in the amniotic fluid produces profound changes in the development of the lungs (Gotsch et al., 2007).

There is a strong group of evidence linking the presence of fetal inflammatory environment and brain injury. The proposed mechanisms for this relation is not only the systemic inflammation but also due to a limited ability of the fetus to buffer the effect of proinflammatory cytokines, mediators and/or oxidative stress (Gotsch et al., 2007).

Consequences of FIRS

Fetuses that experienced the presence of FIRS have a higher rate of diverse neonatal complications. One of the most critical derived consequences is perinatal death. Among other complications, the most common are cerebral palsy and bronchopulmonary dysplasia (Lee et al., 2007). Also, fetal changes in lung development due to the presence of FIRS causes a premature maturation which translates into significant predisposition of developing chronic lung diseases including bronchopulmonary dysplasia. All the proinflammatory effector molecules involved in FIRS can produce white matter damage which will lead to cognitive limitations, behavioural problems, visual-spatial difficulties and cerebral palsy (Gotsch et al., 2007).

Current treatments for FIRS

There is increasing interest into developing approaches to interrupt the course of FIRS and its later morbidities. Some of the used strategies are induction of delivery, use of antimicrobial treatment, administration of anti-inflammatory agents or a combination of these strategies. The generation of a viable clinical approach needs

to fulfill the attenuation of prematurity as well as the presence of intrauterine infection. The use of antimicrobial agents seemed to be promising because it reduced the neonatal morbidities and delayed the onset of parturition, but there was no information on the state of the already ongoing inflammation. It has been proven that the use of anti-inflammatory agents can prevent PTB (Gotsch et al., 2007). Therefore there is an increasing interest into developing selective anti-inflammatory agents as a valuable tool to prevent PTB and neonatal injury.

1.2 Interleukin-6

The immune response and effector phases of immune reactions are regulated by soluble mediators called cytokines. These mediators play a major cellular communication role, acting in a nanomolar-to-picomolar range. The most typical and multifunctional cytokines are IL-6, IL-1 and TNFα. Production of these cytokines is transient and induced by different stimuli in diverse cell types. Some of these stimuli are the presence of virus, bacteria, LPS and esters (Akira, Hirano, Taga, & Kishimoto, 1990). Before PTB, there is a significant increase of IL-6 in amniotic fluid and it has been studied the possibility of using this feature as a possible biomarker for the onset of PTB (Roberto Romero, Avila, Santhanam, & Sehgal, 1990). IL-6 is a member of the long-chain α-helix-bundle cytokines family. In this family, there is a subgroup that uses a common receptor subunit for signal transduction, gp130. This subgroup is denominated as IL-6-type cytokines and it comprises IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic granulocyte colonystimulating factor and cardiotrophin 1 (P C Heinrich, Behrmann, Müller-Newen, Schaper, & Graeve, 1998). IL-6 is a multifunctional cytokine with pleiotropic activities constituted by 184 amino acids arranged into four-helix (Boulanger, Chow, Brevnova, & Garcia, 2003; Romano et al., 1997; Stephanou, Isenberg, Akira, Kishimoto, & Latchman, 1998).

1.2.1. *Function*

IL-6 was originally identified as a T cell-derived molecule that stimulated antibody production and firstly called B cell differentiation factor (BCDF or BSF2).

Therefore one of the major functions of IL-6 is antibody production. IL-6 is also correlated with the regulation of immune response, hematopoiesis and inflammation. In hematopoiesis, IL-6 has a major role in thrombopoiesis by inducing maturation of megakaryocytes and increasing platelet count (Romano et al., 1997). In the brain, IL-6 has been suggested to be involved in reducing food intake and in normal brain aging (Godbout & Johnson, 2004). In inflammation, IL-6 is linked to acute phase response by the induction of production of acute phase proteins in the liver. This marked function has been confirmed by the observation of correlated upregulation of IL-6 with other acute phase proteins like CRP and serum amyloid A (Akira et al., 1990). The ability of IL-6 to induce such a diverse spectrum of functions depends on its ability to stimulate different signaling pathways (Stephanou et al., 1998).

1.2.2. *Interleukin-6 receptors*

Both main receptors of IL-6 signaling belong to the family of hemopoietic receptors, denominated more specifically as class I cytokine receptors (Niemand et al., 2003). The IL-6 receptor (IL-6R) is a low-affinity receptor expressed in several cell types and the first reports showed that the deletion of the intracytoplasmic and membranous sites of the IL-6R did not affect the signal transduction. The IL-6R system consists of two different polypeptide domains: a ligand binding and a signal transducing (Akira et al., 1990). The formation of a complex of IL-6-IL-6R induces the homodimerization of a signal transducer component named gp130, which is the second main receptor of the IL-6 signaling. This signal transducer is shared with all the family of IL-6-related cytokines such as leukemia inhibitory factor, oncostatin M and IL-11. Gp130, unlike IL-6R, is found ubiquitously and its IL-6-mediated activation on IL-6R-lacking cells is through the presence of a soluble form of the IL-6R (sIL6R). This soluble receptor can be found in biological fluids and is generated by shedding of the membrane-bound form due to the action of metalloproteinases of the ADAM family or by mRNA alternative splicing. The presence of this alternative form indicates a negligible function of the intracytoplasmic and transmembrane domains of the IL-6R (Romano et al., 1997; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011).

For activation to occur there must be the generation of an hexameric complex containing two IL-6, two IL-6R or sIL-6R and two gp130 that will assemble sequentially and cooperatively. For the formation of this complex, IL-6 presents in its structure, specific epitope sites for the binding either to IL-6R/sIL-6R or gp130 (Boulanger et al., 2003). After signal transduction, the IL-6R complex follows the typical membrane-protein-transport pathway and undergo endocytosis. This endocytosis is vital for the establishment of homeostasis. For the generation of receptor downregulation and receptor endocytosis, the involvement of the cytoplasmic domain of gp130 is crucial as it is a constitutive response of the dimeric form of gp130. Therefore, after binding, IL-6 is rapidly internalized and degraded in the lysosomal compartment. This activity is confirmed by the fact that to replenish the binding sites it is needed *de novo* protein synthesis (P C Heinrich et al., 1998). The possibility of complex formation between IL-6/sIL-6R generates a potential of generating an IL-6-induced response in all the cells in the body, which could generate an unconstrained response. Nevertheless, there is a soluble form of gp130 (sgp130) which was found in high concentrations in human plasma. This receptor is demonstrated to block IL-6 responses dependent on sIL-6R, letting membranous IL-6R response unaffected in Crohn´s disease patients. This selective response suggests that the activity of sgp130 is specific for the complex IL-6/sIL-6R, and that this soluble receptor is not able to interfere with IL-6 alone. The selective function is explained by the different affinity of either the complex or IL-6 alone against the receptor (Peter C Heinrich et al., 2003; Jostock et al., 2001).

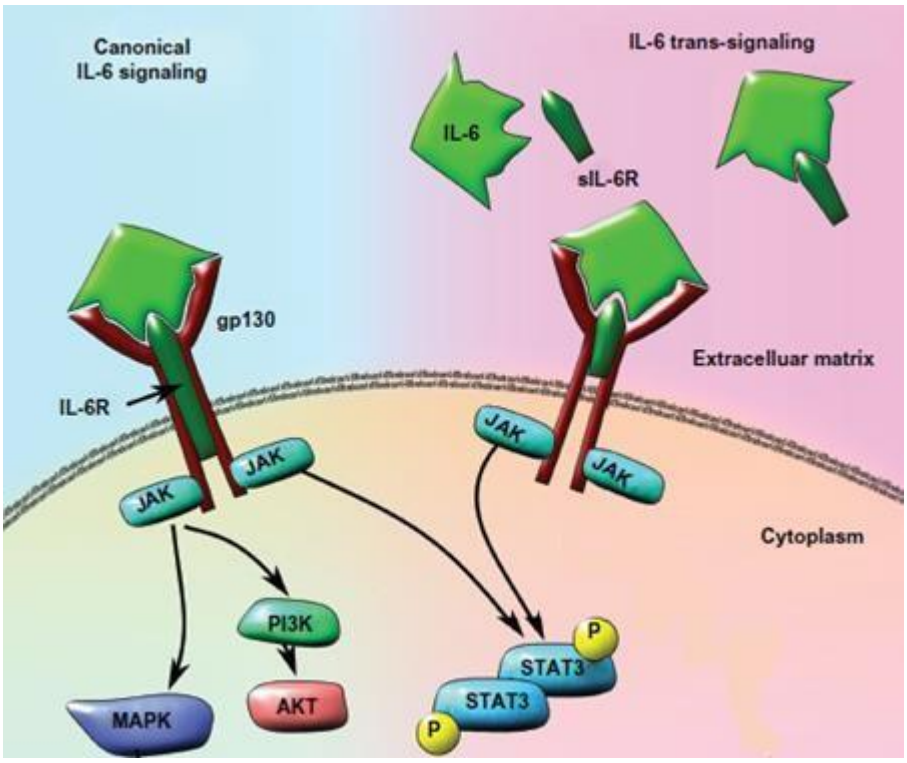


Figure 1.3 | IL-6 signalling pathway. General descriptive model design of the classical and trans-signalling pathway of IL-6 (Modified form: Tawara et al. 2011)

1.2.3. *Signaling pathway*

Studies on the IL-6 gene has shown different conserved regulatory regions in the promoter zone, like binding sites for NFκB, linking this molecule to the regulation of IL-6 induction. As abovementioned, IL-6 signaling pathway is mediated trough gp130 which can be found in cells that contain or not the IL-6R. When the IL-6R is stimulated by IL-6, both receptors (IL-6R and gp130), become associated at the extracellular portion (Akira et al., 1990). This association will induce the formation of a gp130 dimer which will activate Jak kinase by phosphorylating the catalytic site. Afterwards, there will be the production of tyrosine-specific phosphorylation of the signal transducer and activator of transcription 3 (STAT3) (Romano et al., 1997). Upon activation, STAT will generate the formation of homo- and heterodimers, which is a prerequisite for DNA binding. The sequences of DNA that will bind to STAT will depend on the composition of the dimer complex. This opens the possibility of selective gene activation according to the dimer formation. Therefore, once activated,

STAT dimers will translocate to the nucleus by the action of a short motif called nuclear localization sequence and afterward, they will exert their function in the nucleus. This function could be the enhancement, stimulation and repression of target genes. The known genes activated by IL-6 via STAT are acute phase proteins like CRP (P C Heinrich et al., 1998; Stephanou et al., 1998). After activity, STAT3 will get dephosphorylated and return to the cytoplasm to undergo additional rounds of activation (P C Heinrich et al., 1998).

Gp130 contains, in its cytoplasmic region (Tyr759), binding sites for protein tyrosine phosphatase 2 (SHP2). The binding of SHP2 mediates the activation of the Erk MAP kinases (Hirano, Ishihara, & Hibi, 2000). The activation via SHP2 is due by the linking of Gab1 to gp130, which will later recruit SOS to activate Ras and subsequently activating the Ras-Raf-MAPK cascade. This pathway is marked and studied by the initial phosphorylation of Erk1/2, process done merely by the IL-6mediated translocation of SHP2. The activation of Erk1/2 gives IL-6 a role of cell survival maintenance (Peter C Heinrich et al., 2003; Skiniotis, Boulanger, Garcia, & Walz, 2005).

Lastly, IL-6 is related to the prevention of induced apoptosis and cell growth; activity mediated by the activation of the protein kinase B (Akt) pathways (Hirano et al., 2000). This signaling cascade involves the activity of PI3K which, by modifying phosphatidylinositides, will recruit Akt to the plasma membrane for its activation by phosphorylation. Akt will act on the forkhead transcription factor and proapoptotic factor Bad to induce the protection activity (Peter C Heinrich et al., 2003).

After IL-6 activity, there are several mechanisms for signal termination. The first mechanism is the activation of protein tyrosine phosphatases, which in absence of a tyrosine phosphorylated binding partner, inhibits the enzymatic activity. This inhibitory activity is done by direct interaction of this phosphatases (SHP2, PTPε, PTPεC) with JAK1, JAK2 and TYK2. Another mechanism of termination is related to the activation of protein inhibitor of activated STATs (PIAS). These proteins are important transcriptional regulators of the JAK/STAT pathway and its selective activity against STATs depends on the presence of tyrosine phosphorylation of the STAT protein. The last mechanism involves the activation of the suppressor-ofcytokine-signalling (SOCS) proteins. This mechanism acts by inhibiting tyrosine

phosphorylation of gp130, STAT3 and STAT1 (Peter C Heinrich et al., 2003). Recent reports showed how in sustained activation of STAT3, the SOCS3 system gets depleted and the complex of IL-6/IL-6R exerts its action trough EGFR. In this EGFR system, there could be a prolonged and enhanced activation of STAT3 which can generate a pathological phenotype (Wang, van Boxel-Dezaire, Cheon, Yang, & Stark, 2013).

Classical IL-6 signaling pathway

This type of signaling pathway is restrained to the cell lines that present the IL-6R expressed in the membrane. The cells responsive to IL-6 alone are macrophages, neutrophils, some types of T-cells and hepatocytes (Scheller et al., 2011). In this IL-6R-containing cells, IL-6 exerts its activity on the target cells by binding to this receptor. Once together, this ligand/receptor complex binds to gp130 and generates its homodimerization which will result in transduction of the IL-6 signaling pathway (Jostock et al., 2001). The classical signaling pathway has been linked mostly to the generation of regenerative and anti-inflammatory activities (Scheller et al., 2011). The current work suggests that the classical signaling controls central homeostatic processes and immunological outcomes such as acute-phase response, glucose metabolism, hematopoiesis and regulation of neuroendocrine system, as well as hyperthermia, fatigue and loss of appetite (Hunter & Jones, 2015).

Trans-signaling IL-6 pathway

It is known that IL-6R shows a restricted expression. Despite this, there are reports of IL-6 function and cellular activities in the cells lacking this receptor. Examples of the IL-6 activity in cells without the IL-6R are presented in endothelial cells, survival of neurons, osteoclast formation and smooth cell activation. In the case of the cells lacking the expression of membranous IL-6R but containing the ubiquitous gp130, the sIL-6R will form a complex with the molecule of IL-6 and induce the dimerization of gp130 and later IL-6 signaling pathway (Jostock et al., 2001). There are current studies that try to explain the main reason for the existence of this

pathway and there are reports that link the trans-signalling to leukocyte migration, activation and apoptosis into the inflamed tissue (Saavedra Ramírez, Vásquez Duque, & González Naranjo, 2011; Scheller et al., 2011). Studies indicate that the trans-signalling pathway is related to regulation of the liver regeneration and inflammatory response (Scheller et al., 2011). It has been linked to recruitment and apoptosis of leukocytes, maintenance of the effector function of T cells and inflammatory activation of stromal tissues. There is evidence that proves that the activation of the shedding of the IL-6R depends on the presence of inflammatory mediators, thus the presence of the sIL-6R can be used to detect a danger signal (Hunter & Jones, 2015). Current reports showed the presence of a mutation in Ala358 on the IL-6R, which generates a higher blood level of sIL-6R; these individuals seemed to be less susceptible to autoimmune diseases such as rheumatic arthritis. This discovery gives the possibility of an anti-inflammatory role to the sIL-6R by the possibility of the generation of a buffer system formed by the sIL-6R and the sgp130. The presence of this alternative system opens the possibility for the development of different therapeutic approaches (Garbers, Apariciosiegmund, & Rose-john, 2015).

1.2.4. *Role of IL-6 in inflammation*

IL-6 cytokine signaling results in the regulation of diverse complex cellular processes such as proliferation, differentiation and gene activation (P C Heinrich et al., 1998). IL-6 exerts both pro- and anti-inflammatory activities. This dual activity is demonstrated by the ability to stimulate T cell proliferation and their differentiation into cytotoxic T cells, stimulate antibody production and induce acute phase proteins (Niemand et al., 2003). This double function allows IL-6 to be a versatile molecule that can generate either a protective and/or an amplification of the inflammatory reaction (Kristiansen & Mandrup-Poulsen, 2005). Hence, IL-6 has clear proinflammatory effects, but depending on the context, it can also generate an antiinflammatory response for the resolution of its own inflammation (Hunter & Jones, 2015).

a) Pro-inflammatory

Studies on the different functions of IL-6 gave confirmatory evidence on the role of IL-6 in acquired immunity. These studies showed an IL-6-driven amplified leukocyte accumulation at the sites of inflammation by the augmentation of chemokines (Romano et al., 1997). This immunity reaction is started by the generation of a first phase of acute inflammation, which will cause an initial infiltration of neutrophils, monocytes and T-cells. IL-6 plays a major role in neutrophil attraction, which is one of the first pieces of evidence portraying IL-6 as one of the main players in inflammation (Scheller et al., 2011). The pro-inflammatory activity of IL-6 is been widely studied due to the fact that IL-6 leads to the increased synthesis of acute phase reactants such as CRP, serum amyloid A, hypoalbuminemia, increased platelet count and development of AA amyloidosis which will sustain the inflammatory environment (Okuda, 2008). The pro-inflammatory capacity of IL-6 is driven by the activation of STAT3 due to tyrosine phosphorylation. STAT3 activation supports the sustained expression of a subset of inflammatory IL-6-induced proteins (Wang et al., 2013).

b) Anti-inflammatory

There are reports of suppression of TNFα synthesis in activated macrophages mediated by the action of IL-6. These roles are established by the action of similar dimers of STAT to the ones created by the action of IL-10 (El Kasmi et al., 2006). It is reported that this anti-inflammatory role is due to the activation of the major feedback inhibitor system regulated by SOCS3. It is hypothesized that the activation of SOCS3 will modify STAT3-mediated activation of pro-inflammatory stimuli. Other reports have established a crucial role of Erk1/2 in the blocking of STAT3 mediated pro-inflammatory response (Niemand et al., 2003). Under the same perception, Erk1/2 is reported to present a regulatory role against the expression of inflammatory genes in vascular cells (Maeng et al., 2006).

1.2.5. *Diseases related to IL-6*

IL-6 upregulation has been identified in a broad spectrum of syndromes. Several of the aging-related diseases like Alzheimer, dementia, Parkinson, etc., present a profile of IL-6 upregulation due to a normal age-linked increase in IL-6 plasma concentration. This IL-6 rise presents direct effect on the brain, translating in neurotoxicity and affecting cognitive and emotional systems. These results are correlated by the discovery of high IL-6 levels after head trauma and CNS infections (Godbout & Johnson, 2004). Upregulation of IL-6 has been described in many other non-aging related syndromes like diabetes (Kristiansen & Mandrup-Poulsen, 2005), rheumatoid arthritis (Okuda, 2008), cancer (Shinriki et al., 2009), systemic lupus erythematosus, osteoporosis, spondyloarthropathies, juvenile arthritis, Castleman´s disease and Crohn disease (Saavedra Ramírez et al., 2011).

a) Current modulators of IL-6

IL-6 is an attractive therapeutic target because it is a regulator of the acute phase inflammatory response. Also, during an inflammatory episode, IL-6 is highly expressed and upregulated which translates into its categorization as the most highly expressed mediator of inflammation (S. a Jones, Scheller, & Rose-john, 2011).

The major clinically available modulator of the IL-6 activity is Tocilizumab (TOC) which is a humanized anti-IL-6 receptor antibody. TOC acts by inhibiting the induction of biological activity due to IL-6 in cells that express or not the IL-6R, therefore, this antibody is able to competitively inhibit the classical and the transsignalling pathways. This medication has shown excellent improvement in the treatment of rheumatoid arthritis by inhibiting the joint destruction (Okuda, 2008). TOC as well has been proven to decrease capillary vessels and reduced tumor growth in carcinomas (Shinriki et al., 2009).

Some other anti-IL-6 drug is CNTO-328 which is a monoclonal chimeric antibody and is now in clinical trials for prostate, kidney and renal cancer; and a new small protein called avimer. This avimers are less immunogenic because they lack the immunoglobulin domains, and therefore, it is expected to present reduced side

effects. The type of avimer against IL-6 is called AMG-220 and it already presented an IC_{50} in the picomolar range and an *E. coli*-based production. AMG-220 is currently in phase 1 for Crohn´s disease (Tawara et al., 2011).

A current promising approach to modulate the IL-6 signaling is the use of the soluble gp130-Fc form. This molecule was made by coding the extracellular portion of gp130 and fused to the constant region of the human IgG1 heavy chain. This molecule is used merely for investigation but it has shown to inhibit acute phase protein synthesis and the anti-apoptotic effect on Crohn disease patients (Jostock et al., 2001).

1.3. Drug antagonism

Drugs have core molecular properties, called affinity and efficacy, which donate them unique biological activity and helps them generate a wide range of behaviors depending on the physiological and pharmacological context. Both characteristics can be described in terms of energy flow between a modulator, a conduit and a number of guests (T. Kenakin & Miller, 2010). The affinity could be defined as the energy needed for the ligand to bind to the receptor and efficacy could be seen as the property that produces a change of the receptor activity towards the cell, this will be, a physiological response (T. Kenakin, 2007). It is now considered that ligand activity is due to their ability to bind selectively to pre-existent receptor conformation depending on their affinity and generating stability in their preferred state. These receptor states will modify the receptor affinity either for other ligands or effector proteins (Vaidehi & Kenakin, 2010).

As receptors were seen as on-off switches, drugs were classified by their ability to either fully or partially activate (full or partial agonists) or inactivate the receptors (antagonists). The conventional view of antagonists was molecules that occupied the endogenous agonist-binding site to produce a blockade of effect and generating an inoperative receptor. This conventional view classified antagonism as a non-permissive process. After this notion, another vision classified antagonism as

permissive because of the reports that showed molecule ability to block some of the receptor signal. This permissibility gives the antagonist agents some texture (T. Kenakin, 2005).

1.3.1. *Types of antagonism*

It was first thought that receptor activation by the binding of a ligand was able to generate all possible responses of the receptor and therefore the ligand activity was described to have a linear efficacy. It is now known that the receptor ligands can selectively induce a specific behavior in the receptor. This behavior introduces a new characteristic named collateral efficacy. This collateral efficacy is explained by the possibility of the ligands to stabilize different forms of receptor conformations that could later activate just some of the receptor´s repertoire of behaviors. Several reports have shown that different agonists can select or emphasized different and specific signaling pathways (T. Kenakin, 2005).

Antagonism is linked to the turn off of the receptor. Its activity can be done by occupancy of the binding site with no effect and thus, canceling agonist stimulation. This generates a non-permissive antagonism. On the other hand, the effect can be done by the binding of the antagonist to its own site on the receptor and forming a ternary specie which will be formed by the receptor, the agonist and the antagonist. This is termed allosteric modulation and it is a permissive behavior because it can modify the receptor reactivity in different ways such as increasing or decreasing affinity and/or efficacy (T. Kenakin, 2005).

The set of non-permissive antagonists are also called orthosteric modulators which effect on the ligand is done through steric hindrance. The steric hindrance prevents the binding of another agent because they compete for the same space. Orthosteric antagonists will uniformly block the receptor despite any stimulus that the receptor can receive. This allows a state where if there is enough antagonist the block down can reach to basal levels. In the permissive antagonist, we can find the allosteric agents which can block some signal and allow other to pass, donating some texture in the blockade (T. Kenakin, 2005).

The type of effect that the agent will have will also generate their classification in different types of antagonists. There are ligands that have a preference for the inactive state of the receptor and they can even reverse the presence of a constitutive activity of the receptor. These type of ligands are named inverse agonists. Another type of antagonists is neutral antagonists. This second type of antagonists will not promote a response or an inverse agonism because they do not have any preference for either the active of inactive receptor state. Lastly, there are protean agonists which are ligands that can produce activation of the receptor but in an even lower efficacy than the constitutively active species. This type of antagonism is presented just in systems with constitutive activity (T. Kenakin, 2007).

1.3.3. *Molecular mechanism of action of antagonists*

Receptors present a repertoire of behaviors by its ability to form homo- and heterodimers which will later modify the response in the downstream signaling which can translate in different types of phosphorylation, desensitization and internalization. Receptors are dynamic conglomerates and changes as little as 1 Å can lead to profound effects on the activity of receptors (T. Kenakin & Miller, 2010). As stated before, all receptor activity requires the first step of activation in order to avoid cellular overstimulation. A step of activation refers to the capacity of a ligand to lock an active state of the receptor where it can start producing cellular processes.

This “active state” was seeing, based on the basal activity of the receptors, as a single receptor conformation, but it is now known that there are numerous existent actives states for each receptor (Vaidehi & Kenakin, 2010). Thus, the receptor presents various conformational states depending on the system energy levels and it generates “conformational ensembles” that will generate various physiological conditions. The ligands can either induce a certain receptor conformation or have a preference for one of these ensembles (T. Kenakin & Miller, 2010). Its binding, thus, will lock this “preferred” conformation causing a complete bias to produce more receptors in the same state. This activity changes the view of ligand activity to an active process that will bias to a unique ensemble. Therefore, the allosteric modulation presents the possibility of preserving complex biological patterns of

response (T. Kenakin, 2005). The modulation is thought to be due to the effect of order/disorder transitions that mediate long-range allosteric communication and the fact that the binding of molecules reduces the dynamic motion, giving an idea that allosteric agents are able to “locked” specific receptor conformations (T. Kenakin & Miller, 2010). This functional selectivity is seeing even by the action of agonists, where they do not uniformly activate the cell pathways. This is been referred to as biased agonism (T. Kenakin, 2007). It is important to highlight that, for the functional selectivity to occur, it is needed the lock up of specific conformations of the receptor (T. Kenakin & Miller, 2010).

The efficacy on the activity on a receptor will be determined by its rate of conformational change (Vaidehi & Kenakin, 2010). There is the presence of a discontinuity between intensity and duration of action. For an orthosteric antagonist, both are determined by the rate of offset of the molecule and its concentration in the receptor, therefore in this kind of antagonist, the duration is going to be directly linked to the concentration. In the other hand, an allosteric antagonist can produce a saturation of the allosteric binding site. This saturable effect can produce a maximum possible effect of the modulator (T. Kenakin, 2005).

1.3.4. *Allosteric agent*

An allosteric effect is the imposition of an effect on a protein through an interaction of a molecule with a site on the protein distinct from the natural binding locus. The interaction then occurs through the protein and not through steric interaction. The ability of this ligands to generate a large scale of effects on the receptor makes us referred to them as allosteric agents or modulators. The allosteric agent will change the shape of the receptor, probably through conformational selection and stabilization of one or more ensembles of states. The generation of specific states will donate a set of reactivities to other ligands and cellular compartments (T. Kenakin, 2005; Vaidehi & Kenakin, 2010). The possibility of stabilizing different unique receptor conformations through conformational selection opens the possibility of specifically activate a specific stimulus in the cell, and therefore, modify the cellular response (T. Kenakin, 2007). This receptor activity

modulation can be directed towards the ectodomain by modifying the drug effect, the cell membrane by modifying the receptor oligomerization and/or an effect in the cytosol by generating a differential signaling (T. Kenakin & Miller, 2010). At the same time, it opens more responsibility at the time of drug design, because there is a need of deeply understanding of various conformational states to correctly modulate a receptor (Vaidehi & Kenakin, 2010).

One of the most important characteristics of allosteric modulators is the fact that these agents will bind to separate binding sites than the natural agonist. This is the characteristic that allows them to generate a shift on the response effect. The allosteric agents present a unique quality, named probe-dependent effects on the receptor. The term “probe” is related to any molecule that can bind to the receptor and can be used to measure the state of the receptor. This characteristic describes the function that an allosterically modulated receptor is not simply insensitive to stimulation but has a rather modified reactivity to all probes. For the generation of texture signaling response, the allosteric agent should allow agonist to bind to the receptor and thus, produce a different tertiary conformation (T. Kenakin, 2005).

A second property of the allosteric agents is the presence of a saturable nature, which makes possible a limited change in the affinity of the receptor for the stimulus without complete blockade (T. Kenakin, 2005). This is explained graphically by the presence of an asymptote maximum of effect in the point of complete occupancy of the allosteric site. Compared to an orthosteric modulator which can theoretically produce infinite competitive effects generating a dextral displacement of the agonist response curve (T. Kenakin & Miller, 2010). The third allosteric property is related to its possibility of differentially modulate ligand affinity and efficacy. The last feature of allosteric agents is the extremely selectivity for the receptor type because of its unique binding site (T. Kenakin & Miller, 2010). Some unique effects presented in the allosteric agents are: 1) capacity of modifying the interaction sites of large proteins; 2) specifically modulate the receptor function, this can help them modulate one of the signalling pathways while preserving the physiological functions which can lead to reduced side effects; 3) production of texture antagonism which can be confirmed by its property of differentially modulate ligand affinity and efficacy (T. P. Kenakin, 2012).

1.4. Small peptides as therapeutic agents

Structure-based drug design helps combine biological and structural data to create synthetic compounds that will generate a biological function. Thus, proteins can be used as templates to design small molecules or peptides that can either compete for the ligand binding site or that can modulate the conformation of the receptor. For these small peptides to generate its bioactivity they should mimic the protein chains and present a certain level of flexibility to allow the small peptide to adopt the shape and dynamics of the native protein. Studies have shown that small peptides can assume a relevant structure and mimic the biological function of a region of a protein (McDonnell et al., 1996).

1.4.1. *Generation of an antagonist based on a small peptide*

The notion of the use of small peptides is to force the protein to take a desired conformation by inducing conformation restraints. Rational design of specific inhibitors and screening assays are needed in order to generate these small peptides (Chalifour et al., 2003). Because of the receptor conformational entropy, the binding of a small peptide results in a small decrease in entropy due to the adoption of a bioactive conformation. It has been shown that small peptide inhibitors can be effective in inhibiting protein-protein interaction that occurs over large surfaces. This is supported by the theory that in receptor interactions only a small subset of residues within the binding site may be responsible for the majority of the binding energy (McDonnell et al., 1996). Thus, small peptide-driven inhibition can be due to the hindering of specific inhibition of receptor dimerization, by the blockage of the natural ligand binding site or by the generation of specific receptor conformation (Quiniou et al., 2008).

1.4.2. *Allosteric small peptides*

Receptors are formed from different motifs with unique functions. Loop regions of receptors are particularly susceptible to be functionally interfered by the action of small peptides that reproduce its sequence. The reason of this susceptibility is due to the fact that these regions are crucial for the receptor to take certain conformation or for its interaction with other subunits and probes. The generation of small peptides based on those motifs generates modulators which are independent of the ligand binding site (Quiniou et al., 2008). The binding region allows to classify them as allosteric modulators and opens the possibility of generating an allosterictype inhibition and activity. This property will allow these modulators to alter the signaling and downstream responses without inhibiting completely the receptor function and also provide higher selectivity (Anderson, Tejo, Yakovleva, & Siahaan, 2006).

1.4.3. *633 design*

This work is based on the characterization of a small peptide, named 633, which can act as an antagonist of the IL-6R. The design of this peptide could be done given the high importance of the IL-6R/gp130 complex receptor dimerization. This behavior categorizes this complex as a highly dynamic system and shows the presence of several interaction sites within the receptor (Hebert et al., 1996). The 633 peptide is formed by 9 D-amino acids. The decision of making 633 as a Dpeptide was premeditate in order to facilitate stability. There are reports showing the biological activity of peptides formed by D-amino acids and that the presence of this stereochemical entities does not affect the potency of the peptide (Chalifour et al., 2003). 633 was designed by a rational drug design technique. The structure was developed by analyzing the sequence of the extracellular domains of the IL-6R. Once analyzed, the hydrophobic, flexibility and homology domains were identified by crystallography and modeling data. Based on loops and interdomain regions, 633 was designed as a homologous peptide (Quiniou et al., 2008).

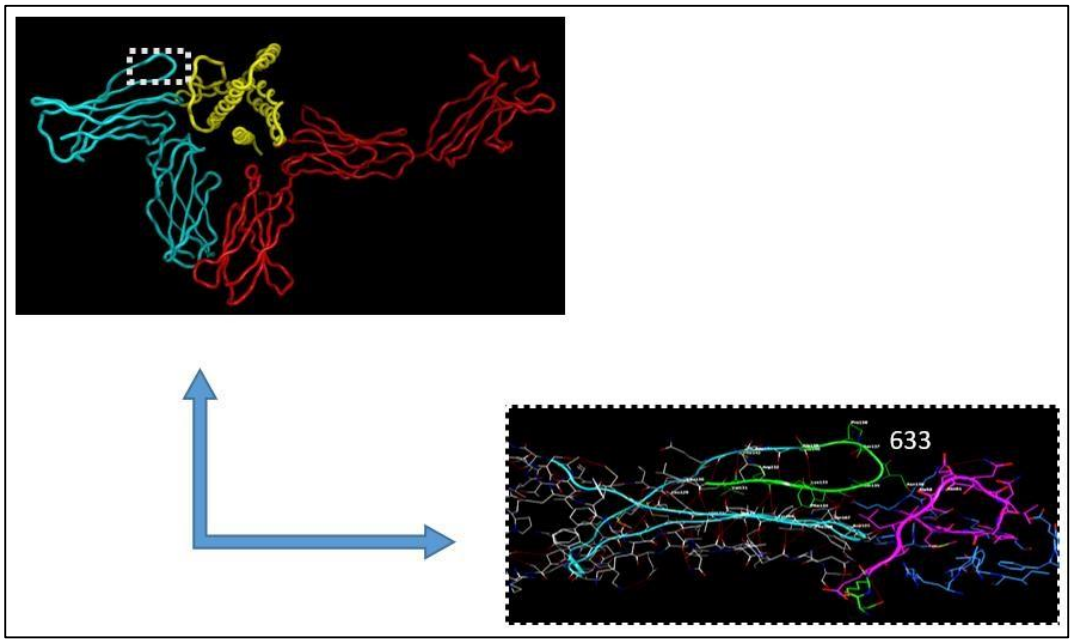


Figure 1.4 | 633 based design . Graphic demonstration of IL -6R motif used as template source for the construction of the 633 small peptide.

HYPOTHESIS

As described above, PTB is an increasing health concern worldwide. The reason why there are not prophylactic measures or treatment is because even in half of the cases the cause is unknown. The only causal link is the presence of an infection that will later translate into the onset of PTB. There are reports showing the prevalent presence of inflammation in the cases of PTB. One of the major regulators of inflammation in PTB is IL-6. It has been shown that IL-6 gets significantly upregulated in amniotic fluid before the onset of PTB, to the point that there are reports trying to use this molecule as a biomarker of the onset of PTB. It is also reported that the IL-6-induced inflammation in the neonate could be the cause of the long-term morbidities that associate to PTB.

IL-6 is a pleiotropic protein that generates its activity by the formation of a hexameric complex formed by 2 molecules of IL-6, IL-6R and gp130. IL-6 presents diverse physiological functions by the activation of three main signaling pathways whose role starts by the phosphorylation of either STAT3, Akt or Erk1/2. IL-6 presents a major role in inflammation by the activation of the STAT3 pathway.

There is currently a clinical available competitive antagonist called Tocilizumab (TOC). This drug is a monoclonal antibody which inhibits all three signaling pathways of IL-6, therefore causing major immunological side effects. The improvement of the structure-based drug design allowed our group to develop a small peptide, named 633, which is an antagonist of the IL-6R. The theory states that this structure-based small peptides are able to differentially modulate the signaling pathways.

We hypothesized that 633 is an allosteric modulator of the IL-6R that can selectively modulate the IL-6 signaling. We think that the specific 633-driven modulation will reduce the inflammation that will later translate into PTB, thus, preventing the onset of PTB and its co-morbidities.

OBJECTIVES

To elucidate the modulation of 633 in the three IL-6 signaling pathways *in vitro*.

To analyze the 633 potential inhibition of inflammation by the generation of dose-response curves measuring the IL-6-induced upregulation of inflammatory cytokines *in vitro*.

To analyze the IL-6-driven inflammatory response in relevant mouse gestational tissues like uterus, placenta and fetal membranes.

To corroborate 633 function by the generation of dose-response curves in relevant mouse gestational tissue.

To analyze 633 behaviour by the generation of a functional antagonism analysis on the generation of inflammatory cytokines *in vitro*.

To validate IL-6 as a key target by the generation of an IL-6-induced PTB model in mice.

To analyze 633 effect on the prevention of prematurity in the IL-6-induced PTB model in mice.

To analyze gene upregulation in gestational tissue and leukocytes of mice exposed to the IL-6-induced PTB model.

To corroborate 633 efficacy on an LPS-induced PTB in mice.

To analyze gene upregulation in gestational tissue and leukocytes of mice exposed to the LPS-induced PTB model.

To analyze protein upregulation in amniotic fluid and maternal plasma of mice exposed to the LPS-induced PTB model.

To validate 633 benefit on the survival and weight of the neonates exposed to the LPS-induced PTB model.

2. MATERIALS AND METHODS

2.1. Animals

Timed pregnant CD-1 mice were obtained from Charles River at gestational day 11 and were allowed to acclimatize in housing cages for 5 days prior to experiments. Mice were housed alone in each cage. Animals were used according to a protocol of the Animal Care Committee of Sainte-Justine Hospital along to the principles of the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. The animals were maintained on standard laboratory chow under a 12:12 light:dark cycle and allowed free access to chow and water.

2.2. Chemicals

The chemical products were purchased to the following companies: Lipopolysaccharides (LPS) of *Salmonella typhimurium* (#L2630, Sigma Saint Louis MO, USA), 633 (Elim Biopharmaceuticals, Hayward, CA), Tocilizumab (TOC, #186639, Hoffmann-La Roche Limitée, Mississaugua, ON, CA), Human IL-6 (#20006, Peprotech, Rocky Hill, NJ, USA), Recombinant Murine IL-6 (#216-16, Peprotech, Rocky Hill, NJ, USA).

2.3. Cell culture

HEK and HEK-blue IL-6 cells were purchased (#hkb-hil6, InvivoGen, San Diego, Ca, USA) and used at passages under 15. Cells were cultured in DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, 50 mg/ml streptomycin. HEK-blue IL-6 cells media was also supplemented with Zeocine (10µl/10ml of DMEM) and Hygromycine B Gold (20µl/10ml of DMEM). Cells were propagated in regular conditions (37°C, 5% CO_2). Passages were done only when cell confluence was at least 85% of the surface of the plate. For cell passage, media was removed and two washes were done with phosphate-buffered saline (PBS, R&D,

Minneapolis, MN, USA). Then, cells were detached with treatment with trypsin for 5 min and complete media was allocated into the new plates with the detached cells.

2.4. Analyzing of IL-6 signaling

For signaling experiments, cells were allowed for two passages before start working. This two passages were done in order to complete stabilization of HEKblue IL-6 cells receptor. Prior to experiment, cells were starved overnight and media was changed 30 min before the beginning of the stimulation. Subsequently to the 30 min, 633 (10^{-6} M) or TOC (10^{-3} M) was added to allow equilibrium for 15 min.

Afterwards, cells were treated with 0.1 µg/ml IL-6 for 10 min. Cell lysis was performed in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors in addition to Laemmli buffer. Samples were stored at -20°C until its use in Western blotting.

2.4.1. *Immunoblotting*

A total of 30 µl of protein obtained with radioimmunoprecipitation assay buffer was loaded onto SDS-PAGE gel and electrotransferred onto polyvinylidene diflouride membranes. After blocking with milk 5%, membranes were incubated with either an antibody (Ab) against phospho-STAT3 (#9134; Cell-Signalling Technology, Danvers, Massachusetts, USA), phospho-AKT (#9271; Cell-Signalling Technology) or phospho-Erk1/2 (#9101; Cell-Signalling Technology). After washing, membranes were incubated 2 h with respective secondary antibody conjugated to HRP (SigmaAldrich). Samples were normalized by following membrane stripping and incubation with Ab against STAT3 (#9132; Cell-Signalling Technology), AKT (#9272; CellSignalling Technology) or Erk1/2 (#4695; Cell-Signalling Technology). ECL (GE Healthcare, Little Chalfont, UK) was used for detection using the ImageQuant LAS500 (GE Healthcare), and densitometric analysis was made with ImageJ.

2.5. IL-6 dose-response curves *in vitro*

For dose-response *in vitro* experiments, cells were allowed for two passages before start working. This two passages were done in order to complete stabilization of HEK-blue IL-6 cells receptor. Prior to experiment, cells were starved overnight and media was changed 30 min before the beginning of the stimulation. Subsequently to the 30 min, 633 (from 10^{-4} to 10^{-12} M) was added to allow equilibrium for 15 min. Afterwards, cells were treated with 0.1 µg/ml IL-6 for 6 h. Cell lysis and mRNA extraction was performed directly into RIBOzol (AMRESCO, Solon OH, USA). Samples were stored at -20°C until its use in quantitative PCR.

2.6. Functional IL-6 antagonism analysis *in vitro*

For the generation of a functional antagonism analysis *in vitro*, cells were allowed for two passages before start working. This two passages were done in order to complete stabilization of HEK-blue IL-6 cells receptor. Prior to experiment, cells were starved overnight and media was changed 30 min before the beginning of the stimulation. Subsequently to the 30 min, 633 (from 10^{-5} to 10^{-10} M) was added to allow equilibrium for 15 min. Afterwards, cells in each set of concentrations of 633 were treated with different concentrations of IL-6 (from 10^{-9} to 10^{-15}) for 6 h. Cell lysis and mRNA extraction was performed directly into RIBOzol (AMRESCO, Solon OH, USA). Samples were stored at -20°C until its use in quantitative PCR.

2.6.1. *Cell RNA extraction*

Extracted cells in RIBOzol were thawed. RNA of cell samples was extracted according to manufacturer´s protocol. Briefly, chloroform was added in a proportion of 1:5 chloroform:RIBOzol, and the samples were centrifuged at 13,000 rpm for 30 min. The aqueous phase was recuperated and 500 µl of isopropanol was added and incubated in -20°C for 20 min. After incubation, samples were centrifuged and the pellet was washed twice with ethanol. Later, the pellet was dried and solubilized in RNAse free water. RNA concentration and purity was assessed with NanoDrop 1000

spectrophotometer. After quantification, 500 µg of pure RNA was used to synthetized cDNA with iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA).

2.7. IL-6 stimulation of tissue *ex vivo*

Timed pregnant CD-1 mice at gestational day 16.5 were sacrificed and fragments of uterus, placenta and fetal membranes were collected on PBS where explants were cut into pieces of 3 mm x 3 mm. After cutting, each explant fragment was cultured in a well of a 12-well plate and covered with DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, 50 mg/ml streptomycin for 30 min at normal growth conditions (37°C, 5% CO_2). Explants were later starved for 30 min with DMEM media. After, explant were exposed with 633 (10^{-6} M) for 15 min to allow equilibrium. Later, explants were stimulated with IL-6 (0.1 µg/ml) for 6 hours and collected directly into RIBOzol (AMRESCO). Solutions were stored at -80°C until RNA extraction.

2.7.1. *IL-6 dose-response curve ex vivo*

Timed pregnant CD-1 mice at gestational day 16.5 were sacrificed and fragments of uterus were collected on PBS where explants were cut into pieces of 3 mm x 3 mm. After cutting, each explant fragment was cultured in a well of a 12-well plate and covered with DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, 50 mg/ml streptomycin for 30 min at normal growth conditions (37°C, 5% CO_2). Explants were later starved for 30 min with DMEM media. After, explant were exposed with 633 (from 10^{-4} to 10^{-12} M) for 15 min to allow equilibrium. Later, explants were stimulated with IL-6 (0.1 µg/ml) for 6 hours and collected directly into RIBOzol (AMRESCO). Solutions were stored at -80°C until RNA extraction.

2.8. Intraperitoneal IL-6- and LPS-induced PTB models

The used model is based on previous literature and confirmed by recent reports which confirmed the significance of the LPS model of PTB (Migale et al., 2016). Timed pregnant CD-1 mice at gestational day 16.5 were anesthetized with isoflurane and received an intraperitoneal (i.p.) injection of either IL-6 (single dose of 1 µg in 100 µl saline), LPS (single dose of 10 µg in 100 µl saline) or vehicle (100 µl). Doses of IL-6 and LPS were selected based on reported documentation (Chang et al., 2011; Elovitz & Mrinalini, 2004; Fidel et al., 1994; Sadowsky, Adams, Gravett, Witkin, & Novy, 2006; Salminen et al., 2008; Urakubo, Jarskog, Lieberman, & Gilmore, 2001) and on *in vivo* dose-response experiments. A total of 100 µl of 633 (1 mg/Kg/12 h), TOC (10 mg/Kg/12 h) or vehicle was injected subcutaneously (s.c.) in the neck 30 min before stimulation with LPS or IL-6 (to allow distribution of drugs to target tissues) and these injections were followed until day 18.5; all doses used were based on reported literature (Nadeau-Vallee et al., 2015; Okuda, 2008; Quiniou et al., 2016). A set of animals, were anesthetized 12 or 24 hours after the LPS or IL-6 injection respectively, and an intracardiac puncture was performed to collect maternal systemic blood and deposited in tubes with EDTA to prevent blood from clotting. Blood plasma was obtained by centrifugation. The remaining blood pellet was treated with red blood cell (RBC) lysis buffer (Norgen Biotek, Thorold ON, CA), according to manufacturer protocol, to isolate white blood cells (WBCs). Plasma, WBCs, amniotic fluid, myometrium, placenta and fetal membrane samples were snap frozen in liquid nitrogen and kept at -80°C for subsequent RNA purification. With the remaining set of animals, delivery was assessed until term (G19-19.5) and in the LPS-induced PTB mice model the weight and survival rate of the neonates was recorded.

2.8.1. *Circulating leukocyte RNA purification*

Isolated WBCs were treated for RNA extraction using a leukocyte RNA purification kit (Norgen Biotek, Thorold, ON, CA). Briefly, WBC pellets were resuspended and vortexed in chloroform and then passed through a RNA-binding column. Several washes were done and RNA was eluted and collected into

RNAsefree tubes. RNA concentration and purity was assessed with NanoDrop 1000 spectrophotometer. After quantification, 500 µg of pure RNA was used to synthetized cDNA with iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA).

2.8.2. *Tissue RNA extraction*

Extracted myometrium, placenta and fetal membrane samples were thawed and deposited in RIBOzol. The obtained samples were crushed with sonicator homogenizer until all tissue was capable of passing through a 1000 µl pipet tip. RNA of different tissue solution sample was extracted according to manufacturer´s protocol. Briefly, chloroform was added in a proportion of 1:5 chloroform:RIBOzol, and the samples were centrifuged at 13,000 rpm for 30 min. The aqueous phase was recuperated and 500 µl of isopropanol was added and incubated in -20°C for 20 min. After incubation, samples were centrifuged and the pellet was washed twice with ethanol. Later, the pellet was dried and solubilized in RNAse free water. RNA concentration and purity was assessed with NanoDrop 1000 spectrophotometer. After quantification, 500 µg of pure RNA was used to synthetized cDNA with iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA).

2.8.3. *Real-time quantitative PCR*

Primers were designed using the National Center of Biotechnology Information Primer Blast. Quantitative analysis of gene expression was performed with Stratagene MXPro3000 (Stratagene) using SYBR Green Master Mix (Bio-Rad). All results were normalized by the value of the expression of 18S with universal primers (Ambion Life Technology, Burlington ON, CA). Dissociation curves were performed to confirmed primer specificity. All primers were previously tested to prove efficacy and selectivity. Genes analyzed include: *IL1B, IL6, IL12, TNFA, PTGER3, CCL2, PTGHS2, RELA, CASP1, OXTR, NFKB, PGES* and *CXCL15.* Primer sequences are shown in table 2.1.

Table 2.1. Primers used for real-time quantitative PCR

Mouse Primers	
IL1B-F: 5´-CAACGATGATGCACTTGCAGA-3´	IL1B-R: 5´-GGAAGGTCCACGGGAAAGAC-3´
IL6-F: 5´-AGATGAAGGGCTGCTTCCAAA-3´	IL6-R: 5´-TCTCTCTGAAGGACTCTGGCT-3´
IL12-F: 5´-TTCTCACCGTGCACATCCAA-3´	IL12-R: 5´-GAGGAGGTAGCGTGATTGACA-3´
TNFA-F: 5´-ATGGCCTCCCTCTCATCAGT-3´	TNFA-R: 5´-TGGTTTGCTACGACGTGGG-3´
PTGER3-F: 5´-ATCATGTGTGTGCTGTCCGT-3´	PTGER3-R: 5´-TCAGGTTGTTCATCATCTGGCA-3´
CCL2-F: 5´-GCTCAGCCAGATGCAGTTA-3´	CCL2-R: 5´-TGTCTGGACCCATTCCTTCT-3´
PTGHS2-F: 5´-TTAGCCCCAATAAGCCCAGG-3´	PTGHS2-R: 5´-TTTCTCTCCCTGATGCGTGG-3´
RELA-F: 5´-TCTGCCCAGACCGCAGTATC-3´	RELA-R: 5´-TTGGTCTGGATTCGCTGGC-3´
CASP1-F: 5´-CACAGCTCTGGAGATCGGTGAA-3´	CASP1-R: 5´-CCACGGCATGCCTGAATAATG-3´
OXTR-F: 5´-TGTGTCTCCTTTTGGGACAA-3´	OXTR-R: 5´-GGCATTTCAGAATTGGCTGT-3´
NFKB1-F: 5´-CCTGCTTCTGGAGGGTGATG-3´	NFKB1-R: 5´-CAGCCTTCTCCCAAGAGTCG-3´
PGES-F: 5´-CGCAAGAAGAAGGCTTTTGCCAAC-3´	PGES-R: 5´-AGCCGAGGAAGAGGAAAGGA-3´
CXCL15-F: 5´-TGCTTTTGGCTTTGCGTTGA-3´	CXCL15-R: 5´-GTCAGAACGTGGCGCTATCT-3´
Human Primers	
IL1B-F: 5´-AGCTGGAGAGTGTAGATCCCAA-3´	IL1B-R: 5´-ACGGGCATGTTTTCTGCTTG-3´
IL6-F: 5´-ACCCCCAGGAGAAGATTCCA-3´	IL6-R: 5´-CACCAGGCAAGTCTCCTCATT-3´
TNFA-F: 5´-AGACCCCTCCCAGATAGATG -3´	TNFA: 5´-GCCTGTAGCCCATGTTGTAG-3´

2.8.4. *Murine ELISA*

The ELISAs were performed with: mouse IL-1β/IL-1F2 Quantikine (#MLB00C; R&D systems) and mouse C-Reactive Proteín/CRP (#MCRP00; R&D systems). Following the manufacturer´s instructions. Briefly, 50 µl of standard curve samples, control, maternal plasma or amniotic fluid were loaded into an antibody precoated 96-well plate and incubated for 2 h at room temperature. Wells were washed five times with washing solution and after were incubated for 2 h with an enzyme linked specific antibody. A second round of five washes was performed and a substrate solution was added and incubated for 30 min. The reaction was stopped and the plate was read at 450 nm with a wavelength correction at 540 nm.

2.9. Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA). Groups were analyzed by one-way ANOVA. Dunnett´s multiple

comparison method was employed, considering as the reference comparison the positive control. A p value <0.05 was considered statistically significant. Data are presented as means ± SEM. Data showed consider a different number of subjects depending on the nature of the experiment, the numbers are pointed in each specific result but any result with less than an n of 3 was not considered.

3. RESULTS

3.1. 633 can modulate IL-6 signaling pathway

To test if 633 was able to bind and regulate IL-6 pathway, we took HEK293 cells that do not express the IL-6R and HEK-Blue IL-6 cells that are transfected to express IL-6R. We exposed the cells with 633, TOC or vehicle for 15 min and then we stimulated them with IL-6 or vehicle for 10 min in order to generate the first actions of IL-6 signaling. Based on the current knowledge of the IL-6 signaling cascade, we analyze the activation of the molecules driving the first induction signal of the three major pathways, named STAT3, Akt and Erk1/2 by immunoblotting (P C Heinrich et al., 1998). After analyzing the response, we noticed that with the presence of IL-6 on the HEK-Blue IL-6 cells, we can observe the presence of the activation in the form of phosphorylation of these three major molecules. We used the already commercially available anti-IL-6R drug, TOC, as a positive control. In the case of TOC, we noticed the complete absence of bands for phosho-STAT3, phospho-Erk1/2 or phospho-Akt, corroborating that the results of the immunoblotting are just due to IL-6 induction. 633 effect is shown to have a significant reduction in the activation of STAT3 (Figure 3.1.A). This reduction was quantified and it is estimated that 633 has an effect by reducing approximately 50% of STAT3 activation. In the case of Erk1/2 and Akt, 633 showed a non-significant tendency to reduce this two pathways (Figure 3.1.B and C).

3.2. 633 can inhibit IL-6-induced cytokine expression

To confirm that the modulation of 633 signalling was enough to inhibit the inflammatory process related to PTB, we analyze the inhibition on the synthesis of pro-inflammatory cytokines, named IL-1β, IL-6 and TNFα in different concentrations of 633 in order to assess its inhibitory effect (Roberto Romero et al., 2015). This determination was made in a dose-response manner either in cells or in mice uteri *ex vivo* explants. Likewise, in order to understand the specific response of the gestational tissue to IL-6, we analyzed all the relevant gestational tissues for PTB. We analyzed the tissue-specific upregulation of pro-inflammatory cytokines and the

modulatory effect of 633, in a concentration of maximum functionality of small peptides. This concentration was corroborated by previous reports (Quiniou et al., 2008). Also, we analyzed the TOC-driven modulation of the upregulation of cytokines by the effect of IL-6 and took the results as a positive control.

3.2.1. *In vitro*

In the analysis of IL-6, IL-1β and TNFα gene upregulation in cells, the results showed a dose-related inhibition of these pro-inflammatory cytokines. This modulatory effect, got to a complete inhibition of the pro-inflammatory cytokine synthesis at the largest doses of 633 (10^{-7} – 10^{-4}). We constructed dose-response curves with this results. Therefore, we were able to determine the IC50 in the three different genes (0.767 nM, 2.072 nM, 0.267 nM; Figure 3.2.; A, B, C respectively).

3.2.2. *Ex vivo*

We tested the effect of IL-6 and 633 directly in relevant gestational tissues related to PTB (myometrium, fetal membranes and placenta). We stimulated tissue explants *ex vivo* and these explants were collected from timed-pregnant mice on gestational day 16.5. After collection, the explants were stimulated with 633 (10^{-6} M) or TOC (10^{-3} M) for 15 min to allow stabilization. Later, the explants were exposed to a pro-inflammatory environment by its stimulation with IL-6 (0.1 µg/ml) for 6 h to allow the signaling pathway machinery to generate mRNA transcripts. Afterward, we analyzed the amount of pro--inflammatory cytokines induced by the IL-6 effect. We noticed a tissue-specific upregulation of proinflammatory cytokines. More specifically, the upregulation of IL-1β in fetal membranes and of TNFα and Casp1 in uterus (Figure 3.3.; A and B). Interestingly, we did not obtain induction of IL-6induced proinflammatory genes in placental explants (Figure 3.3.; C). Then, we tested the inhibitory capacity of 633 in a dose-response manner in *ex vivo* explants. We decided to use uteri explants because of its ability to perform more uniform results given that the number of fetal membranes by mouse was limited. We used the same experimental setting than the previous experiment, with the only difference that we

added different concentrations of 633 (10^{-12} – 10^{-4} M) and the later induction of the tissue explants with IL-6 (0.1 µg/ml) for 6 h. Results indicated that the amount of TNFα is reduced in a dose-response manner by the action of 633 (Figure 3.3.; D).

3.3. 633 reduces efficacy and increases affinity of IL-6

To understand the behavior of 633 with the receptor, we decided to perform a functional antagonism analysis. In this experiment, we tested different concentrations of 633 (from 10^{-5} to 10^{-10} M) against different concentrations of IL-6 (from 10^{-9} to 10^{-15} M). This experiment was done in order to overview a possible allosteric modulatory activity of 633. The function of 633 was studied by the IL-6induced IL-1β upregulation in HEK-Blue IL-6 cells. We observed a non-significant inhibitory tendency in the lower 633 concentrations (from 10^{-10} to 10^{-8} M) and a significant inhibition that lead to almost a complete blockade of the IL-6-induction of IL-1β production in the highest concentrations of 633 (from 10^{-7} to 10^{-10} M). This inhibitory response is seen as a downshift of the IL-6 dose-response curve (Figure 3.4.). We also studied the EC50 in each of the experimental conditions. As seeing in the Table 3.1, the highest concentrations of 633 generate an increase on the IL-6 affinity for the receptor, being significant in the 633 concentration of 10^{-6} M.

Table 3.1. Efficacy and potency indicators analyzed by functional antagonism analysis. Data are representative of 4 samples per group. *p<0.05, **p<0.01 by one-way ANOVA with Dunnett´s multiple comparison test compared to the IL-6/vehicle group.

	Control	**633**					
		10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10_{-10} M
EC_{50}	0.017nM	0.071pM	0.018pM*	0.013nM	0.016nM	1.929pM	1.377pM
$Log\ EC_{50}$	-10.76	-13.15	-13.76	-10.86	-10.80	-11.71	-11.86
St. Error $Log\ EC_{50}$	0.268	2.322	1.252	2.180	0.181	0.351	0.583
E_{max}	9.52	0.95 **	0.65 **	0.45 **	5.25	5.14	8.67
St. Error E_{max}	5.03	0.82	0.48	0.50	0.88	1.36	6.55

3.4. 633 can prevent IL-6-induced preterm birth

We performed *in vivo* experiments in order to confirm that IL-6 is an important target and that 633 was able to counter for the specific IL-6 effect. To analyze this, we performed an animal model of IL-6-induced PTB. For this model we use timed pregnant mice at gestational day 16.5 and injected 633 (1 mg/Kg/12 h), TOC (10 mg/Kg/12 h) or vehicle s.c.; to induce PTB we also inject IL-6 (1 µg/100µl) i.p. Some of the mice were sacrificed 24 h after the IL-6 induction for tissue collection (uterus, fetal membranes, placenta and maternal blood). RNA was extracted from the tissues and gene quantification was done by quantitative RT-PCR. The gene quantification was done based in proteins that are related either to inflammation or to the induction of uterine contractility. The rest of the mice were used to record the time of delivery. We were able to demonstrate that IL-6 is sufficient to induce PTB by showing a significant increase in the percentage of prematurity and having most of the births before the control group (vehicle/vehicle). We noticed that the presence of either 633

or TOC were able to reduce significantly the percentage of prematurity. These two groups performed most of the births at the same time than the control group (vehicle/vehicle). It is important to remark that the presence of TOC produced a slight delay on the timing of parturition, presenting all of the births after day 19.5 (Figure 3.5.; A and B).

3.4.1. *633-mediated gene downregulation*

The analysis of the gestational tissues proved an IL-6-induced upregulation of genes with a specific profile in each gestational tissue. The results presented an upregulation of Casp1 in uterus (Figure 3.6.; A); IL-1β, IL-6, OXTR, PTGER3 and PGES1 in fetal membranes (Figure 3.6.; B); IL-6 and CCL2 in leukocytes (Figure 3.6.; C) and IL-1β, IL-6, TNFα and CCL2 in placenta (Figure 3.6.; D). 633 peptide was able to selectively downregulate, *in vivo*, some of the upregulated genes in fetal membranes, being presented as a significant reduction on IL-1β, IL-6, OXTR and PTGER3 (Figure 3.6.; B); and a reduction tendency of IL-6 in leukocytes (Figure 3.6.; C).

3.5. 633 can prevent LPS-induced preterm birth

To corroborate 633 efficacy, we performed an LPS-induce PTB model in mice. For this model we used timed-pregnant CD1 mice at gestational day 16.5 and injected 633 (1 mg/Kg/12 h), TOC (10 mg/Kg/12 h) or vehicle s.c.; to induce PTB we also inject LPS (10 μg/100μl) i.p. Some of the mice were sacrificed 12 h after the LPS induction for tissue and fluid collection (uterus, fetal membranes, placenta, maternal blood and amniotic fluid). RNA was extracted from the tissues and gene quantification was done by quantitative RT-PCR. The gene quantification was done based in proteins that are related either to inflammation or to the induction of uterine contractility. Fluids like plasma and amniotic fluid were used to quantify inflammatory protein markers by ELISA. The rest of the mice were used to record the time of delivery. The results showed that the effect generated by the exposition of either 633 or TOC were able to significantly reduce the percentage of prematurity. This was

demonstrated by the delay of more than 50% of the births leading them to be presented at term (E19, or determined by the time of delivery of the control group (vehicle/vehicle)). It is important to remark that the presence of TOC produce a slight delay on the timing of parturition of some of the mice, presenting 25% of the births after gestational day 19.5 (Figure 3.7.; A and B).

3.5.1. *633-mediated gene downregulation*

The analysis of the gestational tissues after 12 h of IL-6 induction demonstrated an LPS-induced upregulation of genes. This gene upregulation showed a tendency of increase on gene level in its majority in fetal membranes. The quantification presented an upregulation of Casp1, IL-6, TNFα and connexin 43 in uterus (Figure 3.8.; A); IL-6, TNFα, CCL2, IL-12, Casp1, PTGFR, and PGES1 in fetal membranes (Figure 3.8.; B); IL-6 in leukocytes (Figure 3.8.; C) and IL-1β in placenta (Figure 3.8.; D). 633 peptide was able to significantly downregulate, *in vivo*, some of the LPS-induced upregulated genes in uterus (TNFα), fetal membranes (IL-6, TNFα, CCL2 and PGES1) (Figure 3.8.; A and B); and a reduction tendency of IL-6 in uterus (Figure 3.8.; A).

3.5.2. *633-mediated protein downregulation*

We produced a protein analysis on maternal and gestational fluids (maternal plasma and amniotic fluid) by ELISA. This analysis showed a significant increase of C-reactive protein (CRP) in maternal plasma that can be returned to control levels by the effect of 633 (Figure 3.9.; A). The analysis on gestational tissues displayed a significant upregulation of IL-1β in amniotic fluid that can be negatively counted by the effect of both 633 and TOC (Figure 3.9.; B).

3.6. 633 can ameliorate neonatal outcomes

To study the scopes of the 633 effect, it was important to address the neonatal outcome after the prophylactic treatment. In order to account for this question, we

record the neonatal survival and weight after performing the abovementioned LPSinduced PTB model. On the mice used to record the time of the parturition, we register the number of pups alive in each group and we record the weight of the alive pups with a timing less than 30 min following parturition. We notice a significant increase on the percentage of neonatal survival accounted for the use of 633 compared to the positive control (LPS/vehicle) (Figure 3.10.; A). We also recorded the neonatal weight and we noticed a significant reduction on the weight of the pups in the positive control group (LPS/vehicle) which was significantly returned to the normal weight presented in the negative control (vehicle/vehicle) by the effect of 633 and TOC (Figure 3.10.; B).

FIGURES

Figure 3.1. | 633 modulation of IL-6 signaling

The 633 peptide can downregulate IL-6 signalling pathway. (A-C) HEK293 and HEK-Blue IL-6 cells were stimulated with 633 (10^{-6} M), TOC (10^{-3} M) or vehicle for 15 min. Then, cells were induced by either IL-6 (0.1 µg/ml) or vehicle for 10 min. Obtained cell lysates were blotted against p-STAT3, p-Erk and p-Akt and normalized with STAT3, Erk1/2 and Akt. The histograms show the percentage of STAT3, Erk1/2 or Akt activation. The activation value was obtained by the normalization against the positive control (IL-6/vehicle). Values are presented as means ± SEM. Data are representative of 3 samples per group. *p<0.05, ***p<0.001, ****p<0.0001 by one way ANOVA with Dunnett´s multiple comparison test compared to IL-6/vehicle group.

Figure 3.1.

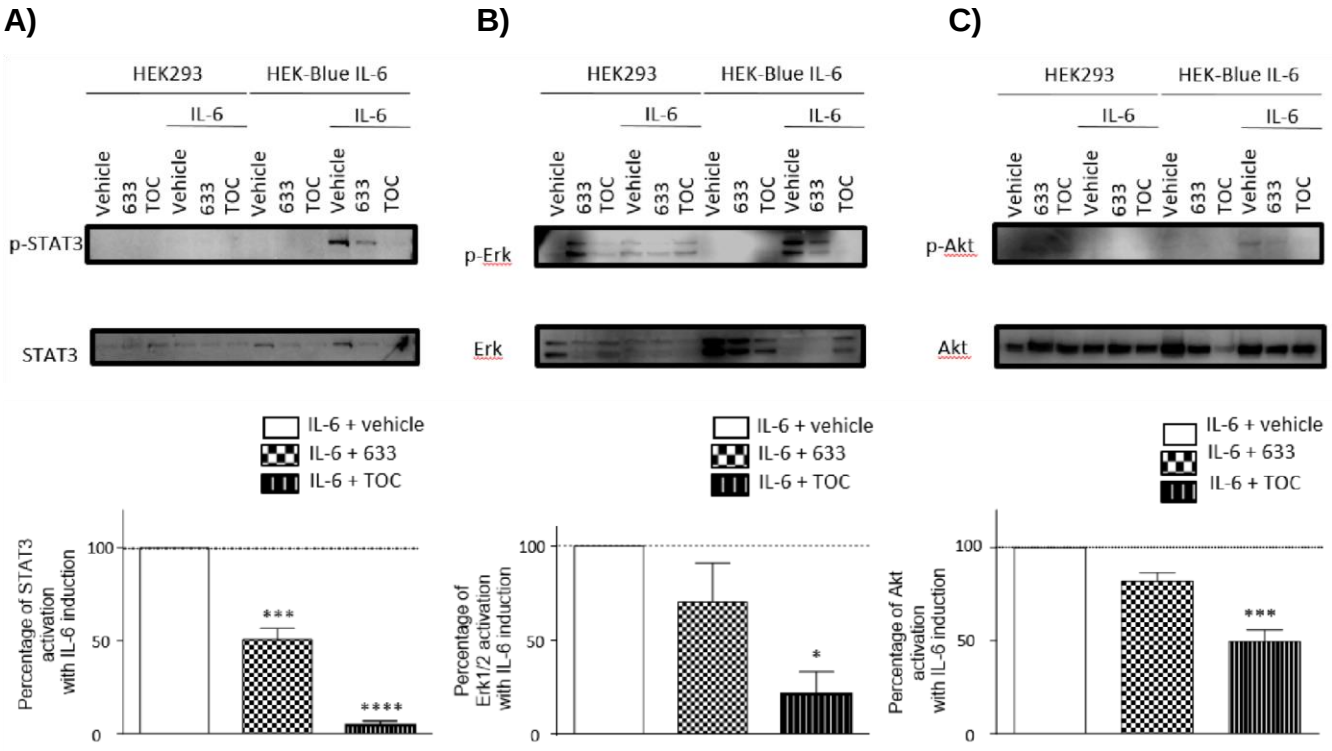

A)
B)
C)
HEK293
HEK-Blue IL-6
IL-6
Vehicle
633
TOC
p-STAT3
STAT3
p-Erk
Erk
p-Akt
Akt
IL-6 + vehicle
IL-6 + 633
IL-6 + TOC
Percentage of STAT3 activation with IL-6 induction
Percentage of Erk1/2 activation with IL-6 induction
Percentage of Akt activation with IL-6 induction
100
50
0
***
****
*

Figure 3.2. | 633 modulation of IL-6-induced proinflammatory cytokine expression *in vitro*

The 633 peptide can inhibit IL-6-induced cytokine expression. (A-C) HEKBlue IL-6 cells were stimulated with different concentrations of 633 (from 10^{-4} to 10^{-12} M) or vehicle. Then, the cells were induced by either IL-6 (0.1 µg/ml) or vehicle for 6 h. HEK cells were as well induced by IL-6 and 633 to confirm that the activity was only IL-6-driven. RNA was extracted and the amount of IL-1β, IL-6 and TNFα mRNA was quantified by quantitative RT-PCR and normalized by the amount of 18S. All values are graphed normalizing by the value of negative control (vehicle/vehicle). Values are presented as means ± SEM. Data are representative of 4-10 samples per group. Curves were made with a non-linear regression fit with three parameters. **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with Dunnett´s multiple comparison test compared to the IL-6/vehicle group.

Figure 3.2.

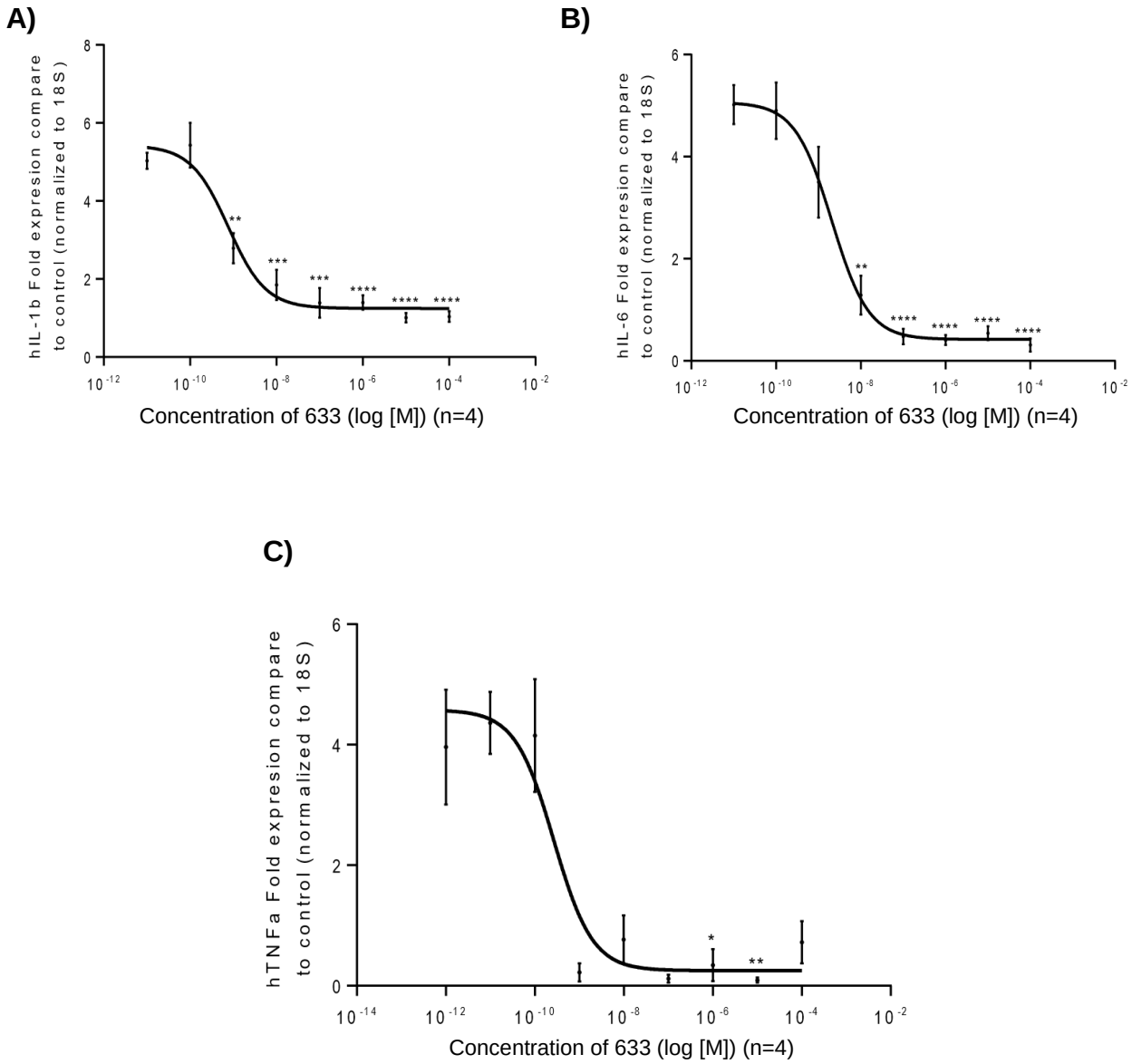
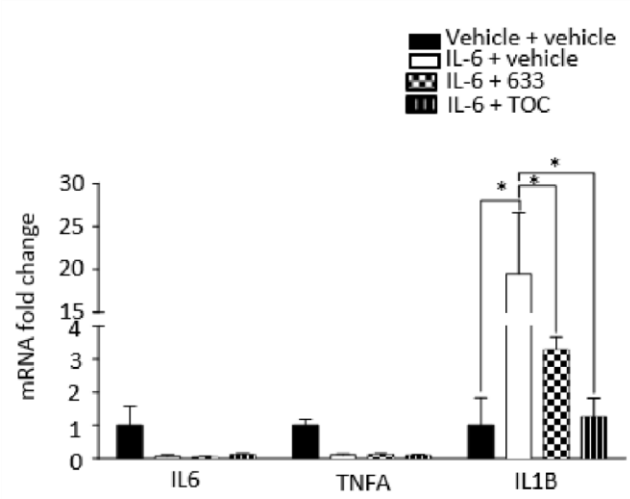

A)
hIL-1b Fold expresion compare to control (normalized to 18S)
Concentration of 633 (log [M]) (n=4)
B)
hIL-6 Fold expresion compare to control (normalized to 18S)
Concentration of 633 (log [M]) (n=4)
C)
hTNFa Fold expresion compare to control (normalized to 18S)
Concentration of 633 (log [M]) (n=4)

Figure 3.3. | 633 modification of IL-6-induced proinflammatory cytokine expression *ex vivo*

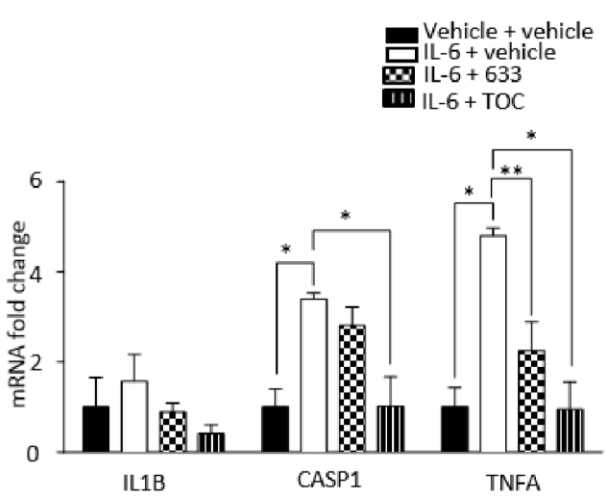
The 633 peptide can inhibit IL-6-induced cytokine expression. (A-C) Fragments of fetal membranes, myometrium and placenta were collected and stimulated with 633 (10^{-6} M) or vehicle, Then, the cultured explants were induced by IL-6 (0.1 µg/ml) or vehicle for 6 h. RNA was extracted and a screening of proinflammatory genes was performed and normalized by the amount of 18S. All values are graphed normalizing by the value of negative control (vehicle/vehicle). (D) Myometrial fragments were stimulated by different concentrations of 633 (from 10^{-4} to 10^{-12} M) or vehicle. Then, the cultured explants were induced by either IL-6 (0.1 µg/ml) or vehicle for 6 h. RNA was extracted and the amount of TNFα mRNA was quantified by quantitative RT-PCR and normalized by the amount of 18S. Values are presented as means ± SEM. Data are representative of 4-10 samples per group. Curve was made with a non-linear regression fit with three parameters. *$p<0.05$, **$p<0.01$ by one-way ANOVA with Dunnett´s multiple comparison test compared to the IL-6/vehicle group.

Figure 3.3.

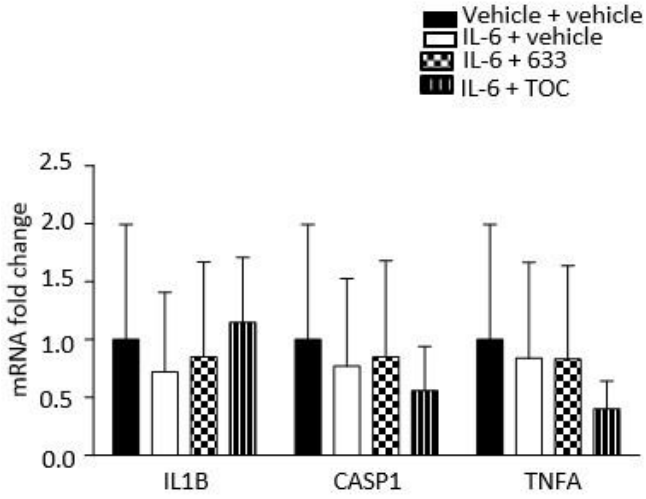
A) Fetal Membranes (n=4)



B) Myometrium (n=4)



C) Placenta (n=4)



D) Myometrium (n=4-10)

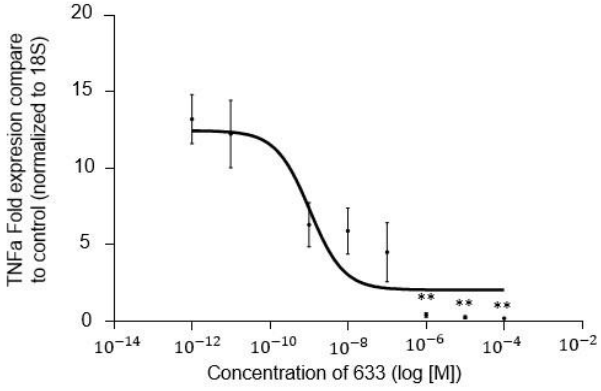


Figure 3.4. | Functional IL-6-induced antagonism analysis *in vitro*

The 633 peptide can reduce IL-6 efficacy. A) HEK-Blue IL-6 cells were stimulated with different concentrations of 633 (from 10^{-5} to 10^{-10} M) or vehicle. Then, the cells were induced with different concentrations of IL-6 (from 10^{-9} to 10^{-15} M) or vehicle for 6 h. HEK cells were as well induced by IL-6 and 633 to confirm that the activity was only IL-6-driven. RNA was extracted and the amount of IL-1β mRNA was quantified by quantitative RT-PCR and normalized by the amount of 18S. All values are graphed normalizing by the value of negative control (vehicle/vehicle). Values are presented as means ± SEM. Data are representative of 4 samples per group. Curves were made with a non-linear regression fit with three parameters.

Figure 3.4.

A)

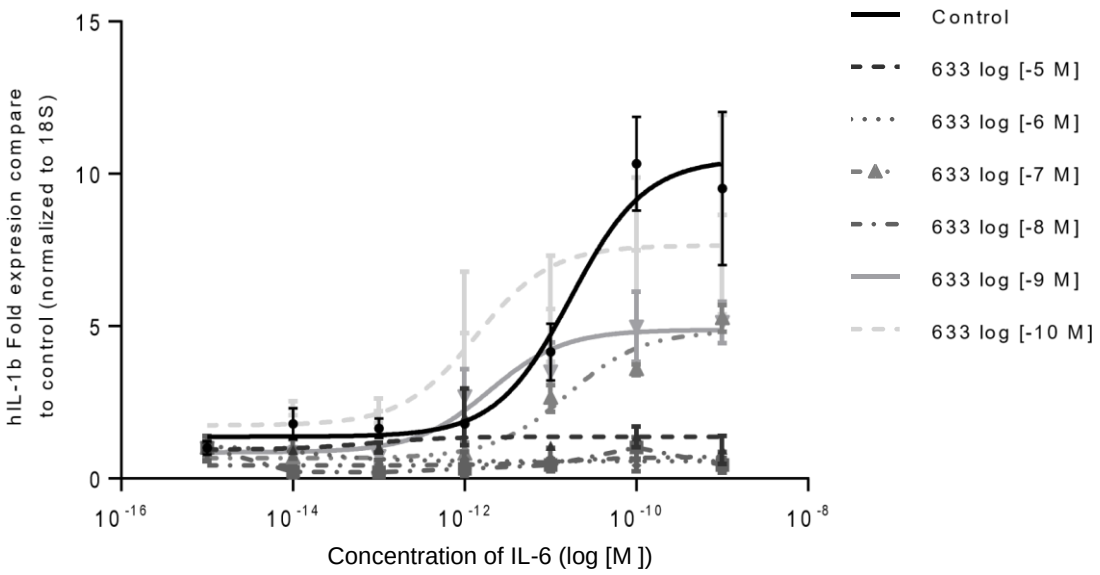
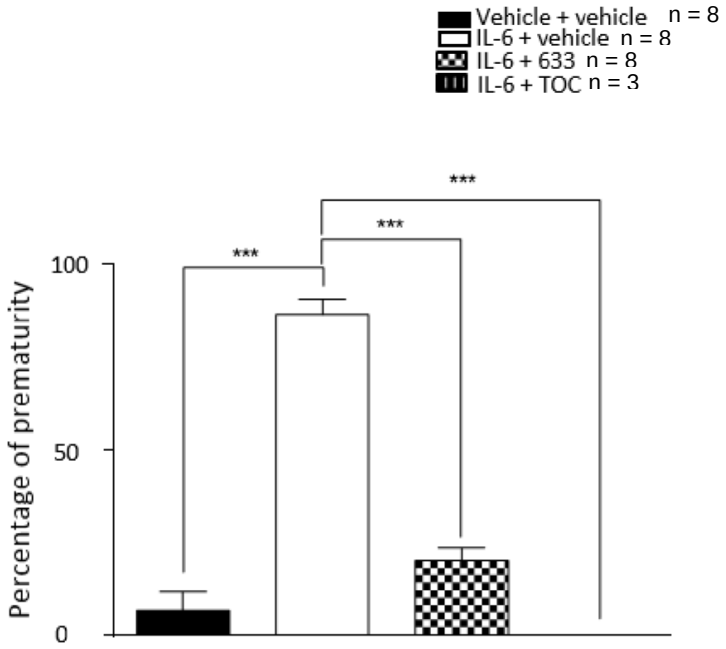

15
10
5
0
hIL-1b Fold expresion compare to control (normalized to 18S)
10 -16
10 -14
10 -12
10 -10
10 -8
Concentration of IL-6 (log [M])
Control
633 log [-5 M]
633 log [-6 M]
633 log [-7 M]
633 log [-8 M]
633 log [-9 M]
633 log [-10 M]

Figure 3.5. | 633 prevention of IL-6-induced preterm birth

633 can prevent IL-6-induced PTB. (A) Percentage of prematurity presented. Control mice were injected i.p. and s.c. with vehicle to mimic the stimulus made by IL-6 induction with an i.p. injection of IL-6 (1 µg IL-6/ 100 µl saline) and s.c. injection of 633, TOC or vehicle. (B) Percentage of animal delivery against the gestational age. Values are presented as means ± SEM. Data are representative of 3-8 samples per group. ***p<0.001 by one-way ANOVA with Dunnett´s multiple comparison test compared to the IL-6/vehicle group.

Figure 3.5.

A)


Vehicle + vehicle n = 8
IL-6 + vehicle n = 8
IL-6 + 633 n = 8
IL-6 + TOC n = 3
***
***
***
100
50
0
Percentage of prematurity

B)

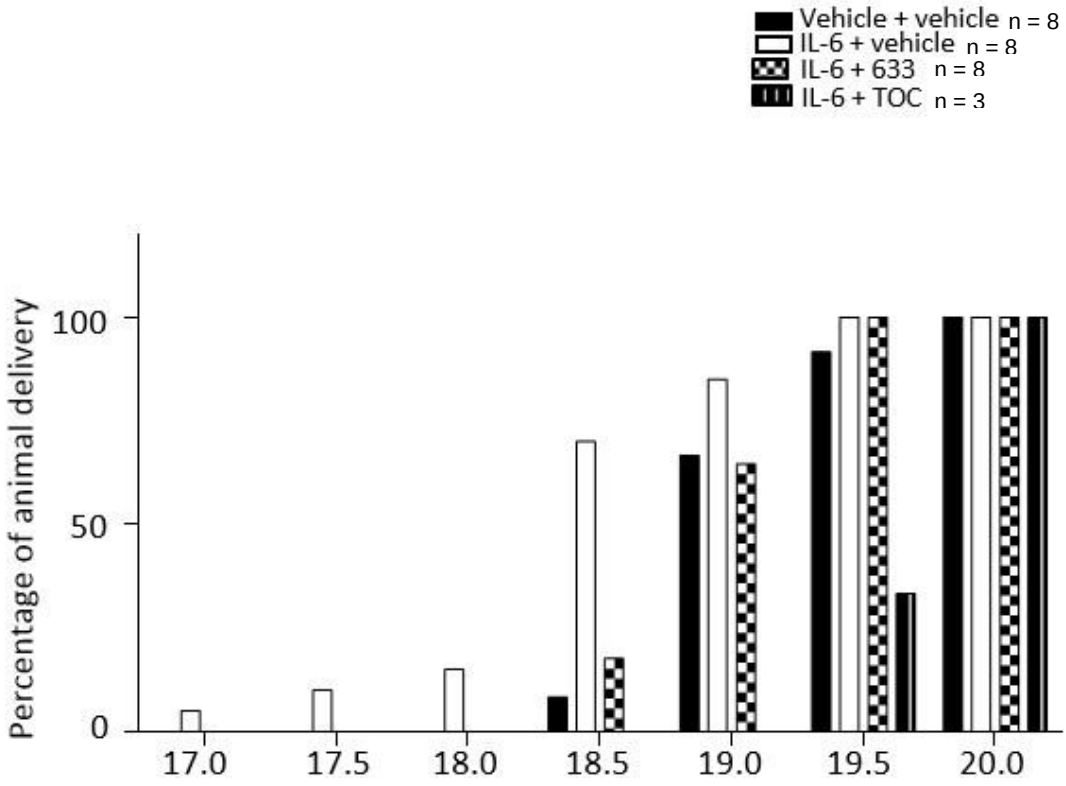
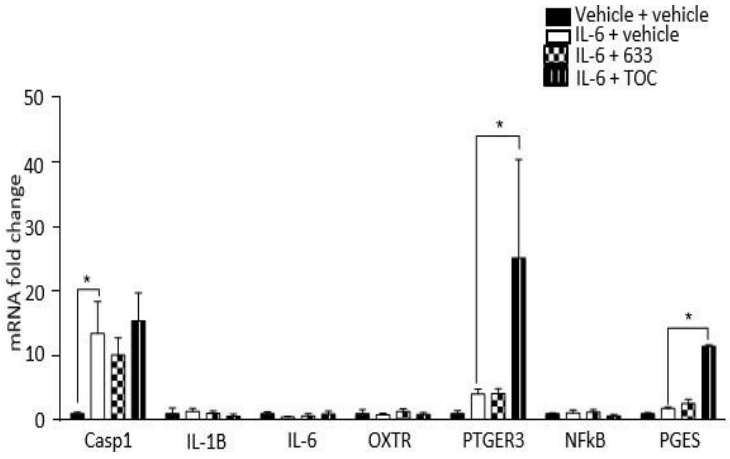

Vehicle + vehicle n = 8
IL-6 + vehicle n = 8
IL-6 + 633 n = 8
IL-6 + TOC n = 3
100
50
0
Percentage of animal delivery
17.0
17.5
18.0
18.5
19.0
19.5
20.0

Figure 3.6. | 633 gene modulation in maternal and gestational tissue on an IL6-induced preterm birth model

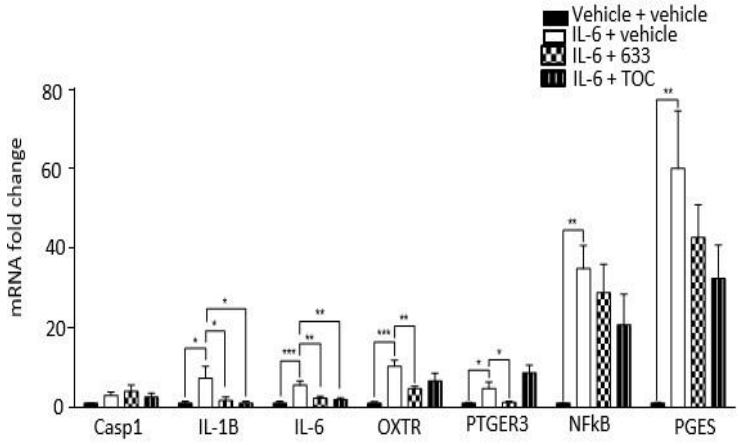
633 can prevent IL-6-induced PTB by selectively inhibiting gene expression in gestational and maternal tissue. (A) Quantitative RT-PCR results in mice uteri extracted on day 17.5 after IL-6 induction. Graph shows the fold increase on the genes correlated with the levels in the control group. (B) Quantitative RT-PCR results in mice fetal membranes extracted on day 17.5 after IL-6 induction. (C) Quantitative RT-PCR results in mice leukocytes extracted on day 17.5 after IL-6 induction. (D) Quantitative RT-PCR results in mice placenta extracted on day 17.5 after IL-6 induction. RNA was extracted and a screening of proinflammatory and uterine activation protein (UAP) genes was performed and normalized by the amount of 18S. All values are graphed normalizing by the value of negative control (vehicle/vehicle). Values are presented as means ± SEM. Data are representative of 2-8 samples per group. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett´s multiple comparison test compared to the IL-6/vehicle group.

Figure 3.6.

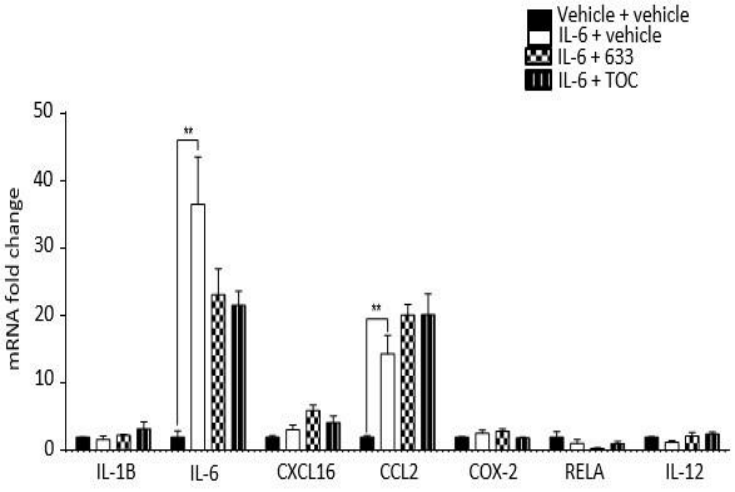
A) Myometrium (n=3-6)



B) Fetal membranes (n=3-6)



C) Leukocytes (n=3-6)



D) Placenta (n=3-6)

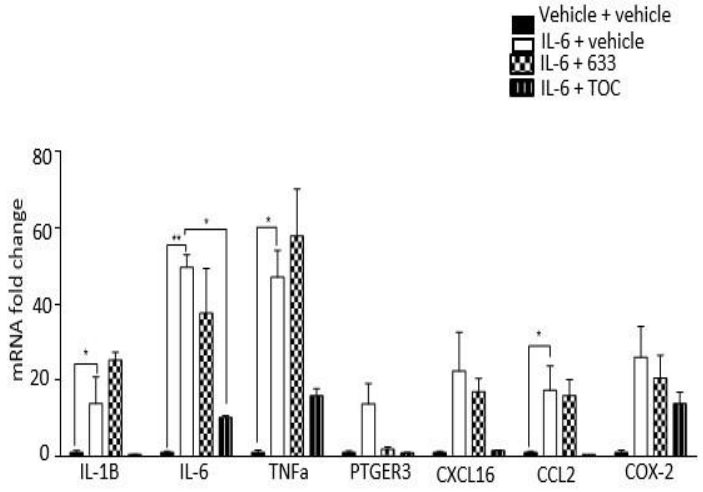
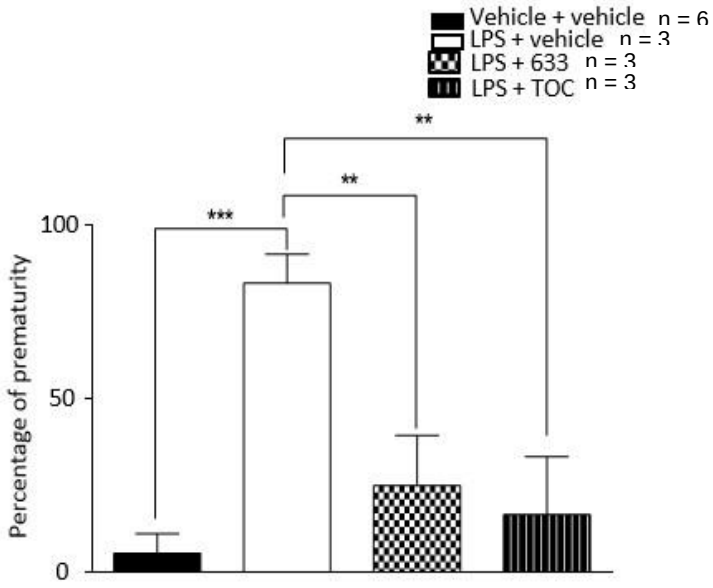


Figure 3.7. | 633 prevention of LPS-induced preterm birth

633 can prevent LPS-induced PTB. (A) Percentage of prematurity presented. Control mice were injected i.p. and s.c. with vehicle to mimic the stimulus of the i.p. injection of LPS (10 µg IL-6/ 100 µl saline) and s.c. injection of 633 (1 mg/Kg/12 h), TOC (10 mg/Kg/12 h) or vehicle. (B) Percentage of animal delivery against the gestational age. Values are presented as means ± SEM. Data are representative of 2-6 samples per group. **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett´s multiple comparison test compared to the LPS/vehicle group.

Figure 3.7.

A)


Vehicle + vehicle n = 6
LPS + vehicle n = 3
LPS + 633 n = 3
LPS + TOC n = 3
**
**
***
100
50
0
Percentage of prematurity

B)

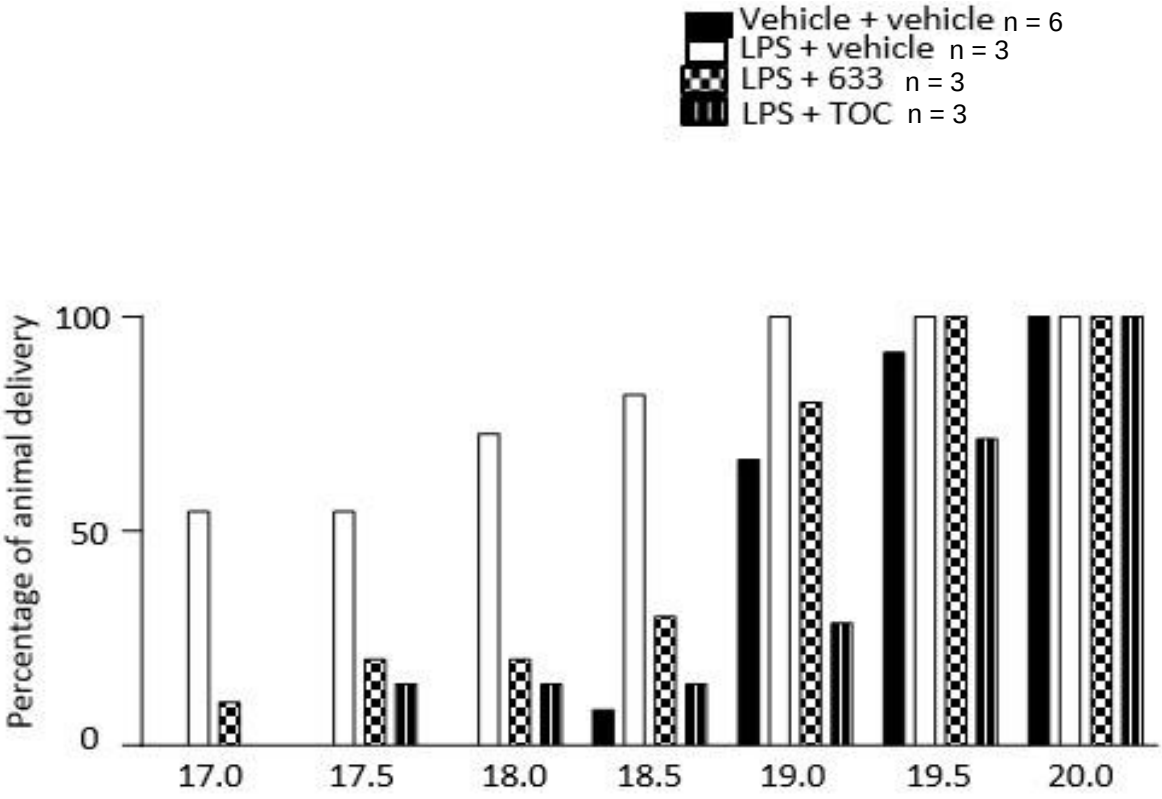
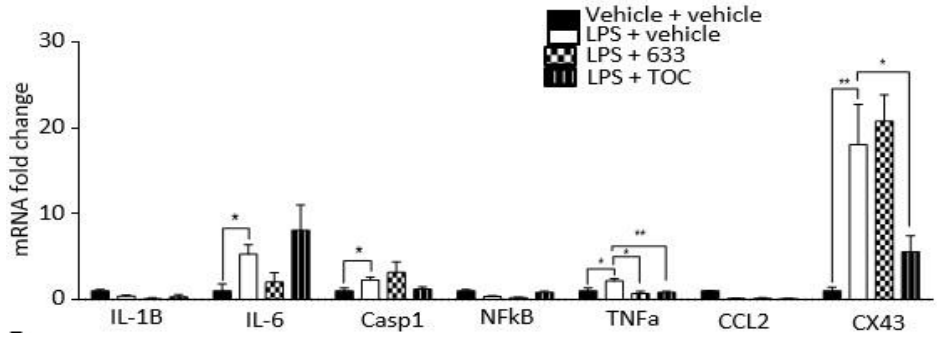

Vehicle + vehicle n = 6
LPS + vehicle n = 3
LPS + 633 n = 3
LPS + TOC n = 3
100
50
0
Percentage of animal delivery
17.0
17.5
18.0
18.5
19.0
19.5
20.0

Figure 3.8. | 633 gene modulation in maternal and gestational tissue on a LPS-induced preterm birth model

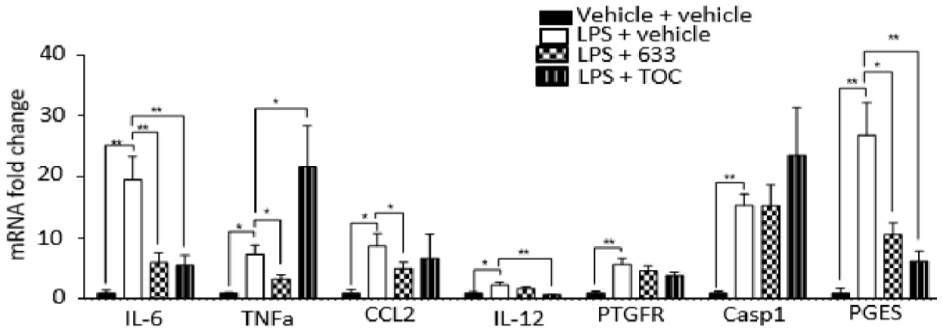
633 can prevent LPS-induced PTB by selectively inhibiting gene expression in gestational and maternal tissue. (A) Quantitative RT-PCR results in mice uteri extracted on day 17 after LPS induction. Graph shows the fold increase on the genes correlated with the levels found in the control group. (B) Quantitative RT-PCR results in mice fetal membranes extracted on day 17 after LPS induction. (C) Quantitative RT-PCR results in mice leukocytes extracted on day 17 after LPS induction. (D) Quantitative RT-PCR results in mice placenta extracted on day 17 after LPS induction. RNA was extracted and a screening of proinflammatory and uterine activation protein (UAP) genes was performed and normalized by the amount of 18S. All values are graphed normalizing by the value of negative control (vehicle/vehicle). Values are presented as means ± SEM. Data are representative of 3-5 samples per group. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett´s multiple comparison test compared to the LPS/vehicle group.

Figure 3.8.

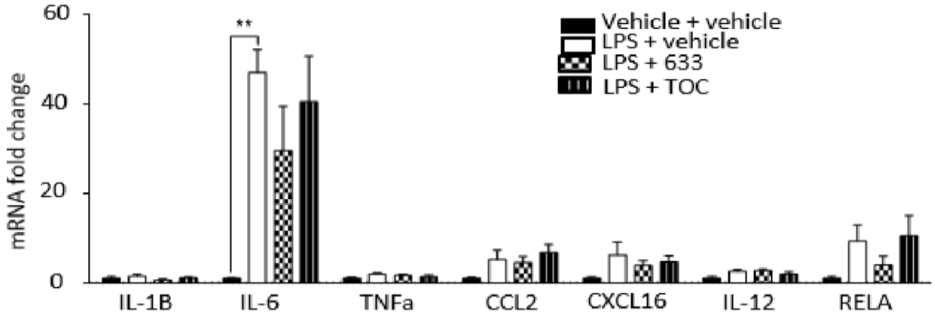
A) Myometrium (n=3-5)



B) Fetal membranes (n=3-5)



C) Leukocytes (n=3-5)



D) Placenta (n=3-5)

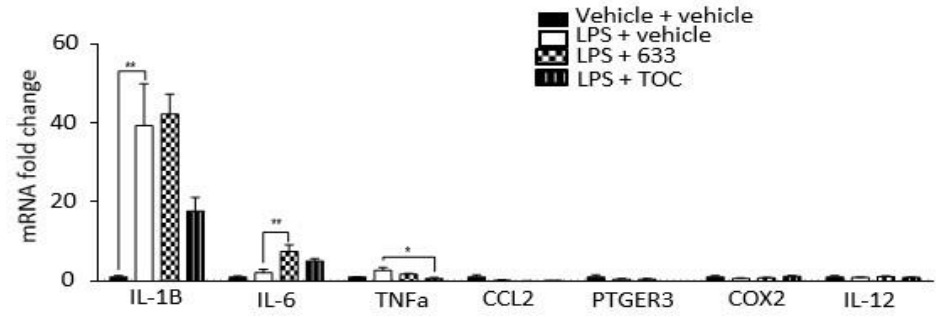
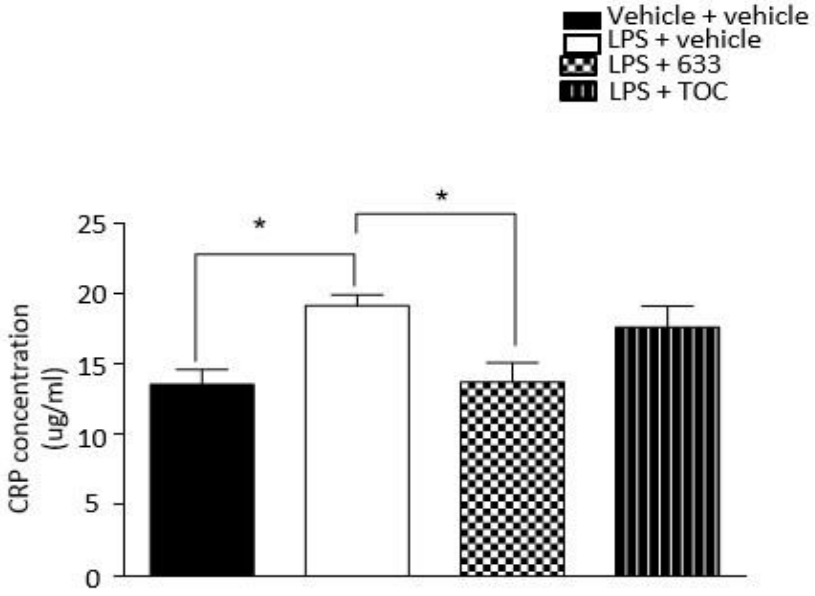


Figure 3.9. | 633 protein modulation in maternal and gestational tissue on a LPS-induced preterm birth model

633 can prevent LPS-induced PTB by selectively inhibiting protein expression in gestational and maternal fluids. (A) CRP quantification in maternal plasma extracted on day 17 after LPS induction. CRP levels were determined by ELISA. (B) IL-1β quantification in amniotic fluid extracted on day 17 after LPS induction. IL-1β levels were determined by ELISA. Values are presented as means ± SEM. Data are representative of 3-6 samples per group. *$p<0.05$, ***$p<0.001$ by one-way ANOVA with Dunnett´s multiple comparison test compared to the LPS/vehicle group.

Figure 3.9.

A) Maternal plasma (n= 3-6)



B) Amniotic fluid (n= 4-5)

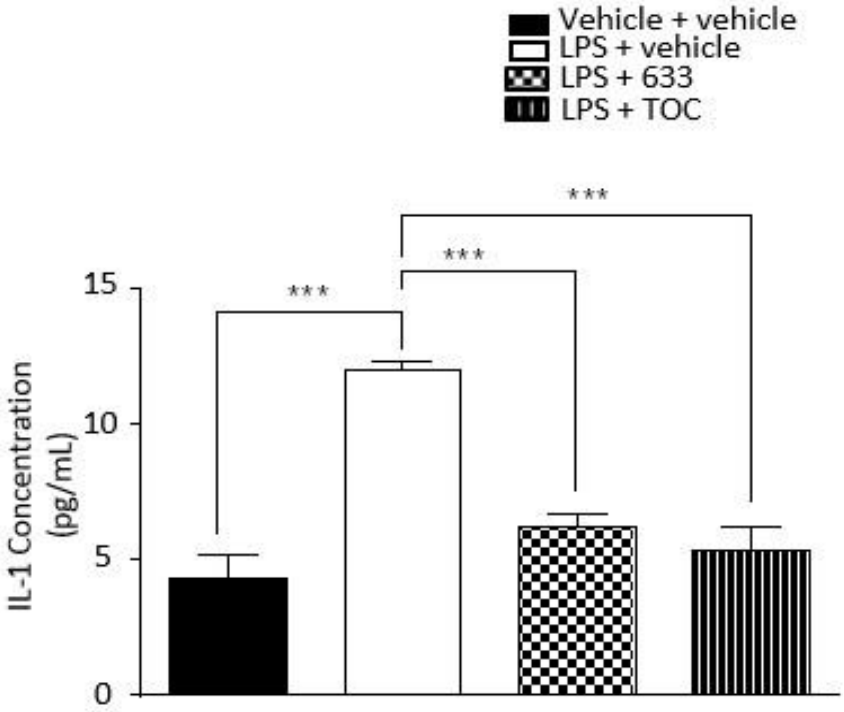
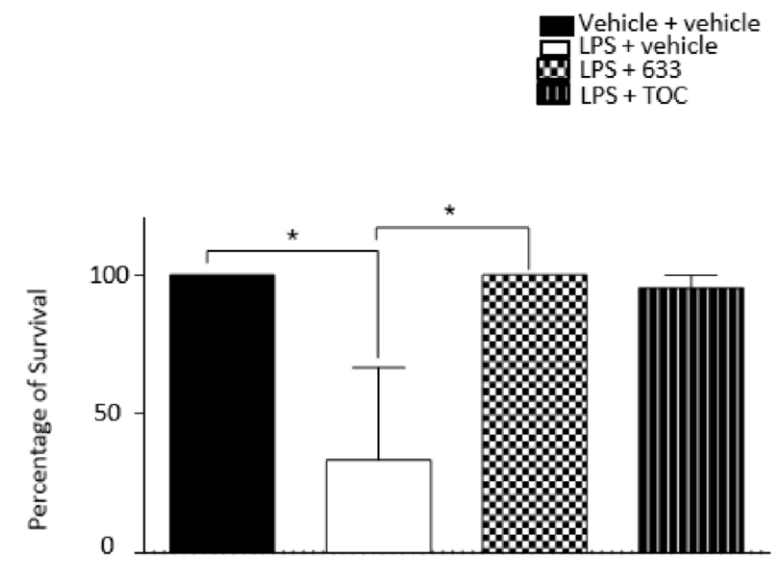


Figure 3.10. | Neonatal outcomes

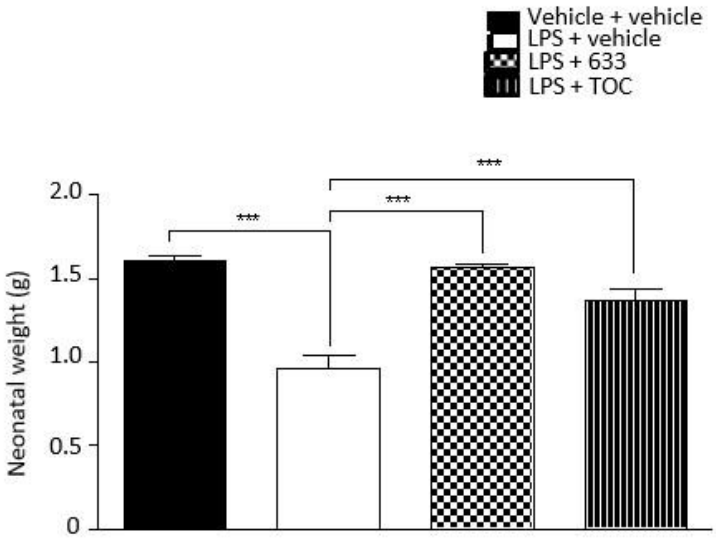
633 can improve neonate outcomes. (A) Percentage of neonatal survival. (B) Neonatal weight. Both measures were done in less than 30 min after parturition. Values are presented as means ± SEM. Data are representative of 3-4 samples per group in the panel A and 35-45 samples per group in the panel B. *p<0.05, ***p<0.001 by one-way ANOVA with Dunnett´s multiple comparison test compared to IL-6/vehicle group.

Figure 3.10.

A) n= number of animals tested = 3-4



B) n= number of pups measured = 35-45



4. DISCUSSION

In this work, we characterized *in vitro, ex vivo* and *in vivo* the function of a small peptide that can be an antagonist of the IL-6R. The characterization was made in a model of PTB because it is well established the importance of inflammation in the onset of PTB. During this work, we also confirmed the importance of IL-6R as a potential target in the context of PTB. We demonstrated that the effect of IL-6 is enough to generate an inflammatory process in the gestational environment and that this inflammation is sufficient to generate the synthesis of UAP and followed by the preparation of the uterus for PTB. We analyzed the modulatory effect of 633 in the IL-6 upstream and pro-inflammatory downstream signaling. We also generated different dose-response curves and a functional antagonism analysis to help us understand the behavior of 633 with the receptor.

4.1. Design of the 633 peptide.

The 633 peptide was designed based on previous reports that showed that with a structure-based design we could design selective small peptides that would selectively modulate the receptor signaling (McDonnell et al., 1996). The conception of these peptides by our group was based on the analysis of flexible motifs of the IL6R and the creation of small D-peptides that mimic these structures (Quiniou et al., 2008). The decision of using D-amino acids in the construction of the peptides was based on the conception of peptide stability and on previous reports that showed that the presence of D-amino acids does not interfere with the peptide activity (Chalifour et al., 2003).

4.2. 633 modulation of IL-6-signalling and its first allosteric characteristic.

Once having the small peptide antagonist, named 633, we decided to generate a valid assay to analyze the capability of 633 to bind the IL-6R and modify the IL-6

activity on it (Hughes, Rees, Kalindjian, & Philpott, 2011). For this, we decide to use cell culture of cells that we knew could work as negative and positive control itself. We used HEK cells that do not express the IL-6R and HEK-Blue IL-6 cells that are specifically transfected to express the IL-6R. We let the cells to interact with either the 633 peptide, TOC to provide an additional negative control inside the HEKBlue IL-6 group or vehicle as a positive control. Afterward, we induced the cells with either with IL-6 to generate and IL-6-driven signaling response or vehicle to add another extra negative control for each different subgroup. We allowed induction for a period of 10 min in order to catch the first wave of activation of the three IL-6 signaling pathways, named as phosphorylation of STAT3, Akt and Erk1/2 (Hirano et al., 2000; Wang et al., 2013). The results showed a differential 633 modulation of the three pathways. We observed how 633 is able to significantly inhibit the STAT3 activation without inhibiting Erk1/2 or Akt. This result gives a really promising approach to the potential of 633 as a therapeutic agent because the STAT3 pathway is characterized to be the one related to the activation and upregulation of acute phase proteins, which are the proteins that will generate an inflammatory response (Levy & Lee, 2002). It is reported that the activation of the STAT3 pathway can be the one influencing the upregulation of other acute pro-inflammatory cytokines like IL-1β or TNFα (Mori et al., 2011). Also, the lack of response in the Erk1/2 and Akt pathway is really promising because the function of this two proteins is mainly cell protective. The complete blockade of IL-6 signaling has already been reported to be deleterious in a context of PTB (Ouellet, Berthiaume, Corriveau, Rola-pleszczynski, & Pasquier, 2013). Based on the known functions of all signaling pathways of IL-6, we know that in the case of Erk1/2, there are reports of the function of Erk1/2 in cell cycle progression like cell proliferation and cell growth. There are also reports relating the Erk1/2 pathway to the prevention of apoptosis (Mccubrey et al., 2007). Some studies even showed how IL-6-driven Erk1/2 activation could be directly related to wound healing (McFarland-Mancini et al., 2010). On the other hand, Akt is also related to cell cycle progression, cell survival, cell differentiation, DNA repair and RNA export (Martelli et al., 2012). Therefore, the selective blocking of STAT3 pathway gives the possibility of having a molecule that will inhibit the generation of a pro-inflammatory environment without affecting the important cell-related functions of IL-6. This response follows the already described allosteric characteristic of probe dependence effect because the binding

of 633 allows IL-6 to bind but modifies the signaling generating a textural response (T. Kenakin, 2005).

4.3. 633 inhibition of IL-6-driven inflammatory response.

Once knowing the potential of 633 to inhibit the signaling pathway related to the generation of acute phase proteins, we decided to analyze the activity of 633 in the capacity of IL-6 to induce other pro-inflammatory cytokines. It is reported that the activation of the IL-6 pathway can generate an unconstrained inflammation that will be described mainly by the presence of IL-6, IL-1β and TNFα (Akira et al., 1990; Kurihara et al., 1990). Thus, we decided to analyze in a dose-response manner the potential of IL-6 to generate an upregulation of the three main pro-inflammatory cytokines in different concentrations of 633. We noticed a dose-response effect of 633 on all three cytokines. This negative modulatory effect reached complete inhibition at the higher concentrations of IL-6 (from 10^{-7} to 10^{-4} M). The presence of a dose-response relationship gives 633 more compelling characteristics. It is important to highlight that the generation of a dose-response analysis is basic to determine the safety and optimal use of medications (Emilien, Meurs, & Maloteaux, 2000). This result allow us to conclude that the modulation driven by the action of the 633 peptide could be enough to reduce the inflammatory effect because this modulation is able to reduce the synthesis of the three main pro-inflammatory cytokines (Gomez-Lopez et al., 2014).

4.4. 633 presents allosteric characteristics in a functional antagonist analysis.

After knowing the 633 negative modulation in a pro-inflammatory context, we wanted to understand further the characteristics of 633 against the IL-6R. There are reports indicating that the use of small peptides designed by the structural analysis of the receptor present allosteric characteristics (Anderson et al., 2006). As stated before, 633 presents the biased agonism by the generation a texture in the response

of the receptor and it gives an idea of a binding site independent of the ligand binding site (T. Kenakin, 2005). We wanted to confirm the allosteric characteristics of 633, thus, we performed a functional antagonism analysis to study the 633-driven modulation of affinity and efficacy of the IL-6 by testing it as an inducer of other proinflammatory cytokines (Baker & Hill, 2007). As result of this analysis, we demonstrated that 633 is able to indistinctively modulate IL-6 affinity and efficacy by reducing IL-6 efficacy and even creating a non-significant reduction tendency in the lower concentrations of 633 (from 10^{-8} to 10^{-10}) and a complete blockage in its highest concentrations (from 10^{-5} to 10^{-7} M). Even though 633 activity blocked the IL-6 stimulatory function to upregulate pro-inflammatory cytokines, the affinity of the natural ligand to the IL-6R, remained unchanged, except in the highest concentrations of 633 (10^{-6} and 10^{-5} M). This modulatory activity gives us another evidence into the possibility of 633 being an allosteric small peptide negative modulator. The belief of the possibility of 633 presenting allosteric capacities from this result comes from the fact that an orthosteric antagonist will have result into a gradual increase in the IL-6 EC50 value (Baker & Hill, 2007).

4.5. IL-6R is a valuable target for PTB and 633 can ameliorate the IL-6-driven detrimental effects.

By this time, we had a promissory molecule that had the ability of specifically modulate the IL-6 signaling pathway and that this functional selectivity was only referred to the downregulation of the IL-6-driven inflammatory response. So, in order to establish the strength of this molecule we needed to validate the target, in this case, the IL-6R. In order to accomplish this, we performed and IL-6-induced PTB model in mice. Based on previous reports, we knew that the intraamniotic injection of IL-6 was not sufficient to generate the onset of PTB (Yoshimura & Hirsch, 2003). Then, in our model, we decided to perform i.p. injections of IL-6. At the same time, we studied the effect of 633 and of TOC used as one of our negative controls. The application of the treatment via s.c. injection and the dosage were determined based on previous studies (Nadeau-Vallee et al., 2015; Wakabayashi et al., 2013b). By the

generation of this model, we validate the IL-6R as an important target in the context of PTB and confirmed the potential of 633 to prevent the onset of PTB. In this model, we observed that consistently, the positive control (IL-6 + vehicle) presented its births before the other groups. We also observed, as seeing in Figure 3.5. B, how the generations of births between the negative control (vehicle + vehicle) and the 633 group (IL-6 + 633) were amazingly similar by presenting must of their births on gestational day 19, which is the normal gestational day for the CD-1 mice. Additionally, we noticed a consistent delay in the TOC group (IL-6 + TOC), which presented an onset of births on gestational day 19.5 and 20. This could be explained by the presence of a complete IL-6 signaling blockade driven by the competitive action of TOC, due to the fact that TOC is a monoclonal antibody. There are reports showing that the absence of IL-6 signaling consistently translated into a parturition delay. This report was made by the study of the parturition age on KO mice for IL-6 (Robertson et al., 2010). Therefore, the results in this model demonstrate that IL-6 is essential for the onset and PTB, by the significant increase of neonatal prematurity (Figure 3.5. A). The results also showed that the complete blockage of IL-6 signaling translates into a significant delay of the onset of parturition and that the 633generated selective modulation of IL-6 signaling allow the IL-6-induced pregnant mice to return to the control levels of prematurity and time of delivery.

A comparison between the IL-6-induced PTB model to other reports made with other cytokines suggests that IL-6 is not a strong PTB inducer. A report made on the effect of IL-1β shows a tendency of causing parturition on gestational day 17.5, which means 24 hours before what was presented in this work (Nadeau-Vallee et al., 2015). Because of these results, we decided to analyze the different maternal and gestational tissues that presented a relevant role for PTB after 24 h of IL-6 stimulation. For its analysis, we performed a screening of either proinflammatory proteins or UAP by quantitative PCR. The results showed a significant increase in several genes mainly in fetal membranes, being IL1B, IL6, PTGER3, OXTR, NFKB and PGES. The upregulation of IL1B, IL6 and NFKB demonstrate the beginning of an inflammatory response. The presence of OXTR, PTGER3 and PGES showed also the beginning of a generation of a prostaglandin-dependent response, which is crucial for the onset of parturition (Behrman & Stith Butler, 2007). The analysis made

on the uterus showed just a significant upregulation of caspase 1 (CASP1), the upregulation of this protein also demonstrate an upstream function of IL-6 compared with the effect of IL-1β. This belief is supported by previous reports on the IL-1β activation process which collocates CASP1 as an upstream protein that will help in the cleavage for the activation of IL-1β (Gotsch et al., 2008). We observed an upregulation of IL6 and CCL2 in leukocytes, which can be explained as a normal response to the generation of an IL-6-generated inflammatory effect and leukocyte recruitment (Hunter & Jones, 2015). Lastly, we analyzed the response on the placenta, and we detected an upregulation of the main pro-inflammatory cytokines, demonstrating, one more time, the capacity of IL-6 to generate an inflammatory response that will later translate in the onset of PTB (Cappelletti et al., 2016). Thanks to the tissue analysis, we also observed a 633-mediated negative modulation of almost all the upregulated genes in fetal membranes, uterus and leukocytes. We also observed no effect of 633 on the gene upregulation in the placenta. Previous reports that worked with small peptides showed the capacity of these to reach placental cells, therefore further studies will need to be made in order to explain the lack of function of 633 in the gene upregulation in placenta (Nadeau-Vallee et al., 2015). We believe that 633, by being a small molecule, is able to cross maternal membranes, which allows it to generate a regulatory effect on fetal membranes (Wakabayashi et al., 2013b).

4.6. IL-6 and 633 direct effect on gestational tissues

After the results obtained in the IL-6-induced PTB model in mice, we decided to review if the activity of IL-6 was done by an-induced systemic inflammation or if IL-6 was able to exert some actions directly in the gestational tissues. In order to solve our question, we decided to perform the IL-6 induction *ex vivo* in the different gestational tissues collected exactly in the same gestational day than the induction by IL-6 in the previous mice model (gestational day 16.5). We decided to work with tissue explants because of the already know limited expression of the IL-6R, therefore, we thought that the use of tissue explants would allow us to have different

types of cells in each explant and it will also better mimic the physiological environment (Calabrese & Rose-John, 2014). We also decided to search just for the upregulation of pro-inflammatory cytokines due to the innate pro-inflammatory nature of IL-6 (Zhang, Shen, Xie, Chu, & Ma, 2015). The results displayed a significant induction of IL1B in fetal membranes and an induction of CASP1 and TNFA in uterus. We did not observed any induction of pro-inflammatory cytokines in placenta. The induction of IL1B, CASP1 and TNFA was expected because of our previous results that showed how IL-6 is able to induce expression of other proinflammatory cytokines and the late response of the IL-6-induced PTB. This response makes us believe that IL-6 acts by generating an induction of other proinflammatory cytokines, which will later generate a strong response in the gestational tissues and provoke the onset of PTB (Nadeau-Vallée et al., 2015). The lack of response in placental tissue was surprising because we expected a strong induction in this tissue, due to the fact that it presents an important blood supply for the fetus and the IL-6R is highly expressed in leukocytes (G. W. Jones et al., 2010). The obtained result drives our current thinking to the possibility that the effect showed in placenta in the IL-6-induced PTB model in mice is due to a direct effect in systemic leukocytes which will later generate another pro-inflammatory molecules that will interact with the placental cells generating a response on this gestational tissue (Barnes, Anderson, & Moots, 2011). In this experiment we also observed a significant 633-driven negative modulation of IL1B in fetal membranes and TNFA in uterus. This results are very promising because this showed us how the activity of 633 can prevent the generation of a pro-inflammatory environment in the relevant tissues for parturition (Menon, 2016). After establishing a reproducible method for the induction of explants *ex vivo*, we decided to generate a dose-response curve in this tissues (Emilien et al., 2000). We decided to perform this dose-response curve just in uteri explants because of the limited amount of fetal membranes found in some of the mice. We really considered the last part because we wanted to have enough tissue from the same animal to tested in all the experimental doses, and thus, to give a trustful result. For all the stated before, we performed a dose-response curve in uteri for the 633 response against the IL-6 induction of TNFA. The obtained curve showed us a negative modulation of the TNFA production in a dose response manner which got into a complete inhibition in the highest concentrations of 633. With this result we confirmed, once again, that 633 is

a valuable and promising molecule to be developed into a potential therapeutic drug (Hughes et al., 2011). At this point, we had a promissory molecule that had the ability of specifically modulate the IL-6 signaling pathway by selectively modulating the secretion of proinflammatory molecules. We knew that IL-6 is crucial for the pathological onset of PTB, and that 633 was able to prevent an IL-6-induced PTB setting by selectively modulating the pro-inflammatory environment in the different gestational tissues (Robertson et al., 2010).

4.7. 633 prevents LPS-induced PTB in mice

Consequently to our results, it was time to test 633 in a more physiologically setting by mimicking an infection (Roberto Romero et al., 2002). In order to generate this mimicked environment, we used LPS in order to generate a response which will activate the TLR-4 and simulate a gram negative infection (Agrawal & Hirsch, 2012). Based on previous reports, we choose to perform an i.p. injection of LPS to generate a systemic response of infection, and in this environment we decided to study analyze the effect of 633 and TOC used as a one of our negative controls (Elovitz & Mrinalini, 2004). The application of the treatment via s.c. injection and the dosage were determined based on previous studies (Nadeau-Vallee et al., 2015; Wakabayashi et al., 2013). By the use of this model, we observed a significant increase in the prematurity of the positive control (LPS + vehicle) and, as seeing in the Figure 3.7.A, how 633 was able to significantly reduce the prematurity (LPS + 633). In this model, we observed that consistently, the positive control (LPS + vehicle) presented its births before the other groups. We also observed, as seeing in Figure 3.5.B, how the onset of births in the 633 group (LPS + 633) were delayed leading to most of the births on gestational day 19, which is the normal gestational day for the CD-1 mice. Additionally, we noticed, as we showed before in the IL-6-induced PTB model, a slight delay in the TOC group (LPS + TOC), compared to the positive control group (LPS + vehicle). The TOC group (LPS + TOC) presented most of their births on gestational day 19.5 and 20. This would confirmed that the presence of a complete IL-6 signaling blockade by the competitive action of TOC, causes an impaired induction of

parturition and settles the importance of IL-6 in both PTB and physiological parturition. Therefore, the results in this model demonstrate that 633 activity is enough to prevent an infection-induced PTB setting and highlights the promising action of 633 to be a potential therapeutic agent used against PTB.

4.8. 633 specific modulation of proteins and genes in maternal and gestational tissues

We decided to take tissues for further analysis 12 hours after LPS induction due to the fact that waiting more days will generate the onset of parturition for most of the animals in the positive control group (LPS + vehicle) and this will limit the type or quantity of tissues that we would be able to collect. After collecting all the tissues, we performed a screening of either proinflammatory proteins or UAP by quantitative PCR. The results, consistently with what was seeing in the IL-6-induced PTB mice model, showed a significant increase in several genes mainly in fetal membranes, being IL6, TNFA, CCL2, IL-12, PTGFR, CASP1 and PGES. The upregulation of IL6, TNFA, CCL2, IL-12 and CASP1 demonstrate the beginning of an inflammatory response. Even the presence of CASP1 showed us the beginning of the induction of IL1B (Gotsch et al., 2008). The presence of PTGFR and PGES showed also the beginning of a generation of a prostaglandin-dependent response, which is crucial for the onset of parturition (Behrman & Stith Butler, 2007). The analysis made on the uterus showed significant upregulation of CASP1, IL-6, TNFA and CX43. This response demonstrated the generation of a pro-inflammatory environment and the beginning of uterine contractility due to CX43 (Behrman & Stith Butler, 2007). We observed an upregulation of IL6 in leukocytes, which can be explained as a normal start response due to the presence of an infection in the body (Akira et al., 1990). Lastly, we analyzed the response on the placenta, and we detected an upregulation of just one pro-inflammatory cytokine, IL1B, this will explained a beginning of an acute inflammatory response that will later translate into the onset of PTB (NadeauVallée et al., 2015). Thanks to this tissue analysis, we observed a 633-mediated negative modulation of different genes in the selected tissues. In this case we observed a

negative modulation mainly of the generation of proinflammatory cytokines and of most of the genes upregulated in fetal membranes. Again, in this model, we were not able to observe a significant regulation or tendency in placenta. Pulling together all the previous results made us believe that maybe in this tissue there are not enough IL-6R and therefore there is not an IL-6-driven response which provokes a lack of response against 633. We think that the results obtained are due to the previous stated fact because 633 is able to modulate consistently the fetal membranes which confirms the ability of 633 to cross feto-maternal interface. The results observed in this model also confirmed the hypothesis of IL-6 being upstream of the action of IL-1β or TNFα in the PTB scenery. This conclusion is stated because of the profile of gene expression in the different tissue only after 12 hours of LPS induction, which is a strong inducer of PTB (Wakabayashi et al., 2013). The early gene expression analysis, as we saw before in the IL-6-induced PTB mice model, showed mainly an upregulation of different kind of inflammatory and pro-parturition genes in fetal membranes, a lack of response in placenta and an IL-6-driven systemically response in leukocytes. This makes us believe that the blockage of IL6 pathway is extremely important in the cases of PTB because this molecule exerts its actions upstream on the parturition cascade, and therefore, it will prevent all the comorbidities that come along with this pathology. In the LPS-induced PTB mice model, we also generate the analysis of protein secretion in the different maternal and gestational fluids important for PTB. We noticed a significant upregulation of CRP levels in maternal plasma and of IL-1β levels in amniotic fluid. We also observed the capacity of 633 of significantly modulate the upregulation of both proinflammatory proteins. The effect of 633 was able to return this group levels (LPS + 633) into the negative control levels (vehicle + vehicle). We believe that this effect may be due to the modulation of the STAT3 signaling pathway by the 633 binding to the receptor. STAT3 activation is known to produce the pathway that will generate the production of acute phase proteins which include both CRP and pro-inflammatory cytokines (Stephanou et al., 1998). The secretion of this proteins will cause a later induction of more inflammatory proteins and it will generate the onset of PTB. It is interesting to remark the presence of a negative modulation in the TOC group (LPS + TOC) in the IL-1β upregulation in amniotic fluid. This response makes us believe that the IL-6-driven secretion of IL-1β in amniotic fluid is not generated by direct stimulation of IL-6 in the fetal side because

TOC is a very large molecule that is not able to cross fetomaternal membranes (Wakabayashi et al., 2013). Therefore, this IL-1β stimulations can be due to a downstream signaling cascade that started with the effect of IL-6.

4.9. 633 improves neonatal survival and weight.

Lastly, we wanted to know if the effects of 633 were sufficient to help in the state of the neonate. In order to have a first conception of this, we keep track of the survival rate of the neonates and to the neonate weight. We observed a significant reduction of the neonate survival in the positive control group (LPS + vehicle) and the capacity of 633 to return the survival to 100%, as seeing in the negative control group (vehicle + vehicle). We also observed a decrease on the neonatal survival rate in the TOC group (LPS + TOC). This result showed us that even though TOC was able to return the birth timing to the physiological age or even delayed it for a day, TOC was not capable of rescuing the neonates. This could be explained either by the inability of TOC to cross fetomaternal membranes, which will explained that TOC prevented the generation of uterine contractility but it did not prevent the creation of a pro-inflammatory environment in the fetal compartment (Wakabayashi et al., 2013). The second explanation will be related to the complete blockage of TOC in the IL-6 signaling. This will highlight the importance of selectively modulating the IL6 signaling and the importance of the other two IL-6 pathways for the maintenance and health of the neonate (Han & Holtzman, 2000; Injury et al., 2011; Mihara, Hashizume, Yoshida, Suzuki, & Shiina, 2012).

5. CONCLUSION

In this work, we were able to demonstrate, by the use of different assays and models, the potential of a novel small peptide that can selectively act as an antagonist of the IL-6R. We showed how 633 can selectively modulate the IL-6 signaling pathway and how this modulation can translate into an inhibition of the secretion of other pro-inflammatory cytokines, thus, preventing the generation of an inflammatory environment. We corroborate the ability of 633 of reducing the IL-6 efficacy and even increase its affinity at the highest 633 doses. We proved the importance of the action of IL-6 in PTB by performing an IL-6-induced PTB mice model that confirmed how the presence of IL-6 provoked an increase in the prematurity percentage. We also confirm the efficacy of 633 in preventing the IL-6induced PTB by selectively modulating the gene upregulation in the different maternal and gestational tissues. We settle 633 efficacy in a more physiological model of PTB by generating its induction with the action of LPS. We observed the ability of 633 to prevent LPS-induced prematurity by selectively modulating gene and protein expression in different tissues. Lastly, we studied the effects of 633 on the neonate outcomes and observed a 633-driven rescue of the prematurity rate and the neonatal weight.

For all the different observations we can conclude that 633 is a valuable therapeutic agent against IL-6R and that its use can significantly reduce the IL-6driven inflammatory morbidities. The results indicate that 633 presents some allosteric properties like biased signaling and a diferential modulation of affinity and efficacy. We believe that thanks to the specific regulatory activity of 633, this molecule has tremendous potential to be used as a therapeutic drug against PTB and that its action can be beneficial for the prevention of PTB and its later comorbidities.

6. FUTURE DIRECTIONS

This work sets the basis of a potential negative modulator, called 633, that can be used as a therapeutic agent in scenarios of inflammation-linked PTB. We showed a first characterization of this promising molecule. Therefore, it will be important to do some extra studies to confirm the capacity of this molecule in order to take it further into clinical phases to make it become a commercially available drug. The information currently needed will be:

- To analyze additionally the binding capacity of 633 in the receptor.
- To confirm the lack of activity of 633 on other IL-6 functions different than the pro-inflammatory by analyzing some of the downstream active molecules.
- To understand the tissues reached by 633, this could be done by labeling the small peptide and tracking the localization in tissues *ex vivo.*
- To study 633 specificity by analyzing its effect into KO mice or tissue.
- To try 633 effect on other types of models like the use of LTA and RU846 in order to validate its effect in different types of infectious and non-infectious models.
- To study further the effect of 633 on the neonates by analyzing histologically the most important organs, called lungs, brain and heart.
- To study the effect of 633 later on the development of the enfant by the generation of functional and behavioural studies.

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