Immune-modulation by the placenta and its dysregulation in preeclampsia: role of syncytiotrophoblast microparticles and cytokines

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissentschaftlichen Fakultät
der Universität Basel

von
Anurag Kumar Gupta
aus Indien

Basel, 2005

Genehmigt von der Philosophisch-Naturwissentschaftlichen Fakultät auf Antrag von:

Dr. Sinuhe Hahn

Professor Markus Rüegg

Professor Peter Erb

Professor Wolfgang Holzgreve

Basel, den 22.April 2005

Prof. Dr. Hans-Jakob Wirz
Dekan

Table of content

Acknowledgments

This work was performed in the Laboratory for Prenatal Medicine, University Women`s Hospital/ Department of Research, University of Basel, Switzerland from December 2001 to March 2005.

My heartfelt gratitude goes to Dr. Sinuhe Hahn and I feel grateful to have worked with a person like him. He has been a great source of encouragement in cultivating my scientific enthusiasm and has been instrumental in developing my scientific intellect. His aptitude for science, his warmth and thoughtfulness has generated a lot of respect for him. I must thank him for giving me liberty to design my experiments and all the essential inputs and for his constant support. His encouragements and creative ideas and his firm faith in me are the foundation on which this thesis is built. I feel privileged to have worked as a part of his team.

I would like to extend my gratitude to Prof. Wolfgang Holzgreve, who gave me the opportunity to perform this work in his lab.

I owe sincere thanks to Dr. Corinne Rusterholz for sharing her knowledge, experience, and for reviewing this thesis. Her vital suggestions in this work gave me opportunity to develop a critical outlook for research.

A very vivacious, supportive and friendly lab atmosphere made all the difference. I fondly remember all my lab mates, present and past, who have contributed to a very amiable relation amongst us. N. Chiodetti, L. Dudler, T. Babochkina, S. Hristoskova, I. Haari, G. De Napoli, V. von Wartburg, S. Seelman, D. Huang, T. Meier and S. Shilpa have been great labmates and people to be around with. I would like to extend my thanks to all Drs. A. Schoeberlain, X.Y. Zhong, S. Mergathaler, C.S.K. Reddy, B. Zimmermann, Y. Li for their prompt help and guidance. Zestful moments spent celebrating birthday parties and lab outings will be treasured.

Vivian had always been there with her affections. She has been very generous in taking all the pains to help me in the all-official and non-official work. I appreciate her support and care.

Sachin and Arundhati are very special to me; they have been like family members, making home away from home, and always ready to help me in every possible way. Sudip, Rejina, Ratnesh, Richa, Navratna, Syam, Deepti have been very affectionate ; wonderful moments shared with them in Basel will always make me nostalgic and stay as ever cherished memories.

I am lucky to have such caring and zestful friends as Akanksha and Abhijeet. Together we have spent some quality time, memorable in every sense.

Nidhi is an adorable sister, who has been very caring and extensively supported me in every decision that I made. I thank my brothers, Alok, Ajit, and Manish, and all my

Bhabhis for their love and kind support. I would also like to extend my thanks to all my family members for sharing wonderful moments.

All this wouldn't have been possible without the love, support, blessing and sacrifices of my parents and my Chache and Chachiji. They have always supported me in all my endeavors, from whom I have learned the essential virtues of honesty and truthfulness, guiding me through life.

I also thank Daddy and Ma, for their kind love, support, encouragement and patience. Devam, is a great friend and a wonderful brother.

I feel blessed to share the bonding of immense love with Divya, and our lives together. She has always been the best friend and a source of inspiration. Her strength and faith in me guided me in difficult moments. Anything that I say falls short of the feelings that I store for her. Her valuable suggestions should be admired for shaping up this thesis.

Above all I thank that omnipresent, for without whose blessings this task could not have reached its finale.

SYMBOLS AND ABBREVIATIONS USED

1-MT	**1-methyl-DL-tryptophan**
CRH	**Cortico-releasing hormone**
h	**hour**
HUVEC	**Human umbilical venous endothelial cells**
IDO	**Indoleamine 2,3, dioxygenase**
IFNν,	**interferon gamma**
IL-2	**interleukin-2**
IL-6	**interleukin-2**
IL-8	**interleukin-6**
L	**Litre**
L-trp	**L-tryptophan**
MηCD	**Methy η cyclo dextrin**
mSTBM	**Mechanical STBM**
Mg	**Magnesium**
mg	**milligram**
min	**minute**
ml	**milliliter**
mM	**milli molar**
NETs	**Neutro**
nM	**nanomolar**
nm	**nanometer**
PLAP	**Placental like alkaline phosphatase**
pSTBM	**Placental perfusion STBM**
PBLs	**Peripheral blood lymphocytes**
PBMCs	**Peripheral blood mononuclear cells**
PBS	**Phosphate buffered saline**
PHA	**Phytohaemagglutinin**
PI	**PMA and ionomycin**
PMA	**Phorbol 12-myristate 13-acetate**
pmol	**picomole**
RBC ghosts	**Red blood cell microparticles**
STBM	**Syncytiotrophoblast microparticles**
TNFζ	**Tissue necrosis Factor ζ**
U	**Unit**
VE-CM	**STBM free villous explant conditioned medium**
v/v	**volume/volume**
w/v	**weight/volume**

Summary

The present study focuses on exploring the role of placental syncytiotrophoblast microparticles (STBM) during normal pregnancy and preeclampsia. In particular, the qualitative properties of the STBM were examined and the effects of STBM and placentally derived cytokines were studied on immune cells.

Three STBM methods were used to generate qualitatively different STBM:

A. Villous explant culture (vSTBM)

B. Perfusion of a single placental cotyledon (pSTBM)

These two methods were used for the first time. STBM were also prepared by third well established method:

C. Mechanical dissection of the villous tissue (mSTBM)

The qualitative assessment of the three STBM reveled that all the three STBM preparations are morphologically similar. But biochemically these three STBM preparations differ in the presence of syncytiotrophoblast protein PLAP, nucleic acids (DNA and RNA) and lipids. Functionally the three STBM behaved differently on endothelial cell cultures in that all the STBM preparations inhibited endothelial cell proliferation in different manner. Only mSTBM induced apoptosis in the endothelial cells further confirming that these three STBM preparations also differ biologically.

Comparative study of the three STBM preparations and placentally derived cytokines on purified T cell response in terms of T cell activation, proliferation, cytokine production and apoptosis further confirmed that the three STBM preparations differ in their qualitative nature. These differences among three STBM preparations are attributable to their mode of preparation. Placentally derived cytokines and STBM mostly reduced T cell responses. These findings suggest that placental factors help in immune modulation, which is essentially required for successful pregnancy completion.

The effects of the placentally derived factors were also examined on the innate immune response. For the first time we were able to show that placentally derived factors IL-8 and STBM were able to generate neutrophil extracellular traps (NETs). This is the first indication where physiological signals have been shown to generate NETs. Furthermore, massive presence of the neutrophil NETs were observed in the preeclamptic placenta, which might make a revolutionary change in our understanding of the pathogenesis of this pregnancy related disorder.

Introduction.....

1. Introduction

Viviparity is a unique characteristic of mammals. An embryo is a mating product of histo-incompatible individuals in an outbred population. It can be compared with a semi-allogenic graft which has to be tolerated over all the gestational period [1]. Gestational outcomes avoiding fetal defects or loss, maternal infection, or morbidity are contingent upon an intimate association between mother and developing fetus that nurtures the fetus without provoking maternal immune responses. The process of nurturing new individuals in this way necessitates exquisite integration and coordination of several complex biological processes, including metabolic, endocrine, vascular, and immune functions. Almost certainly, ancestral mammals evolved fundamental mechanism(s) to allow successful viviparity [2].

Although pregnancy seems a happy symbiosis between mother and genetically different fetus but reality of this symbiosis is very complicated. Success of any pregnancy hinges on mother's embracing her allogenic fetus immunologically rather than attacking it. Perturbations to the feto-maternal symbiosis can lead to the miscarriage early in the pregnancy or premature birth later. Premature birth can also be triggered by a condition called preeclampsia.

1.1. Eclampsia and Pre-eclampsia: a historical view

Humans are the only mammals to suffer frequent eclampsia and preeclampsia [3, 4]. Eclampsia is an easily recognizable event and has been described in the medical literature as long as 4200 years ago (Egyptian Papyrus, Indian Atharva Veda, Petric Papyrus, Chinese literature). 2000 years ago Celsus described seizures in pregnant women, which abated with delivery. Since the condition seemed to arise without warning it was termed eclampsia from the Greek word for "lighting". The etiology of eclampsia typically consisted of the sudden onset of convulsions in women nearing delivery with approximately one-third ending in maternal death or fetal death *in utero*. It is that spectacular complication which prompted the first cesarean sections on dying convulsive parturients, at least 2000

years ago; to try to save the newborns lives. In today's modern obstetrics, eclampsia has artificially disappeared. Obstetricians either induce labor or cesarean section in women presenting with severe preeclampsia or threatening eclampsia [5]. Preeclampsia affects about 7-10% of the first pregnancies and is an important cause of maternal perinatal morbidity or mortality. Preeclampsia is a multisystem disease of pregnancy of unknown cause. It is a maternal syndrome, which is characterized by increased blood pressure, edema, proteinuria and abnormal clotting, liver and renal functions all of which may be due to the release of placental toxic factors into the mother’s circulation. The only effective therapy to this complication is to facilitate the delivery (induction, cesarean section) [6-7].

1.2. Clinical features of preeclampsia

Preeclampsia is diagnosed when a woman with chronic hypertension develops new onset proteinuria after 20 weeks of gestation. This usually occurs over several days to weeks, but may occur more quickly. The following maternal signs or symptoms characterizes severe preeclampsia:

- Blood pressure greater than 160/110
- Severe headache
- Visual problems
- Protenuria
- Fluid in the lungs
- Low urine production (less than 500 ml in 24 h)
- Low platelet count
- Liver abnormalities

1.3. Pathogenesis of preeclampsia

It should be emphasized that the causes of preeclampsia remain unknown. Therefore, an attempt to define pathophysiological data in one causal framework represents another one of the many hypotheses proposed to explain the pathogenesis of preeclampsia. These theories can summarized in the following flow chart **(Fig: 1)**.

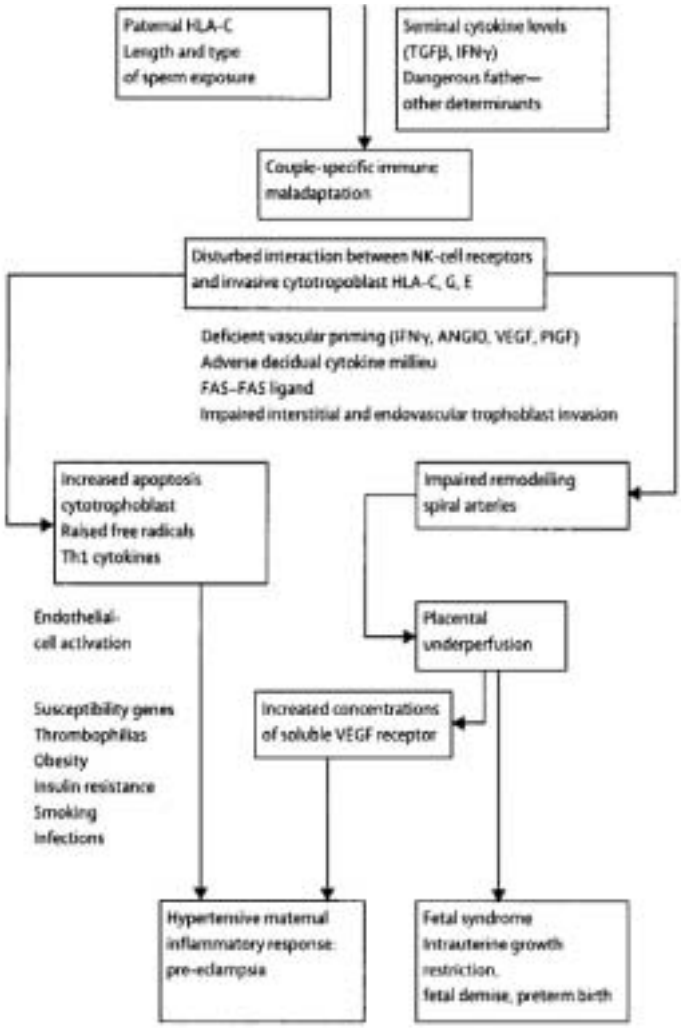


Figure: 1. Hypothetical cause and pathogenesis of preeclampsia. TGF=transforming growth factor. IFN=interferon. VEGF=vascular endothelial growth factor. PIGF=placental growth factor. ANGIO=angiopoietin 2. Adapted from Sibai B et al. Lancet 2005.

Preeclampsia is caused by presence of the placenta or the maternal response to placentation. However, it is now clear that poor placentation is not the cause of preeclampsia, but rather a powerful predisposing factor. Once poor placentation is established it leads to the maternal syndrome, depending on the extent to which it causes inflammatory signals and the nature of the maternal response to those signals [8]. Preeclampsia is characterized by abnormal vascular response to placentation that is associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system, and

endothelial-cell dysfunction. [8, 9]. Several studies have suggested that women who develop preeclampsia are at increased risk of cardiovascular complications later in life. Indeed, many risk factors and pathophysiological abnormalities of preeclampsia are similar to those of coronary-artery disease [10-12].

The cause of preeclampsia are often described as two opposing schools of thoughts—the vascularists, for whom ischaemia-reperfusion leads to oxidative stress and vascular disease, and the immunologists, who see preeclampsia as a maternal–paternal immune maladaptation (ie, a maternal alloimmune reaction triggered by a rejection of the fetal allograft). Recently it has been proposed that the difference between vascular and immune events is no longer reasonable in view the molecules secreted within the immune system. Most, if not all, cytokines are equipped with pleiotropic effects, of which action on the vascular endothelium and smooth muscle, coagulation, and other immune cells are most relevant to preeclampsia [13]. Following theories have been described to explain the pathogenesis of preeclampsia.

1.3.1. Placentation and the immune theory of preeclampsia

This theory includes abnormal placentation, placental oxidative stress and immunological factors that can be summarized as follows:

1.3.1.1. Impaired trophoblast invasion and differentiation

In human pregnancy implantation of the embryo occurs by two physiological invasions of the cytotrophoblast inside the uterine wall. First, cytotrophoblast cells stream out of the tips of the anchoring villi and penetrate the trophoblast shell and overlying syncytiotrophoblast to form cytotrophoblast columns that develop into the cytotrophoblast shell. Trophoblast cells continue to migrate into the decidua. After an apparent long pause (6–8 weeks) at the end of first trimester (14–16th week) of gestation, a second very deep trophoblast invasion colonize the placental bed's myometrium. Once the cytotrophoblast shell makes contact with spiral-artery openings, trophoblast cells stream into arterial lumina to form intraluminal plugs. Endovascular trophoblast cells replace the endothelium of spiral arteries and then invade the media, resulting in destruction of the medial elastic,

muscular, and neural tissue. Trophoblast cells become incorporated into the vessel wall, and the endothelial lining is finally reconstituted. As a result, these vessels undergo transformation from small muscular arterioles to large capacitance vessels of low resistance [14, 15].

Trophoblast differentiation during spiral arteries invasion involves alteration in expression of the number of different classes of molecules, including cytokines, adhesion molecules, extracellular matrix molecules, metalloproteinases, and the class Ib major histocompatibility complex molecule, HLA-G [16-19]. During normal differentiation, invading trophoblasts alter their adhesion molecule expression from those that are characteristic of epithelial cells (integrin ζ6/η1, ζv/η5, and E-cadherin) to those of endothelial cells (integrin ζ1/η1, ζv/η3, and VE-cadherin), a process referred to as pseudo-vasculogenesis [16] **(Fig: 2)**.

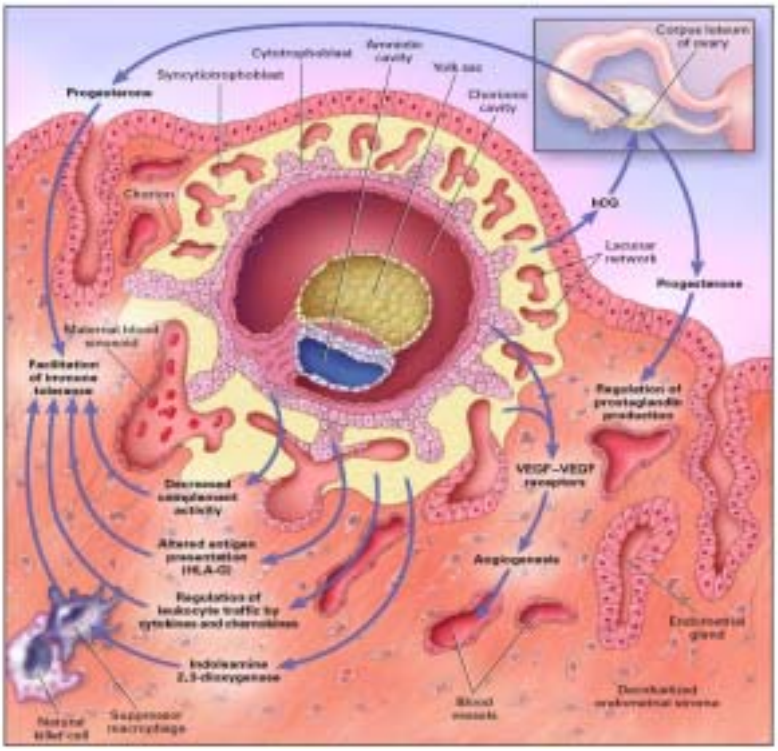


Figure: 2. Trophoblast differentiation and maintenance of early pregnancy. The diagram shows an implanted embryo (approximately 14 days after conception) and the processes necessary for the maintenance of an early pregnancy. VEGF denotes vascular endothelial growth factor, and hCG human chorionic gonadotropin. Adapted from Norwitz ER et. al. 2001, NEJM.

The proper trophoblast invasion and switching in the adhesion molecules allows increased blood flow (i.e., oxygen, nutrients) to the fetus. It has been proposed that the trophoblasts obtained from women with preeclampsia do not show upregulated adhesion molecules expression or pseudo-vasculogenesis [18]. The impairment of pseudo-vasculogenesis limits the cytotrophoblast infiltration only to the decidual portion of the spiral arteries and fails to penetrate the myometrial portion and inadequate transformation of spiral arteries around 14–16th week of the gestation, inducing poor vascular exchanges between the mother and the placenta [20-22] **(Fig. 3)**. The rise of blood pressure in the human mother is then probably a compensatory mechanism to increase the exchanges and try to save the fetus from poor supplies. Whether this adaptation is maternally driven or a response to fetal stress signals is yet unknown.

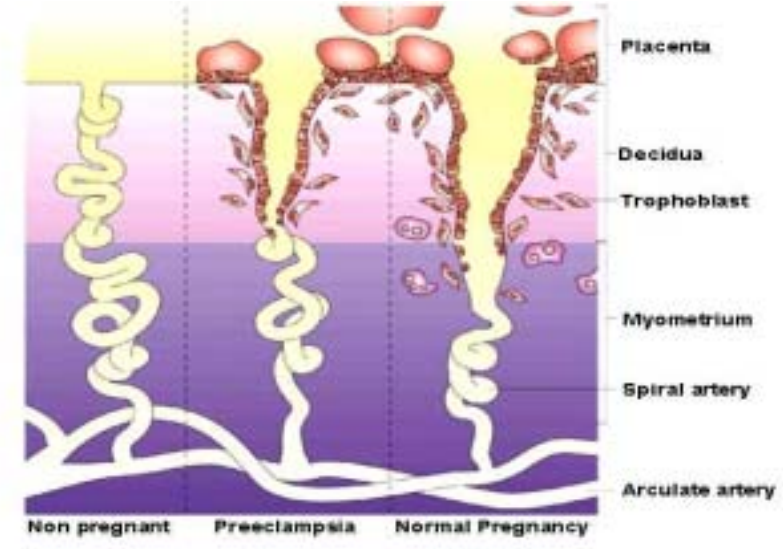


Figure: 3. A comparison between uninvaded arteries (non-pregnant), normal pregnancy and pathological conditions of pregnancy such as preeclampsia is shown. Note that the extent and depth of trophoblast invasion is less in pathological compared with normal pregnancy, which results in inadequate transformation of the spiral arteries in the former. This results in reduced blood flow to the feto-placental unit, which leads to poor fetal growth. Adapted from Moffett-King A, 2002, Nat. Rev. Immunol.

The primary event that contributes to failed trophoblast differentiation is unknown but placental oxidative stress and immunologic factors might play a role.

1.3.1.2. Placental oxidative stress

The trigger for the placental oxidative stress has yet to be identified. As described above defective remodeling of the endometrial spiral arteries is the most widely recognized predisposing factor for preeclampsia [23]. As a result, perfusion of the intervillous space is impaired compared with normal pregnancies, leading to the general assumption that the placental changes are induced by hypoxia [24]. However, no direct measurements are available to confirm that this is indeed the case. By contrast, pregnancy at high altitude is one of the few situations in which the oxygen tension in the maternal arterial supply to the placenta is known to be reduced [25, 26]. Comparison of the placental histological changes seen in these two situations indicates that many of the features that characterize the preeclamptic placenta, for example, the increased incidence of infarction, cannot be explained on the basis of hypoxia alone. It has been proposed that defective remodeling results in the retention of vasoreactivity in the myometrial segments of spiral arteries and that this leads to intermittent perfusion of the intervillous space and hence to fluctuating oxygen concentrations within the placenta [27]. Such fluctuations in oxygen tension could provide the basis for an ischemia-reperfusion type insult [27]. It has recently been demonstrated that oxidative stress occurs when hypoxic placental tissues are reoxygenated *in-vitro*, which is consistent with an ischemia-reperfusion injury [28]. Collectively, these findings indicate that hypoxia-reoxygenation (H/R) may represent a suitable model system for investigating the generation of placental oxidative stress in preeclampsia. Indeed, oxidative stress can cause apoptosis in numerous cell lines *in-vitro* [29, 30], and ischemia-reperfusion can also induce apoptosis in the heart and brain *in-vivo* [31]. Therefore, it is speculated that, preeclampsia may result from oxidative stress in the preeclamptic placenta leading to increased apoptosis or even necrosis [32]. Oxidative stress can lead to atherosis (lipid-laden cells in the wall of the arteriole), fibrinoid necrosis, thrombosis, sclerotic narrowing of arterioles, and placental infarction [33-36] although all of these lesions are not uniformly found in patients with preeclampsia, there appears to be a correlation between the severity of the disease and the extent of the lesions [37, 38]. It has been

proposed that the placenta, which is in oxidative stress may elaborate soluble factors into the maternal bloodstream, such as sFlt-1 (soluble Fms-like tyrosine kinase 1) which might play an important role in regulating the maternal vasculature during pregnancy, and proinflammatory cytokines that further alter maternal vascular endothelial cell function and lead to the characteristic signs and symptoms of preeclampsia [39].

1.3.1.3. Immunological factors

The focus on immunological factors as a possible cause of placental abnormality is based upon the observation that prior exposure to paternal/fetal antigens appears to protect against preeclampsia. This is illustrated by the following examples:

In a report of 1,011 consecutive women who delivered on an obstetric unit, the incidence of pregnancy-induced hypertension was 11.9% among primigravidae, 4.7% among same-paternity multigravidae, and 24% among new-paternity multigravidae [40].

The length of sexual cohabitation before conception is inversely related to the risk of preeclampsia, suggesting that prolonged exposure to paternal sperm antigens may be protective [40-43].

The incidence of preeclampsia is higher in women using barrier contraceptives and in those conceiving by intrauterine insemination with donor rather than partner sperm [44, 45], although this has not been a consistent finding [46].

Growing reports on the length of frequent sexual exposure and cohabitation of the couples in the reduction of preeclampsia have now confirmed that the preeclampsia is a disease of first pregnancy and long exposure of the paternal antigens reduces the frequency of the preeclampsia [47-49]. In summary, to reduce the risk of preeclampsia/eclampsia, it is better for the human female to avoid conception soon after initiating sexual relations with a new partner, regardless of her gravidity. Biological explanations for the observations mentioned above remain unclear, but the idea of preeclampsia being a graft rejection of the fetal–maternal allograft is a promising approach.

1.3.1.3.1. Preeclampsia: graft rejection of the feto–maternal allograft

Since half of the fetal genome is derived from the father, the fetus synthesizes antigens considered to be foreign by the maternal immune system therefore, mother should reject it, but this generally does not occur [50]. In his classic 1953 paper, Medawar proposed the concept of the "fetal allograft" to explain the immune relationship between mother and fetus for successful pregnancy out come. In this model, three hypotheses were proposed by the authors: 1) that the conceptus lacked immunogenicity; 2) that there was a significant lowering of immune response during pregnancy; 3) and that there is the elaboration of an immune barrier by the placenta [51]. Through the years, the third hypothesis, suggesting the existence of an immune barrier elaborated by the placenta, have acquired considerable attention. Originally, this barrier was presumed to be passive or neutral but later the placenta was shown to be a site of active tolerance. It is presumed that fetal cells and molecules are released into the maternal blood during proliferation of trophoblastic cells [52], following tissue ruptures that occur at the terminal extremity of the growing chorionic villi. The whole immune system comes into contact with these potential fetal immunogens. Therefore, tolerance to the semi-allogenic fetus by the maternal immune system seems mainly an active mechanism whereby fetal tissues are prevented from being recognized as foreign [53].

Means of fetal tolerance: Human beings have a particularly extensive placental invasion, possibly because of the long intrauterine period needed for fetal brain development [54]. The mucosal lining of the uterus is transformed from the endometrium in the non-pregnant state to the decidua in pregnancy. A major leukocyte infiltration is the major cellular characteristic of this change [55]. The process begins in the luteal phase before potential implantation. During early pregnancy, natural-killer cells in the uterus (probably derived from those in the blood) accumulate as a dense infiltrate around the invading cytotrophoblast cells. From mid-gestation onwards, these killer cells progressively disappear, which coincides with cytotrophoblast invasion, since human placentation is complete by about 20 weeks' gestation [55]. Natural-killer cells affect both trophoblast invasion

and vascular changes in the maternal placental bed [55]. The uterine natural-killer cells produce several cytokines that are implicated in angiogenesis and vascular stability, including vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and angiopoietin 2 [56]. Trophoblast-cell invasion into the decidua with its massive leukocyte infiltration and the subsequent arterial transformation results in close tissue contact between allogeneic cells. Then it should be consider that what immune mechanisms allow this deeply controlled trophoblast invasion? In this regard many mechanisms have been proposed by large number of studies, locally at the placental level, many different mechanisms are involved, working alone or in interaction with each other. These mechanisms are briefly summarized below:

- In contrast to classical HLA-A and HLA-B, syncytiotrophoblast expresses HLA-G, which is a very particular set of MHC class I molecules [57] whereas HLA-C seems weakly expressed [58, 59]. Recently, HLA-G has been found to bind the KIRs (killer-cell immunoglobulin-like receptors) of NK cells, as the other HLA molecules do. This interaction appears to block the cytotoxicity of these cells **(Fig: 4A)** and may contribute to the tolerance of the conceptus by reducing NK cell activity [60].
- During implantation, the endometrium secretes LIF (leukemia inhibitory factor) whereas the blastocyst expresses at its surface the LIF-receptor (LIF-R) [61-63] **(Fig: 4B)**. This is absolutely required for blastocyst implantation. During pregnancy, LIF is synthesized by the decidua—the maternal part of the placenta—and by Th2 lymphocytes whereas the syncytiotrophoblast expresses LIF-R [64] **(Fig: 4C)**. The exact function of the LIF/LIF-R molecules during pregnancy is still unknown but the binding of LIF on its receptor might favor trophoblastic growth and differentiation [65].
- Placental trophoblast epithelium express complement inhibitory molecules, such as MCP (membrane complement protein), DAF (decay accelerating factor) **(Fig: 4D)**, or other factors (i.e. Crry in rodents) [66-68]. These molecules may play an important role in protecting the semiallogeneic human conceptus from maternal complement mediated attack.

- Indoleamine 2, 3 dioxygenase (IDO) is produced by the syncytiotrophoblast and reduces close-range immune cell reactions. A proposed explanation for its role in tolerance of the fetus is that IDO could act by catalyzing tryptophan destruction in maternal immune cells that are localized in the placental area **(Fig: 4E)**; this tryptophan deprivation might reduce or inhibit some immune cell responses [69].
- The Fas/FasL system is in involved in cellular turnover, tumor cell elimination, antiviral responses or protection of tissues against activated lymphocytes. It might be active in controlling trophoblastic growth and elimination of cells endangering trophoblastic cells **(Fig: 4F)** [70, 71]. Fas present on trophoblasts does not seem to transduce apoptotic signals in these cells but could play a role in limiting the proliferation of the trophoblast cells [72].
- Annexins are membrane-associated proteins that are expressed in both normal and malignant cells and can also be secreted by the placenta [73]. Recently, Aarli and Matre showed that annexin II can partially inhibit lymphoproliferation of, and IgG and IgM secretion, by maternal immune cells [74] **(Fig: 4G).** Thus, this molecule might be involved in protection of the fetus against the maternal immune system.
- Syncytiotrophoblast, cytotrophoblast, decidual macrophages and both maternal and fetal endothelial cells consistently expressed IL-4 and can also influence cytokine production by the maternal cells, which can change Th1/Th2 balance [75, 76]. Trophoblast cells generally direct Th1/Th2 cytokine balance to a Th2-type response in the endometrium during pregnancy and reduce the production of pro-inflammatory molecules that can endanger fetus survival [77-80].
- Alternatively' activated macrophages that are able to exert anti-inflammatory influences occur naturally in placenta [81]. Alternative macrophage molecules are induced by IL-4 and inhibited by IFNν, while classical macrophages are induced by IFNν and inhibited by IL-4. Alternative macrophages produce little to no NO and free oxygen derivatives but produce more of the anti-inflammatory molecules IL-10 and IL-1-R-antagonist [82]. These observations

suggest that suppressive macrophages seems to be the first line of defense cells that, if they function successfully can reduce Th1 immunity [82].

- Deposition of semen in the female genital tract provokes a cascade of cellular and molecular events that resemble a classic inflammatory response. The critical seminal factor seems to be seminal-vesicle-derived transforming growth factorη1 (TGFη1)—it initiates a postmating inflammatory reaction, allowing an increased ability to sample and process paternal antigens, and a strong type-2 immune reaction. By initiating a type-2 immune response towards paternal antigens, seminal TGFη1 may inhibit the induction of type-1 responses against the semi-allogenic conceptus that are thought to be associated with poor placental development and fetal growth [83].
- The diversity of νι T cells in maternal tissue during pregnancy contrasts with a limited TCR subtype diversity in the non-pregnant state [80, 84]. Many other T, NK and NKT cells are present in the deciduas; the identification of their roles requires further research.
- Production of OX-2 by trophoblast and decidua, a glycoprotein which pushes Th1/Th2 balance toward Th2, also inhibits macrophages and decreases fgl2, a pro-thrombinase able to trigger abortion by placental thrombosis and activation of the complement, [85].

In summary, maternal tolerance of the feto-placental graft is the result of the integration of numerous mechanisms of various origins and modes of action; however not all of these mechanisms may really be important for the success of the pregnancy.

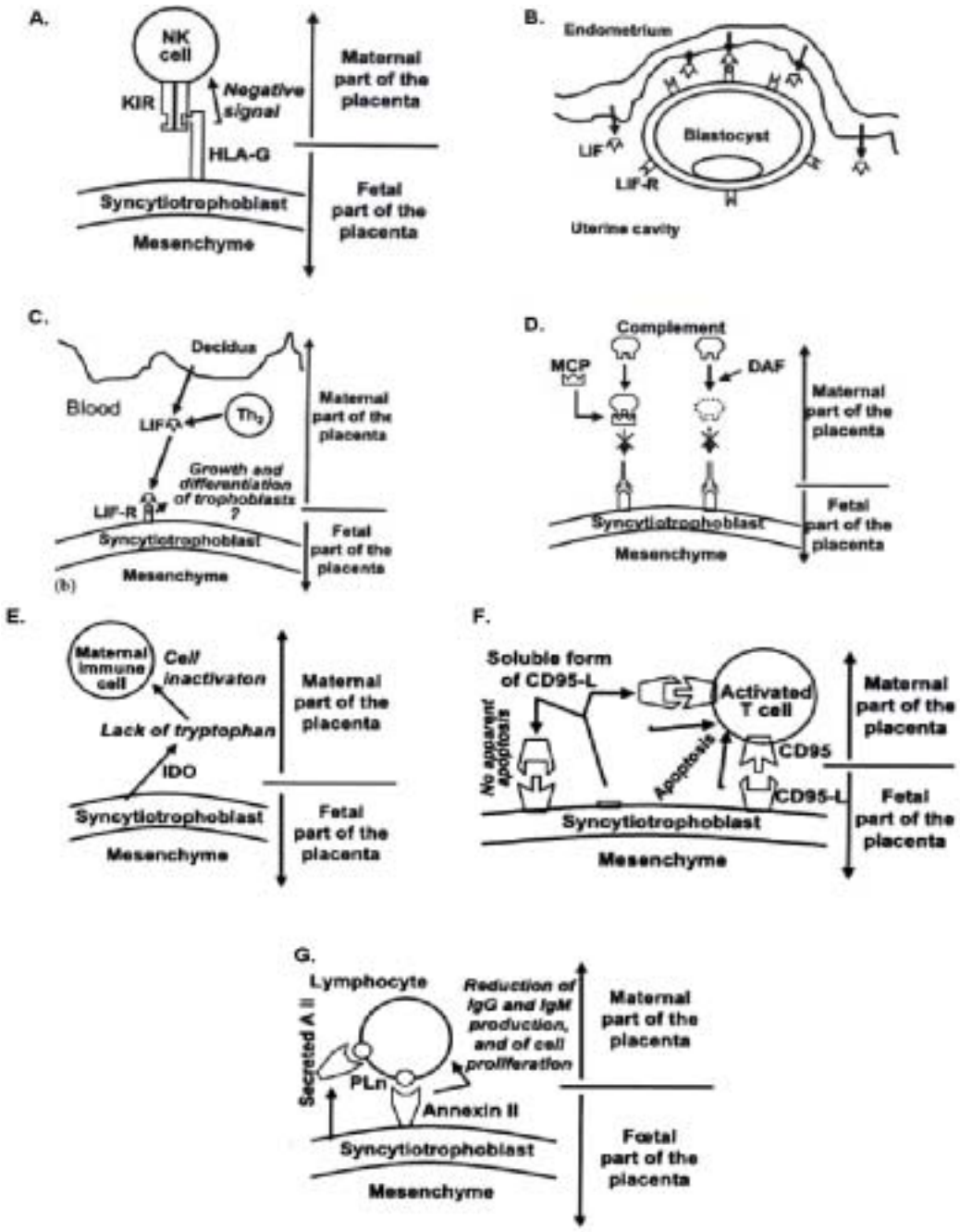


Figure: 4. Means of fetal tolerance during pregnancy. (A) HLA-G. HLA-G may inhibit cytolytic activity of NK cells by binding their KIR receptors. **(B)** LIF (by endometrium) and its receptor (on blastocyst) are essential for implantation. **(C)** After implantation, LIF could influence the growth and differentiation of trophoblasts . **(D)** Complement. Inhibition of complement (by destruction or binding) would reduce its availability to harm fetal tissues. **(E)** IDO is produced by the trophoblast and catabolizes tryptophane at short range and may so inhibit close maternal immune cells. **(F)** Syncytiotrophoblastic CD95-L (membrane or soluble form) can induce apoptosis in maternal immune cells, but not syncytiotrophoblastic cells, expressing CD95. **(G)** The Annexin

II secreted by the placenta could inhibit lymphoproliferation and secretion of maternal immunoglobulins. Adapter from Thellin O. et al., 2000, Curr. Opion. Immunol.

Means of fetal rejection: By definition, fetal rejection happens when critical tolerance mechanisms are absent or severed. A most important inducible way of rejection involves a Th1/Th2 imbalance toward Th1, increasing the production of Th1 cytokines, notably the pro-inflammatory IFNν , TNFζ and IL-2 [86]. The increase in the Th1 cytokines has been shown to be associated with preeclampsia [87]. Th1 cytokines can activate inflammatory macrophages and NK cells into lectin-activated killers (LAK), which then exert cytolytic capacities. Th1 cytokines can also act directly on trophoblastic cells, reducing OX-2 production and increasing their production of fgl 2 (pro-thrombinase) [84]. Fgl 2 can then induce clotting, which blocks the circulation of maternal blood in the placenta (thrombosis), thus the vital exchanges between the maternal and fetal bloods [88]. This leads to the death of fetal tissues. Thrombin-triggered IL-8-production in endothelial cells may attract and activate neutrophils, which attack feto-placental structures. Th1 cytokines also stimulate the production of immunoglobulins able to activate the complement cascade. Failure to reduce complement capacity contributes to the abortion process. TNFζ and IFNν, able to act directly on syncytiotrophoblasts, appear to be the effectors inducing spontaneous abortions in stress situations leading to coagulation, arrest of the local maternal blood flow, and aggression of the feto-placental structures [89]. Hormones or inhibitory molecules can counteract the placental hormones which sustain Th2-type immune responses.

1.3.2. Placental debris hypothesis-syncytiotrophoblast shedding

It has been known since the end of the 19th century that fragments of trophoblast break away from the surface of the placenta and enter the maternal circulation **(Fig: 5)**.

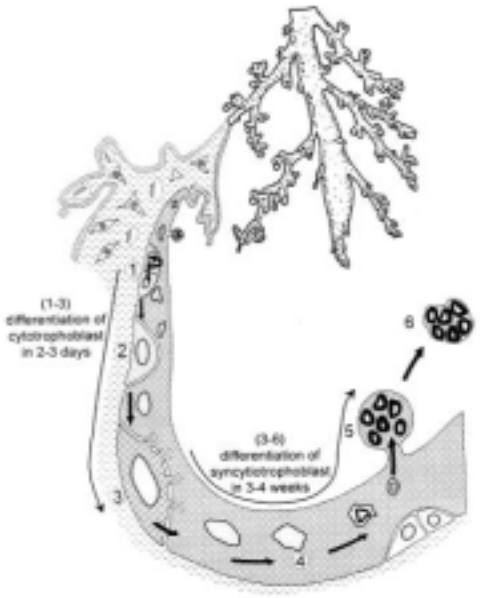


Figure: 5. Schematic representation of trophoblast turnover and trophoblastic apoptosis at the villous surface. Cytotrophoblast nuclei surrounded by high concentrations of ribosomes are transferred into syncytiotrophoblast by syncytial fusion. In the course of about three weeks the syncytially incorporated nuclei undergo apoptotic changes, accompanied by degranulation of the surrounding cytoplasm. Finally, they are extruded by apoptotic knotting and shedding.the density of point shading represents the density of the ribosomes (based on Huppertz et al., 1998).

It has been proposed that this shedding occurs in normal pregnancy and is significantly increased in preeclampsia. On the basis of increased shedding of STBM during preeclampsia the following models has been proposed for normal and preeclamptic pregnancies [90] **(Fig: 6)**.

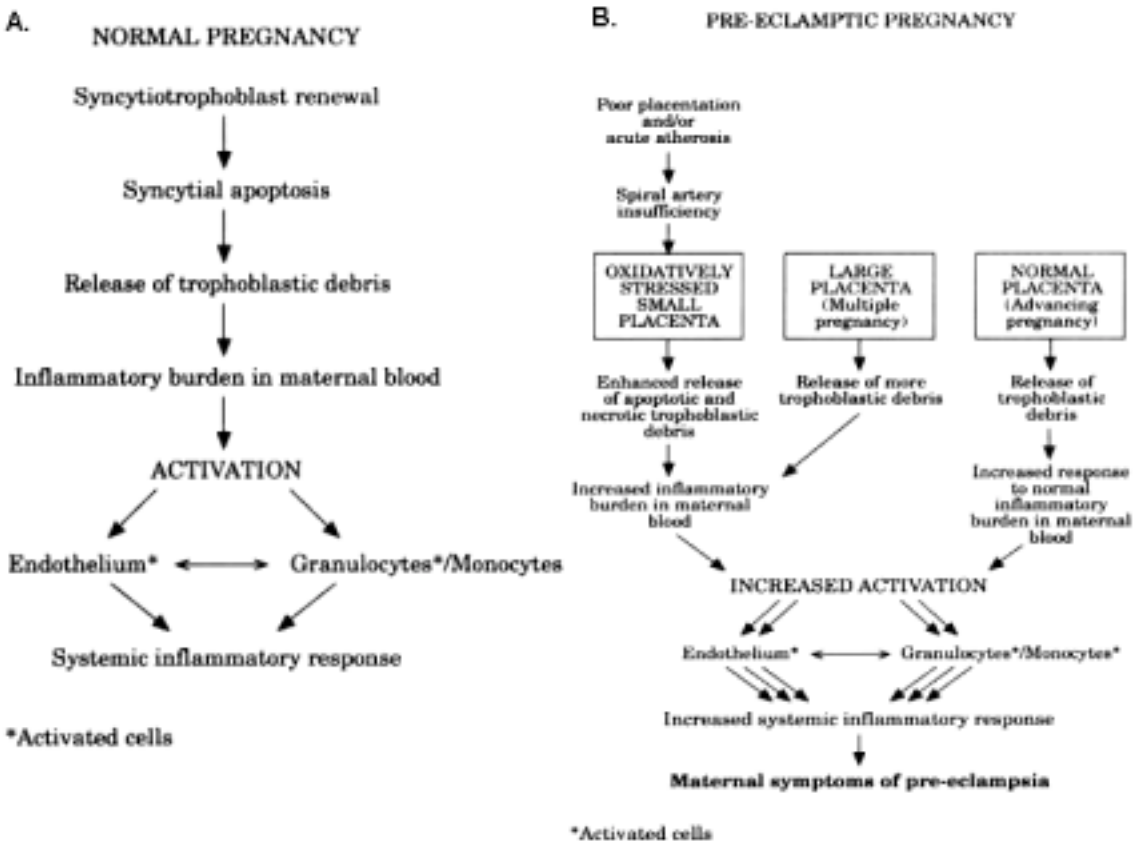


Figure: 6. A proposed model for the inflammatory response during normal and preeclamptic pregnancies. (A) low burden of trophoblastic release during normal pregnancy leads to mild inflammatory response. **(B)** Increased trophoblastic release during preeclampsia due to placental abnormalities leads to increased inflammatory response.

These "cellular" trophoblast fragments are readily detectable in uterine vein blood sampled at the time of caesarean section, both in preeclampsia and normal pregnancy, there are very few in peripheral blood samples taken from the same women [91], probably because they become trapped in the capillaries of the lungs. Thus, these fragments are unlikely to be the cause of systemic leukocyte and endothelial cell activation. The subcellular syncytiotrophoblast microparticles (which would circulate freely) are also shed into the maternal circulation. Using trophoblast-specific antibodies increased amount of microparticles released during preeclampsia have been detected in the maternal plasma in comparison to the plasma of normal pregnant women [92]. The search for syncytiotrophoblast debris, have revealed that levels of both cytokeratin (a cytoskeletal protein of trophoblast)

and fetal cell-free DNA are also significantly increased in preeclampsia compared with normal pregnancy [93-95]. Thus, there is a whole spectrum of syncytiotrophoblast debris in the maternal circulation.

Syncytiotrophoblast debris are the product of syncytiotrophoblast apoptosis: The reason for the shedding of this debris was not at first apparent. However, the proposal by Huppertz et al., [96] made clear that apoptosis plays a central role in the formation of syncytiotrophoblast from the underlying villous cytotrophoblast, and that it causes controlled cell fragmentation in order to allow continuous renewal of the syncytial surface of the chorionic villi, offering an explanation for the shedding of subcellular debris as part of a normal apoptotic process. Furthermore, it has been shown that this process is increased in preeclampsia [97, 98]. To what extent apoptosis or necrosis contribute in the debris seen in preeclampsia is not certain but this may have important consequences for the effects it may have on the maternal immune system.

The syncytiotrophoblast microparticles (STBM) that were detected in the maternal plasma were annexin V positive. This finding confirmed that STBM are product of apoptosis [99]. These experiments have shown apoptotic microparticles to be present in the plasma of normal pregnancy, with significantly higher levels in preeclampsia. However, it appears that particularly in preeclampsia there are more apoptotic microparticles than trophoblast microparticles [99]. It appears that large amounts of apoptotic particles during preeclampsia may have originated not only from the placenta but also from maternal leukocytes and endothelial cells that might also undergo apoptosis as a part of the systemic inflammatory response. Further detection of annexin V positive STBM in uterine vein and peripheral vein blood of normal pregnant and preeclamptic women revealed that levels of STBM were higher in the uterine vein than the peripheral vein samples [100]. Further evidence for this was obtained by looking for fetal and total cell-free DNA in similar samples. Fetal DNA was found to be higher in the uterine vein circulation whereas total DNA (fetal plus maternal) was the same in uterine vein and peripheral vein samples [101].

Why increased STBM shedding? The reason why levels of syncytiotrophoblast debris are significantly increased in preeclampsia is unknown. It has been believed since long time that the rate of apoptosis is thought to be increased in preeclampsia as a result of placental oxidative stress [97, 98], which is frequently, although not universally, increased in the condition. In 2000, Formigli et al proposed a chimerical type of cell death that is regarded as a truncated form of apoptosis with an incomplete execution, which is followed by degeneration via necrosis. This combination of both forms has been termed “aponecrosis” [102]. Recently it has been proposed that in preeclampsia not only apoptosis is increased, but it seems as if the whole turnover of villous trophoblast from proliferation via fusion to apoptosis is enhanced [103]. This may shed new light on the increase in syncytiotrophoblast apoptosis. The higher rate of proliferation seems to be linked to a higher rate of fusion, because a multilayered cytotrophoblast compartment is not a feature of preeclampsia [103]. With a higher rate of proliferation, much more material is introduced into the syncytiotrophoblast via fusion, and it is hypothesized that this multinucleated layer has to increase its apoptotic release of material to counterbalance the higher input. Thus the increase in apoptosis found in preeclampsia may not be a sign of damage but simply a sign of a higher traffic or turnover of villous trophoblast due to an adaptive process. The question now is whether the syncytiotrophoblast is able to adjust a new physiologic balance between input and output. If there is too much input to be compensated by apoptotic release, the apoptotic machinery of the syncytiotrophoblast may be overwhelmed and material may be released from the syncytiotrophoblast in a nonapoptotic manner [103]. Huppertz et al have recently shown similar apoptotic features in preeclampsia as observed by Formigli et al, and have proposed villous trophoblast in preeclampsia likely undergoes aponecrosis [103].

However, as preeclampsia may also occur with a perfectly normal placenta as in twin pregnancy, in these cases, it may just be the larger placental mass which contributes to the increased shedding of debris.

1.3.2.1. STBM and maternal inflammatory response

As STBM cannot be readily retrieved from maternal plasma samples, the *in-vitro* isolation of STBM have been performed by mechanical dissection of the term placenta [104]. It has been shown that mechanically prepared STBM from normal full term placenta disrupt the morphology, inhibit the proliferation of cultured endothelial cell monolayers *in-vitro* [105, 106]. Further it has been shown that the STBM prepared from the preeclamptic placenta also showed same levels of inhibition in proliferation of endothelial cells as shown by the STBM prepared from normal placenta [107]. Furthermore STBM perfused ex-*vivo* into pre-constricted small subcutaneous fat arteries alter their relaxation response [108]. It has, therefore, been proposed that the increased shedding of STBM into the maternal blood of women with preeclampsia could be responsible for the manifest dysfunction of the vascular endothelium [108]. The endothelial dysfunction caused by STBM may induce release of pro-inflammatory factors [109]. These inflammatory factors can activate lymphocytes *in-vitro* [109].

Increased STBM in preeclampsia could participate in pathogenesis by enhancing the inflammatory stimulus with or without specific immune recognition. STBM might interact with the maternal innate immune system to stimulate the inflammatory response in pregnancy. Monocytes and neutrophils binding to STBM results in raised production of TNFζ and IL-12, and superoxide radicals, respectively [110-112]. Many other placental factors seen in the maternal circulation during healthy pregnancy are increased in preeclampsia. These include several inflammatory cytokines, corticotropin-releasing hormone, free-radical species, and activin A; all could stimulate the maternal inflammatory response [113]. More recently it has been shown that monocytes bind STBM both *in-vivo* and *in-vitro* that can stimulate the production of the proinflammatory cytokines TNFζ and IL-12 *in-vitro* [114]. In particular TNFζ, with its ability to activate endothelial cells, cause microvascular protein leakage, and reduce acetylcholine-induced vasorelaxation, has received a lot of attention as a having a potential key role. Increased TNFζ amounts in the preeclamptic placenta are probably produced by villous stromal cells, especially macrophages, but sources other than the

placenta seem to contribute to raised plasma TNFζ and IL-6 concentrations seen in the disease [27, 115]. IL-12 derived from monocytes or macrophages is important in driving Th1 reactions in preeclampsia. IL-12 is a potent stimulus of IFNν release by natural-killer cells and naive T cells. Importantly, IFNν efficiently primes monocytes for further IL-12 release that triggers a feed-forward cycle, which could explain the very rapid deterioration in some severely ill preeclamptic patients [110, 111, 116].

It is known since long time that STBM can inhibit proliferation of PHA stimulated lymphocyte *in-vitro* [117, 118]. Recent studies have described the methods of purification of STBM from the maternal serum and have shown that shed STBM express FasL and have shown that exposure of T lymphocytes (Jurkat cells) to such STBM induced FasL mediated apoptosis and down regulation of CD3-zeta expression [119]. These evidences suggest that during normal pregnancy T cell response is impaired to suppress Th1 immune response. Thus, it can be interpreted that during preeclampsia excessive shedding of STBM might stimulate innate immune system that will induce Th1 response as discussed above.

1.3.3. Systemic endothelial dysfunction and inflammation

It is still uncertain whether preeclampsia is caused by the damaged ischaemic or reperfused placenta or by the inappropriate or exaggerated maternal inflammatory response towards the presence of the trophoblast, although the endothelium is associated with the pathophysiology of disease. The inflammatory response during preeclampsia is not confined to the disease only but is also present in normal pregnancy, albeit in a milder form. This "mild" inflammatory response appears to cause no harm and may even be beneficial to the pregnancy [120]. Preeclampsia develops when the normal inflammatory response in pregnancy becomes exaggerated in response to excessive stimuli and triggers the endothelial dysfunction and inflammation leading to the maternal symptoms [121].

The feto-placental unit initiates the pro-inflammatory stimulus. However, a fetus is not necessary as preeclampsia can occur in molar pregnancies where there is a large placental mass but no fetus [122]. If some or all of the placenta is left

behind, the maternal symptoms of preeclampsia may persist after the delivery of the preeclamptic pregnancy [123]. The factor must be such that it is present in the placenta in normal pregnancy and is shed into the maternal circulation where it can interact with leukocytes and/or endothelium to stimulate the inflammatory response [124]. This same factor would then be shed into the maternal circulation in increased amounts in preeclampsia to cause the exaggerated inflammatory response seen there. The excess release of this factor could be due to either oxidative stress resulting from a intermittent placental blood supply, as seen in the placentae of some cases of preeclampsia or may be the product of a larger placental mass such as in twin or molar pregnancy [121, 124]. There are many factors produced by the placenta (although not necessarily exclusive to the placenta), which are found in the maternal circulation in normal pregnancy, with increased levels in preeclampsia. These include STBM, various pro-inflammatory cytokines [125, 126] and activin A [127], all could be potential stimulators of the maternal inflammatory response.

All of the clinical features of preeclampsia can be explained as maternal responses to generalized endothelial dysfunction [120, 124,]. Endothelial dysfunction leads to hypertension, increased vascular permeability which results in edema and proteinuria.

Laboratory evidence supporting generalized endothelial dysfunction in preeclamptic women includes:

- Increased concentrations of circulating cellular fibronectin, factor VIII antigen, and thrombomodulin [128-130].
- Impaired flow-mediated vasodilation [108, 131, 132] and impaired acetylcholine mediated vasorelaxation [132].
- Decreased production of endothelial-derived vasodilators such as NO and prostacyclin and increased production of vasoconstrictors such as endothelins and thromboxanes.
- Enhanced vascular reactivity to angiotensin II [133].
- Serum from preeclamptic women causes endothelial activation *in-vitro* [134].

- Impaired endothelial function can be demonstrated by brachial artery flow-mediated dilation three years after a preeclamptic pregnancy [135]. It is unknown whether this is a cause or effect of the preeclamptic pregnancy.

These evidence suggests that endothelial dysfunction and inflammation is associated with the pathogenesis of preeclampsia.

Design of the study

In this study, we have explored the role of STBM and cytokines in the normal pregnancy and preeclampsia. To date all studies largely made use of mechanically prepared STBM, from normal full term placenta, which are most likely to be generated by a process of necrosis and might not mimic physiological conditions [105-109].

In this study, we hypothesized that not only the quantity of the STBM as well as their quality might also play an important role in the pathogenesis of preeclampsia. It has been proposed that STBM produced from normal or preeclamptic placenta display similar effects on endothelial cell cultures [107]. Therefore, to have qualitative difference among the STBM we prepared STBM from normal term placentae by three different methods:

A) Placental villous tissues from freshly delivered placentae were mechanically dissected as reported earlier and referred as mSTBM [104, 105].

B) Villous explants were cultured in conditions, which maintain overall tissue integrity and support the physiologic turnover of the syncytiotrophoblast, including apoptotic/aponecrotic shedding and referred as vSTBM [103].

C) Single placental cotyledons were perfused separately on both the maternal and fetal sides and the maternal washes were collected and referred as pSTBM [136, 137].

Further, the morphological, biochemical, functional properties of the STBM generated by the different methods were compared. After characterization of the different STBM preparations their effects were analyzed on various cell types to understand their role during normal pregnancy and preeclampsia.

The placentally derived soluble factors generated after villous explant culture were also prepared and was analyzed for the presence of cytokines to study their effects on immune cells.

AIM of the study

The broad goal of this work was to understand the role of placentally derived STBM and cytokines during normal pregnancy and preeclampsia. In particular the three STBM preparations were characterized qualitatively to mimic most physiologic STBM. Further the effects of all the three STBM preparations and placentally derived soluble factors were studied on activation, proliferation, cytokine production, apoptosis induction of different cell types such as endothelial, T cells and neutrophils.

Results.....

2. Results

2.1. Morphological and Biochemical Characterization of the STBM

In order to understand the functional aspects of the STBM we characterized them morphologically and biochemically. STBM were prepared by three different methods: 1) villous explant culture (vSTBM), 2) perfusion of a placental cotyledon (pSTBM), and 3) mechanically dissected STBM (mSTBM). The STBM depleted villous explant conditioned medium (VE-CM) from villous explant cultures was also collected considering it is a rich source of placentally derived soluble factors. As mentioned earlier that most of the previous studies have made use of mSTBM therefore, we first characterized our two new STBM preparations. mSTBM were also used in parallel to the other two STBM preparations.

2.1.1. Origin and Morphology of the STBM

2.1.1.1. STBM are membrane fragments

To examine whether the STBM prepared by the three methods are products of the membrane, we carried out FACS analysis of the STBM after labeling with membrane specific fluorescent dye PKH-26, which with its aliphatic tails incorporates into lipid regions of the cell membrane. Our FACS analysis of all the three STBM preparations revealed that >95% STBM were the products of the plasma membrane **(Fig: 7)**.

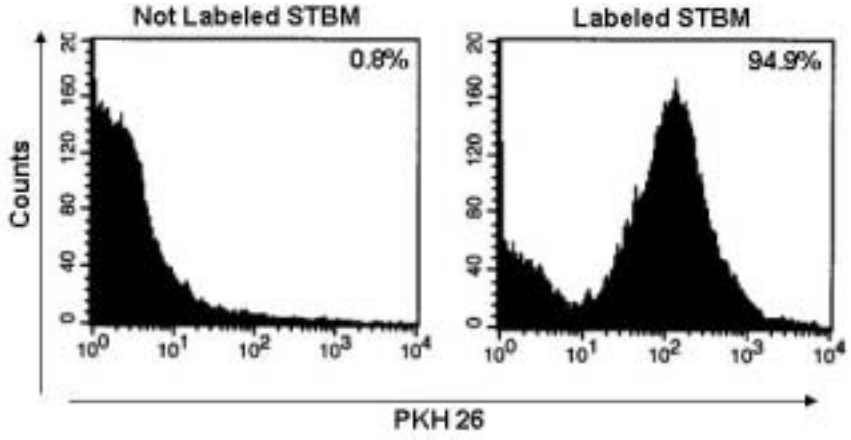


Figure: 7. FACS analysis of the STBM. STBM preparations were stained with PKH-26 dye and analyzed using FACS as described in materials and methods. This analysis indicated the STBM are the products of plasma membrane. A single representative analysis of mSTBM is shown.

2.1.1.2. STBM are the product of syncytiotrophoblast layer of the placenta

To confirm that the STBM are products of outermost layer of the placental villous tree i.e. syncytiotrophoblast, we examined these for the presence of PLAP, a GPI-anchored enzyme present in syncytiotrophoblast membrane, using an ELISA assay. STBM were captured with an anti-PLAP antibody and the endogenous phosphatase activity of the immobilized particles was measured. This analysis indicated that membrane associated PLAP was found in all three STBM preparations **(Fig: 8)**, with almost 4-fold higher concentration being present in the mechanically prepared particles.

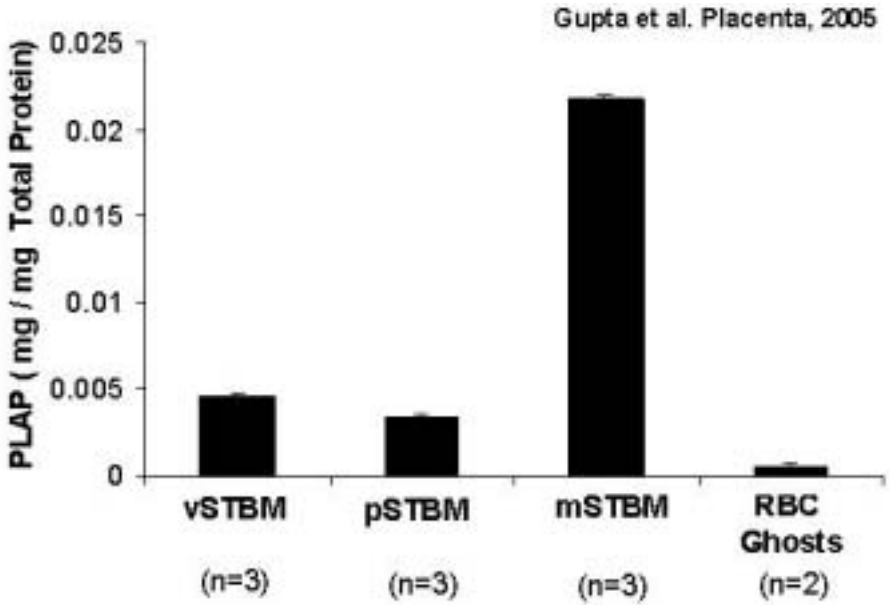


Figure: 8. Concentrations of PLAP protein in each of the STBM preparations. Equivalent amounts of STBM, as assessed by total protein content, were examined for PLAP by ELISA. The figure represents an examination of STBM preparations from 3 different placentae. RBC microparticles (RBC ghosts) were used as a negative control to confirm that PLAP is largely associated with the STBM only.

2.1.1.3. STBM preparations are morphologically similar

The STBM prepared by three different methods were examined using scanning electron microscopy. This analysis demonstrated that all three STBM preparations produced micro-particles with sizes ranging from 200-600 nm in diameter and that these particles were morphologically similar **(Fig: 9)**. Although the SEM analysis was not quantitative, the data suggest that the three preparations vary in the quantity of particles produced relative to the amount of starting material, with the greatest number being produced by the mechanical method. Mechanically prepared particles also had a tendency to aggregate in clumps **(Fig: 9C)**, a feature not observed with particles prepared by the other two methods.

Gupta et al. Placenta, 2005

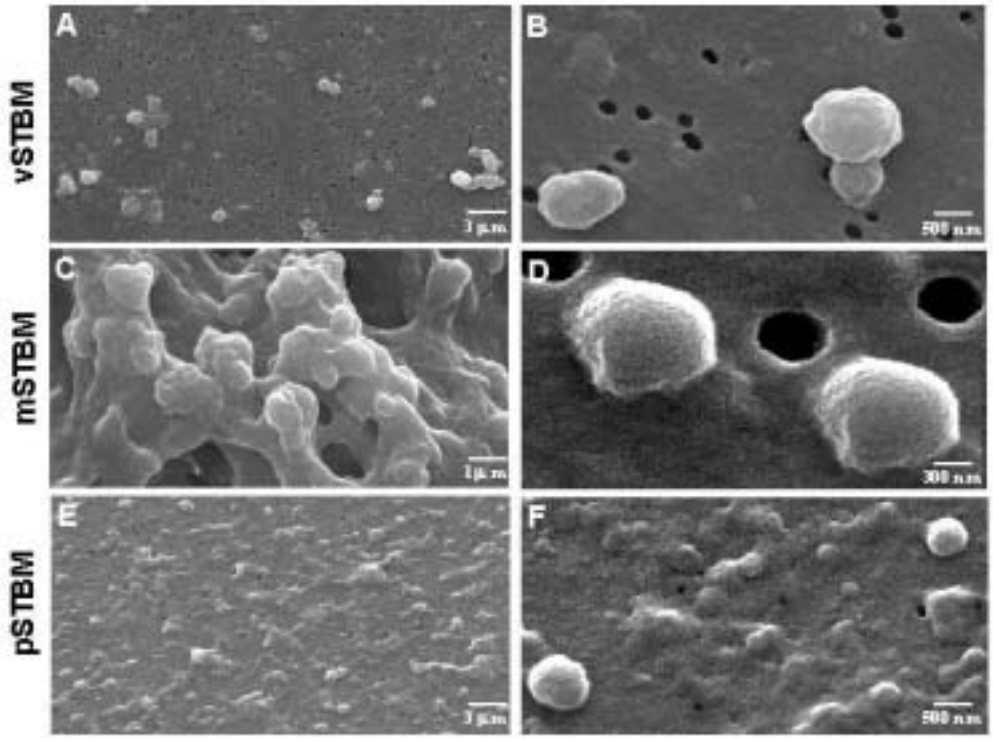


Figure: 9. Scanning electron micrographs of different STBM preparations. (A-B) vSTBM from explants, (C-D) mSTBM, (E-F) pSTBM. mSTBM tend to aggregate in clumps (C).

2.1.2. Biochemical properties of the STBM

2.1.2.1. STBM are rich in RNA and DNA

It has been demonstrated that the amount of fetal nucleic acids is increased in the maternal circulation during preeclampsia [94, 95,138]. It is possible that if fetal nucleic acids are associated with the STBM then their increased release during preeclampsia might contribute in increased nucleic acids concentration in the maternal circulation, therefore, DNA and RNA concentrations in the STBM preparations were quantified. The amount of fetal DNA in the different STBM preparations, obtained from male placentae, was measured by a Taqman® real-time PCR assay for a Y chromosome specific sequence (SRY) [138], whilst the presence of fetal mRNA was quantified by quantitative RT-PCR assay for CRH (corticotropin releasing hormone) gene, which is expressed in the placenta [139]. This analysis revealed that all STBM preparations contain both fetal DNA and mRNA, although the levels of each of these fetal analytes differ in the three preparations **(Table 1)**.

Gupta et al. Clin. Chem., 2004.

STBM	DNA (SRY)	mRNA (CRH)
Villous explant culture	94530.5 (9444-158135)	4986 (1232-10500)
Mechanical dissection	33 (307-7745)	121144.5 (17868-331607)
Placental perfusion	620 (268-2975)	222352.5 (98190-275981)

Table 1. Concentration of fetal DNA and CRH mRNA in different preparations. Six placentae were used for each STBM preparation. Circulatory fetal DNA (SRY locus) and mRNA (CRH) concentrations were determined by real-time PCR and real-time RT-PCR, respectively, and are represented as copies/mg of STBM. Values are given as median and range, in brackets.

In this regard, highest concentration of fetal DNA was detected in STBM prepared by vSTBM **(Fig: 10A)**, whereas the highest concentration of fetal RNA was present in pSTBM **(Fig: 10B)**.

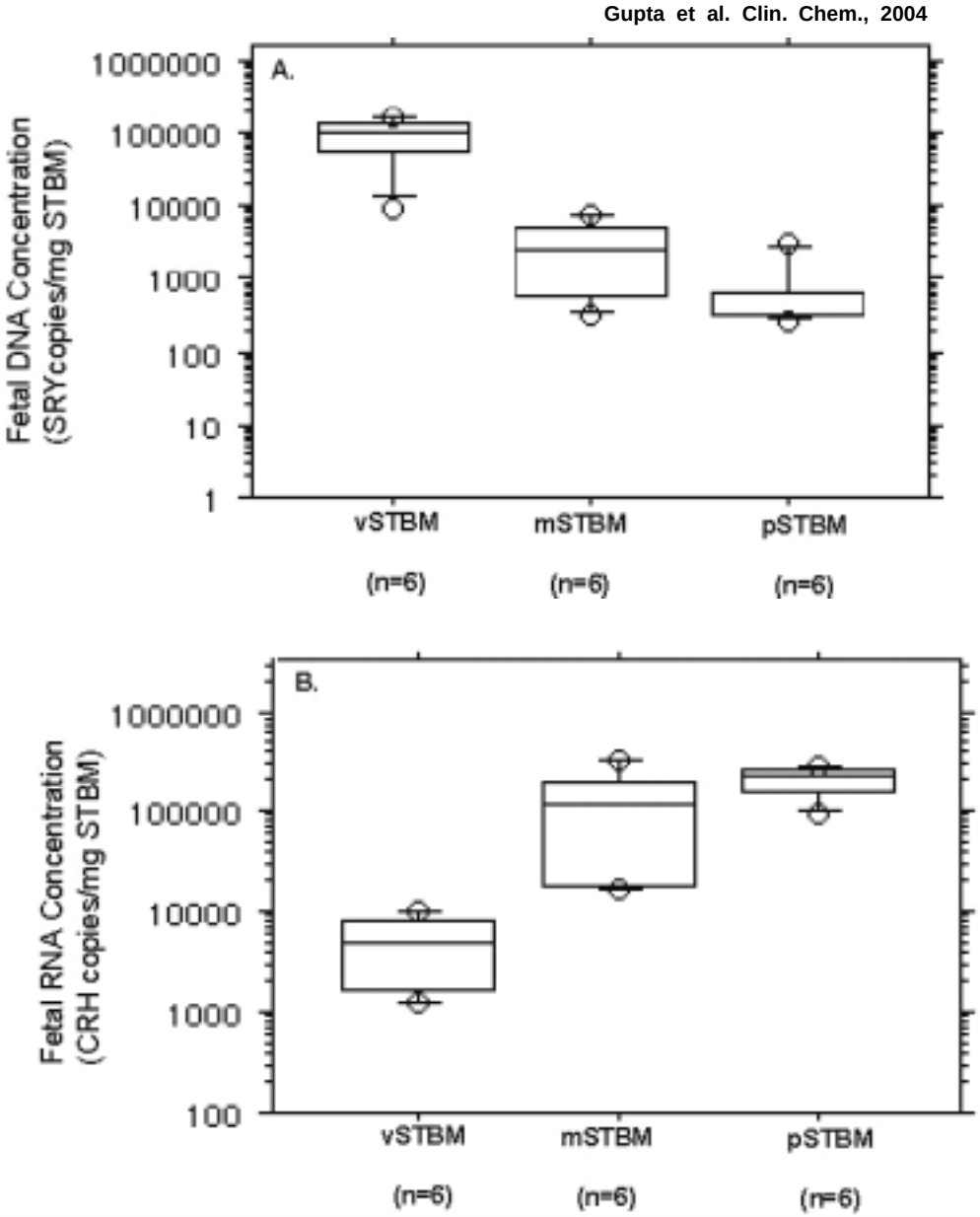


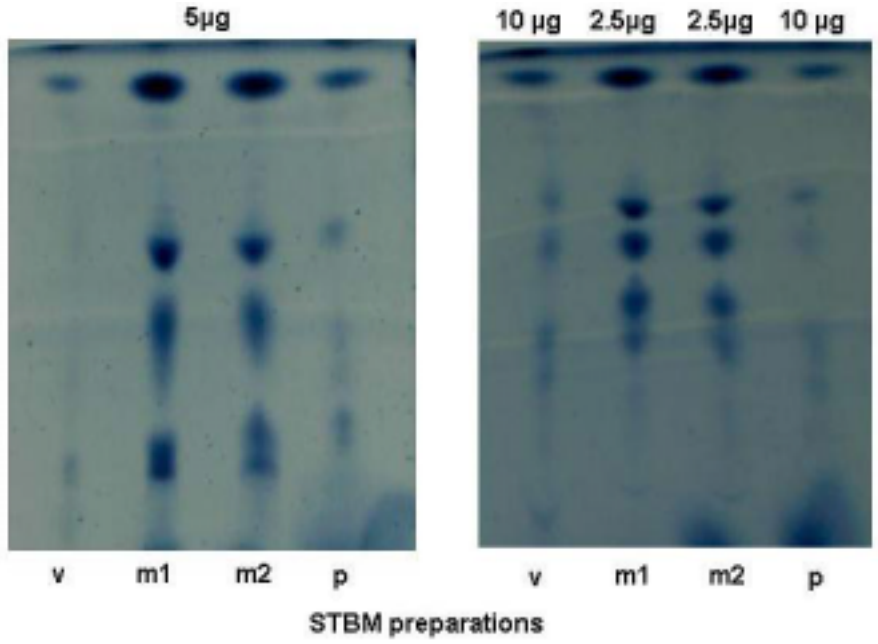
Figure: 10. Box-plot illustration of circulatory fetal DNA and mRNA concentrations in STBM preparations. Fetal DNA (SRY locus; A) and mRNA (CRH; B) concentrations were determined byreal-time PCR and real-time RT-PCR, respectively, and are represented as copies/mg of STBM. Six placentae were used for each STBM preparation. Box plots indicate median value (line in box), 75th and 25th percentiles (limits of box). Upper and lower horizontal bars indicate 10th and 90th percentiles, respectively. Open circles indicate outliners.

2.1.2.2. Lipid analysis of the STBM

Preeclampsia and atherosclerosis share similar symptoms and lipids have been shown to play an important role in the pathogenesis of both diseases [140]. As STBM are the products of syncytiotrophoblast cell layer of the villous tree, they should be rich in the membrane lipids, and therefore, may contribute in the pathogenesis of preeclampsia. Lipid content was examined in all the STBM preparations using Thin layer chromatography (TLC) and mass spectrometry.

All three preparation were applied to TLC the plate as mentioned in the materials and method. Around 6 spots were observed on the run front of the TLC plate. All the spots were identified by mass spectrometry and were products of cholesterol esters normally present in the plasma membrane [141]. The spot at the top of the TLC plate was identified as cholesterol. This analysis revealed that the lipid concentration in the mSTBM is relatively higher than the other two STBM preparations **(Fig: 11A)**. This indicates the possibility of mSTBM to be richer in membrane materials at equal protein concentration. These cholesterol lipids and cholesterol ester present in the mSTBM could be depleted in a dose dependent manner using a known compound Methyl-η-cyclodextrin (MηCD) that can deplete cellular membrane cholesterol [142] **(Fig: 11B)**.

A.



B.

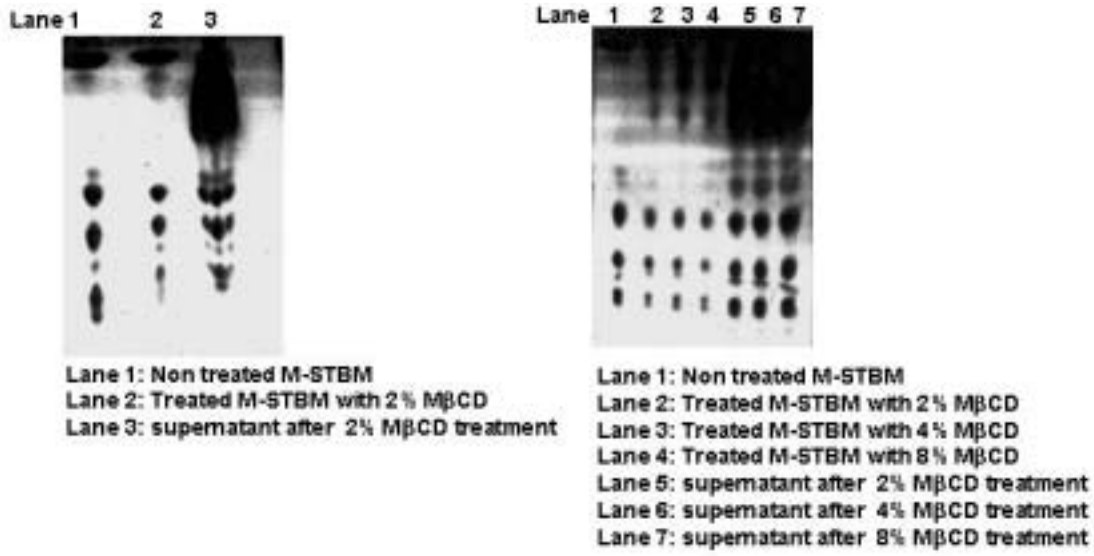


Figure: 11. Lipid analysis in the STBM preparations. (A). Lipids were extracted from all the three STBM preparations and TLC was carried out. TLC analysis revealed that mSTBM are rich in the lipid content than the other two preparations. **(B).** The lipids from the STBM could be depleted in a dose dependent manner using MηCD, depleted lipids could be observed in the supernatant.

2.1.2.3. Particle free nucleic acids and cytokines produced by the placenta

Placentally derived soluble factors might play an important role in the pathogenesis of preeclampsia. Therefore, STBM depleted VE-CM collected from the villous explant cultures. VE-CM was analyzed for the presence of nucleic acids and cytokines. In comparison to fetal RNA very high levels of fetal DNA were detected in the VE-CM **(Fig: 12)**. These levels of nucleic acid may correspond to the *in-vivo* situation where majority of the fetal DNA is cell free and the fetal mRNA species may be largely associated with membrane particles, as reported previously [143]. Supernatants collected from other two STBM preparations were also analyzed for the presence of nucleic acid and cytokines but we could not detect any nucleic acid and cytokines in these samples, which may be depends upon the mode preparation of the different STBM.

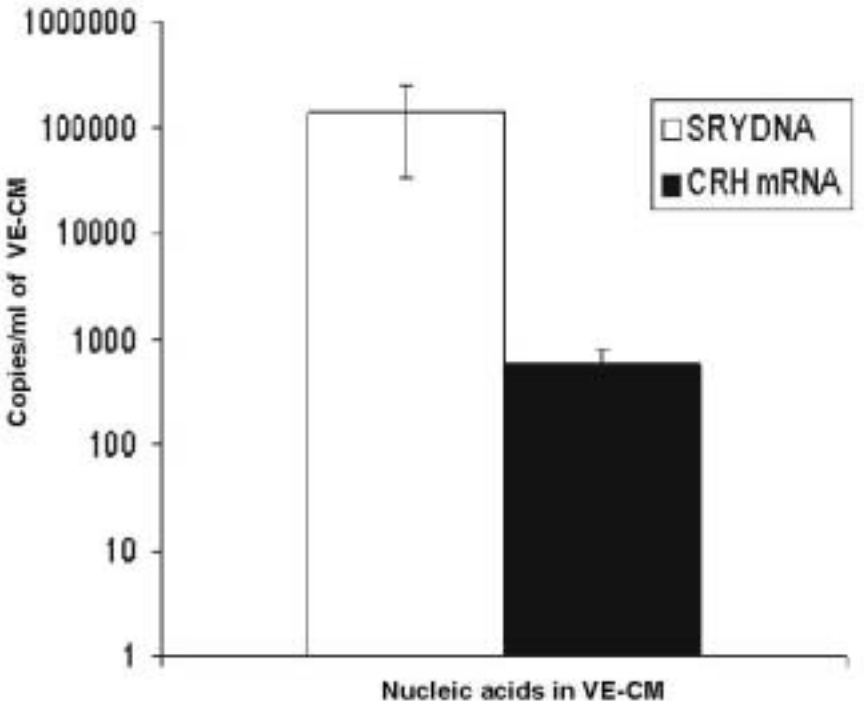
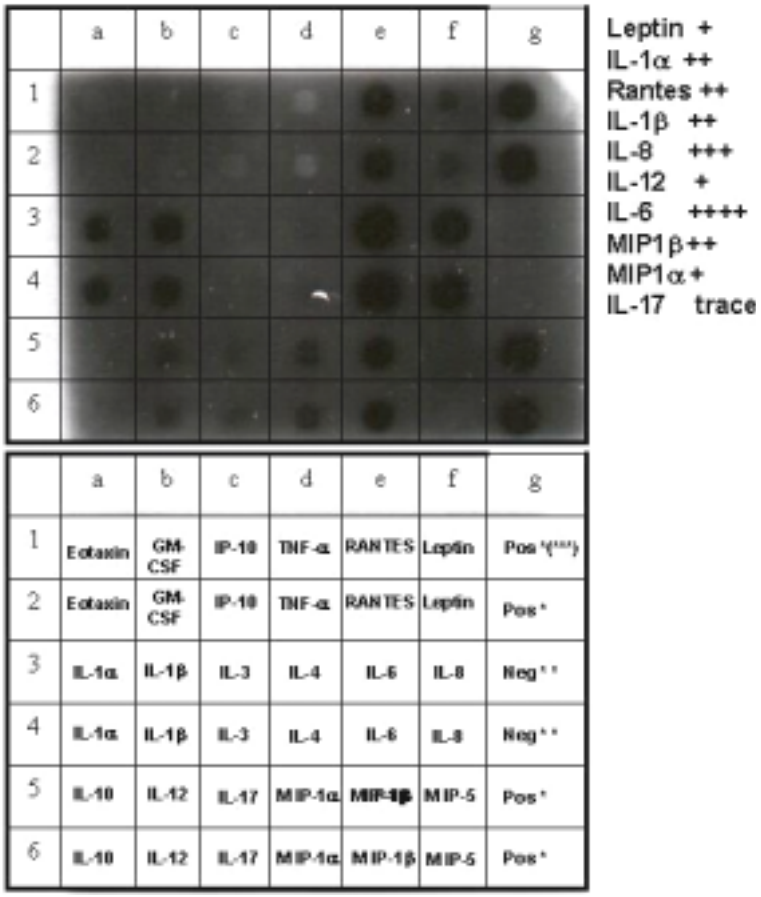


Figure: 12. Analysis of nucleic acids in the culture supernatant. DNA and RNA in the VE-CM were analyzed using real-time PCR assays as described in materials and methods. This assay suggested that majority of the DNA produced by the placenta is cell and particle free.

Further, placentally derived cytokines were identified in the VE-CM using a multiplexed protein assay designed to enable the simultaneous detection of 18 cytokines within individual samples. **(Fig: 13)**. This analysis indicated that placenta produced some inflammatory cytokines such as IL-1 and IL-6 and chemokines such as IL-8, RANTES, MIP1ζ, and MIP1η. If released in large quantities from the placenta into the maternal circulation, these inflammatory molecules and chemokines may activate endothelial cells and innate immune system as observed during preeclampsia [144, 145]. Our cytokine assay also revealed two white spots in column D row 1 and 2 that were TNFζ spots. The intensity of the spots reveals that high levels of TNFζ are present in the VE-CM.



	a	b	c	d	e	f	g
1	Eotaxin	GM-CSF	IP-10	TNF-α	RANTES	Leptin	Pos *(***)
2	Eotaxin	GM-CSF	IP-10	TNF-α	RANTES	Leptin	Pos *
3	IL-1α	IL-1β	IL-3	IL-4	IL-6	IL-8	Neg * *
4	IL-1α	IL-1β	IL-3	IL-4	IL-6	IL-8	Neg * *
5	IL-10	IL-12	IL-17	MIP-1α	MIP-1β	MIP-5	Pos *
6	IL-10	IL-12	IL-17	MIP-1α	MIP-1β	MIP-5	Pos *

Figure: 13. Detection of the cytokines present in the VE-CM. 11 cytokines were identified in VE-CM using a multiplexed protein assay. This analysis identified IL-6, IL-8, Leptin, IL-1ζ, IL-1η, RANTES, MIP1ζ, MIP1η, IL-12, IL-17, in the VE-CM which was collected after the villous explant culture further suggesting it is rich source of cytokines. White spots corresponds to TNFζ.

2.1.3. Conclusion

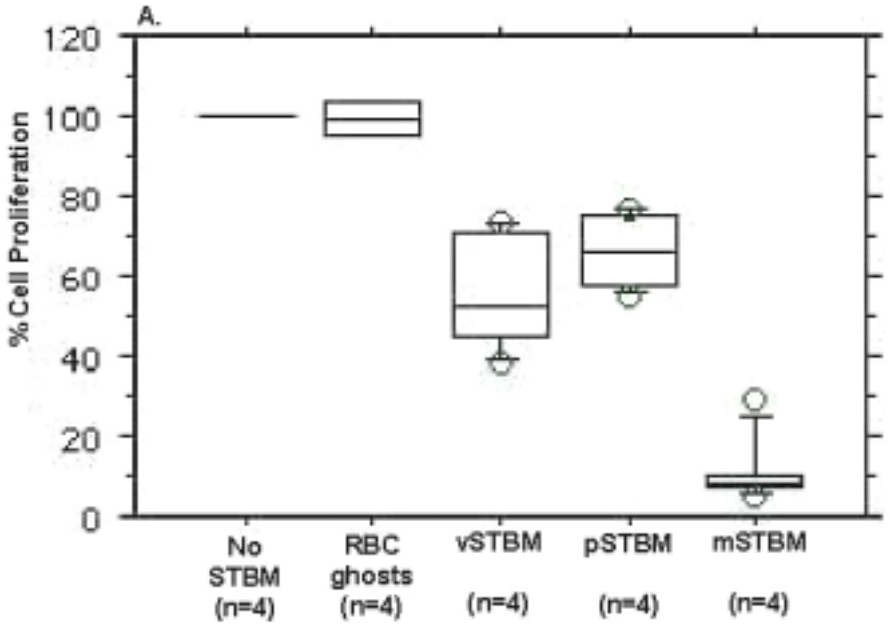
These observations confirmed that the STBM are membranous particles, which have originated from the syncytiotrophoblast. These three preparations are morphologically similar but differ from each other biochemically in terms of the presence of STBM associated, PLAP, nucleic acids, and lipids. Further, it can also be concluded that the placentally derived VE-CM is rich in DNA and cytokines.

2.2. Functional characterization of the STBM

2.2.1. Inhibition of HUVEC proliferation by STBM preparations

In order to functionally characterize the three different STBM preparations, we used HUVEC, a well-characterized endothelial cell model. These cells have been previously used to assess the growth inhibition properties of mSTBM [105]. The effects of equal amounts of STBM, as determined by protein content, were compared on HUVEC growth. Particles prepared from red blood cells (RBC ghosts) were used as a control. mSTBM inhibited HUVEC proliferation up to 90% at 12 σg STBM concentration while the other two STBM preparations at similar dose showed only a 30-50% inhibition of endothelial cell proliferation **(Fig: 14A)**. All STBM preparations inhibited HUVEC proliferation in a dose dependent manner **(Fig: 14B)**. When the dose of vSTBM or pSTBM was increased to 50 σg/well, a further reduction in HUVEC proliferation could be observed. However the high degree of inhibition of proliferation achieved with the mechanically produced STBM could not be obtained with the other preparations.

Gupta et al. Placenta, 2005



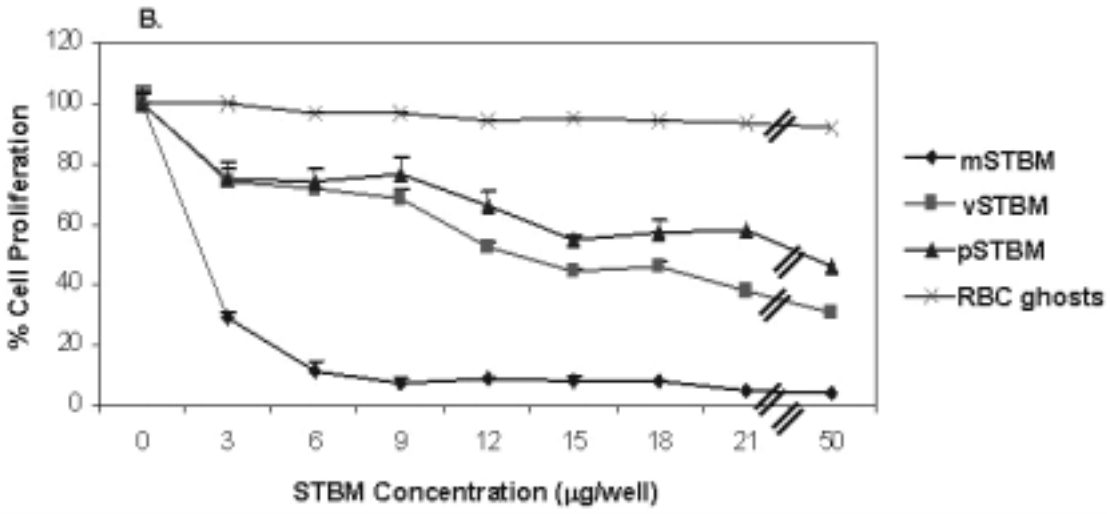
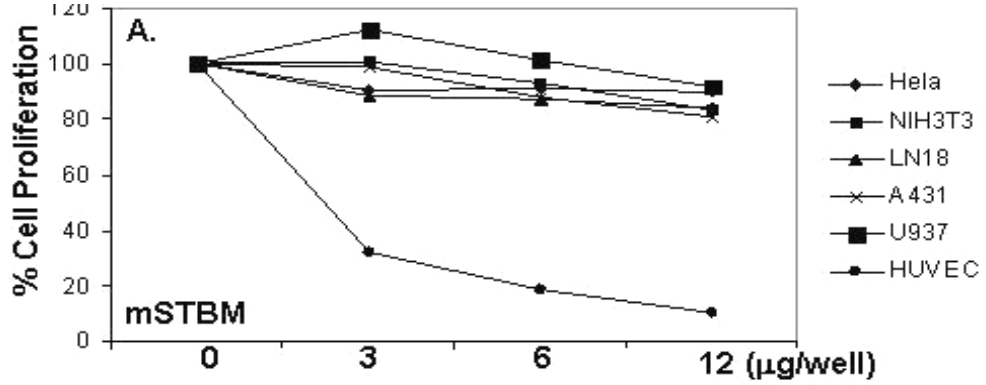


Figure: 14. Inhibition of endothelial cell proliferation by STBM. (A) Sub-confluent HUVEC cells were incubated for 40 h with 12 σg/well STBM from different STBM. Cell proliferation is represented as the percentage of cell proliferation relative to untreated control as 100% cell proliferation. A representative analysis of STBM preparations from 4 placentae is illustrated **(B)** A representative experiment from one placenta preparation showing dose dependent effect of various STBM preparations on inhibition of HUVEC proliferation

2.2.2. Effects of STBM preparations on cell line proliferation

To investigate whether STBM were able to inhibit cell proliferation of other cell types or whether this effect was specific for HUVEC proliferation, the effects of all the STBM preparations on adherent and non adherent cell types namely U937 (nonadherent) and A431, HELA, NIH-3T3, LN-18 (adherent) was assessed. It was observed that cell proliferation was not significantly reduced by any of the STBM preparations in any of these cell lines **(Fig: 15A-15C)**, unlike the effect that was observed with HUVEC.

Gupta et al. Placenta, 2005



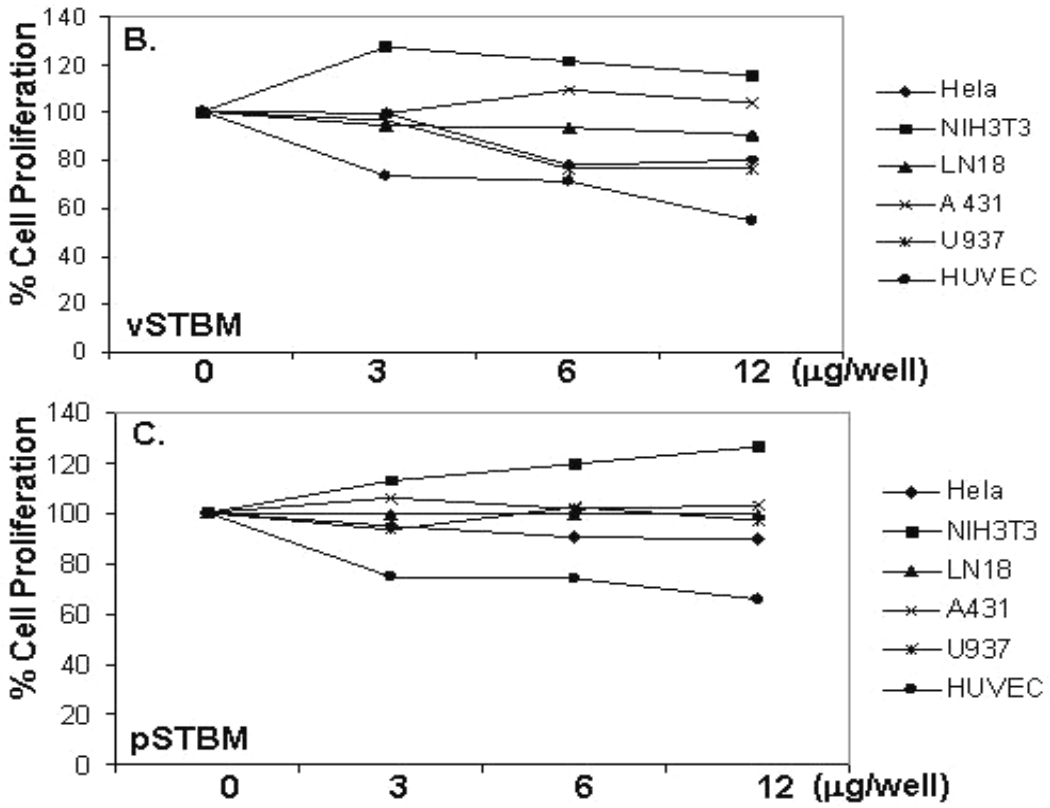


Figure: 15. Effects of different STBM preparations on cell proliferation comparing HUVEC and other cell types. Each point is mean of three replicates and expressed as the percentage of cell proliferation relative to untreated control as 100% cell proliferation. **(A)** Treatment with mSTBM, **(B)** Treatment with vSTBM, **(C)** pSTBM. A representative experiment from one STBM of each preparation is shown.

2.2.3. Mechanically derived STBM disrupt HUVEC monolayer

When HUVEC were incubated together with the three different STBM preparations at a dose of 12 σg/well, only the mSTBM caused disruption of the cell monolayer, leading to cell detachment **(Fig: 16)**. The kinetics of detachment was found to be dose dependent. It was observed that with a 12 σg dose of mSTBM, the disruption of the HUVEC monolayer started after 12h of incubation, and resulted in the detachment of almost 90% cells within the next 12h. At low concentrations (3 and 6 σg), detachment started after treatment periods exceeding 12h but eventually resulted in 80-90% HUVEC detachment at 24h. In contrast, the other two STBM preparations, even at higher concentration (50 σg/well)

neither disrupted the continuity of the HUVEC monolayer nor caused any cell detachment.

Gupta et al. Placenta, 2005

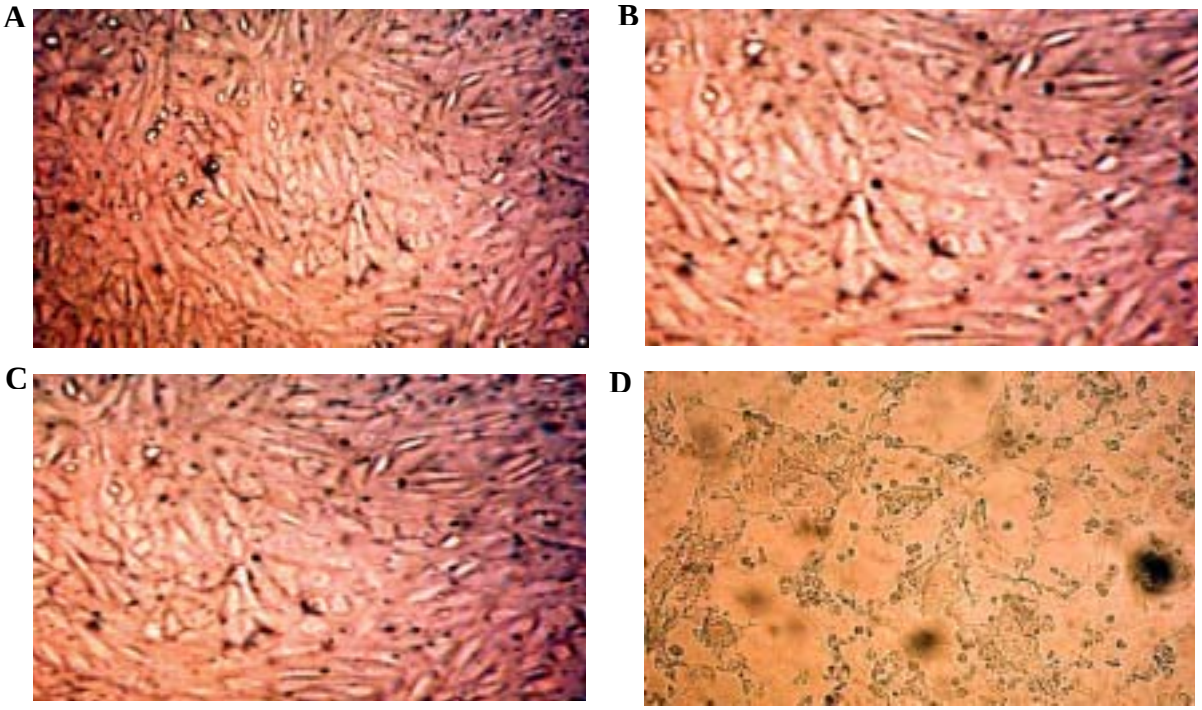


Figure: 16. Effects of different STBM preparations on HUVEC monolayer integrity. (A) Intact confluent HUVEC monolayer incubated 24 h without STBM, **(B)** with 12 σg vSTBM, **(C)** with 12 σg pSTBM **(D)** Disrupted HUVEC monolayer following incubation with 12 σg of mSTBM. Magnification is 400x.

2.2.4. Mechanically derived STBM induce apoptosis

As described above, all three STBM preparations inhibited HUVEC proliferation, albeit not to the same extent. Furthermore only mSTBM caused detachment and disruption of the HUVEC monolayer. For this reason HUVEC apoptosis was examined after the treatments with STBM preparations. The analysis indicated that mSTBM caused approximately 3-5 fold higher levels of HUVEC apoptosis than the untreated control cultures **(Fig: 17)**. No induction of apoptosis was evident in the cultures treated with the two other STBM preparations, even at high concentration (50σg/well), or RBC ghost particles. HUVEC treated with 5 σM

staurosporine (a well known inducer of apoptosis) for 3h were used as a positive control.

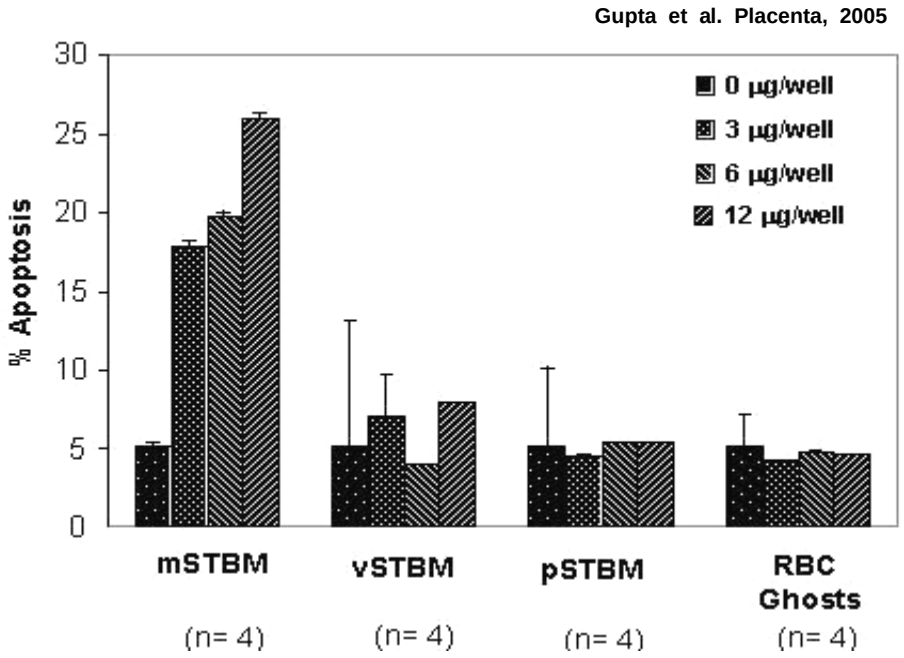


Figure: 17. mSTBM induce HUVEC apoptosis. Confluent HUVEC were incubated with various concentrations of the different STBM preparations and apoptosis was measured. 5 σM staurosporine was used as positive control. Apoptosis is expressed as percentage relative to staurosporine-induced apoptosis, which was designated as 100%. The results of four different STBM preparations are illustrated.

2.2.5. Lipid depletion from mSTBM reduced their ability to inhibit HUVEC proliferation

It has been proposed that *in-vitro* excessive amount of cholesterol can induce cell death by restricting cell processes [146]. As we noticed above that mSTBM are rich in cholesterol and were able to induce HUVEC apoptosis. We, therefore, hypothesized that may be this excessive amount of cholesterol present in the mSTBM is responsible for the low HUVEC proliferation, therefore, we depleted lipids from mSTBM by incubating them with MηCD and then HUVEC proliferation was measured. The cholesterol depletion significantly but not completely restored HUVEC proliferation suggesting that the high cholesterol levels present in mSTBM are partly responsible for the low HUVEC proliferation

(Fig: 18) further suggesting that high cholesterol may drives the cell towards apoptosis.

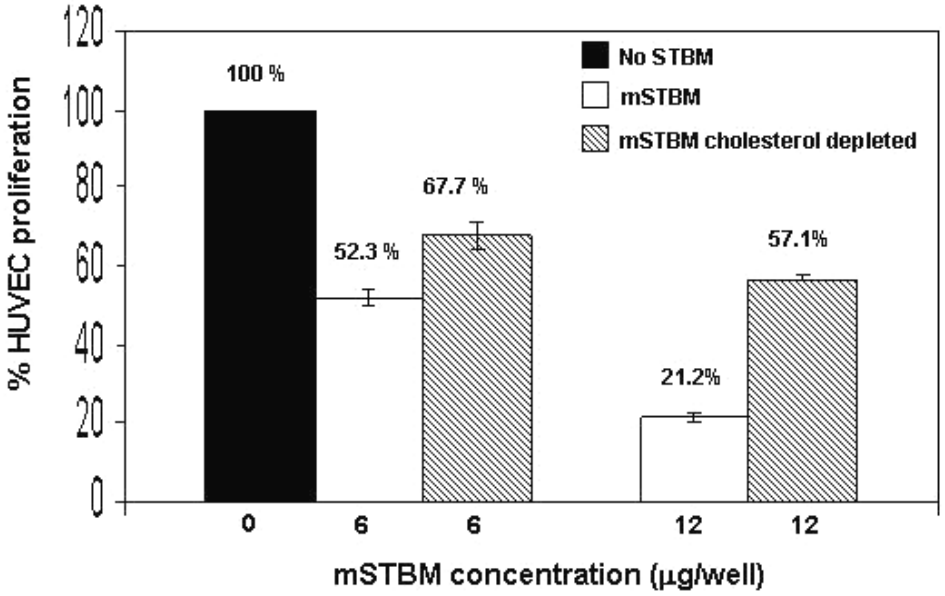


Figure: 18. Analysis of lipid depletion on HUVEC proliferation. Lipids were depleted from the mSTBM using MηCD as described in materials and methods and then HUVEC proliferation was measured. HUVEC proliferation was significantly but not completely restored after lipid depletion in the mSTBM.

2.2.6. Conclusion

This functional characterization of the three STBM preparations suggests that the three STBM preparations are biologically different from each other and differ in their effects of HUVEC cultures. In particular the effects observed by vSTBM and pSTBM are quite similar in contrast to mSTBM, which induced HUVEC apoptosis. These observations suggest that the mode of preparation greatly affects their functional properties.

2.3. Effects of placentally derived factors (STBM and soluble factors) on the T cells

It has been reported that mechanically derived STBM and placental homogenates can inhibit proliferation of PHA stimulated lymphocyte *in-vitro* [117, 118]. It has also been shown that maternal serum derived STBM can induce Jurkat T cell apoptosis *in-vitro* [119]. Therefore, in this study we examined the effects of three

biologically different STBM preparations and VE-CM on purified T cells, which is in contrast to the previous studies that have used a mixed lymphocyte culture and crude homogenates prepared from the placenta.

2.3.1. Effects of STBM on T cells

2.3.1.1. STBM did not activate T cells

T cells enriched from peripheral blood samples were cultured with the 3 different STBM preparations for 24h to examine whether they activated these cells. The FACS analysis of early activation marker CD69 expression indicated that none of the STBM preparations upregulated CD69 expression after the treatments **(Fig: 19).** We also observed that STBM treatment did not affect the viability of T cells, as >90% cells were viable after treatments as measured by tryptan blue dye exclusion test. This analysis indicates that STBM preparations were not able to activate T cells.

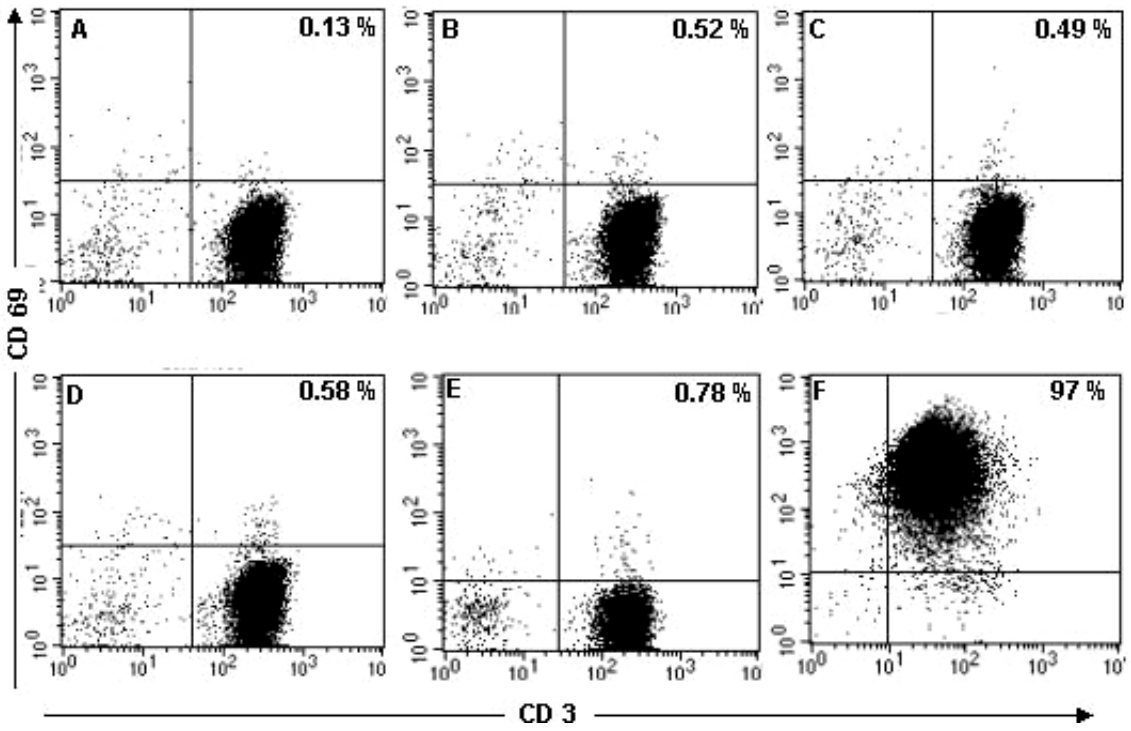


Figure: 19. Analysis of T cell activation after incubation with different STBM preparations. Enriched T cells were incubated separately with each STBM preparations (300σg/ml each) alone for 24h. FACS analysis was carried out to check the expression of the early T cell activation

marker CD69. Non-treated T cells **(A),** T cells treated with pSTBM **(B),** vSTBM **(C),** mSTBM **(D),** and RBC ghosts **(E)** showed basal level of CD69 expression. T cells stimulated with PMA and ionomycin for a 3h period of time showed significant increase in the CD69 expression on T cells **(F).**

2.3.1.2. STBM preparations affect T cell proliferation differently

In order to see the effects of the three STBM preparations on purified T cells we used PMA and ionomycin in a combination to synergistically activate T cells in a receptor and APC-independent manner [147].

This analysis revealed that vSTBM and mSTBM significantly inhibited T cell proliferation **(Fig: 20)**. On the other hand, pSTBM significantly enhanced T cell proliferation **(Fig: 20)**. This observation suggests that the three STBM preparations affect the proliferative response of activated T cells differently.

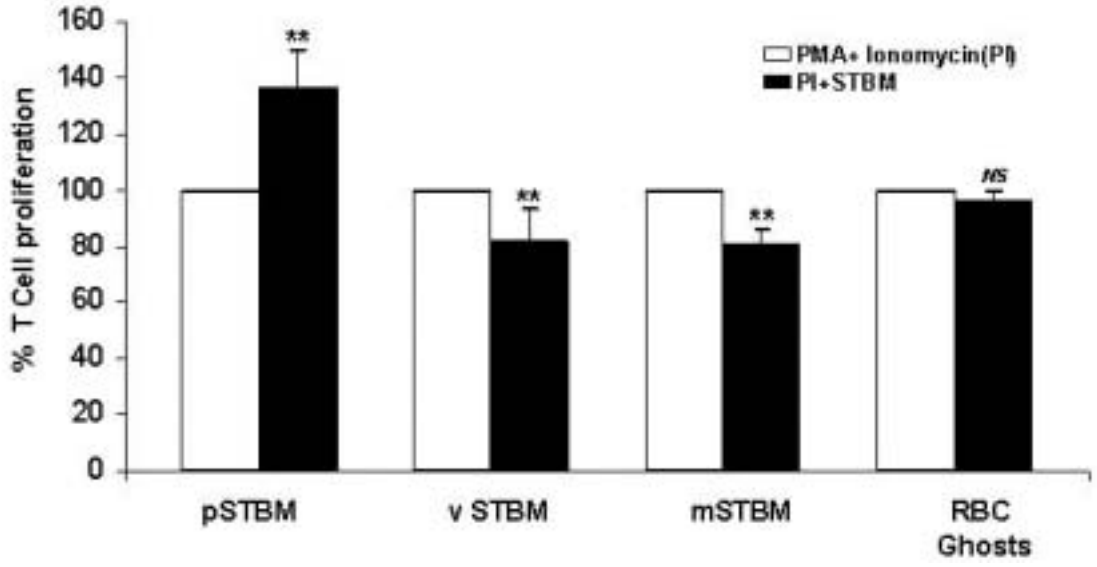


Figure: 20. Analysis of T cell proliferation after incubation with different STBM preparations. $1x10^5$ T cells were stimulated with PMA and ionomycin in the presence of each STBM preparations (300σg/ml each) for 72 h. T cell proliferation was measured using a commercial ELISA. pSTBM significantly induced T cell proliferation while vSTBM and mSTBM reduced T cell proliferation in a significant manner (** P value = 0.005). Cell proliferation is represented as the percentage of cell proliferation relative to PMA and ionomycin induced T cell proliferation (referred as 100% proliferation). An analysis of STBM preparations from 6 placentae is illustrated as mean ∂ S.D.

2.3.1.3. STBM preparations affect IL-2 and IFNν production differently

Three STBM preparations showed different effects on T cell proliferation; therefore, the effects of STBM treatments were observed on cytokines production. It was found that mSTBM and vSTBM significantly reduced the IL-2 production at both mRNA and protein levels, while the IL-2 production in the presence of pSTBM preparations was not affected **(Table 2)**. In a similar manner, mSTBM and vSTBM significantly reduced the IFNν production at both mRNA and protein levels, while this appeared to be moderately enhanced by pSTBM **(Table 2)**. In all experiments we used RBC ghosts) as a negative control. These did not affect any of the parameters we examined including cytokine production **(Table 2)** or T cell proliferation **(Fig 20)**. This suggests that the observed change in cytokine production with the different STBM preparations is specific to syncytiotrophoblast micro-particles.

Cytokine		% mRNA	% Protein	P value
IL-2	mSTBM	34.8 ± 5.1	25.5± 5.1	P= 0.0051
	vSTBM	78.2 ± 15.5	77.6 ± 9.8	P= 0.0051
	pSTBM	101.6 ± 6.8	99.5 ± 4.3	P= NS
	RBC ghosts	101 ± 2.8	100.5 ± 2.5	P= NS
IFNγ	mSTBM	88.6 ± 6.0	75.1 ± 8.7	P= 0.0051
	vSTBM	91.8± 4.1	79.6± 7.7	P= 0.0051
	pSTBM	102.8± 2.2	108.1 ± 6.7	P= 0.0203
	RBC ghosts	100.3 ± 2.1	101.1 ± 2.8	P= NS

Table:2. Cytokine production by the activated T cells after the STBM treatment. $1x10^6$ T cells were stimulated with PMA and ionomycin in the presence of each STBM preparations (300σg/ml each) for 72 h. After incubation cells were collected for total RNA isolation and culture supernatant was saved for cytokine analysis using ELISA. Total RNA was reverse transcribed and relative IL-2 and IFNν mRNA expression was measured using ÷÷C_T method as described in the materials and method. Cytokines production described here is relative to the cytokines levels produced by PMA and ionomycin stimulated T cells, which was considered as 100% cytokine production for both mRNA and protein measurement. An analysis of STBM preparations from 6 placentae is illustrated as mean ∂ S.D. P value represented here are same for the RNA and protein production. NS; Non significant.

2.3.1.4. STBM reduced CD69 expression

To investigate whether the reduced levels of IL-2 and IFNν expression may be due to the reduced activation of T cells following co-incubation with STBM, T cells were activated in the presence of the three STBM preparations and then CD69 expression was examined. Data indicated that all three STBM preparations reduced T cell activation mediated by PMA and ionomycin, albeit to different degree, in that CD69 expression was reduced after the STBM treatments **(Fig: 21).** The reduction in CD69 expression due to treatment with pSTBM was not as great as that caused by treatment with mSTBM or vSTBM preparations **(Fig: 21).**. These results suggest that the reduction in T cell activation, caused by mSTBM and vSTBM may be responsible for the reduced IL-2 and IFNν expression and low T cell proliferation under these conditions.

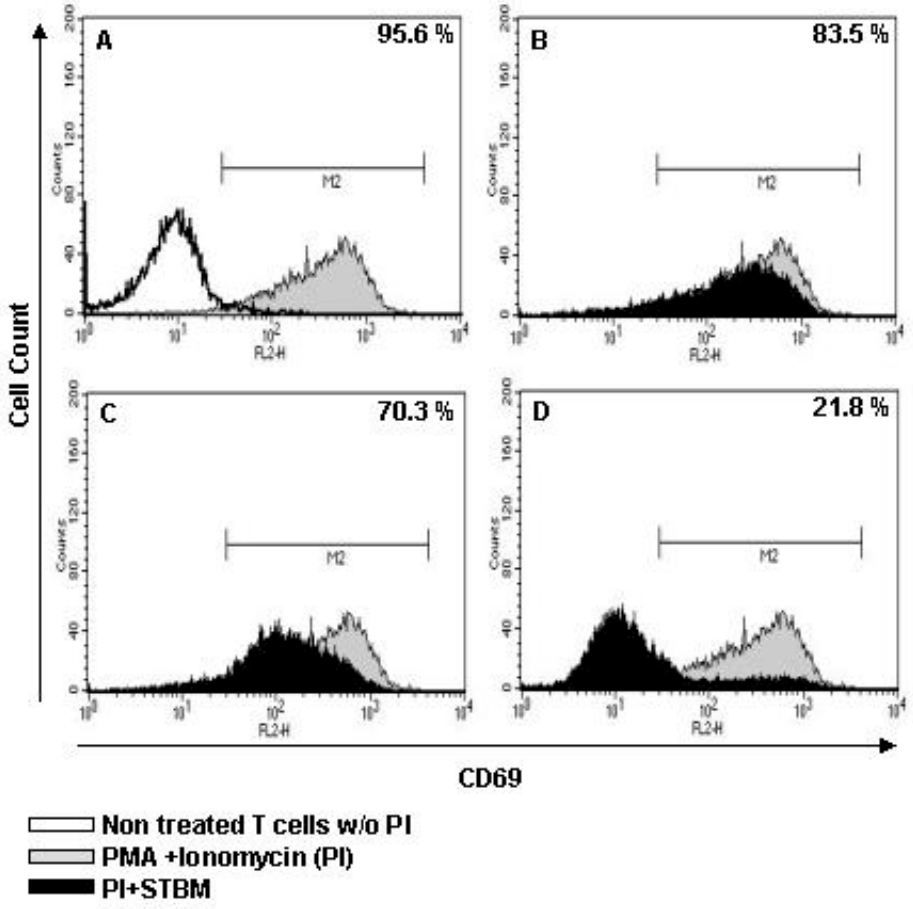


Figure: 21. Analysis of CD69 expression on activated T cell after incubation with different STBM preparations. Enriched T cells ($1x10^6$) were stimulated with PMA and ionomycin in the presence of each STBM preparations (300σg/ml each) for 24 h. FACS analysis was carried out to check CD69 expression on activated T cells after STBM co-incubation. 20,000 gated events were acquired. PMA and ionomycin activated T cells showed significant increase of CD69 expression on T cells **(A)**, whereas all the STBM preparations, namely pSTBM **(B),** vSTBM **(C),** and mSTBM **(D)** reduced CD69 expression on PMA and ionomycin activated T cells.

2.3.1.5. STBM do not cause T cell apoptosis

It has been reported that maternal serum derived STBM caused FasL mediated apoptosis of the Jurkat T cell lymphoma cell line [119]. In order to examine whether reduced cytokine production and impaired proliferation of T cells is associated with T cell apoptosis. T cell apoptosis was assessed using the annexin V assay, which recognizes phosphatidylserine on the outer leaflet of apoptotic cell membranes [148]. Interestingly, this analysis showed that none of the three different STBM preparations induced apoptosis in T cells **(Fig: 22)**. The vSTBM and mSTBM preparations reduced apoptosis of the activated T cells to a small extent **(Fig: 22)**, which may be a consequence of impaired T cell activation under these conditions.

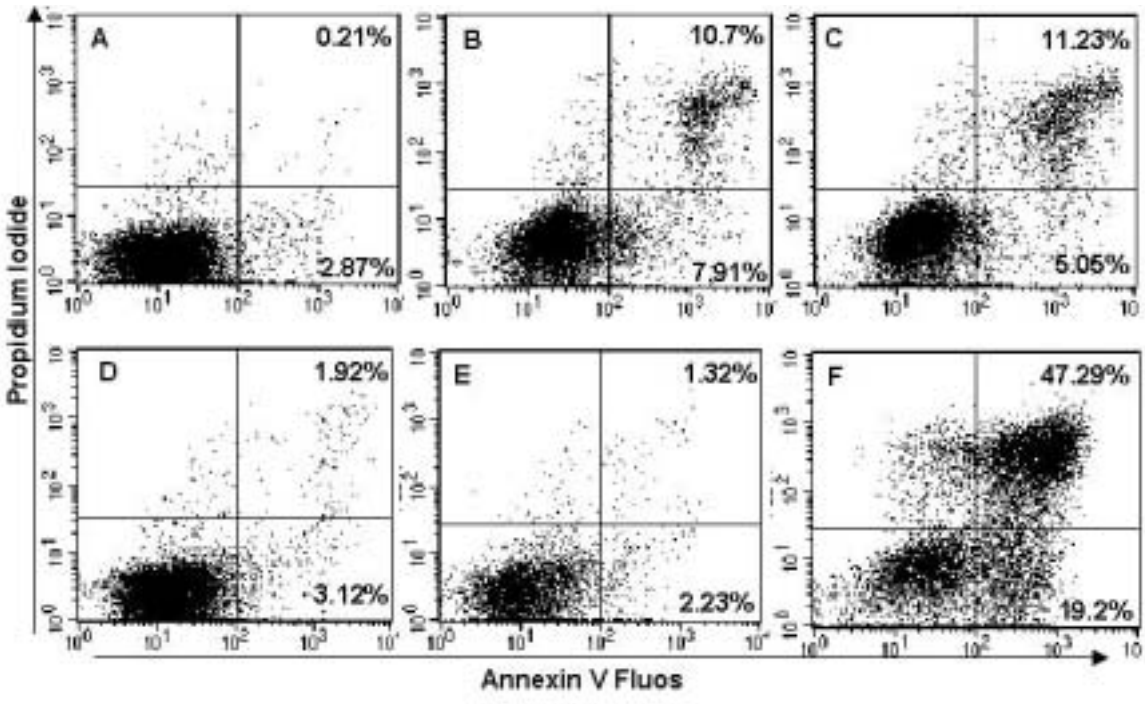


Figure: 22. Measurement of T cell apoptosis by annexin V binding assay after incubation with different STBM preparations. All three STBM preparations (300σg/ml each) were incubated with PMA and ionomycin stimulated T cells for 48h. Annexin V binding assay in combination with propidium iodide was performed to assess T cell apoptosis. The analysis indicated that none of the STBM preparation induced significant T cell apoptosis. After 48h incubation a basal level of apoptosis was observed in the non-treated T cells **(A).** Upon activation with PMA and ionomycin this basal level of apoptosis increased 3 fold **(B).** Further activated T cells incubated with different STBM preparations showed reduced apoptosis. pSTBM **(C),** vSTBM **(D),** and mSTBM **(E).** When activated T cells were incubated with 5σM staurosporine for period of 3h it induced massive apoptosis of T cells **(F).** Cells in the lower right corner represent apoptotic cells and the cells in upper right corner are either late apoptotic or necrotic cells.

2.3.2. Effects of placentally-derived soluble factors on T cells

Our cytokine analysis, described above, detected 10 cytokines in placentally derived particles free VE-CM. Therefore, we examined the effects of placentally derived soluble factors on T cell response.

2.3.2.1.VE-CM did not activate T cells

To examine whether VE-CM can activate T cells we cultured T cells with VE-CM for 24h. Data, obtained by FACS analysis of early activation marker CD69 expression indicated that VE-CM did not cause T cell activation **(Fig: 23).** VE-CM treatment did not affect the viability of T cells, as >90% cells were viable after treatment as measured by tryptan blue dye exclusion test.

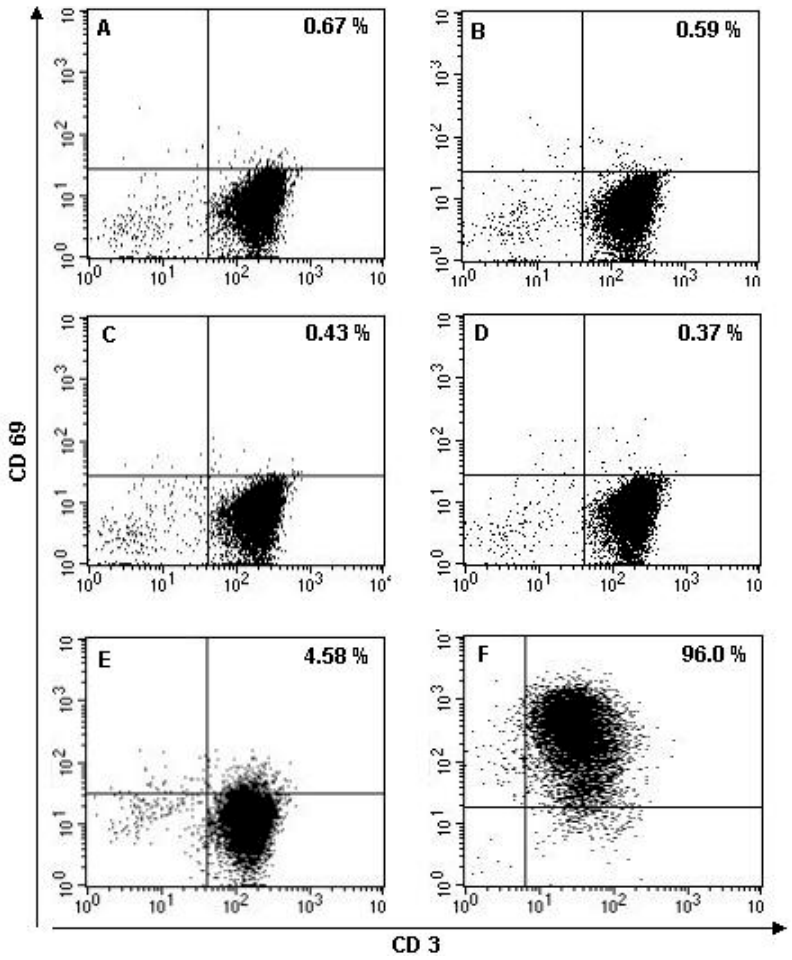


Figure: 23. Analysis of T cell activation after incubation with VE-CM. Enriched T cells were incubated with varying concentration of VE-CM alone for 24h. FACS analysis was carried out to check the expression of the early T cell activation marker CD69. Non-treated T cells **(A),** T cells treated with 20% **(B),** 40% **(C),** 60% **(D),** and 100% VE-CM **(E)** showed negligible levels of CD69 expression. T cells stimulated with PMA and ionomycin for a 3h period of time showed significant increase in the CD69 expression on T cells **(F).**

2.3.2.2. VE-CM inhibited T cell proliferation

When PMA and ionomycin activated T cells were incubated with varying concentrations of VE-CM, T cell proliferation was significantly inhibited in a dose dependant manner **(Fig: 24).** At high doses of the VE-CM T cell proliferation was completely inhibited. In this retrospect we examined whether the culture supernatant influenced cytokine production in a similar manner.

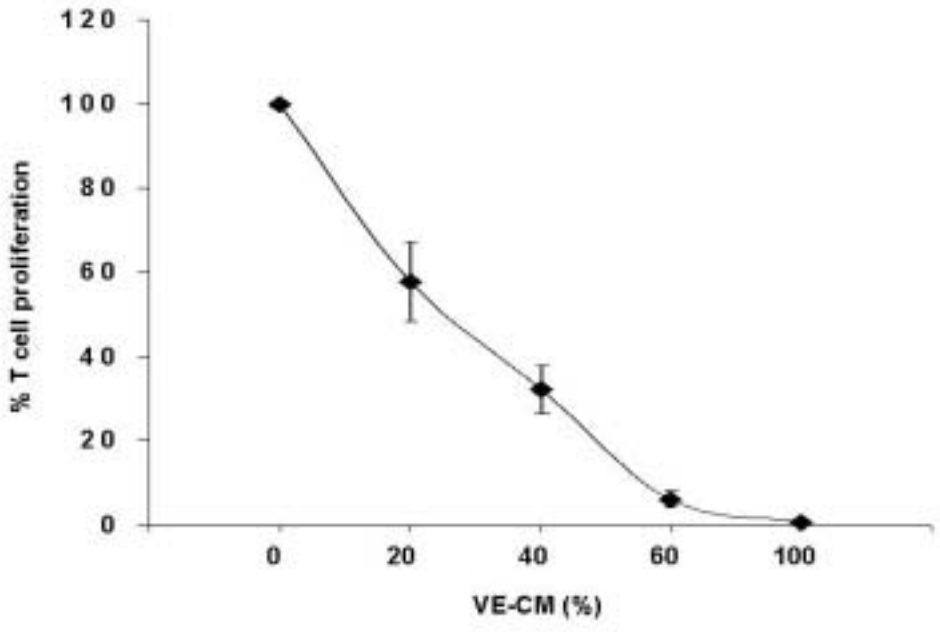


Figure: 24. Analysis of T cell proliferation after incubation with VE-CM. $1x10^5$ T cells were stimulated with PMA and ionomycin in the presence of varying concentration of VE-CM for 72 h. T cell proliferation was measured using a commercial ELISA. VE-CM reduced T cell proliferation significantly in a dose dependent manner. Cell proliferation is represented as the percentage of cell proliferation relative to PMA and ionomycin induced T cell proliferation (referred as 100% proliferation). An analysis of 8 placentae preparations is illustrated as mean ∂ S.D.

2.3.2.3 Reduced T cell proliferation by VE-CM is specific to placentally derived factors

In order to examine that the reduced T cell proliferation caused by VE-CM is specific to placentally derived factors and is not due to the low nutrient concentrations or high salt concentration ($MgSO_4$), used in the villous explant cultures. We examined various controls and then measured T cell proliferation. None of the control revealed low T cell proliferation **(Fig: 25)**. This observation suggests that low T cell proliferation by the VE-CM is specific to placentally derived factors.

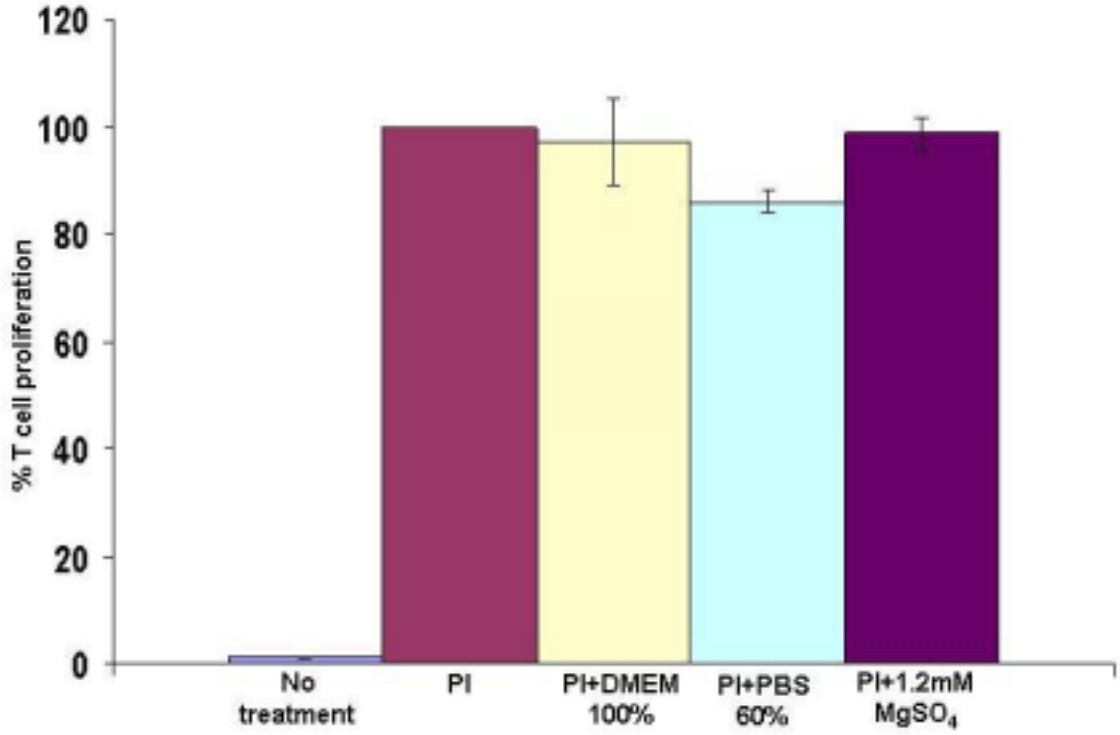


Figure: 25. Analysis of T cell proliferation after incubation with various control groups. $1x10^5$ T cells were stimulated with PMA and ionomycin (PI) in the presence of various control groups for 72 h. T cell proliferation was measured using a commercial ELISA. None of the control group reduced T Cell proliferation. Cell proliferation is represented as the percentage of cell proliferation relative to PI induced T cell proliferation (referred as 100% proliferation). An analysis of 4 control preparations is illustrated as mean ∂ S.D.

2.3.2.4 VE-CM reduced IL-2 and IFNν production by activated T cells

T cells were stimulated by PMA and ionomycin in the presence of VE-CM for 72h, after which the supernatant was collected and the levels of IL-2 and IFNν protein were measured using ELISA. It was observed that the VE-CM significantly reduced the IL-2 production as well as IFNν production in a dose dependent manner after the treatments **(Fig: 26A, 26B)**, which suggests that the placentally derived soluble factors are immunosuppressive in nature and reduce Th1 type immunity. Cytokines production was also measured in the various control groups mentioned above. None of the control group reduced cytokines production by T cells.

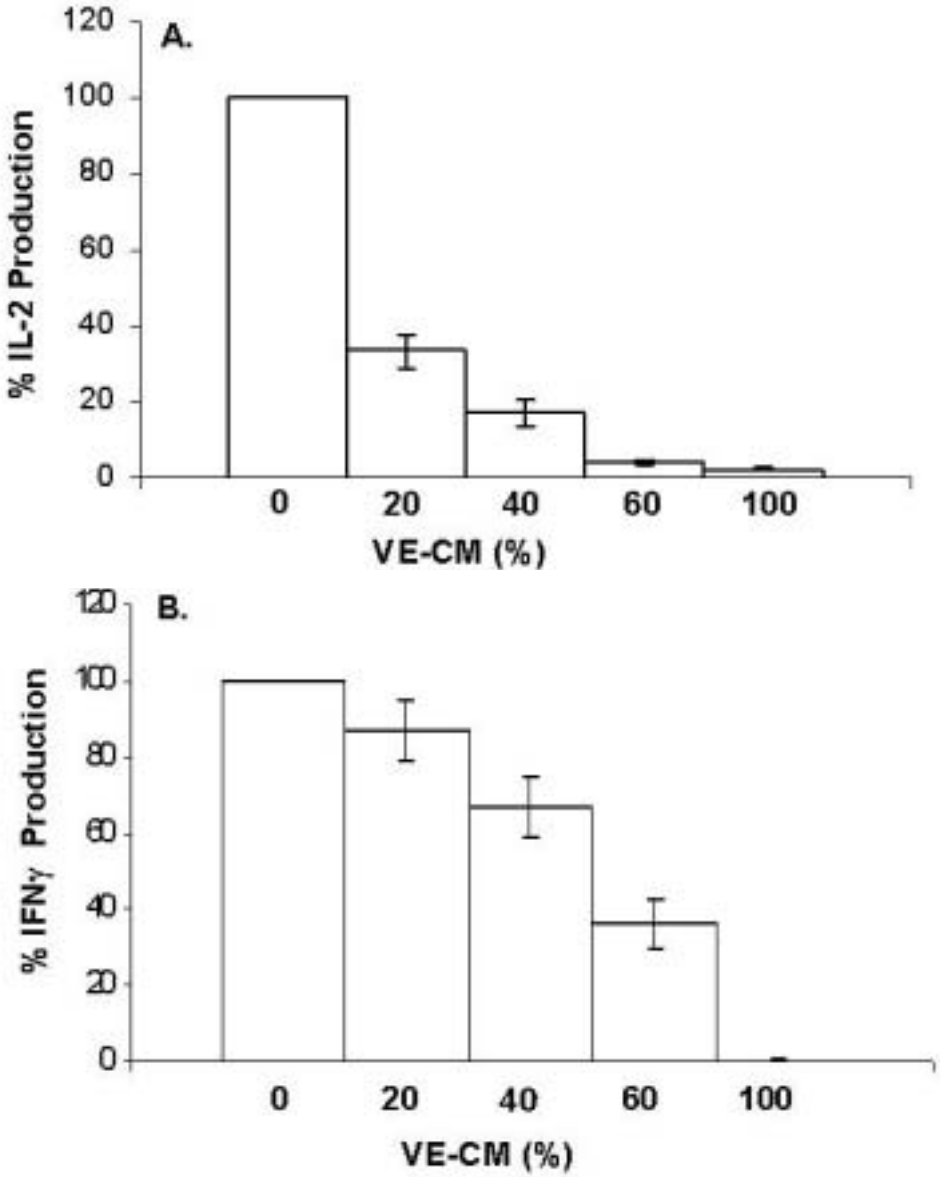


Figure: 26. Analysis of cytokines production by T cells after incubation with VE-CM. $1x10^6$ T cells were stimulated with PMA and ionomycin in the presence of varying concentration of VE-CM for 72 h. Supernatant was collected after the incubation and IL-2 and IFNν levels were measured using ELISA. VE-CM reduced IL-2 **(A)** and IFNν **(B)** production. Cytokine production is represented as the percentage of cytokine production relative to PMA and ionomycin mediated cytokine production (referred as 100% production). An analysis of 8 placentae preparation is illustrated as mean ∂ S.D.

2.3.2.5. VE-CM reduced CD69 expression

To investigate whether the reduced levels of IL-2 and IFNν expression may be due to the reduced activation of T cells following co-incubation with VE-CM, we stimulated T cells in the presence of VE-CM and then CD69 expression was examined. Note that the VE-CM reduced T cell activation by PMA and ionomycin in dose dependent manner **(Fig: 27)**. It is likely that the reduction in the CD69 expression, and hence, reduction in T cell activation, caused by VE-CM may directly be correlated with the reduction of IL-2 and IFNν expression under these conditions.

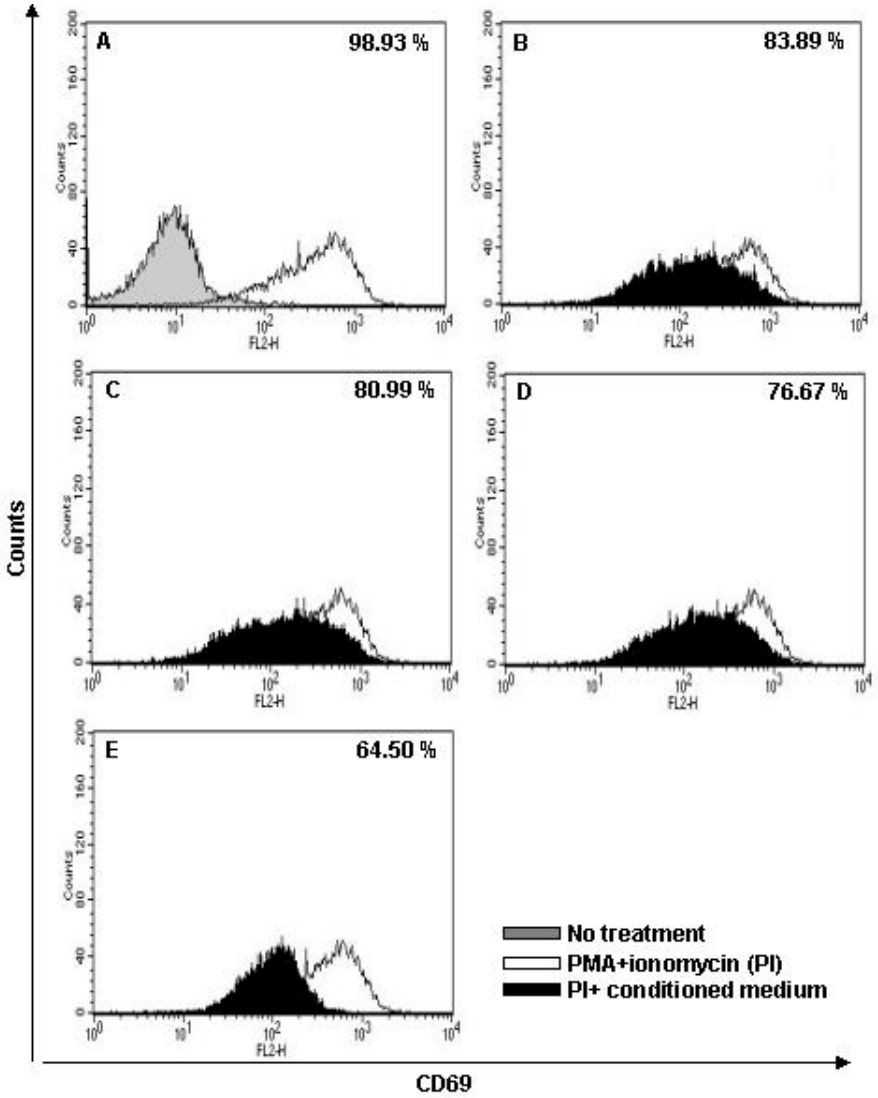


Figure: 27. Analysis of CD69 expression on activated T cell after incubation with VE-CM. $1x10^6$ enriched T cells were stimulated with PMA and ionomycin in the presence of VE-CM for

24 h. FACS analysis was carried out to examine CD69 expression on activated T cells, 20,000 gated events were acquired. PMA and ionomycin activated T cells showed significant increase of CD69 expression on T cells **(A)**, whereas increasing concentration of VE-CM, namely 20% **(B),** 40% **(C),** 60% **(D),** and 100% VE-CM **(E)** significantly reduced CD69 expression on PMA and ionomycin activated T cells.

2.3.2.6. VE-CM did not induce T cell apoptosis

We examined whether reduced cytokine production and low proliferation of T cells upon treatment with VE-CM is associated with T cell apoptosis. T cell apoptosis was examined using the annexin V assay. FACS analysis showed that VE-CM did not induce apoptosis in T cells **(Fig: 28).**

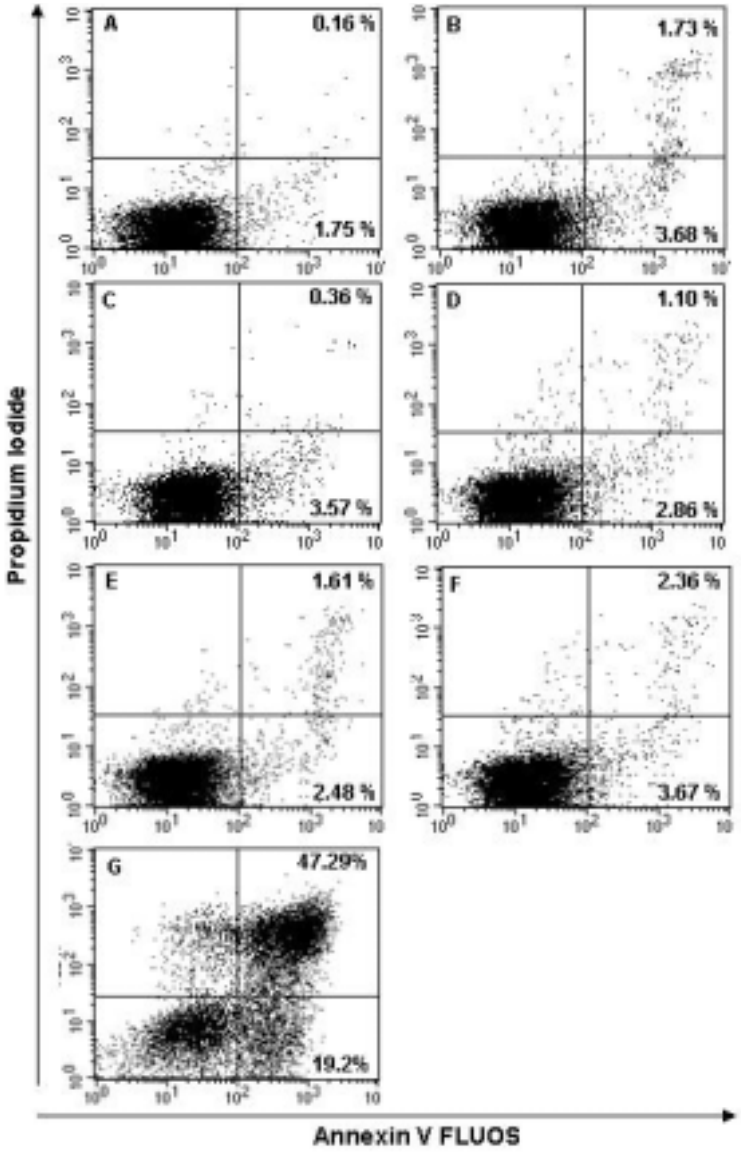


Figure: 28. Measurement of T cell apoptosis by annexin V binding assay after incubation with VE-CM. T cells were activated and incubated for 24h in the presence of VE-CM. Annexin V binding assay in combination of propidium iodide was performed to assess T cell apoptosis using FACS. The analysis indicated that VE-CM did not induce T cell apoptosis. After 24h incubation a basal level of apoptosis was observed in the non-treated T cells **(A).** Upon activation with PMA and ionomycin this basal level of apoptosis increased 2.5 fold **(B).** Further activated T cells incubated with 20% **(C)**, 40% **(D)**, 60% **(E)**, 100% VE-CM **(F)**, showed either lower or similar levels of apoptosis as observed in the PMA and ionomycin activated T cells. When 5σM of staurosporine was added to activated T cell cultures for period of 3h, it induced massive apoptosis of T cells **(G).** Cells in the lower right corner represent apoptotic cells and the cells in upper right corner are either late apoptotic or necrotic cells.

2.3.2.7. IDO is not responsible for low T cell proliferation

It has previously been proposed that IDO mediated depletion of the L-tryptophan at the feto-maternal interface is responsible for the reduced T cell proliferation [69]. To examine whether IDO is responsible for the low T cell proliferation mediated by VE-CM, we added L-tryptophan and the inhibitor of IDO 1-methyl-DL-tryptophan (1-MT) to the T cell cultures and measured T cell proliferation. The addition of L-tryptophan and 1-MT did not significantly alter the T cell proliferation **(Fig: 29)**. This observation suggest that under these conditions, the inhibition of T cell proliferation is not mediated by IDO, but is rather due to some unknown factors.

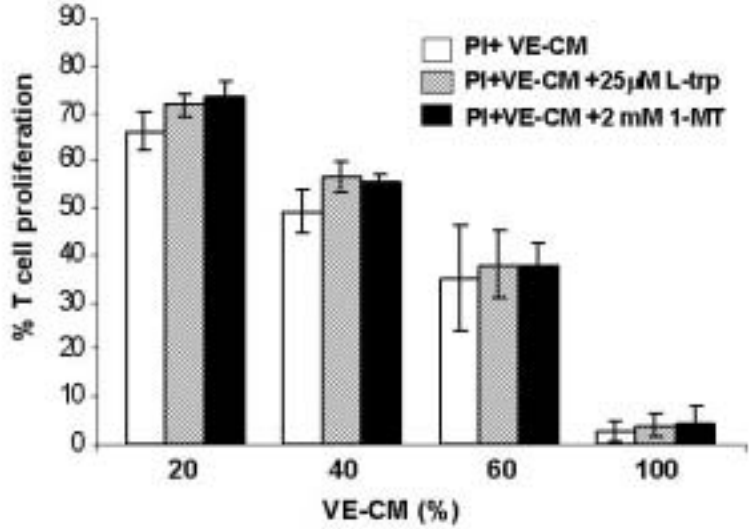


Figure: 29. Effects of L-trp and 1-MT on T cell proliferation. $1x10^5$ T cells were stimulated with PMA and ionomycin in the presence of varying concentration of VE-CM for 72 h with or

without 25σM L-tryptophan or 2mM 1-MT. T cell proliferation was measured using a commercial. Addition of L-trp or inhibitor of IDO, 1-MT, could not reverse low T cell proliferation mediated by VE-CM. Cell proliferation is represented as the percentage of cell proliferation relative to PMA and ionomycin mediated T cell proliferation (referred as 100% proliferation). Data represent mean ∂ S.D. of 5 separate experiments with triplicate assay.

2.3.3. Conclusion

These observations suggest that the placentally derived factors can modulate T cell response *in-vitro*. Different STBM, which differ in their mode of preparations and biological properties, can modulate T cell response in terms of their proliferation, and activation. Overall these observations suggest that the placentally derived factors are inhibitory in nature and can reduce T cell response.

2.4. Effects of placentally-derived STBM and soluble factors on neutrophils

Activation of neutrophils by the placental factors has been shown during normal pregnancy and preeclampsia [144, 145]. It has recently been shown that upon activation by inflammatory signals such as that mediated by IL-8 (a member of the chemokine superfamily of proinflammatory cytokines), neutrophils generate extracellular DNA containing fibrous lattices, termed NETs (neutrophil extracellular traps), which are used by these cells to ensnare and kill bacteria [149, 150]. Also it has previously been observed that maternal cell-free DNA levels are significantly increased in the circulation of pregnant women with preeclampsia [94, 95, 138]. Intrigued by the observation that neutrophils can release DNA in an extracellular form, we examined whether placentally derived inflammatory factors can similarly activate neutrophils to generate NETs, and whether the presence of such NETs was increased in preeclampsia.

2.4.1. STBM and placental IL-8 activated neutrophils

In order to examine the activation of neutrophils by placentally derived factors *in-vitro*, we assessed CD11b up-regulation on neutrophils after treatments with VE-CM and STBM. CD11b expression was significantly increased in isolated

neutrophils activated with PMA **(Fig: 30A)**, or rIL-8 **(Fig: 30B)**. Similarly, treatment with VE-CM lead to a significant increase in CD11b expression **(Fig: 30C)** on neutrophils. vSTBM **(Fig: 30D)** and mSTBM **(Fig: 30E)** also induced CD11b expression.

As our cytokine analysis, mentioned above, detected IL-8 (a known activator of neutrophils) in VE-CM. Therefore, we quantified IL-8 levels in the STBM preparations and VE-CM. Results from ELISA indicated that IL-8 was detectable in VE-CM, as well as in highly purified vSTBM preparations. Negligible levels of IL-8 were detected in mSTBM **(Table: 3)**.

Further to determine whether the activation of neutrophils by these placentally derived factors is mediated by IL-8, we used an anti IL-8 blocking antibody. This antibody significantly, but not completely blocked CD11b expression induced by the VE-CM **(Fig: 30F)**, suggesting that IL-8 is largely responsible for the neutrophil activation under these conditions. On the other hand, the induction of CD11b expression by the two different STBM preparations occurred in an IL-8 independent manner, as this effect was not hindered by co-incubation with the IL-8 blocking antibody **(Fig.: 30G)**. To summarize, these observations clearly demonstrate that placentally derived factors (IL-8 and STBM) can individually activate neutrophils.

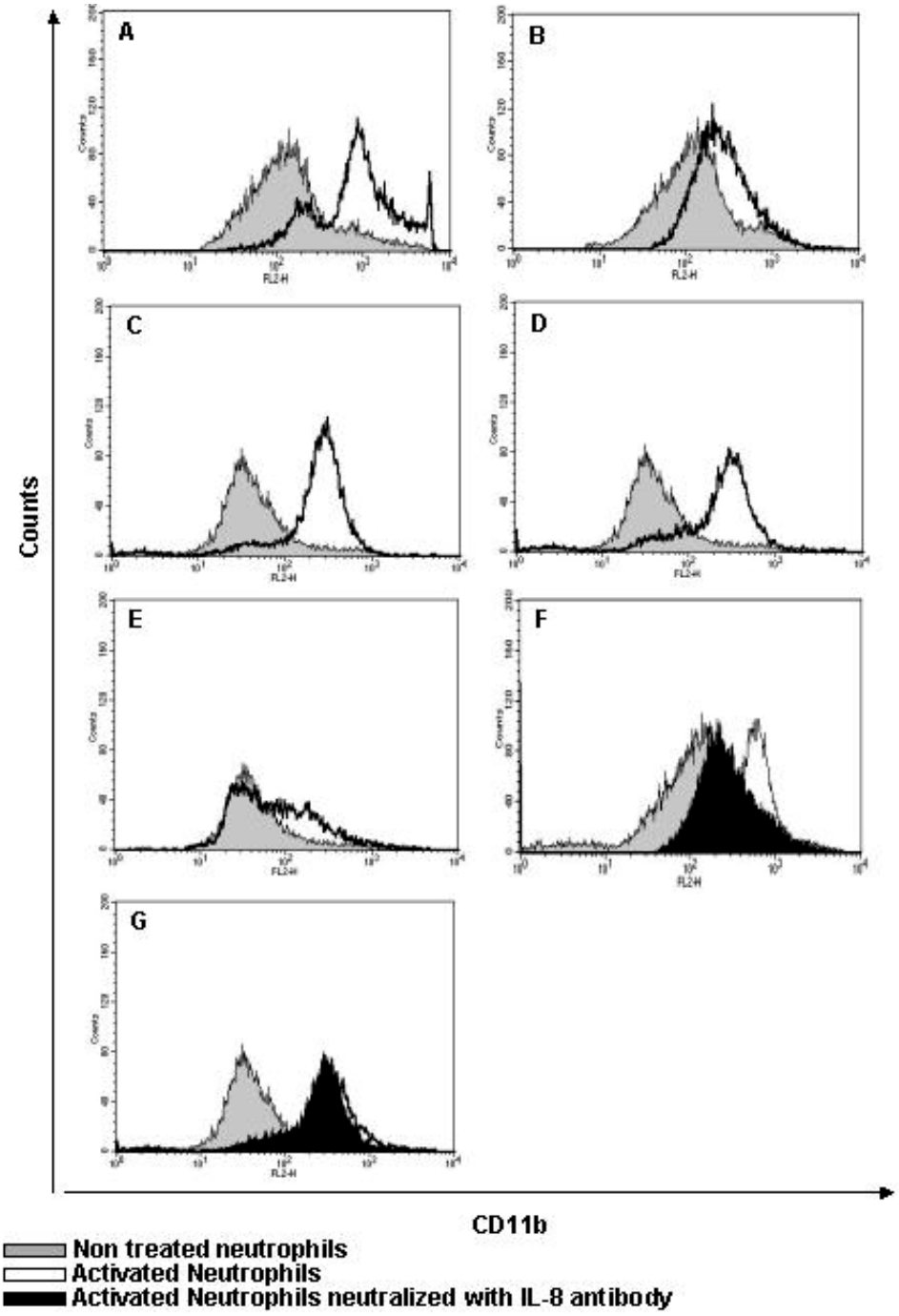


Figure: 30. FACS analysis of CD11b up-regulation on activated neutrophils. **(A).** Neutrophils activated with 25nM PMA for 30 min showed significant up-regulation of CD11b. Similarly CD11b expression is up-regulated when neutrophils were activated with 10ng/ml rIL-8 **(B),** with 20% VE-CM **(C),** 150 σg/ml vSTBM **(D)** and mSTBM **(E)**. Neutralizing IL-8 with 2.5ug/ml IL-8 neutralizing antibody in VE-CM could significantly reverse the effect of soluble IL-8 on CD11b up-regulation **(F),** while neutralizing IL-8 in vSTBM did not affect CD11b expression **(G).**

IL-8 concentration in the different STBM and VE-CM

mSTBM (ng/mg of total protein)	vSTBM (ng/mg of total protein)	*VE-CM (ng/ml of medium)
0.017 (0.013-0.021)	5.5 (3.1-10.9)	39.9 (22.7- 76.2)

* STBM free conditioned medium of villous explants cultures

Table:3. Quantification of IL-8 present in the VE-CM and STBM. IL-8 was quantified using a commercial ELISA kit. Significant levels of IL-8 are present in the vSTBM as well as in the VE-CM, while the mSTBM contain traces of IL-8. Data in the table is expressed in median and range and was calculated from 6 samples of each preparation.

2.4.2. Preeclamptic plasma moderately activated neutrophils

ELISA revealed that the IL-8 concentrations were also elevated in the plasma of preeclamptic pregnant women when compared to those with uncomplicated pregnancies **(Fig: 31A)** When neutrophils were incubated with normal and preeclamptic plasma, only preeclamptic plasma samples slightly increased CD11b expression **(Fig. 31B)** than that attained by the normal plasma samples **(Fig. 31C)**. Neutrophils with the normal plasma samples did not show any CD11b upregulation. The degree of neutrophils activation attained by the preeclamptic plasma samples is very low in comparison to those obtained by STBM and VE-CM. This low activation of neutrophil activation by the preeclamptic plasma samples is may be due to very low concentration of IL-8 and STBM present in these samples.

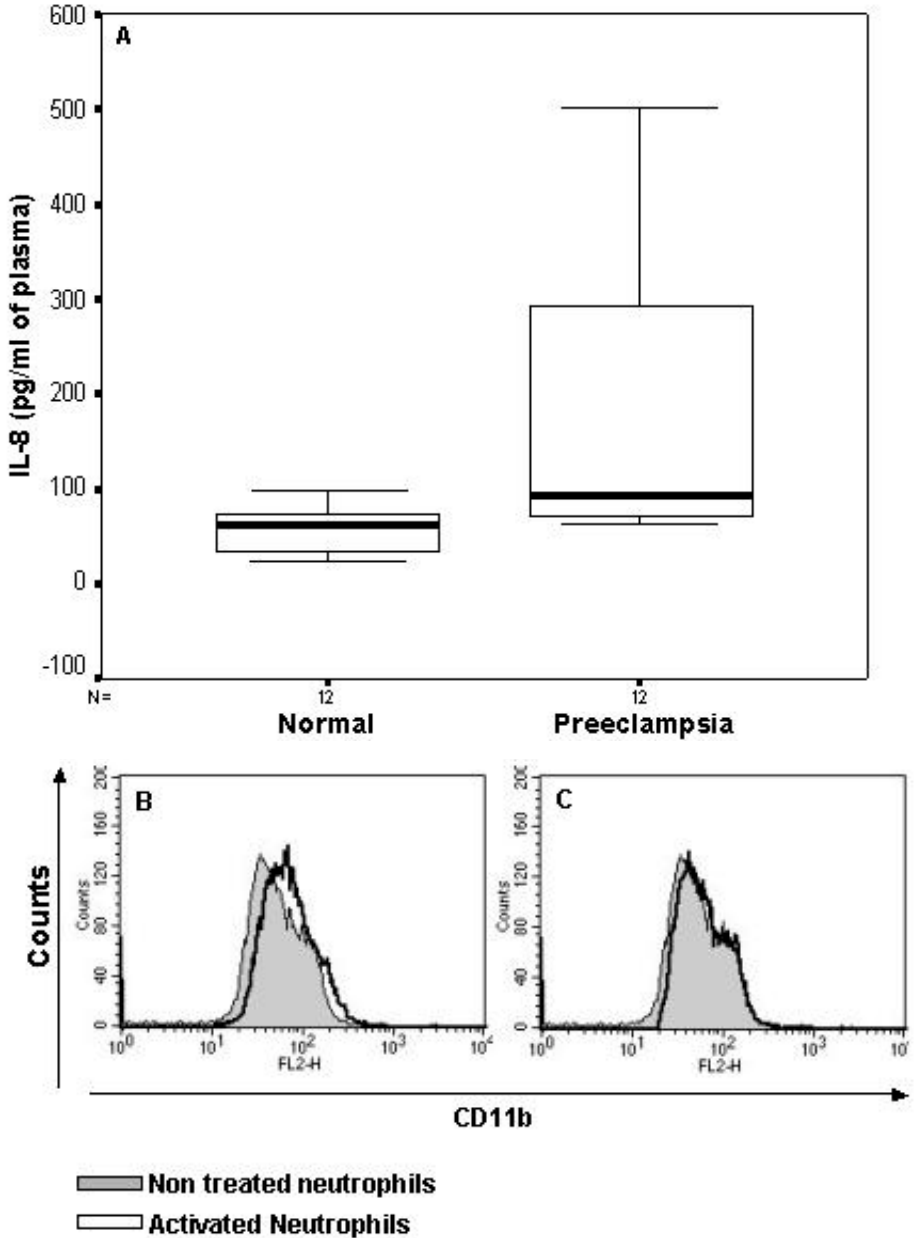


Figure: 31. IL-8 quantification in the maternal plasma samples. 12 plasma samples of each normal pregnancy, and preeclampsia were analyzed for presence of IL-8 using a commercial ELISA kit. The higher levels of soluble IL-8 were present in the preeclampsia samples **(A)**. FACS Analysis of CD11b up-regulation on neutrophils treated with plasma samples. $1x10^6$ Neutrophils were resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100 U/ml penicillin, 100σg/ml streptomycin and incubated with 60% plasma either normal or preeclamptic for 3 h. CD11b up-regulation was measured by FACS. Very little up-regulation of CD11b was observed when neutrophils were activated with preeclamptic plasma **(B)** and normal plasma **(C)**.

2.4.3. Placentally-derived IL-8 induced DNA rich neutrophil NETs formation by activated neutrophils

We investigated whether placentally derived stimuli can also trigger NETs formation. Our observations, using scanning electron microscopy and fluorescence microscopy clearly indicated that these placentally derived factors induced NETs formation **(Fig: 32)**. Non-stimulated neutrophils remained round **(Fig: 32A, 32J)**, while neutrophils incubated with preeclamptic plasma, **(Fig: 32B, 32K)**, VE-CM **(Fig: 32C, 32L)**, mSTBM **(Fig: 32D, 32M)**, and vSTBM **(Fig: 32E, 32N)** generated NETs. The NETs produced by either the VE-CM or STBM were comparable to those observed using known inducers of NETs, PMA **(Fig: 32F, 32O)** or rIL-8 **(Fig: 32G, 32P)**, and were morphologically similar to those described by Brinkmann et al [149], consisting of smooth fibers with a 15-17nm diameter and globular domains of 25-28nm diameter **(white arrows Fig. 32H, and 32Q)**. Although, NETs formation by VE-CM could be inhibited by the IL-8 neutralizing antibody **(Fig: 32I and 32R)**, this antibody did not reduce NETs formation by either of the two STBM preparations. Therefore, these data suggest that the formation of NETs can occur in IL-8 dependent and independent manners and that the generation of NETs may be a general phenomenon associated with neutrophil activation. Furthermore, the major structural component of the NETs was DNA as they could be stained using the DNA binding dye, sytox green, **(Fig: 32J- 32R),** and a brief treatment with DNase I resulted in disintegration of the NETs.

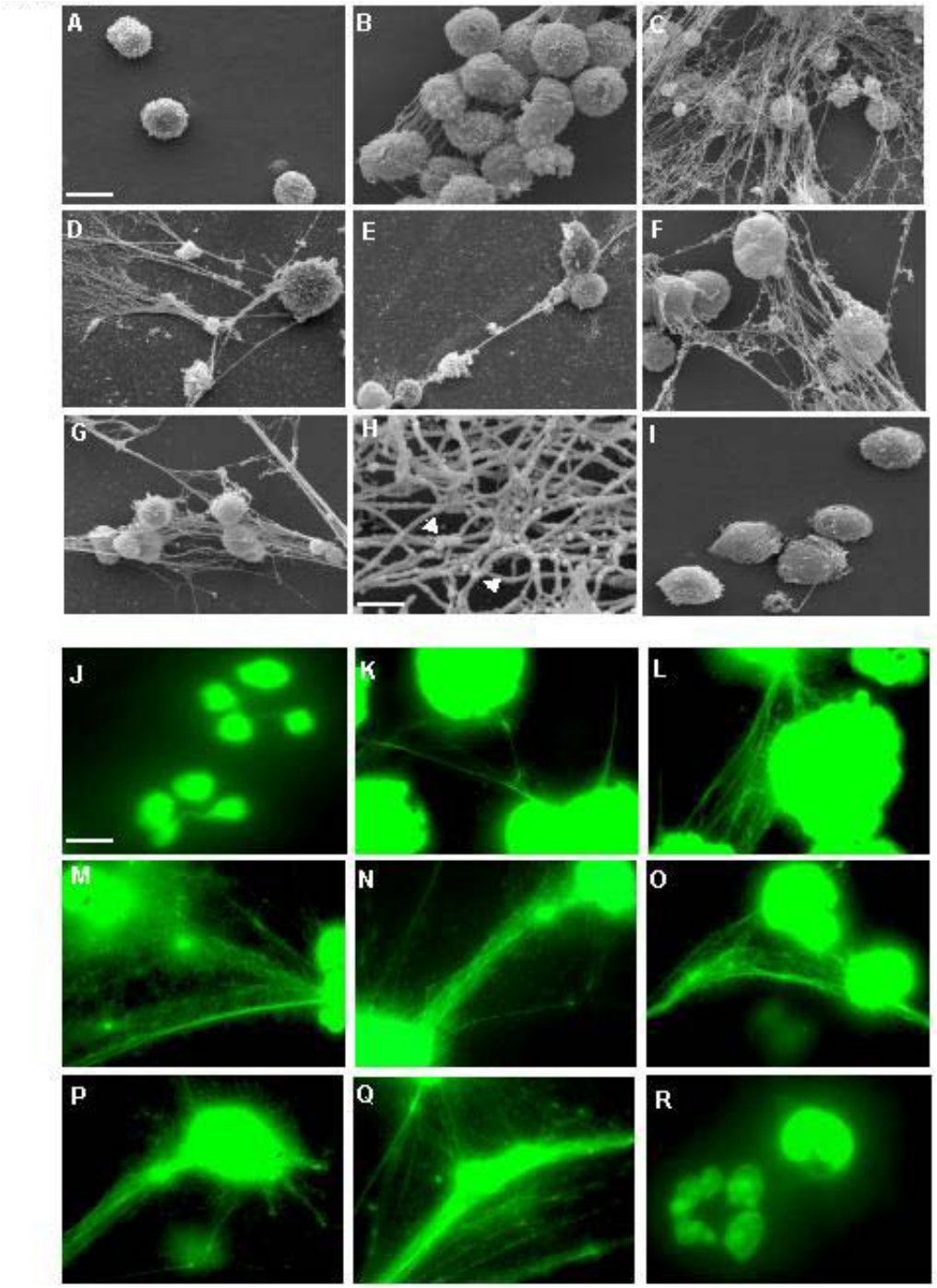


Figure: 32. Analysis of activated neutrophils using electron and fluorescent microscopy. Non-treated and treated cell were fixed and stained with sytox green DNA binding dye for fluorescence microscopy. Nonactivated neutrophils are round and devoid of NETs **(A and J**). Upon activation with 60% preeclamptic plasma **(B and K)** 60% VE-CM **(C and L),** mSTBM **(D and M) ,** vSTBM **(E and N),** 25nM PMA **(F and O)**, and with 10ng/ml rIL-8 **(G and P)**, neutrophils generated NETs within 30 min of incubation. High resolution SEM revealed that NETs consist of smooth fiber and globular domains **(white arrow, H).** NETs formation was reduced significantly

when soluble IL-8 present in the VE-CM was neutralized with 2.5σg/ml IL-8 neutralizing antibody **(I and R)**. NETs were rich in DNA **(J-R)**. Bars in the figures A-G, H, I and J-R and are 5σm, 300nm, 5σm and 50σm, respectively.

2.4.4. Neutrophil NETs trap STBM

Our studies revealed that STBM contain fetal RNA and DNA, and that these small particles (200nm-600nm) have a tendency to make clusters (shown above [151, 152]). Here we observed that NETs entrapped clusters of both mSTBM **(Fig: 33A, 33B white arrows)** and vSTBM **(Fig: 33C 33D, white arrows)** as revealed by scanning electron microscopy. This observation suggests that NETs not only capture large microorganisms such as bacteria (>2 σm), [149] but also ensnare very small (<200nm) inflammatory particles such as STBM **(Fig: 33D)**.

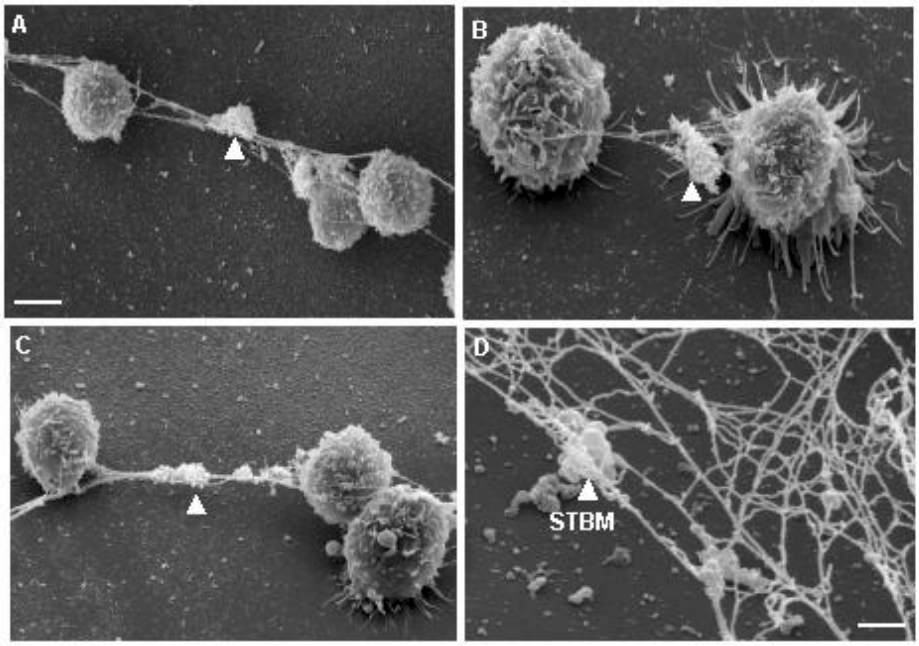


Figure: 33. Entrapment of the STBM by neutrophil NETs. 150σg/ml vSTBM or mSTBM were incubated with neutrophils for 30 min. Cells were then fixed for SEM analysis. SEM analysis show that both mechanical **(white arrows, A, B)** and villous explant STBM **(white arrows, C D)** clusters are trapped in the neutrophil NETs. Bars in the figures A-C, and D, and are 5σm, 1σm, respectively.

2.4.5. Generation of the neutrophil NETs is dose and time dependent

Fluorometric quantification of NETs induction by PMA **(Fig: 34A)**, STBM (mSTBM or vSTBM) **(Fig: 34B)**, and VE-CM **(Fig: 34C)** using a DNA binding dye (sytox green) [149], indicated dose dependent increases in NETs formation by these treatments. This analysis also indicated that though NETs were generated within 5 min of incubation with high doses of PMA or VE-CM, but only formed within 30 min of incubation with STBM **(Fig: 34D)**. This implies that the kinetics for NETs induction may differ between the IL-8 dependent and independent pathways.

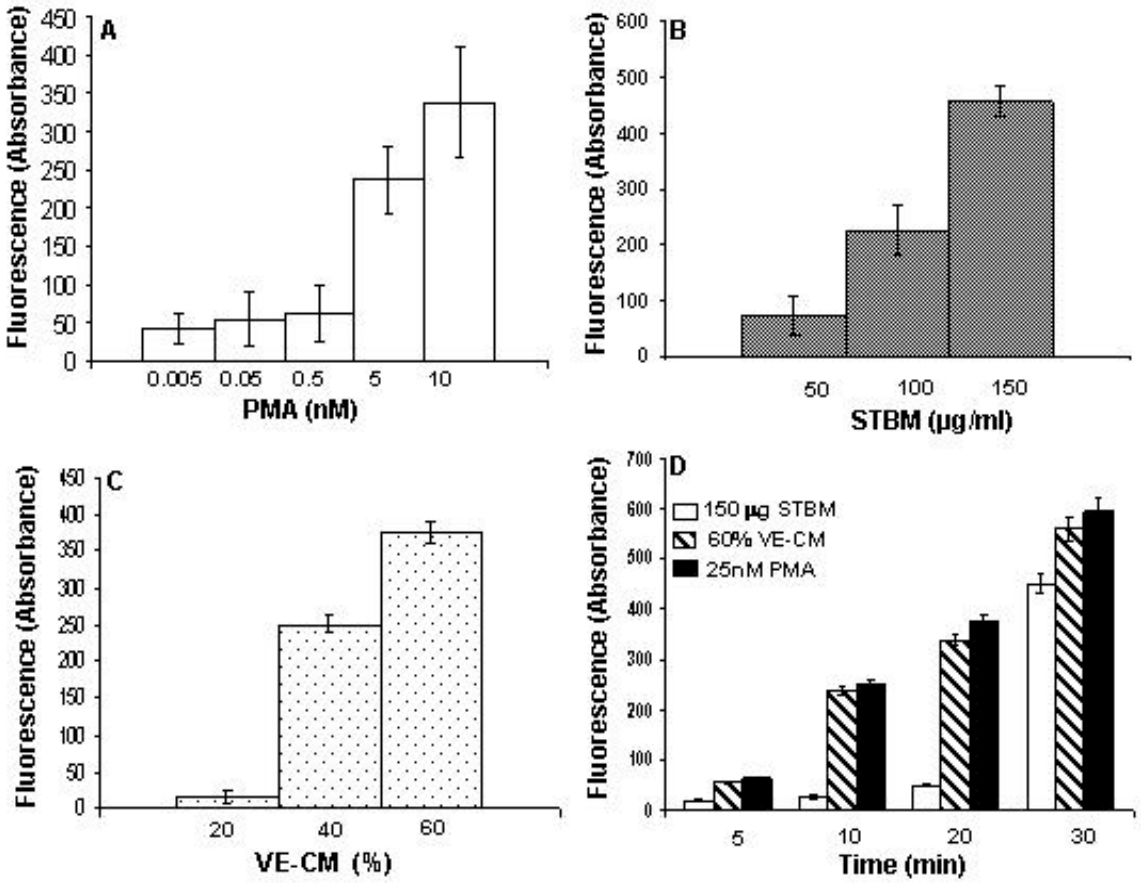


Figure: 34. Quantification of NETs formation by activated neutrophils. NETs formation by neutrophils activated with PMA **(A),** STBM **(B),** and VE-CM **(C)** induced DNA release in dose and time dependent **(D)** manner. The values represented for the STBM response were obtained after subtracting the values obtained for the DNA present in the STBM alone. The error bars in the figures represent ∂ standard deviation of the means.

2.4.6. Increased presence of neutrophil NETs in preeclamptic placenta

Because STBM, and possibly placentally derived IL-8, are released directly by the syncytiotrophoblast into the maternal circulation in the intervillous space of the placentae, we examined for the presence of neutrophil NETs in cryosections of placental villous tissue. The presence of NETs, as detected by combined staining for DNA and neutrophil-derived elastase indicated that their number was quite small in normal placental tissue **(Fig: 35A-35C)**. This is to be expected, as STBM are released during normal pregnancy, especially at term. It furthermore, appears that these NETs also confined in close contact with the syncytiotrophoblast, possibly at the site of immediate release of STBM, as this shedding may involve a budding-off process [96].

In preeclamptic placentae, the number of NETs was found to be increased and they appear to fill the entire intervillous space areas. This is evident from a haze over the entire inter-villous area when stained with the neutrophil elastase and DNA binding dye (DAPI) **(Fig: 35D-35F)**.

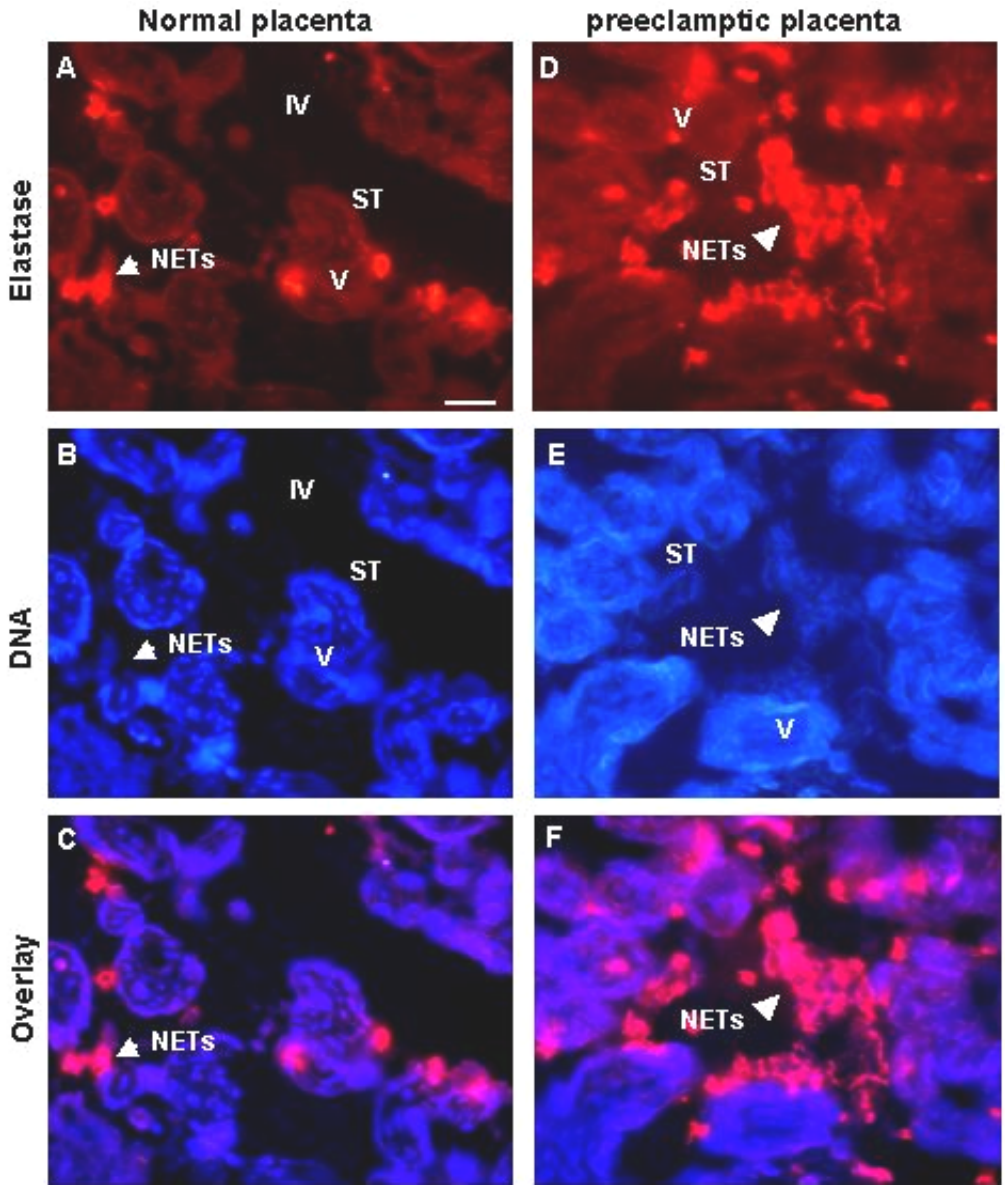


Figure: 35. Analysis of placental tissue sections of normal (A to C) and preeclamptic placenta (D to F). (A) Immunofluorescence staining of the neutrophil elastase reveals localization of neutrophil elastase within the villi or adjacent to the syncytiotrophoblast in the normal placenta. **(B)** Elastase staining largely overlaps with staining for DNA**. (C)** The overlay indicates that the DNA is closely associated with the neutrophil elastase. **(D)** Staining for neutrophil elastase, DNA **(E)**, revealing a haze over large portions of the intervillous space and their overlay **(F)** in the preeclamptic placenta reveals that the NETs are largely localized within the intervillous space. Bar in the figures is 50σm.

It has been shown that histone proteins are the major component of the NETs [149]; therefore, NETs within the placenta were also examined for the presence of

histones. The NETs observed within the intervillous space were also shown to be rich in histones, which largely overlapped with DNA staining (**Fig: 36**). These observations indicate that NETs are rich in DNA, histones, and neutrophil elastase and are similar to those described previously [149].

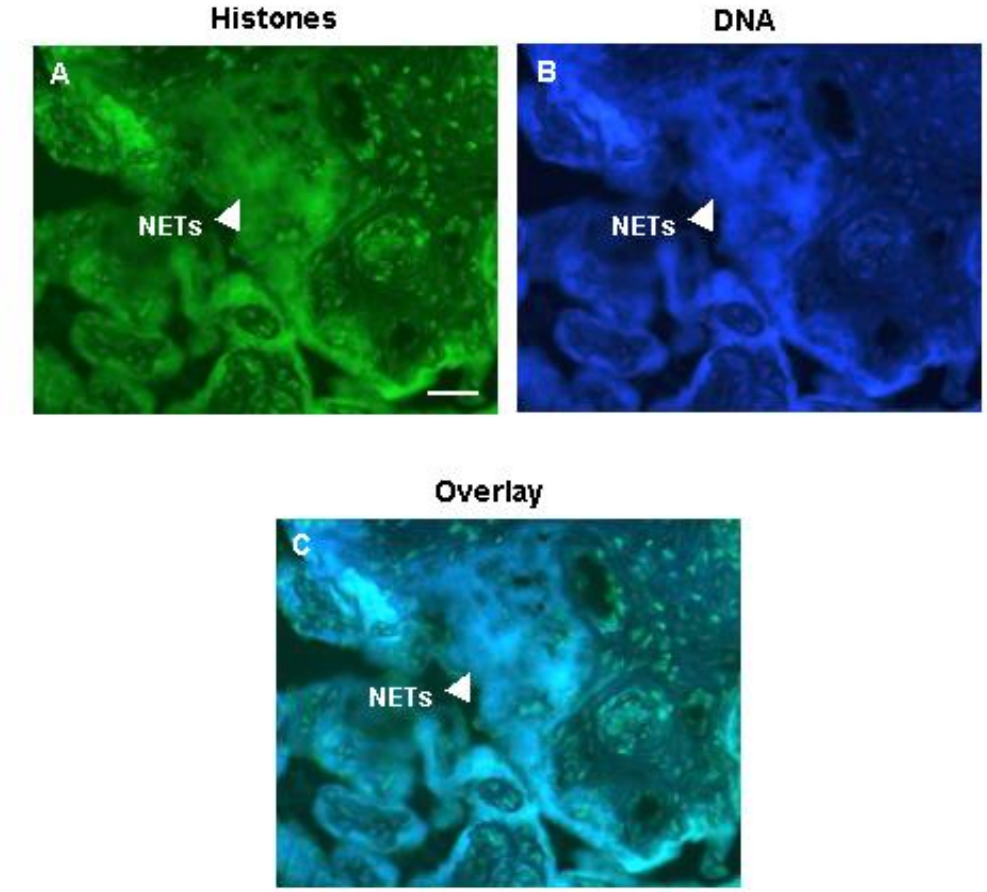


Figure: 36. Analysis of tissue sections from preeclamptic placenta. (A) Immunofluorescence staining of the histones reveals that the NETs are rich in histones. **(B)** Histones staining largely overlaps with the DNA staining. **(C)** The overlay indicates that the histones are associated with the DNA and the NETs are largely localized within the intervillous space and are rich in DNA and histones. Bar in the figures is 50σm.

2.4.7. The NETs formation ends with the death of neutrophil

Activated neutrophils were stained with the non-cell-permeant DNA binding dye, sytox green, and directly observed under the fluorescence microscope without fixing. This dye stained the whole cell surface, which was releasing extracellular DNA containing NETs. This observation suggests that the NETs are coming out from the whole cell surface and cell still is alive. When the NETs producing cell

was observed continuously for 3-4 min, slowly the NETs were disappeared. Later sytox green dye entered in the cells and stained nucleus concomitantly suggesting that the cell is no more viable **(Fig: 37A-37L)**. This interesting observation suggests that this NETs formation is a general neutrophils activation phenomenon, which ends with the death of the activated neutrophil. It is not clear that how the NETs generated by neutrophils are disintegrated but it is evident by these observations that the NETs formation occurs much more quickly than apoptosis, and that stimuli (IL-8 and PMA) known to prolong life of neutrophils in fact induce NETs formation [149].

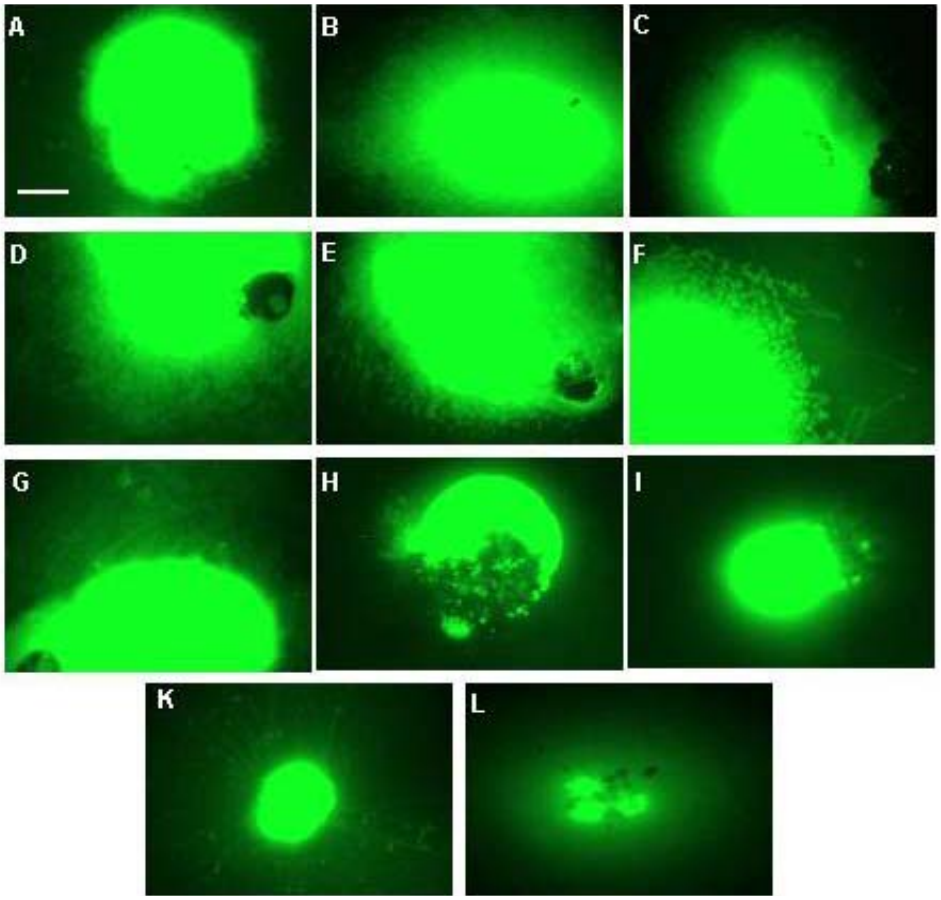


Figure: 37. Fluorescent microscopical analysis of NETs formation after DNA staining. Neutrophils were incubated with 60% VE-CM for 30 min and stained with 10σM sytox green DNA binding dye for 5 min and observed under the microscope. NETs were released from the entire cell surface and spread in all direction as dye could stain DNA in the NETs **(A-H)**. The NETs were then dismantled in 3-4 min and later nuclear staining revealed that the cell is dead now **(I-L)**. The bar in the figures is 50σm.

2.4.8. Conclusion

These data suggest that the placentally derived STBM and IL-8 can individually activate neutrophil and can induce them to generate NETs, which are rich in DNA, histones and neutrophil elastases. Furthermore, massive numbers of neutrophil NETs are present in the preeclamptic placenta further suggesting that these NETs may play a role in the pathogenesis of preeclampsia.

Discussion.....

3. Discussion

This study was designed to study the role of placentally derived factors (STBM and cytokines) during normal pregnancy and preeclampsia. It has been reported earlier that the STBM produced either from normal or preeclamptic placenta showed similar effects on the endothelial cells cultures [107]. Therefore, in this study STBM and cytokines were prepared from normal term placentae. It has also been proposed that the excessive shedding of STBM in maternal circulation leads to endothelial dysfunction during preeclampsia, which suggests that quantity of the shed STBM is responsible for this maternal syndrome [105-109]. In contrast to previous studies in this study, we, therefore, hypothesized that STBM produced during normal pregnancy and preeclampsia may differ in their quality, which might help us to explain the pathogenesis of preeclampsia. To prepare qualitatively different STBM, we prepared STBM by three different preparatory methods:

A. mSTBM were prepared by mechanical dissection of villous tissues, these STBM are known since long time [105-109]. Based on the mode of preparation we assumed that this method should produce STBM that are rich in necrotic particles.

In addition to the above-mentioned STBM for the first time we prepared STBM by two new methods:

B. vSTBM were prepared by villous explant culture based on methods described by Huppertz et al. [103], which will produce STBM of aponecrotic nature.

C. pSTBM were prepared from the placental cotyledon perfusion as described earlier [136, 137] assuming that most of the STBM produced by this procedure should be apoptotic.

We hypothesized that these STBM may mimic physiologic STBM that are released by the placenta into the maternal circulation during normal pregnancy and preeclampsia. Therefore, in this study we have biochemically and functionally characterized STBM preparations and have examined their effects on immune cells. In addition to the STBM, the effects of placentally derived cytokines were also examined on immune cells.

3.1. Characterization of the STBM preparations

Characterization of these preparations revealed that all three STBM preparations (including mechanical one) contained the syncytiotrophoblast specific membrane protein PLAP **(Fig: 8)**, thereby confirming their origin as *bona fide* STBM. Their morphological examination also confirmed that STBM prepared by three different methods are of similar sizes **(Fig: 9)**. The finding that the concentration of PLAP is about 4-5 fold higher in the mSTBM compared to the other two STBM preparations suggests that mSTBM contains a higher amount of syncytiotrophoblast-derived material. One important observation made in this study is that all STBM preparations contain both fetal DNA and mRNA, although the levels of each of these fetal analytes differ in the three preparations. In this regard, the highest concentration of fetal DNA was detected in vSTBM, whereas the highest concentration of fetal RNA was present in pSTBM **(Fig: 10 and Table: 1)**. Although we have taken great care to harvest as many of the STBM as possible by the use of high-speed ultra-centrifugation, we were still able to detect considerable amounts of fetal DNA in VE-CM **(Fig: 12)**. The high levels of particle free fetal DNA in VE-CM may be extrapolated to the release of fetal DNA into maternal plasma [138], then it is possible that the major proportion of circulatory fetal DNA may exist in a completely particle free form. On the other hand, very little fetal RNA was detected in VE-CM. Again, provided that the observations we have made with *in-vitro* generated STBM correspond to the *in-vivo* situation, then it is possible that fetal mRNA species may be largely associated with membrane particles, as has been previously reported [143]. It is also likely, that these few mRNA species present in the cleared VE-CM are associated with very small micro-particles, which are not effectively harvested by high-speed ultra-centrifugation. The difference observed in fetal DNA and mRNA content in the three STBM preparations may be due to the manner in which these particles are generated, in that those obtained by perfusion or *in-vitro* villous explant culture are generated predominantly by apoptotic cell turn-over [96, 103], in contrast to STBM isolated by mechanical disruption where STBM may involve release by necrotic pathways.

In this context it is worth noting that the release of STBM differs in normal pregnancy to that occurs in preeclampsia [96, 103, 153]. In normal pregnancy, the shedding of placental particles occurs continuously as part of the self-renewal of the syncytiotrophoblast monolayer, a process that involves apoptosis of the aged nuclei and fusion of cytotrophoblast cells [96, 103]. In preeclampsia, this process is altered in that syncytiotrophoblast apoptosis rates are significantly increased, which has been suggested to contribute to the elevated release of STBM, possibly by apo-necrotic pathways [98, 103, 153]. As our data suggests that the vSTBM are rich in DNA concentration than the other two STBM preparations this suggest that shedding of such STBM by apo-necrosis might contribute in the increased DNA concentrations during preeclampsia.

Based on these observations it can also be hypothesized that pSTBM may be regarded as being the closest representatives of those generated under normal physiological conditions, in that STBM are collected directly from the intervillous space, the site where they would normally enter the maternal circulation. The presence of fetal DNA and mRNA species in all three STBM preparations, especially in those obtained by perfusion of the maternal compartment of the placenta near to physiological conditions implies that cell-free fetal nucleic acids may similarly be STBM-associated *in-vivo*. This facet, however, needs to be confirmed by the analysis of STBM isolated from maternal blood samples, currently a technically demanding undertaking. Furthermore, provided that circulatory fetal nucleic acids are indeed associated with STBM *in-vivo*, then it is possible that the analysis of the fetal DNA and RNA content of STBM in the maternal circulation in normal and pathological pregnancies may yield new insight into the underlying mechanisms leading to their release by the syncytiotrophoblast. It may also provide a new strategy for the enrichment of these fetal analytes from maternal blood samples.

Functional characterization of the three STBM preparations indicated that all preparations altered HUVEC proliferation in a dose dependant manner, with the mSTBM showing the greatest degree of growth inhibition. While it has previously been reported that mSTBM can inhibit HUVEC proliferation [92, 105, 106], this

is the first study that has examined the potential effects of two other forms of *in-vitro* generated STBM on endothelial cell growth **(Fig: 14A, 14B)**. The inhibition of cell proliferation by all three STBM preparations was specific to HUVEC only **(Fig: 15A-C)**. Although a slight effect on the proliferation of some of the cell lines was observed upon treatments with the STBM preparations, these results were similar to the observations made previously by Samarson et al [105].

Differential HUVEC cell proliferation upon treatment with the STBM preparations suggest that the three STBM populations differ in their biological properties. Whereas vSTBM and pSTBM reduce cell growth without causing a loss of endothelial cell integrity, mSTBM massively disrupted the HUVEC monolayer **(Fig: 16)** and triggered endothelial cell death **(Fig: 17)**. From these observations it is however unclear whether the onset of apoptosis preceded detachment of the endothelial cells from the culture dish or occurred once the monolayer had become disrupted. Since a previous study has reported that the anti-proliferative activity of mechanically derived STBM might be due to the interaction of adhesion molecules on STBM with endothelial cells [154], it is tempting to propose that apoptosis follows cell detachment induced by competitive binding to the gelatin-bound ligands. Nevertheless the observation that increased amounts of STBM (up to 50 σg/well) from the two new preparations do not achieve the high degree of growth inhibition triggered by mSTBM strongly suggests that the quality of the STBM produced rather than their quantity is determining for altering HUVEC proliferation and induction of cell death.

These observations, therefore, indicates that the three STBM preparations differ functionally with regard to their effect on HUVEC cultures that raise the issue of which of the different STBM preparations is closest to normal physiology. STBM prepared either from villous explant cultures or by placental perfusion may more closely mimic the physiological status of apoptotic release, as the micro-particles released by these methods are more likely to result from normal syncytiotrophoblast turnover than the STBM isolated by physical disruption of villous integrity like the mechanical preparation [153], which may lead to necrotic particle release. A remarkable feature of vSTBM and pSTBM is that they do

inhibit HUVEC proliferation in a dose dependent manner without the induction of endothelial cell apoptosis; this suggests that these STBM might alter endothelial cell function rather than cell integrity. This is in good agreement with the *in-vivo* findings, where evidence of endothelial cell activation in preeclampsia is observed from increased plasma levels of von Willebrand factor, fibronectin, sVCAM-1 and sE-selectin [155, 156]. Furthermore, in preeclampsia endothelial function is altered as myometrial arteries from women suffering with the disorder exhibit an attenuated endothelium-dependent vasodilatory response [157]. As none of these observations suggest an increased endothelial cell turnover and/or massive endothelial cell apoptosis, the possible physiological role of particles having similar properties to those exhibited by mSTBM will have to be further addressed. Therefore we conclude that investigations studying *in-vitro* effect of placental STBM on endothelial cells will have to consider the mode in which these micro-particles are prepared.

In an effort to understand the qualitative difference of the three STBM preparations, lipid analysis of STBM preparations was carried out. This analysis revealed that in comparison to the two new STBM preparations, mSTBM are rich in lipids such as cholesterol esters **(Fig: 11A)**. It has been proposed that the excessive amount of the cholesterol can induce inflammatory reaction [146. 158]. Therefore, depletion of cholesterol in mSTBM was carried out using MηCD **(Fig: 11B),** and HUVEC proliferation was measured after co-incubation with lipid-depleted mSTBM. This cholesterol depletion in mSTBM resulted into the significant restoration of HUVE proliferation, which suggests that presence of high cholesterol content in mSTBM is partially responsible for the reduced HUVEC proliferation **(Fig: 18)**.

These observations summarizes that the three STBM preparations differ biologically and these differences are attributable to their mode of preparation.

3.2. Placentally derived factors and T cell response

In-vitro generated STBM have been shown to modulate lymphocyte activities, in that STBM can inhibit lymphocytes proliferation and can induce apoptosis in

Jurkat T cell line [109, 118,119]. These studies largely made use of STBM prepared by mechanical dissection of villous tissue or STBM prepared form maternal serum. In contrast to these studies, in this study the effect of three qualitatively different STBM preparations, characterized above, and VE-CM was examined on purified T cells.

This comparative study revealed that the three different STBM preparations and VE-CM are not able to activate T cells on their own *in-vitro* **(Fig: 19, 23)**. This observation suggests that in the presence of antigen presenting cells (APCs), STBM might have antigenic potential. It might also be possible that the effects of STBM on T cells is mediated via an intermediate step, such as STBM mediated activation of endothelial cells, which then leads to the production of inflammatory molecules which subsequently act on the T cells. Evidence for such a mechanism is partially obtained from previous studies, which indicated that supernatants from endothelial cells treated with STBM activate PBLs *in-vitro* [109, 159]. As these studies have made use of PBLs it is most likely that observed effects are specific to leukocytes (neutrophils) not to T cells.

This study, however, do indicate that mSTBM and vSTBM can influence the response of PMA and ionomycin activated T cells, in that these two STBM preparations significantly reduced T cell proliferation **(Fig: 20)**, as well as IL-2 and IFNν production **(Table: 2)**. This phenomenon was associated with reduced CD69 expression in these T cells **(Fig: 21)**. On the other hand, pSTBM enhanced T cell proliferation and lead to a moderate increase in the cytokine production **(Fig: 20, Table: 2)**.

One common facet observed with all the three STBM preparations is that none of the STBM preparations induced apoptosis in activated T cells **(Fig: 22)**. It is in contrast to the data where maternal serum STBM have been shown to cause FasL mediated apoptosis of Jurkat T cells, which might be explained by the fact that this transformed T cell line is highly receptive to pro-apoptotic signals [119], or these particles differ qualitatively than the placentally derived STBM and may be contaminated with the particles that are of maternal origin. Differential T cell proliferation and cytokine production (IL-2 and IFNν) that was observed, however

do emphasize that the three STBM preparations are functionally and qualitatively different. The effects examined with pSTBM are intriguing as they behave largely opposite to the other two STBM preparations. In this context, it should be noted that it is similar to that observed with HUVEC cultures where these STBM also exhibited different activities. These differences exhibited by three STBM preparations on T cells may reflect upon the mode whereby these different particles are generated as described above where the mSTBM may be produced by the necrosis and other two new STBM preparations are rich in apoptotic/apo-necrotic particles.

To examine the effects of placentally derived factors on purified T cells, activated T cells were cultured in the presence of VE-CM. VE-CM reduced proliferation of the activated T cells **(Fig: 24)** and decreased IL-2 and IFNν production **(Fig: 26)** in a dose dependent manner. It was also observed that T cell activation was reduced upon treatment with VE-CM **(Fig: 27)** and this treatment did not induce any T cell apoptosis **(Fig: 28)**. These observations suggest that low T cell proliferation and reduced cytokines production observed with VE-CM treatments might be associated with the reduced T cell activation.

An important mechanism for the low T cell proliferation *in-vitro* is via the IDO system, which suppresses T-cell responses by limiting the availability of tryptophan in local tissue microenvironments [160]. However, in context of our study, it has been shown that IDO expression by tumor cells did not interfere with their ability to stimulate naive T cells to enter cell cycle and to express early activation markers such as CD69 [161]. *In-vitro* studies have shown that addition of IFNν to placental villous explant cultures can induce IDO expression in the villous tissue. The villous explant homogenates obtained form such cultures can inhibit the proliferation of PHA stimulated PBMC *in-vitro* [162]. In contrast to these studies we did not induce IDO expression in villous explant culture with IFNν and did not use villous explant homogenate to study T cell response. Rather we used placental micro-particles free conditioned medium still, we observed significant inhibition of the T cell proliferation. Further to rule out the role of IDO in the low T cell response in our conditions, we added L-tryptophan or

inhibitor of IDO, 1-MT, to the T cell cultures that could not recover reduced T cell proliferation **(Fig: 29)**. These observations strongly suggest that the inhibition of T cell proliferation is not mediated by the IDO. Therefore, it can be hypothesized that IDO is a cytoplasmic enzyme [163], present in the syncytiotrophoblast [164, 165], acts locally by deletion of activated T cells at the site of feto-maternal interface but not distally. Furthermore, CD69 expression was not affected by the IDO [162], this suggests that the reduction in CD69 expression on activated T with VE-CM involves some unknown factors produced by the placenta. As we could observe a significant reduction in the IL-2 and IFNν production by the activated T cells after the treatment with VE-CM this suggest that placentally derived soluble factors are immuno-modulatory in nature.

In summary this data suggests that the placentally derived STBM and soluble factors are immunosuppressive in nature. The effects of STBM on T cells largely depend upon the quality of the STBM. These features should, therefore, be taken into consideration when using *in-vitro* prepared STBM as a model to study their potential role in normal pregnancy and preeclampsia. We could observe a significant reduction in T cell proliferation *in-vitro* by an IDO independent mechanism and reduced Th1 cytokine production by the activated T cells after the treatment with placentally derived factors. These observations further confirm that the normal pregnancy is associated with the reduced Th1 immunity as proposed earlier [86, 166, 167]. Therefore, these data strengthen the fact that T cell response during pregnancy is tightly regulated to achieve successful pregnancy.

3.3. Placentally derived factors and innate immune response

It has been proposed that the maternal innate immune system is activated during pregnancy [110]. It has been speculated that monocytes are the likely target of the innate immune system that are activated by placentally derived soluble factors, cytokine, enzymes, lipids and free radicals [110]. In this study we have focused upon the polymorphonuclear cells i.e. neutrophils. Evidence suggests that STBM can activate neutrophils *in-vitro* [112]. Recent observations suggested that activated neutrophils generate extracellular DNA containing networks (NETs), which trap

and kill bacteria [149]. In this study for the first time we have demonstrated that these NETs can be induced by physiological factors, such as STBM and placental cytokine, IL-8. This is the first evidence, which suggests that NETs induction is not solely mediated by foreign objects, such as bacteria. In this regard, we observed that the placentally derived cytokine IL-8 and STBM efficiently activated neutrophils individually **(Fig: 30).** Here, We show that upon activation with different STBM and VE-CM, neutrophil NETs were generated **(Fig: 32)**. These observations also provides the first evidence implicating neutrophil NETs in preeclampsia in that NETs were induced by plasma samples from cases with preeclampsia **(Fig: 32B, 32K).** NETs formation with the VE-CM could be blocked by the IL-8 neutralizing antibody, which suggests the placentally derived IL-8 is responsible for the NETs formation **(Fig: 32I and 32R)**. These NETs were able to entrap STBM **(Fig: 33)**. This NETs formation occurred in time and dose dependent manner **(Fig: 34)**. These observations suggest that placentally derived factors can induce NETs formation in an IL-8 dependent and independent manner. Furthermore, DNA, histones and elastase rich NETs were found to occur with high frequency directly in the intervillous space of preeclamptic placentae **(Fig: 35, 36)**. This latter observation may be of considerable importance regarding the etiology of this disorder, which is widely accepted to involve inadequate perfusion of the placenta [168], in that it provides new evidence that the underlying hypoxic lesion can occur directly at the feto-maternal interface, and need not be distally located to the placental villi, such as the maternal spiral arteries.

By their ability to trap and immobilize large bacteria such as S. *aurens* [149], it is very likely that the presence of these neutrophil NETs, especially when present in such large numbers as we have observed in preeclampsia **(Fig: 35, 36)**, will severely disrupt maternal blood through the intervillous space. This reduction in perfusion will therefore lead to a condition of oxidative stress directly at this critical site of feto-maternal exchange.

Previous studies have suggested that the oxidative stress resulting from placental hypoxia is instrumental in mediating the downstream events that lead to the maternal syndrome of preeclampsia [23], including an imbalance in the VEGF-R1

system and ensuing endothelial cell damage [169, 170]. In this context, it should be noted that placental hypoxia per se may not be the main contributor to oxidative stress, but rather, that this may result from periods of hypoxia and re-oxygenation [28]. The phenomenon we have described here would be very amenable to such a proposed model, in that the fluctuation in blood flow through the intervillous space would be mediated by the rapid generation and clearance of NETs, leading to a sudden influx of oxygen rich maternal blood into these hypoxic tissues and ensuing oxidative stress.

Furthermore, as enhanced interactions between vascular tissue and neutrophils have been demonstrated in ischemic tissues [171, 172], it is possible that such a mechanism may operate in preeclampsia [173], contributing to the generation of the NETs that we have described here. Therefore, it is possible that these conditions may lead to the formation of a cascade with endless loop character, whereby the initial hypoxic condition and associated oxidative stress brought about by inadequate modification of the spiral arteries, will lead to neutrophil activation and NETs formation, which will then lead to further hypoxia / re-oxygenation or oxidative stress conditions. Although enticing, these speculations need to be confirmed, by further studies, perhaps, using *in-vitro* placental villous culture systems [174].

With regard to the previous observations concerning cell-free DNA in preeclampsia [94, 95], these data are also of considerable interest, as the major component of NETs is extracellular DNA. Therefore, the generation of these NETs in preeclampsia may contribute to the increased levels of maternal circulatory cell-free DNA detected in this disorder [94, 95]. Furthermore, as the levels of circulatory cell-free DNA correlated very well with the severity of the disorder, this could be indicative of increased NETs formation, which would support our proposal that these novel extracellular entities generated by neutrophils may play an important role in the underlying etiology of preeclampsia.

Based on the above data where placentally derived factors activated neutrophils and induced NETs formation following model can be proposed for the pathogenesis of preeclampsia.

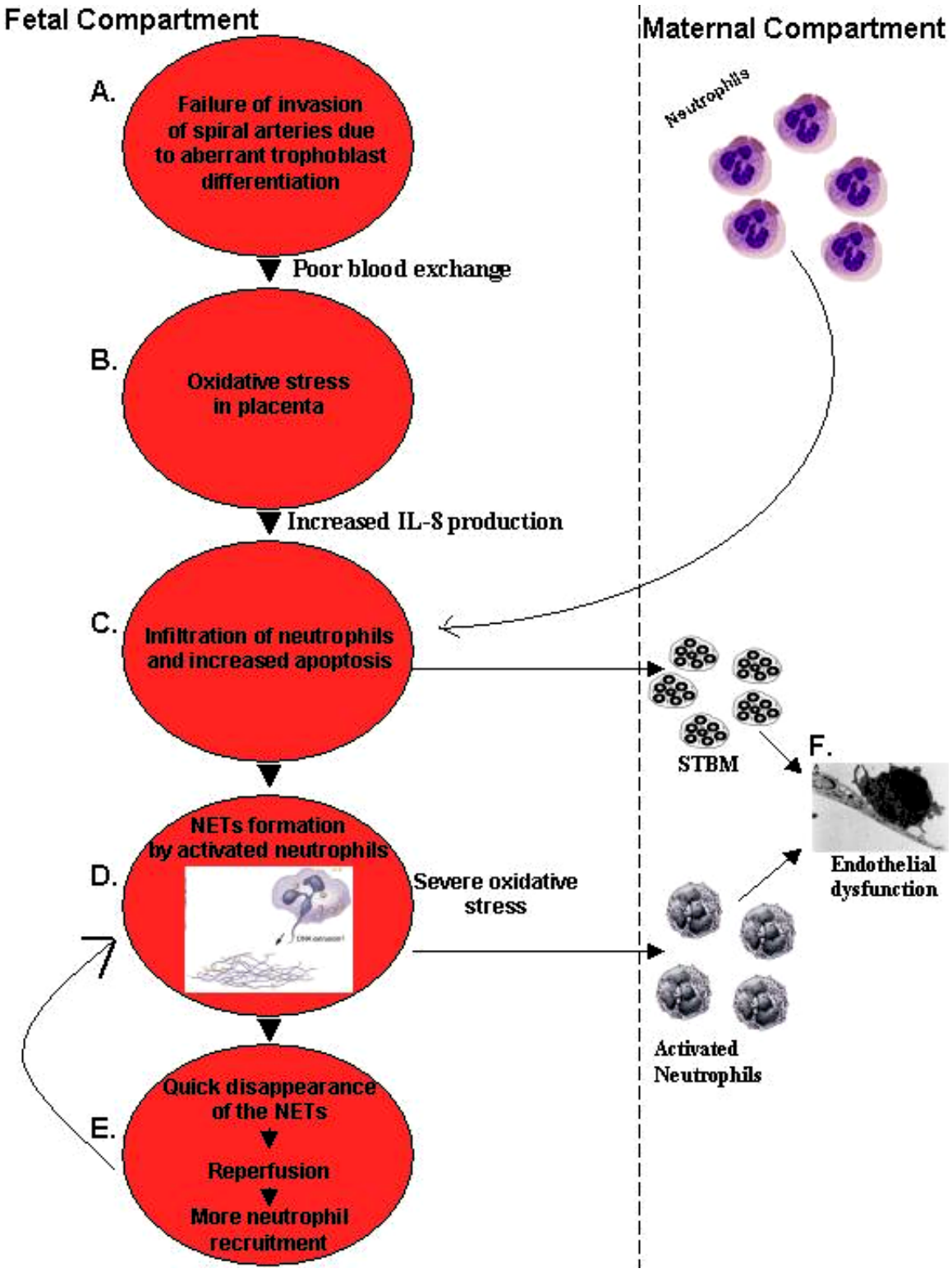


Figure: 38. A proposed model for the pathogenesis of preeclampsia. A. Aberrant trophoblast differentiation reduces invasion of the trophoblast cells into the myometrial spiral arteries, which results in the poor vascular exchange and reduced blood supply to placenta. **B.** Reduced blood supply leads to oxidative stress in placenta. **C.** Oxidative stress results into the increased apoptosis

and increased shedding of STBM, which will then enter into the maternal circulation and will lead to endothelial dysfunction. More neutrophil infiltration will occur due to increased IL-8 production by hypoxic tissue. **D.** Activated neutrophils will then generate NETs within the placenta which will further induce oxidative stress by blocking blood flow in the intervillous space, some activated neutrophils might escape from the placenta and will enter into the maternal circulation and may cause endothelial dysfunction. **E.** Quick disappearance of the NETs will reperfuse placenta, which will then lead to hyperoxia within the placenta. This reperfusion will further recruit more neutrophils, which will then again form NETs. In this manner an endless loop of the hyperoxia and hypoxia will continue. **F.** Endothelial dysfunction caused by STBM and activated neutrophils.

Finally to conclude, these all data summarized here provides an insight that not only the quantity of the STBM but also their quality play an important role in the pathogenesis of preeclampsia. Furthermore, it can be interpreted by these data that placentally derived factors are immuno-modulatory in nature. In regard to maternal innate immune response, our data do provide some evidences that placentally derived factors are able to activate innate immune system, therefore, it can be proposed that excessive activation of maternal innate immune system leads to preeclampsia. Though not studied here, but it can hypothesized by these data that the Th1 immune response observed during preeclampsia is secondary to activation of maternal innate immune system. Lastly, our novel and important observations regarding the generation of neutrophil NETs within the preeclamptic placenta provides better understanding of the pathogenesis of this pregnancy related disorder.

Methods.....

4. Methods

4.1. Collection of Samples

This study was approved by the Cantonal Institutional Review Board of Basel, Switzerland. Written informed consent was requested in all instances. Placentae and umbilical cords were obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies and processed within 30-90 min of collection.

For T cells and neutrophils isolation blood samples were obtained from healthy donors at the blood donation center, Swiss Red Cross, Basel.

Maternal blood samples were collected from pregnant women with manifest preeclampsia. Preeclampsia was defined by blood pressure of at least 140/90 mm Hg in two determinations 4 h apart or by a diastolic blood pressure of more than 110 mm Hg and an associated proteinuria of at least 300 mg in 24 h after 20 weeks of gestation. Control maternal blood samples drawn from normotensive pregnant women at term who all delivered healthy babies.

4.2. Preparation of syncytiotrophoblast micro-particles

4.2.1. Villous explant culture

Villous tissue was isolated by dissection of the placentae and removal of the decidua. Following a wash in sterile phosphate buffered saline (PBS), the villous tissue was cut into pieces of 1-2 mm. Explants were cultured in 100 mm culture dishes (Corning NY, USA) in Dulbecco Modified Eagle's Medium (DMEM) : F-12 Nutrient Mixture (1:1) (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 1% antimycotic/antibiotics (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA), 10% fetal calf serum (FCS), 25 U/ml heparin (Roche Diagnostics, Germany), 50 U/ml aprotinine (Fluka Chemicals AG, Buchs, Switzerland) and 2 mM $MgSO_4$ for 72 h at 37°C in 5% CO_2. After incubation the culture medium was collected and stored at -70°C.

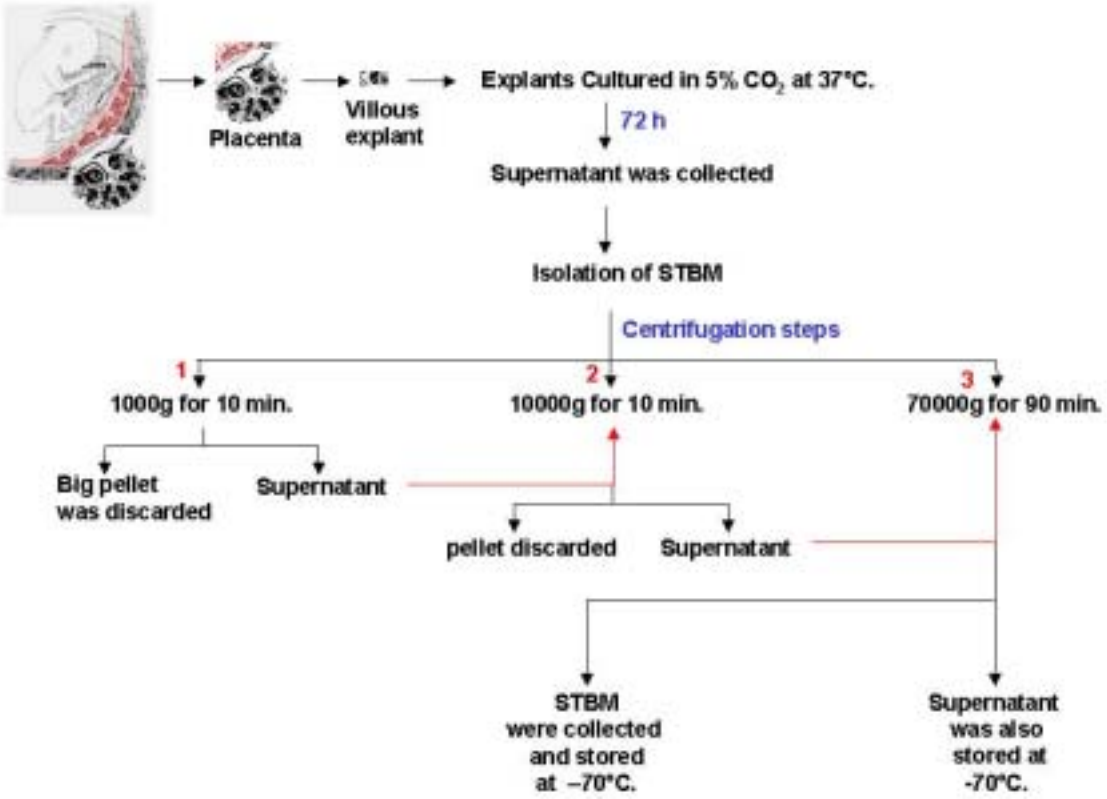


Figure: 39. A schematic representation of preparation and isolation of vSTBM. From all the preparations after initial procedures the supernatant was subjected to three centrifugation steps. The isolated STBM and culture supernatant stored aliquoted at -70°C.

4.2.2. Mechanical dissection

STBM were prepared by a slight modification of methods described previously [99, 120]. We used a smaller volume of 0.15M NaCl and a higher speed centrifugation step to ensure greatest yield. Briefly, villous tissue was washed three times in PBS containing 100 mM $CaCl_2$ then chopped with scissors and rinsed in 100 ml 0.15 M NaCl supplemented with 1% antimycotic/antibiotics overnight at 4°C. After rinsing, tissues were discarded and the supernatant was collected.

4.2.3. Placental perfusion

STBM were prepared using a well-described perfusion system [132, 133]. Briefly, the intervillous space (maternal compartment) and the villous vasculature (fetal

compartment) of an isolated placental cotyledon were perfused separately in open systems i.e. without recirculation at flow rates of about 12 and 6 ml/min, respectively. Perfusion medium was composed of NCTC-135 tissue culture medium diluted with Earle`s buffer (1:1) with the addition of glucose (1.33 g/l), dextran 40 (10 g/l), 4% BSA, heparin (2500 IU/l) and clamoxyl (250 mg/l). After 30 min of initiation of the perfusion, the perfusate from the maternal side was collected and stored at -70°C.

4.2.4. Isolation of STBM

To isolate STBM, supernatants from all the three preparations were subjected to a three-step centrifugation at 4°C: 1000 x g for 10 min, 10000 x g for 10 min and 70000 x g for 90 min. The final pellet was collected, washed with PBS and resuspended in 1 ml sterile PBS containing 5% sucrose and stored at –20°C until use. Red blood cell micro-vesicles (RBC ghosts) were prepared as described previously by Khalfoun et al. [175] and used as a negative control in all experiments.

4.3. Protein and PLAP (placental alkaline phosphatase) quantification of STBM preparations

The protein content in each STBM preparation was quantified with the advanced protein assay reagent (Cytoskeleton Inc. Denver, CO, USA) according to manufacturer's instructions. The amount of PLAP protein was determined using an ELISA. In brief, a 96 well microtiter EIA plate (Nunc, Denmark) was coated with anti PLAP antibody (mouse monoclonal Ab-5, Neomarkers Inc. Fremont, CA, USA) for 2 h at RT. After washing with PBS/0.05% Tween 20, the STBM samples were added and incubated for 2 h at RT. After 4 consecutive washes alkaline phosphatase yellow substrate (pNPP, Sigma chemicals, St. Louis, MO, USA) was added and incubated for 1 h in the dark. Subsequently the plate was read at 405 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and the data was analyzed using Soft Max Pro Software. A dilution series of purified PLAP (Sigma Chemicals, St. Louis, MO, USA) was used as a standard.

4.4. Scanning electron microscopy (SEM) of STBM

For SEM examination 3 σg STBM of each preparation were diluted in 25 σl PBS, then transferred onto 0.2 σm polycarbonate membrane (Whatman, New Jersey, USA) under the application of vacuum and dehydrated with an acetone series (30%, 50%, 70%, 100%, and 100% acetone for 10, 10, 10, 10 and 15 min, respectively). Then specimens were coated with 2nm platinum and analyzed using a Philips XL-30 ESEM scanning electron microscope (ZMB, University of Basel).

4.5. FACS analysis of the STBM

To confirm that the STBM were the products of the syncytiotrophoblast membrane, PKH-26 GL membrane dye (Sigma Chemicals, USA) were used and FACS were used to detect the STBM. 50 σg of the STBM were diluted in 1ml of Diluent C, which was provided with the dye. Dilute dye in the diluent C at 20 σM and add it to the diluted STBM at 10σM concentration (1:1 STBM: Dye both in diluent C). Incubate for 5 min and then add equal volumes (2ml) of heat inactivated FCS, incubate two more minutes then add equal volumes of PBS (4ml). Pellet downs the particles at 2000g. Wash three times using 5ml PBS. Finally resuspend the labeled particles in 1ml PBS and carryout FACS. As particles are very small, therefore, change FSC and SSC scale to log scale.

4.6. DNA and RNA detection in the STBM

For the quantitation of the placentally derived DNA and RNA in the STBM SRY and CRH mRNA were amplified using real-time PCR. DNA was extracted from STBM using High Pure PCR template preparation kit (Roche Diagnostic). Total RNA was isolated using High Pure RNA isolation kit (Roche Diagnostic) and eluted in 50 µL of elution buffer. cDNA was reverse transcribed from 500ng of total RNA using a commercial reverse transcription system (Promega). Real-time quantitative PCR and real-time quantitative RT-PCR were used for all DNA and mRNA quantifications as described earlier [138, 176, 177]. The real-time PCR and real-time RT-PCR reactions were set up according to the manufacturer's

instructions (Applied Biosystems) in a reaction volume of 25 µL. Each sample was analyzed in duplicate, and the corresponding calibration curve was run in parallel with each analysis. Absolute concentrations of *CRH* mRNA and SRY DNA were expressed as copies/mg of STBM.

PCR primers and probes sequences were:

Forward Primer SRY: TCC TCA AAA GAA ACC GTG CAT

Reverse primer SRY: AGA TTA ATG GTT GCT AAG GAC TGG AT

Probe SRY: TCC CCA CAA CCT CTT

Forward Primer CRH: 5'-GCC TCC CAT CTC CCT GGA T-3'

Reverse primer CRH: 5'-TGT GAG CTT GCT GTG CTA ACT G-3'

Probe CRH: 5'-(FAM) TCC TCC GGG AAG TCT TGG AAA TGG C (TAMRA)-3'

Oligo CRH: 5`-GGA GCC TCC CAT CTC CCT GGA TCT CAC CTT CCA CCT CCT CCG GGA AGT CTT GGA AAT GGC CAG GGC CGA GCA GTT AGC ACA GCA AGC TCA CAG CA-3`

4.7. Lipid analysis of the STBM

Lipids in the STBM were identified using thin layer chromatography and Mass spectrometry.

4.7.1. Thin layer chromatography of the STBM

100 µg of the each STBM preparation was extracted with chloroform. After centrifugation for 1 min the lipid containing lower organic layer was collected and applied to the pre-coated TLC plate. TLC was carried out in the pre-saturated chamber. The running buffer consisted of chloroform: Methanol: water: Ammonium hydroxide- 65:30:3:2 (v/v/v/v). The TLC run was monitored until the top of the plate. The separated lipids were visualized staining in mosatin stain [4% ammonium molybdate tetrahydrate (w/v) + 0.08% cesium sulfate tetrahydrate (w/v) + 30% H_2SO_4 (10%, v/v)] and charring at 200°C.

4.7.2. Mass spectrometry of the STBM

A large TLC was run for the mass spectroscopy analysis and 8 spots were scrapped out from the plate after the TLC run is over. The lipids from the Silica gel were extracted using methanol and then stored for the mass spectrometry analysis at Biozentrum, University of Basel.

4.8. HUVEC and Cell lines proliferation assay

HUVEC were isolated from normal term human umbilical cord by the method described by Jaffe et al. [178] and stored in liquid nitrogen until use. Cells from passage 2 to passage 7 were used. To assess HUVEC proliferation the cell proliferation kit (Roche Diagnostics, Basel, Switzerland) was used, which measures BrdU incorporation during DNA synthesis. In brief $1.5x10^4$ cells were plated onto 1% gelatin coated 96 well microtiter plates (Nunc, Denmark) in E-SFM (endothelial serum free medium) medium supplemented with 20 ng/ml human recombinant bFGF (basic fibrobalst growth factor) and 10 ng/ml human recombinant EGF (epidermal growth factor, GIBCO Invitrogen Life Technologies, Grand Island, NY, USA). After 6 h, the culture medium was replaced with STBM or RBC ghost particles diluted in DMEM:F-12 medium supplemented with 1 % antimycotic/antibiotics, 10% FCS, 50 U/ml aprotinin, 25 U/ml heparin (DMEM:F12 complete medium). The cells were then incubated for 22 h, followed by an additional incubation with 10 σM of BrdU for a further 18 h. The amount of incorporated BrdU was detected using an ELISA according to the manufacturer's instructions.

U-937 (human histiocytic monocyte like lymphoma [179] was used as nonadherent control cell line. A431 (human epidermal squamous carcinoma [180], HELA (human epithelial cervix carcinoma [181], NIH-3T3 (Swiss mouse embryo fibroblasts [182], and LN-18 (human brain malignant glioma cell [183] were used as adherent control cell lines. U-937, NIH-3T3, HELA, A431, and LN-18 cells (each $1.5x10^4$ cells/well) were grown in RPMI 1640 (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with Glutamax, 10% FCS,

and 100 U/ml penicillin, 100 σg/ml streptomycin. With all adherent cells RPMI 1640 medium was replaced with STBM diluted in DMEM:F-12 complete medium 6 h after seeding. For the nonadherent cells, the STBM preparations were added directly to the RPMI 1640 medium 6h after the plating. All cells were incubated for the same amount of time and cell proliferation was measured as described above.

4.9. HUVEC apoptosis

Apoptosis was measured using a commercial cellular DNA fragmentation ELISA kit (Roche Diagnostics, Germany). This assay measures the release of BrdU labeled DNA fragments into the cytoplasm during apoptosis. In brief, $2x10^4$ HUVEC were plated in 96 well microtiter plates (Nunc, Denmark) in E-SFM culture medium supplemented with 20 ng/ml bFGF and 10 ng/ml EGF. After 6 h 10 σM BrdU was added and incubated for a further 18 h. Following BrdU labeling, the cell monolayer was washed with sterile PBS, and STBM containing DMEM:F12 complete medium was added for 24 h. In positive control wells, apoptosis was induced with 5 σM staurosporine (Sigma Chemicals, St. Louis, MO, USA), a potent inducer of apoptosis, for 3 h. The detection of BrdU labeled apoptotic DNA fragments was performed according to the manufacturer's instructions.

4.10. Isolation and enrichment of T lymphocytes

PBLs were isolated from whole blood by centrifugation over a ficoll paque plus gradient (Amersham Biosciences, Uppsala, Sweden) for 20 min at RT. $CD4^+$ and $CD8^+$ T cells were enriched together with specific magnetic microbeads (Miltenyi Biotech, Gladbach, Germany) using MACS mini columns (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. The purity of the enriched T lymphocytes routinely reached >90%, as confirmed by flow cytometry.

4.11. T cell proliferation assay

Cell proliferation was assessed using the "Cell Proliferation Kit" (Roche Diagnostics, Basel, Switzerland), which measures BrdU incorporation during DNA synthesis. T cells ($1x10^5$) were cultured in triplicates in 96 well plate (Nunc, Denmark) for 72 h at 37 °C in 5% CO_2 in the presence or absence of STBM or RBC ghost particles as indicated in the figure legends. The culture medium used was RPMI 1640 medium (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS, 2mM L-glutamine (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, 100 σg/ml streptomycin (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA). 50 ng/ml PMA (Sigma Chemicals, St. Louis, MO, USA) and 1σM ionomycin (Sigma Chemicals, St. Louis, MO, USA) were used as stimuli. In the last 18 h of culture 10 σM of BrdU was added to the cells.

4.12. Total RNA isolation and Real Time PCR

Total RNA was isolated from T lymphocytes using High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). cDNA was reverse transcribed from 500 ng of total RNA using a reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions. For the RT-PCR analysis, 25 σl reaction mixture consisted of 2 σl of cDNA, 1x Taqman® universal master mix (Applied Biosystems, Foster City, CA, USA) and 1x pre-developed Taqman® assay reagents (PDAR, Applied Biosystems, Foster city, CA, USA), containing gene specific probes and primers for IL-2 or IL-4 or IFNν or 18s RNA. For these experiments the ABI Prism 7000 system (Applied Biosystems, Foster city, CA, USA) was used. 18s RNA was used as an internal control to correct for any unequal sample loading. It has previously been shown that the amount of 18sRNA per cell does not vary significantly with cell activation [184]. Thermal cycler conditions comprised of 2 min at 50°C, 10 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 min at 60°C.

The change in cytokine mRNA expression was assayed by normalization to the 18sRNA internal control. In order to obtain the fold difference the data was analyzed using the $\div\div C_T$ method described previously by [185], where $\div\div C_T$ = (cytokine C_T - 18sRNA C_T) $_{stimulated}$ - (cytokine C_T - 18sRNA C_T) $_{unstimulated}$.

4.13. IFNν and IL-2 ELISA

IFNν and IL-2 levels produced by T cells after incubation with culture upernatant or STBM were measured using commercial human IFNν and IL-2 ELISA kits (Ebioscience Inc, San Diego, CA, USA) according to the manufacturer's instructions. The ELISA plate was read at 450 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and the data was analyzed using Soft Max Pro Software.

4.14. FACS analysis of for T cell activation

FACS analysis was performed to examine CD69 expression and apoptosis of T cells following co-incubation with ulture upernatant nd different STBM preparations. Analysis of CD69 expression was performed using directly labeled monoclonal antibodies to CD3 and CD69 (BD Biosciences, Basel, Switzerland). In brief, $1x10^6$ T cells were harvested, washed and incubated with the antibodies for 30 min at 4°C, following which they were washed again prior to two color flow cytometry using a Becton Dickinson FACScan flow cytometer (BD Biosciences, Basel, Switzerland). 20,000 gated events were acquired and data was analyzed using Cell Quest Pro ® software (BD Biosciences, Basel, Switzerland). Apoptosis of T cells after STBM treatment was measured using the Annexin-V-FLUOS kit (Roche, Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's instructions.

4.15. Isolation of neutrophils

Human neutrophils were isolated using a dextran-ficoll method [186]. In brief, the mononuclear cells were depleted from whole blood by centrifugation over a ficoll

paque plus gradient (Amersham Biosciences). Neutrophils containing RBC layer was resuspended in HBSS medium (GIBCO) and 6% Dextran T-500 was added to 1% final concentration. RBCs were allowed to settle, neutrophils containing upper layer was collected, remaining RBCs were lysed the pure neutrophil population was obtained after washing with HBSS.

4.16. IL-8 ELISA

IL-8 levels were measured in the samples using a commercial human IL-8 ELISA kit (R&D systems, Minneapolis, USA,), detection sensitivity 1.5-7.5pg/ml, according to the manufacturer's instructions. ELISA plate was read at 450 nm with wavelength correction of 562nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and the data was analyzed using Soft Max Pro Software.

4.17. FACS analysis for neutrophils activation

$1x10^6$ neutrophils were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2mM L-glutamine (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, 100σg/ml streptomycin (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) and incubated with PMA, rIL-8, pre-eclamptic plasma, STBM (mechanical or villous explant) or culture supernatant for 60 min except the pre-eclamptic plasma where the cells were incubated for 3h. After incubation cells were collected washed and stained with directly conjugated mAb against CD15 (FITC) and CD11b (PE) for 20 min at 4°C. Appropriate isotype match control were also used. Later cells were washed and analyzed using a Becton Dickinson FACScan flow cytometer (BD Biosciences, Basel, Switzerland). A minimum of 20,000-gated events were acquired and analyzed with the Cell Quest Pro software (BD Biosciences, Basel, Switzerland).

4.18. Quantification of DNA release from activated neutrophils

Freshly isolated neutrophils were seeded into 96-well plates and stimulated either with PMA at concentrations between 0.005 - 10 nM or with 50-150σg/ml STBM (mechanical or villous explant) or with 20-60% culture supernatant for 30 min. After the incubation, Sytox Green (Molecular Probes, USA), a non cell-permeant DNA binding dye, was added to the cells at a final concentration of 10 µM to detect extracellular DNA. Non-stimulated neutrophils were used a control. The plates were read in a fluorescence microplate reader SpectraMAX Gemini (Molecular Devices, Sunnyvale, CA, USA) with a filter setting of 485 (excitation)/ 538(emission) and the data was analyzed using Soft Max Pro Software.

4.19. Immunofluorescence assays

Neutrophils were seeded on glass coverslips treated with 0.001% polylysine, allowed to settle and either treated with rIL-8 (10ng/ml, R&D Systems), PMA (25 nM), STBMs (150 σg/ml each preparation), culture supernatant (60%) or left unstimulated. Cells were fixed with 4% PFA, blocked overnight (10% FCS, 1% bovine serum albumin, 0.05% Tween 20 in PBS). For DNA detection, sytox green (shown), DAPI were used. Specimens were analyzed with a Zeiss Axioplan 2 imaging fluorescent microscope.

4.20. Examination of NETs formation using Scanning electron microscopy

Neutrophils were seeded on 12mm 0.001% polylysine coated coverslips and incubated with PMA, rIL-8, pre-eclamptic plasma, STBMs or culture supernatant for 30 min and were fixed with 2.5% glutaraldehyde, postfixed using repeated incubations with 1% osmium tetroxid / 1% tannic acid, dehydrated with a graded ethanol series. After dehydration and critical-point drying, the specimens were coated with 2 nm platinum and analyzed in a Philips XL-30 ESEM scanning electron microscope at ZMB, Biozentrum, University of Basel.

4.21. Histology of the placental tissues

Placental villous tissue samples were embedded in Tiise-Tek ® OCT embedding medium (Digitana) and stored at -80°C prior to analysis. Tissue sections were cut at 8 σm, air-dried and post fixed with acetone for 5 min and then rehydrated using TBS buffer (Tris buffered saline + 0.05% Tween 20 + 0.2% BSA). For immunostainings rehydrated samples were incubated with primary antibodies specific to elastase (Calbiochem) and histones (Chemicon), for 1h, which were then detected with secondary antibody conjugated to Texas Red or FITC. DNA was stained with DAPI. The antibodies diluted according to the manufacturer's instructions.

4.22. Statistical analysis

Wherever needed, Mann-Whitney test was used to calculate the statistical significance of the differences between experimental groups. Statistical significance was set at P{0.05.

References……..

5. References

1. Thellin O, Coumans B, Zorzi W, Igout A, Heinen E. Tolerance to the foeto-placental 'graft': ten ways to support a child for nine months. Curr.Opin.Immunol. 2000; **12**:731-7.

2. Mellor AL, Munn DH. Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. Annu.Rev.Immunol. 2000; **18**:367-91.

3. Redman CW. Eclampsia still kills. Br.Med.J.(Clin.Res.Ed) 1988; **296**:1209-10.

4. Douglas KA, Redman CW. Eclampsia in the United Kingdom. BMJ 1994; **309**:1395-400.

5. Roberts JM. Preeclampsia: not simply pregnancy-induced hypertension. Hosp.Pract.(Off Ed) 1995; **30**:25-6.

6. Brown MA. Pre-eclampsia: a case of nerves? Lancet 1997; **349**:297-8.

7. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am.J.Obstet Gynecol 1999; **180**:499-506.

8. Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. Placenta 2003; **24 Suppl A**:S21-S27.

9. Report of the National High Blood Pressure Education Program, Working group report on high blood pressure in pregnancy. Am.J.Obstet Gynecol 2000; **183**:S1–S22.

10. Ramsay JE, Stewart F, Green IA, Sattar N. Microvascular dysfunction: a link between pre-eclampsia and maternal coronary heart disease. BJOG 2003; **110**:1029–1031.

11. Wilson BJ, Watson MS, Prescott GJ *et al*. Hypertensive diseases of pregnancy and risk of hypertension and stroke in later life: results from cohort study. BMJ 2003; **326**:845.

12. Haukkamaa L, Salminen M, Laivuori H, Leinonen H, Hiilesmaa V, Kaaja R. Risk for subsequent coronary artery disease after preeclampsia. Am.J.Cardiol. 2004; **93**:805-8.

13. Chaouat G, Ledee-bataille N, Zourbas S *et al*. Implantation: can immunological parameters of implantation failure be of interest for pre-eclampsia? J.Reprod.Immunol. 2003; **59**:205-17.

14. Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br J Obstet Gynaecol 1986; **93**:1049-59.

15. Pijnenborg R, Anthony J, Davey DA *et al*. Placental bed spiral arteries in the hypertensive disorders of pregnancy. Br J Obstet Gynaecol 1991; **98**:648-55.

16. Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J.Clin.Invest 1993; **91**:950-60.

17. Zhou Y, Damsky CH, Fisher SJ. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? J.Clin.Invest 1997; **99**:2152-64.

18. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science 1994; **266**:1508-18.

19. Lim KH, Zhou Y, Janatpour M *et al*. Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. Am.J.Pathol. 1997; **151**:1809-18.

20. Roberts JM, Redman CW. Pre-eclampsia: more than pregnancy-induced hypertension. Lancet 1993; **341**:1447-51.

21. Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. Br.J.Obstet Gynaecol. 1994; **101**:669-74.

22. Pijnenborg R, Vercruysse L, Verbist L, Van Assche FA. Interaction of interstitial trophoblast with placental bed capillaries and venules of normotensive and pre-eclamptic pregnancies. Placenta 1998; **19**:569-75.

23. Hubel CA. Oxidative stress in the pathogenesis of preeclampsia. Proc.Soc.Exp.Biol.Med. 1999; **222**:222-35.

24. Ekblad U, Erkkola R, Uotila P. The effect of acute hypoxia on prostaglandin release in perfused human fetal placenta. Prostaglandins 1987; **33**:553-60.

25. McAuliffe F, Kametas N, Krampl E, Ernsting J, Nicolaides K. Blood gases in pregnancy at sea level and at high altitude. BJOG. 2001; **108**:980-5.

26. Zhong XY, Wang Y, Chen S *et al*. Circulating fetal DNA in maternal plasma is increased in pregnancies at high altitude and is further enhanced by preeclampsia. Clin.Chem. 2004; **50**:2403-5.

27. Hung TH, Charnock-Jones DS, Skepper JN, Burton GJ. Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia. Am.J.Pathol. 2004; **164**:1049-61.

28. Hung TH, Skepper JN, Charnock-Jones DS, Burton GJ. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ.Res. 2002; **90**:1274-81.

29. Warren MC, Bump EA, Medeiros D, Braunhut SJ. Oxidative stress-induced apoptosis of endothelial cells. Free Radic.Biol.Med. 2000; **29**:537-47.

30. Carmody RJ, Cotter TG. Signalling apoptosis: a radical approach. Redox.Rep. 2001; **6**:77-90.

31. Tomei LD, Umansky SR. Apoptosis and the heart: a brief review. Ann.N.Y.Acad.Sci. 2001; **946**:160-8.

32. Redman CW, Sargent IL. The pathogenesis of pre-eclampsia. Gynecol.Obstet.Fertil. 2001; **29**:518-22.

33. Robertson WB, Brosens I, Dixon HG. The pathological response of the vessels of the placental bed to hypertensive pregnancy. J.Pathol.Bacteriol. 1967; **93**:581-92.

34. Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. Obstet.Gynecol.Annu. 1972; **1**:177-91.

35. Gerretsen G, Huisjes HJ, Elema JD. Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. Br.J.Obstet.Gynaecol. 1981; **88**:876-81.

36. De Wolf F, Robertson WB, Brosens I. The ultrastructure of acute atherosis in hypertensive pregnancy. Am.J.Obstet.Gynecol. 1975; **123**:164-74.

37. Salafia CM, Pezzullo JC, Ghidini A, Lopez-Zeno JA, Whittington SS. Clinical correlations of patterns of placental pathology in preterm pre-eclampsia. Placenta 1998; **19**:67-72.

38. Walker JJ. Pre-eclampsia. Lancet 2000; **356**:1260-5.

39. Wang X, Athayde N, Trudinger B. A proinflammatory cytokine response is present in the fetal placental vasculature in placental insufficiency. Am.J.Obstet.Gynecol. 2003; **189**:1445-51.

40. Robillard PY, Hulsey TC, Perianin J, Janky E, Miri EH, Papiernik E. Association of pregnancy-induced hypertension with duration of sexual cohabitation before conception. Lancet 1994; **344**:973-5.

41. Koelman CA, Coumans AB, Nijman HW, Doxiadis II, Dekker GA, Claas FH. Correlation between oral sex and a low incidence of preeclampsia: a role for soluble HLA in seminal fluid? J.Reprod.Immunol. 2000; **46**:155-66.

42. Wang JX, Knottnerus AM, Schuit G, Norman RJ, Chan A, Dekker GA. Surgically obtained sperm, and risk of gestational hypertension and pre-eclampsia. Lancet 2002; **359**:673-4.

43. Einarsson JI, Sangi-Haghpeykar H, Gardner MO. Sperm exposure and development of preeclampsia. Am.J.Obstet.Gynecol. 2003; **188**:1241-3.

44. Klonoff-Cohen HS, Savitz DA, Cefalo RC, McCann MF. An epidemiologic study of contraception and preeclampsia. JAMA 1989; **262**:3143-7.

45. Smith GN, Walker M, Tessier JL, Millar KG. Increased incidence of preeclampsia in women conceiving by intrauterine insemination with donor versus partner sperm for treatment of primary infertility. Am.J.Obstet.Gynecol. 1997; **177**:455-8.

46. Mills JL, Klebanoff MA, Graubard BI, Carey JC, Berendes HW. Barrier contraceptive methods and preeclampsia. JAMA 1991; **265**:70-3.

47. Marti JJ, Herrmann U. Immunogestosis: a new etiologic concept of "essential" EPH gestosis, with special consideration of the primigravid patient; preliminary report of a clinical study. Am.J.Obstet.Gynecol. 1977; **128**:489-93.

48. Eskenazi B, Fenster L, Sidney S. A multivariate analysis of risk factors for preeclampsia. JAMA 1991; **266**:237-41.

49. Robillard PY, Hulsey TC. Association of pregnancy-induced-hypertension, pre-eclampsia, and eclampsia with duration of sexual cohabitation before conception. Lancet 1996; **347**:619.

50. Bouma GJ, van Caubergh P, van Bree SP *et al*. Pregnancy can induce priming of cytotoxic T lymphocytes specific for paternal HLA antigens that is associated with antibody formation. Transplantation 1996; **62**:672-8.

51. BILLINGHAM RE, BRENT L, MEDAWAR PB. Activity acquired tolerance of foreign cells. Nature 1953; **172**:603-6.

52. Reading JP, Huffman JL, Wu JC *et al*. Nucleated erythrocytes in maternal blood: quantity and quality of fetal cells in enriched populations. Hum.Reprod. 1995; **10**:2510-5.

53. Petraglia F, Florio P, Nappi C, Genazzani AR. Peptide signaling in human placenta and membranes: autocrine, paracrine, and endocrine mechanisms. Endocr.Rev. 1996; **17**:156-86.

54. Martin RD. Scaling of the mammalian brain: the maternal energy hypothesis. News Physiol Sci 1996; **11**: 149–156.

55. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol 2002; **2**: 656–663.

56. Croy BA, He H, Esadeg S, Wei Q *et al.* Uterine natural killer cells; insight into their cellular and molecular biology from mouse modeling. Reproduction 2003; **126**: 149–160.

57. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990; **248**:220-3.

58. King A, Boocock C, Sharkey AM *et al*. Evidence for the expression of HLAA-C class I mRNA and protein by human first trimester trophoblast. J.Immunol. 1996; **156**:2068-76.

59. Hammer A, Hutter H, Dohr G. HLA class I expression on the materno-fetal interface. Am.J.Reprod.Immunol. 1997; **38**:150-7.

60. Moreau P, Paul P, Rouas-Freiss N, Kirszenbaum M, Dausset J, Carosella ED. Molecular and immunologic aspects of the nonclassical HLA class I antigen HLA-G: evidence for an important role in the maternal tolerance of the fetal allograft. Am.J.Reprod.Immunol. 1998; **40**:136-44.

61. Ace CI, Okulicz WC. Differential gene regulation by estrogen and progesterone in the primate endometrium. Mol.Cell Endocrinol. 1995; **115**:95-103.

62. Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. J.Reprod.Fertil. 1994; **101**:421-6.

63. Senturk LM, Arici A. Leukemia inhibitory factor in human reproduction. Am.J.Reprod.Immunol. 1998; **39**:144-51.

64. Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat.Med. 1998; **4**:1020-4.

65. Kojima K, Kanzaki H, Iwai M *et al*. Expression of leukaemia inhibitory factor (LIF) receptor in human placenta: a possible role for LIF in the growth and differentiation of trophoblasts. Hum.Reprod. 1995; **10**:1907-11.

66. Holmes CH, Simpson KL, Wainwright SD *et al*. Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy. J.Immunol. 1990; **144**:3099-105.

67. Purcell DF, McKenzie IF, Lublin DM *et al*. The human cell-surface glycoproteins HuLy-m5, membrane co-factor protein (MCP) of the complement system, and trophoblast leucocyte-common (TLX) antigen, are CD46. Immunology 1990; **70**:155-61.

68. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator crry in fetomaternal tolerance. Science 2000; **287**:498-501. Krasnow JS, Tollerud DJ, Naus G, DeLoia JA. Endometrial Th2 cytokine expression throughout the menstrual cycle and early pregnancy. Hum.Reprod. 1996; **11**:1747-54.

69. Munn DH, Zhou M, Attwood JT *et al*. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998; **281**:1191-3.

70. Runic R, Lockwood CJ, LaChapelle L *et al*. Apoptosis and Fas expression in human fetal membranes. J.Clin.Endocrinol.Metab 1998; **83**:660-6.

71. Coumans B, Thellin O, Zorzi W *et al*. Lymphoid cell apoptosis induced by trophoblastic cells: a model of active foeto-placental tolerance. J.Immunol.Methods 1999; **224**:185-96.

72. Payne SG, Smith SC, Davidge ST, Baker PN, Guilbert LJ. Death receptor Fas/Apo-1/CD95 expressed by human placental cytotrophoblasts does not mediate apoptosis. Biol.Reprod. 1999; **60**:1144-50.

73. Aarli A, Kristoffersen EK, Jensen TS, Ulvestad E, Matre R. Suppressive effect on lymphoproliferation in vitro by soluble annexin II released from isolated placental membranes. Am.J.Reprod.Immunol. 1997; **38**:313-9.

74. Aarli A, Matre R. Suppression of immunoglobulin secretion by soluble annexin II. Scand.J.Immunol. 1998; **48**:522-6.

75. Krasnow JS, Tollerud DJ, Naus G, DeLoia JA. Endometrial Th2 cytokine expression throughout the menstrual cycle and early pregnancy. Hum.Reprod. 1996; **11**:1747-54.

76. Moraes-Pinto MI, Vince GS, Flanagan BF, Hart CA, Johnson PM. Localization of IL-4 and IL-4 receptors in the human term placenta, decidua and amniochorionic membranes. Immunology 1997; **90**:87-94.

77. Lim KJ, Odukoya OA, Ajjan RA, Li TC, Weetman AP, Cooke ID. Profile of cytokine mRNA expression in peri-implantation human endometrium. Mol.Hum.Reprod. 1998; **4**:77-81.

78. Lin HY, Lodish HF. Receptors for the TGF-beta superfamily: multiple polypeptides and serine/threonine kinases. Trends Cell Biol. 1993; **3**:14-9.

79. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunol.Today 1997; **18**:478-82.

80. Clark DA, Arck PC, Chaouat G. Why did your mother reject you? Immunogenetic determinants of the response to environmental selective pressure expressed at the uterine level. Am.J.Reprod.Immunol. 1999; **41**:5-22.

81. Goerdt S, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity. 1999; **10**:137-42.

82. Becker S, Daniel EG. Antagonistic and additive effects of IL-4 and interferon-gamma on human monocytes and macrophages: effects on Fc receptors, HLA-D antigens, and superoxide production. Cell Immunol. 1990; **129**:351-62.

83. Robertson SA, Ingman WV, O'Leary S, Sharkey DJ and Tremellen KP. Transforming growth factor beta—a mediator of immune deviation in seminal plasma. J Reprod Immunol 2002; **57**: 109–128

84. Clark DA, Yu G, Levy GA, Gorczynski RM. Procoagulants in fetus rejection: the role of the OX-2 (CD200) tolerance signal. Semin.Immunol. 2001; **13**:255-63.

85. Clark DA, Ding JW, Yu G, Levy GA, Gorczynski RM. Fgl2 prothrombinase expression in mouse trophoblast and decidua triggers abortion but may be countered by OX-2. Mol.Hum.Reprod. 2001; **7**:185-94.

86. Hill JA, Polgar K, Anderson DJ. T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. JAMA 1995; **273**:1933-6.

87. Makhseed M, Raghupathy R, Azizieh F, Al Azemi MM, Hassan NA, Bandar A. Mitogen-induced cytokine responses of maternal peripheral blood lymphocytes indicate a differential Th-type bias in normal pregnancy and pregnancy failure. Am.J.Reprod.Immunol. 1999; **42**:273-81.

88. Ghanekar A, Mendicino M, Liu H *et al*. Endothelial induction of fgl2 contributes to thrombosis during acute vascular xenograft rejection. J.Immunol. 2004; **172**:5693-701.

89. Clark DA, Croitoru K. TH1/TH2,3 imbalance due to cytokine-producing NK, gammadelta T and NK-gammadelta T cells in murine pregnancy decidua in success or failure of pregnancy. Am.J.Reprod.Immunol. 2001; **45**:257-65.

90. Sargent IL, Germain SJ, Sacks GP, Kumar S, Redman CW. Trophoblast deportation and the maternal inflammatory response in pre-eclampsia. J.Reprod.Immunol. 2003; **59**:153-60.

91. Johansen M, Redman CW, Wilkins T, Sargent IL. Trophoblast deportation in human pregnancy--its relevance for pre-eclampsia. Placenta 1999; **20**:531-9.

92. Knight M, Redman CW, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br.J.Obstet Gynaecol. 1998; **105**:632-40.

93. Schrocksnadel H, Daxenbichler G, Artner E, Steckel-Berger G, Dapunt O. Tumor markers in hypertensive disorders of pregnancy. Gynecol.Obstet.Invest 1993; **35**:204-8.

94. Lo YM, Leung TN, Tein MS *et al*. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin.Chem. 1999; **45**:184-8.

95. Zhong XY, Holzgreve W, Hahn S. Circulatory fetal and maternal DNA in pregnancies at risk and those affected by preeclampsia. Ann.N.Y.Acad.Sci. 2001; **945**:138-40.

96. Huppertz B, Frank HG, Kingdom JC, Reister F, Kaufmann P. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. Histochem.Cell Biol. 1998; **110**:495-508.

97. Leung DN, Smith SC, To KF, Sahota DS, Baker PN. Increased placental apoptosis in pregnancies complicated by preeclampsia. Am.J.Obstet.Gynecol. 2001; **184**:1249-50.

98. Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am.J.Obstet Gynecol 2002; **186**:158-66.

99. Kumar, S., Lo, D.Y.M., Smarason, A.K., Chan, L.Y.S., Linton, E.A., Redman, C.W.G.,Sargent, I.L. Pre-eclampsia is associated with increased levels of circulatingapoptotic microparticles and fetal cell-free DNA. J. Soc. Gynecol. Invest. 2000; **7** (Suppl.): 181a.

100. Bretelle F, Sabatier F, Desprez D *et al*. Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction. Thromb.Haemost. 2003; **89**:486-92.

101. Ganshirt-Ahlert D, Basak N, Aidynli K, Holzgreve W. Fetal DNA in uterine vein blood. Obstet.Gynecol. 1992; **80**:601-3.

102. L. Formigli, L. Papucci and A. Tani *et al.*, Aponecrosis Morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis, *J Cell Physiol* **182** (2000), pp. 41–49.

103. Huppertz B, Kingdom JC. Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis. J.Soc.Gynecol.Investig. 2004; **11**:353-62.

104. Smith NC, Brush MG, Luckett S. Preparation of human placental villous surface membrane. Nature 1974; **252**:302-3.

105. Smarason AK, Sargent IL, Starkey PM, Redman CW. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. Br.J.Obstet Gynaecol. 1993; **100**:943-9.

106. Cockell AP, Learmont JG, Smarason AK, Redman CW, Sargent IL, Poston L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br.J.Obstet Gynaecol. 1997; **104**:235-40.

107. Cooper JC. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. Br.J.Obstet Gynaecol. 1994; **101**:559.

108. Cockell AP, Poston L. Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. Hypertension 1997; **30**:247-51.

109. von Dadelszen P, Hurst G, Redman CW. Supernatants from co-cultured endothelial cells and syncytiotrophoblast microvillous membranes activate peripheral blood leukocytes in vitro. Hum.Reprod. 1999; **14**:919-24.

110. Sacks G, Sargent I, Redman C. An innate view of human pregnancy. Immunol.Today 1999; **20**:114-8.

111. Sacks GP, Redman CW, Sargent IL. Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy: an intracellular flow cytometric analysis of peripheral blood mononuclear cells. Clin.Exp.Immunol. 2003; **131**:490-7.

112. Aly AS, Khandelwal M, Zhao J, Mehmet AH, Sammel MD, Parry S. Neutrophils are stimulated by syncytiotrophoblast microvillous membranes to generate superoxide radicals in women with preeclampsia. Am.J.Obstet Gynecol 2004; **190**:252-8.

113. Hahn S, Holzgreve W. Fetal cells and cell-free fetal DNA in maternal blood: new insights into pre-eclampsia. Hum.Reprod.Update. 2002; **8**:501-8.

114. Germain, S.J., Knight, M., Sooranna, S.R., Redman, C.W.G., Sargent, I.L. Interaction of circulating syncytiotrophoblast microvillous fragments with maternalmonocytes in normal and pre-eclamptic pregnancies. J. Soc. Gynecol. Invest. 2002; **9** (Suppl.)**:** 259A.

115. Benyo DF, Smarason A, Redman CW, Sims C, Conrad KP. Expression of inflammatory cytokines in placentas from women with preeclampsia. J.Clin.Endocrinol.Metab 2001; **86**:2505-12.

116. Saito S, Sakai M. Th1/Th2 balance in preeclampsia. J.Reprod.Immunol. 2003; **59**:161-73.

117. Arkwright PD, Rademacher TW, Boutignon F, Dwek RA, Redman CW. Suppression of allogeneic reactivity in vitro by the syncytiotrophoblast membrane glycocalyx of the human term placenta is carbohydrate dependent. Glycobiology 1994; **4**:39-47.

118. Thibault G, Degenne D, Girard AC, Guillaumin JM, Lacord M, Bardos P. The inhibitory effect of human syncytiotrophoblast plasma membrane vesicles on in vitro lymphocyte proliferation is associated with reduced interleukin 2 receptor expression. Cell Immunol. 1991; **138**:165-74.

119. Gercel-Taylor C, O'Connor SM, Lam GK, Taylor DD. Shed membrane fragment modulation of CD3-zeta during pregnancy: link with induction of apoptosis. J.Reprod.Immunol. 2002; **56**:29-44.

120. Roberts JM, Taylor RN, Goldfien A. Clinical and biochemical evidence of endothelial cell dysfunction in the pregnancy syndrome preeclampsia. Am.J.Hypertens. 1991; **4**:700-8.

121. Redman CW, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. Placenta 2000; **21**:597-602.

122. CHUN D, BRAGA C, CHOW C, LOK L. TREATMENT OF HYDATIDIFORM MOLE. J.Obstet.Gynaecol.Br.Commonw. 1964; **71**:185-91.

123. Piering WF, Garancis JG, Becker CG, Beres JA, Lemann J, Jr. Preeclampsia related to a functioning extrauterine placenta: report of a case and 25-year follow-up. Am.J.Kidney Dis. 1993; **21**:310-3.

124. Redman CW, Sargent IL. Preeclampsia and the systemic inflammatory response. Semin.Nephrol. 2004; **24**:565-70.

125. Vince GS, Starkey PM, Austgulen R, Kwiatkowski D, Redman CW. Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. Br.J.Obstet.Gynaecol. 1995; **102**:20-5.

126. Perkins AV, Linton EA, Eben F, Simpson J, Wolfe CD, Redman CW. Corticotrophin-releasing hormone and corticotrophin-releasing hormone binding protein in normal and pre-eclamptic human pregnancies. Br.J.Obstet.Gynaecol. 1995; **102**:118-22.

127. Muttukrishna S, North RA, Morris J *et al*. Serum inhibin A and activin A are elevated prior to the onset of pre-eclampsia. Hum.Reprod. 2000; **15**:1640-5.

128. Taylor RN, Crombleholme WR, Friedman SA, Jones LA, Casal DC, Roberts JM. High plasma cellular fibronectin levels correlate with biochemical and clinical features of preeclampsia but cannot be attributed to hypertension alone. Am.J.Obstet.Gynecol. 1991; **165**:895-901.

129. Hsu CD, Iriye B, Johnson TR, Witter FR, Hong SF, Chan DW. Elevated circulating thrombomodulin in severe preeclampsia. Am.J.Obstet.Gynecol. 1993; **169**:148-9.

130. Friedman SA, Schiff E, Emeis JJ, Dekker GA, Sibai BM. Biochemical corroboration of endothelial involvement in severe preeclampsia. Am.J.Obstet.Gynecol. 1995; **172**:202-3.

131. McCarthy AL, Woolfson RG, Raju SK, Poston L. Abnormal endothelial cell function of resistance arteries from women with preeclampsia. Am.J.Obstet.Gynecol. 1993; **168**:1323-30.

132. Pascoal IF, Lindheimer MD, Nalbantian-Brandt C, Umans JG. Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. J.Clin.Invest 1998; **101**:464-70.

133. Granger JP, Alexander BT, Bennett WA, Khalil RA. Pathophysiology of pregnancy-induced hypertension. Am.J.Hypertens. 2001; **14**:178S-85S.

134. Roberts JM, Edep ME, Goldfien A, Taylor RN. Sera from preeclamptic women specifically activate human umbilical vein endothelial cells in vitro: morphological and biochemical evidence. Am.J.Reprod.Immunol. 1992; **27**:101-8.

135. Chambers JC, Fusi L, Malik IS, Haskard DO, De Swiet M, Kooner JS. Association of maternal endothelial dysfunction with preeclampsia. JAMA 2001; **285**:1607-12.

136. Schneider H, Panigel M, Dancis J. Transfer across the perfused human placenta of antipyrine, sodium and leucine. Am.J.Obstet Gynecol 1972; **114**:822-8.

137. Di Santo S, Malek A, Sager R, Andres AC, Schneider H. Trophoblast viability in perfused term placental tissue and explant cultures limited to 7-24 hours. Placenta 2003; **24**:882-94.

138. Zhong XY, Laivuori H, Livingston JC *et al*. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am.J.Obstet Gynecol 2001; **184**:414-9.

139. Shibasaki T, Odagiri E, Shizume K, Ling N. Corticotropin-releasing factor-like activity in human placental extracts. J.Clin.Endocrinol.Metab 1982; **55**:384-6.

140. Var A, Kuscu NK, Koyuncu F *et al*. Atherogenic profile in preeclampsia. Arch.Gynecol.Obstet. 2003; **268**:45-7.

141. Brugger B, Erben G, Sandhoff R, Wieland FT, Lehmann WD. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc.Natl.Acad.Sci.U.S.A 1997; **94**:2339-44.

142. Hao M, Mukherjee S, Maxfield FR. Cholesterol depletion induces large scale domain segregation in living cell membranes. Proc.Natl.Acad.Sci.U.S.A 2001; **98**:13072-7.

143. Ng EK, Leung TN, Tsui NB *et al*. The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin.Chem. 2003; **49**:727-31.

144. Greer IA, Dawes J, Johnston TA, Calder AA. Neutrophil activation is confined to the maternal circulation in pregnancy-induced hypertension. Obstet Gynecol 1991; **78**:28-32.

145. Clark P, Boswell F, Greer IA. The neutrophil and preeclampsia. Semin.Reprod.Endocrinol. 1998; **16**:57-64.

146. Munro S. Lipid rafts: elusive or illusive? Cell 2003; **115**:377-88.

147. Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 1985; **313**:318-20.

148. Verhoven B, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J.Exp.Med. 1995; **182**:1597-601.

149. Brinkmann V, Reichard U, Goosmann C *et al*. Neutrophil extracellular traps kill bacteria. Science 2004; **303**:1532-5.

150. Lee WL, Grinstein S. Immunology. The tangled webs that neutrophils weave. Science 2004; **303**:1477-8.

151. Gupta AK, Rusterholz C, Huppertz B *et al*. A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. Placenta 2005; **26**:59-66.

152. Gupta AK, Holzgreve W, Huppertz B, Malek A, Schneider H, Hahn S. Detection of Fetal DNA and RNA in Placenta-Derived Syncytiotrophoblast Microparticles Generated in Vitro. Clin.Chem. 2004; **50**:2187-90.

153. Huppertz B, Kingdom J, Caniggia I *et al*. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta 2003; **24**:181-90.

154. Kertesz Z, Linton EA, Redman CW. Adhesion molecules of syncytiotrophoblast microvillous membranes inhibit proliferation of human umbilical vein endothelial cells. Placenta 2000; **21**:150-9.

155. Deng L, Bremme K, Hansson LO, Blomback M. Plasma levels of von Willebrand factor and fibronectin as markers of persisting endothelial damage in preeclampsia. Obstet.Gynecol. 1994; **84**:941-5.

156. Heyl W, Handt S, Reister F *et al*. Elevated soluble adhesion molecules in women with pre-eclampsia. Do cytokines like tumour necrosis factor-alpha and interleukin-1beta cause endothelial activation. Eur.J.Obstet.Gynecol.Reprod.Biol. 1999; **86**:35-41.

157. Ashworth JR, Warren AY, Baker PN, Johnson IR. Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. Br.J.Obstet.Gynaecol. 1997; **104**:1152-8.

158. Fan J, Watanabe T. Inflammatory reactions in the pathogenesis of atherosclerosis. J.Atheroscler.Thromb. 2003; **10**:63-71.

159. Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. Am.J.Obstet Gynecol 1998; **179**:80-6.

160. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol.Today 1999; **20**:469-73.

161. Mellor AL, Keskin DB, Johnson T, Chandler P, Munn DH. Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses. J.Immunol. 2002; **168**:3771-6.

162. Kudo Y, Boyd CA, Sargent IL, Redman CW. Tryptophan degradation by human placental indoleamine 2,3-dioxygenase regulates lymphocyte proliferation. J.Physiol 2001; **535**:207-15.

163. Kudo Y, Boyd CA. Human placental indoleamine 2,3-dioxygenase: cellular localization and characterization of an enzyme preventing fetal rejection. Biochim.Biophys.Acta 2000; **1500**:119-24.

164. Yamazaki F, Kuroiwa T, Takikawa O, Kido R. Human indolylamine 2,3-dioxygenase. Its tissue distribution, and characterization of the placental enzyme. Biochem.J. 1985; **230**:635-8.

165. Kudo Y, Boyd CA, Spyropoulou I *et al*. Indoleamine 2,3-dioxygenase: distribution and function in the developing human placenta. J.Reprod.Immunol. 2004; **61** :87-98.

166. Dresser DW. The potentiating effect of pregnancy on humoral immune responses of mice. J.Reprod.Immunol. 1991; **20**:253-66.

167. Hill JA, III, Choi BC. Immunodystrophism: evidence for a novel alloimmune hypothesis for recurrent pregnancy loss involving Th1-type immunity to trophoblast. Semin.Reprod.Med. 2000; **18**:401-5.

168. Fisher SJ. The placental problem: Linking abnormal cytotrophoblast differentiation to the maternal symptoms of preeclampsia. Reprod.Biol.Endocrinol. 2004; **2**:53.

169. Maynard SE, Min JY, Merchan J *et al*. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J.Clin.Invest 2003; **111**:649-58.

170. Levine RJ, Maynard SE, Qian C *et al*. Circulating angiogenic factors and the risk of preeclampsia. N.Engl.J.Med. 2004; **350**:672-83.

171. Grisham MB, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am.J.Physiol 1986; **251**:G567-G574.

172. Zweier JL, Kuppusamy P, Lutty GA. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. Proc.Natl.Acad.Sci.U.S.A 1988; **85**:4046-50.

173. Tsukimori K, Maeda H, Ishida K, Nagata H, Koyanagi T, Nakano H. The superoxide generation of neutrophils in normal and preeclamptic pregnancies. Obstet Gynecol 1993; **81**:536-40.

174. Rusterholz C, Gupta AK, Huppertz B, Holzgreve W, Hahn S. Soluble factors released by placental villous tissue: Interleukin-1 is a potential mediator of endothelial dysfunction. Am.J.Obstet.Gynecol. 2005; **192**:618-24.

175. Khalfoun B, Degenne D, Crouzat-Reynes G, Bardos P. Effect of human syncytiotrophoblast plasma membrane-soluble extracts on in vitro mitogen-induced lymphocyte proliferation. A possible inhibition mechanism involving the transferrin receptor. J.Immunol. 1986; **137**:1187-93.

176. Ng EK, Leung TN, Tsui NB *et al*. The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin.Chem. 2003; **49** :727-31.

177. Li Y, Zhong XY, Kang A, Troeger C, Holzgreve W, Hahn S. Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome. J.Soc.Gynecol.Investig. 2003; **10**:503-8.

178. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J.Clin.Invest 1973; **52**:2745-56.

179. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int.J.Cancer 1976; **17**:565-77.

180. Giard DJ, Aaronson SA, Todaro GJ *et al*. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J.Natl.Cancer Inst. 1973; **51**:1417-23.

181. SCHERER WF, SYVERTON JT, GEY GO. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J.Exp.Med. 1953; **97**:695-710.

182. Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J.Virol. 1969; **4**:549-53.

183. Diserens AC, de Tribolet N, Martin-Achard A, Gaide AC, Schnegg JF, Carrel S. Characterization of an established human malignant glioma cell line: LN-18. Acta Neuropathol.(Berl) 1981; **53**:21-8.

184. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J.Biochem.Biophys.Methods 2000; **46**:69-81.

185. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; **25**:402-8.

186. Weiss J, Kao L, Victor M, Elsbach P. Oxygen-independent intracellular and oxygen-dependent extracellular killing of Escherichia coli S15 by human polymorphonuclear leukocytes. J.Clin.Invest 1985; **76**:206-12.

Placenta (2005), **26**, 59–66
doi:10.1016/j.placenta.2004.04.004

A Comparative Study of the Effect of Three Different Syncytiotrophoblast Micro-particles Preparations on Endothelial Cells

A. K. Gupta[a,1], C. Rusterholz[a,1], B. Huppertz[b], A. Malek[c], H. Schneider[c], W. Holzgreve[a] and S. Hahn[a,*]

[a] Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, University of Basel, CH-4031 Basel, Switzerland; [b] Department of Anatomy, University Hospital, RWTH, 52057 Aachen, Germany; [c] University Women's Hospital, Inselspital, CH-3010 Bern, Switzerland

Paper accepted 2 April 2004

Pre-eclampsia is a pregnancy-associated multi-system disorder of unknown etiology, characterized by damage to the maternal endothelium. The latter facet has been suggested to be mediated in part by elevated shedding of inflammatory placental syncytiotrophoblast micro-particles (STBM) into the maternal circulation. In this study, we have examined STBM prepared by three different methods: mechanical dissection, in vitro placental explant culture and perfusion of placental cotyledons. All three preparations yielded morphologically similar STBM, as confirmed by scanning electron microscopy, and all contained syncytiotrophoblast-specific proteins as determined by the presence of placental alkaline phosphatase. The functional properties of the three STBM preparations were examined on human umbilical vein endothelial cells (HUVEC), where the mechanically prepared particles were found to inhibit proliferation to the greatest extent. Furthermore, only mechanically prepared STBM lead to the detachment and apoptosis of HUVEC cells. Our study, therefore, suggests that STBM prepared from placental perfusion or in vitro explant culture are biologically different from mechanically prepared ones, and may provide a better approximation of physiologically produced placental micro-particles.
Placenta (2005), **26,** 59–66

INTRODUCTION

Pre-eclampsia is a multi-system disorder of the second half of pregnancy unique to humans, which remains a major cause of maternal and perinatal morbidity worldwide [1]. Its symptoms include high blood pressure, proteinuria and edema. Recent research has indicated that these diverse arrays of symptoms may be the result of an excessive maternal systemic inflammatory response to pregnancy, involving activation of the innate immune system and damage of the maternal endothelial compartment [2].

Although the origin of the disorder is still unknown, the central role played by the placenta in its pathogenesis is well established [3–5]. In particular, it has been suggested that the generalized maternal endothelial dysfunction that affects pre-eclamptic women is caused directly or indirectly by circulating factors of placental origin [6]. In this regard, syncytiotrophoblast membrane micro-particles (STBM) shed from the placenta at the feto-maternal interface have drawn considerable attention. In normal pregnancy, trophoblast cells and STBM are spontaneously released from the placenta as a sign of normal placental physiologic turnover [7–9] and this phenomenon has been associated with the mild inflammatory state accompanying pregnancy [10]. Significantly elevated amounts of STBM have been found in the circulation of women with pre-eclampsia [11]. This increased shedding of sub-cellular material parallels the raise in circulating cell-free fetal DNA [12,13], demonstrating the increased placental dysfunction associated with the disorder [14].

Mechanically prepared STBM from normal full term placenta disrupt the morphology, inhibit the proliferation and induce cell death of cultured endothelial cell monolayers in vitro [15]. Furthermore, STBM perfused ex vivo into pre-constricted small subcutaneous fat arteries alter their relaxation response [16]. It has, therefore, been proposed that the increased shedding of STBM into the maternal blood of women with pre-eclampsia could be responsible for the manifest dysfunction of the vascular endothelium [10].

In order to study the effect of STBM in greater detail, we have prepared micro-particles from normal term placentae by three different methods.

(A) Placental villous tissues from freshly delivered placentae were mechanically dissected as reported earlier [15,17].

* Corresponding author. Tel.: +41-61-265-9224/9595; fax: +41-61-265-9399.
E-mail address: shahn@uhbs.ch (S. Hahn).
[1] Both authors contributed equally to this work.

0143–4004/$–see front matter

(B) Villous explants were cultured in conditions which maintain overall tissue integrity and support the physiologic turnover of the syncytiotrophoblast, including apoptotic shedding [18].

(C) Single placental cotyledons were perfused separately on both the maternal and fetal sides and the maternal washes were collected [19,20].

The morphological similarity between the micro-particles generated by the different methods was confirmed using scanning electron microscopy. Their functional properties were compared by characterizing their effects on endothelial cell proliferation and viability. Our studies demonstrate that mechanically prepared STBM have very similar activities to those described previously [15], while the two in vitro and placental perfusion STBM preparations are functionally different.

MATERIALS AND METHODS

Preparation of syncytiotrophoblast micro-particles

This study was approved by the Cantonal Institutional Review Board of Basel, Switzerland. Written informed consent was requested in all instances. Placentae and umbilical cords were obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies and processed within 30–90 min of collection.

In vitro explant culture. Villous tissue was isolated by dissection of the placentae and removal of the decidua. Following a wash in sterile phosphate buffered saline (PBS), the villous tissue was cut into pieces of 1–2 mm. Explants were cultured in 100 mm culture dishes (Corning NY, USA) in Dulbecco Modified Eagle's Medium (DMEM):F12 Nutrient Mixture (1:1) (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 1% antimycotic/antibiotics (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA), 10% fetal calf serum (FCS), 25 U/ml heparin (Roche Diagnostics, Germany), 50 U/ml aprotinin (Fluka Chemicals AG, Buchs, Switzerland) and 2 mM $MgSO_4$ for 72 h at 37 °C in 5% CO_2. After incubation the culture medium was collected and stored at −20 °C.

Mechanical dissection. STBM were prepared by a slight modification of methods described previously [15,17]. We used a smaller volume of 0.15 M NaCl and a higher speed centrifugation step to ensure greatest yield. Briefly, villous tissue was washed three times in PBS containing 100 mM $CaCl_2$ then chopped with scissors and rinsed in 100 ml 0.15 M NaCl supplemented with 1% antimycotic/antibiotics overnight at 4 °C. After rinsing, tissues were discarded and the supernatant was collected.

Placental perfusion. STBM were prepared using a well-described perfusion system [19,20]. Briefly, the intervillous space (maternal compartment) and the villous vasculature (fetal compartment) of an isolated placental cotyledon were perfused separately in open systems i.e. without recirculation at flow rates of about 12 and 6 ml/min, respectively. Perfusion medium was composed of NCTC-135 tissue culture medium diluted with Earle's buffer (1:1) with the addition of glucose (1.33 g/l), dextran 40 (10 g/l), 4% BSA, heparin (2500 IU/l) and clamoxyl (250 mg/l). After 30 min of initiation of the perfusion ($n = 4$), the perfusate from the maternal side was collected and stored at −70 °C.

Isolation of STBM

To isolate STBM, supernatants from all the three preparations were subjected to a three-step centrifugation at 4 °C: 1000 × g for 10 min, 10 000 × g for 10 min and 70 000 × g for 90 min. The final pellet was collected, washed with PBS and resuspended in 1 ml sterile PBS containing 5% sucrose and stored at −20 °C until use. Red blood cell micro-vesicles (RBC ghosts) were prepared as described previously by Khalfoun et al. [21] and used as a negative control in all experiments.

Protein and PLAP (placental alkaline phosphatase) quantification of STBM preparations

The protein content in each STBM preparation was quantified with the advanced protein assay reagent (Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer's instructions. The amount of PLAP protein was determined using an ELISA. In brief, a 96-well microtiter EIA plate (Nunc, Denmark) was coated with anti-PLAP antibody (mouse monoclonal Ab-5, Neomarkers Inc., Fremont, CA, USA) for 2 h at RT. After washing with PBS/0.05% Tween 20, the STBM samples were added and incubated for 2 h at RT. After four consecutive washes, alkaline phosphatase yellow substrate (pNPP, Sigma chemicals, St. Louis, MO, USA) was added and incubated for 1 h in the dark. Subsequently, the plate was read at 405 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and the data were analyzed using Soft Max Pro Software. A dilution series of purified PLAP (Sigma Chemicals, St. Louis, MO, USA) was used as a standard.

Scanning electron microscopy (SEM) of STBM

For SEM examination, 3 μg STBM of each preparation was diluted in 25 μl PBS, then transferred onto 0.2 μm polycarbonate membrane (Whatman, New Jersey, USA) under the application of vacuum and dehydrated with an acetone series (30%, 50%, 70%, 100%, and 100% acetone for 10, 10, 10, 10 and 15 min, respectively). Then specimens were coated with platinum and analyzed using a Philips XL-30 ESM scanning electron microscope (ZMB, University of Basel).

Cell proliferation assay

HUVEC were isolated from normal term human umbilical cord by the method described by Jaffe et al. [22] and stored in liquid nitrogen until use. Cells from passage 2 to passage

7 were used. To assess HUVEC proliferation, the cell proliferation kit (Roche Diagnostics, Basel, Switzerland) was used, which measures BrdU incorporation during DNA synthesis. In brief, 1.5×10^4 cells were plated onto 1% gelatin-coated 96-well microtiter plates (Nunc, Denmark) in E-SFM (endothelial serum free medium) medium supplemented with 20 ng/ml human recombinant bFGF (basic fibroblast growth factor) and 10 ng/ml human recombinant EGF (epidermal growth factor, GIBCO Invitrogen Life Technologies, Grand Island, NY, USA). After 6 h, the culture medium was replaced with STBM or RBC ghost particles diluted in DMEM:F12 medium supplemented with 1% antimycotic/antibiotics, 10% FCS, 50 U/ml aprotinin, 25 U/ml heparin (DMEM:F12 complete medium). The cells were then incubated for 22 h, followed by an additional incubation with 10 μM of BrdU for a further 18 h. The amount of incorporated BrdU was detected using an ELISA according to the manufacturer's instructions.

U-937 (human histiocytic monocyte like lymphoma [23]) was used as nonadherent control cell line. A431 (human epidermal squamous carcinoma [24]), HELA (human epithelial cervix carcinoma [25]), NIH-3T3 (Swiss mouse embryo fibroblasts [26]), and LN-18 (human brain malignant glioma cell [27]) were used as adherent control cell lines. U-937, NIH-3T3, HELA, A431, and LN-18 cells (each 1.5×10^4 cells/well) were grown in RPMI 1640 (GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with Glutamax, 10% FCS, and 100 U/ml penicillin, 100 μg/ml streptomycin. With all adherent cells, RPMI 1640 medium was replaced with STBM diluted in DMEM:F12 complete medium 6 h after seeding. For the nonadherent cells, the STBM preparations were added directly to the RPMI 1640 medium 6 h after the plating. All cells were incubated for the same amount of time and cell proliferation was measured as described above.

HUVEC apoptosis

Apoptosis was measured using a commercial cellular DNA fragmentation ELISA kit (Roche Diagnostics, Germany). This assay measures the release of BrdU-labeled DNA fragments into the cytoplasm during apoptosis. In brief, 2×10^4 HUVEC were plated in 96-well microtiter plates (Nunc, Denmark) in E-SFM culture medium supplemented with 20 ng/ml bFGF and 10 ng/ml EGF. After 6 h, 10 μM BrdU was added and incubated for a further 18 h. Following BrdU labeling, the cell monolayer was washed with sterile PBS, and STBM containing DMEM:F12 complete medium was added for 24 h. In positive control wells, apoptosis was induced with 5 μM staurosporine (Sigma Chemicals, St. Louis, MO, USA), a potent inducer of apoptosis, for 3 h. The detection of BrdU-labeled apoptotic

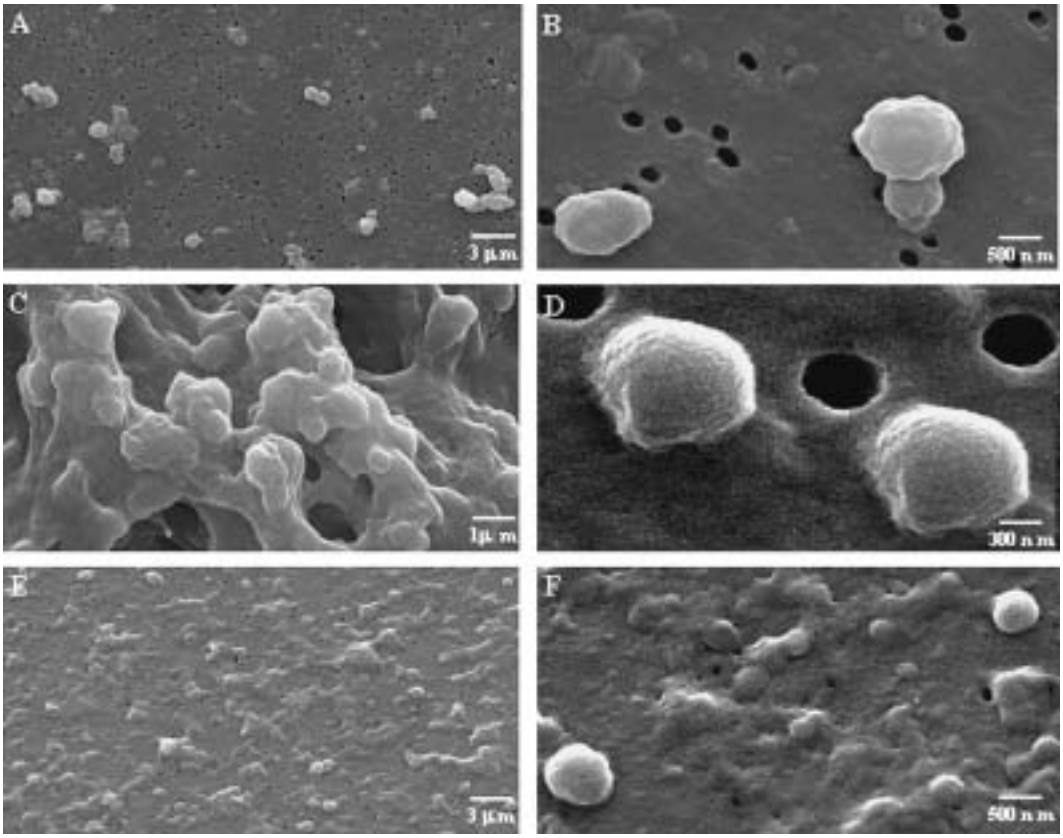


Figure 1. Scanning electron micrographs of different STBM preparations. (A–B) STBM from explants, (C–D) mechanical STBM, (E–F) STBM derived from placental perfusion. Mechanically prepared STBM tend to aggregate in clumps (C).

DNA fragments was performed according to the manufacturer's instructions.

RESULTS

STBM preparations are morphologically similar

The STBM prepared by three different methods were examined using scanning electron microscopy. This analysis demonstrated that all three STBM preparations produced micro-particles with sizes ranging from 200 to 600 nm in diameter and that these particles were morphologically similar (Figure 1). Although the SEM analysis was not quantitative, the data suggest that the three preparations vary in the quantity of particles produced relative to the amount of starting material, with the greatest number being produced by the mechanical method. Mechanically prepared particles also had a tendency to aggregate in clumps (Figure 1C), a feature not observed with particles prepared by the other two methods.

To confirm that syncytiotrophoblast membraneous material was present in the STBM preparations, we examined these for the presence of PLAP, a GPI-anchored enzyme present in syncytiotrophoblast membrane, using an ELISA assay. STBM were captured with an anti-PLAP antibody and the endogenous phosphatase activity of the immobilized particles was measured. This analysis indicated that membrane-associated PLAP was found in all three STBM preparations (Figure 2), with the greatest concentration being present in the mechanically prepared particles.

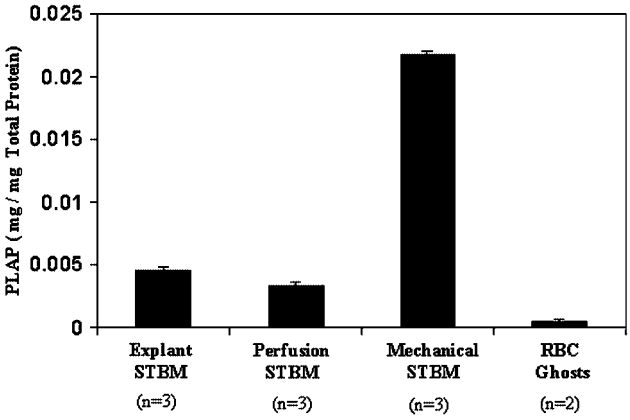


Figure 2. Concentrations of PLAP protein in each of the STBM preparations. Equivalent amounts of STBM, as assessed by total protein content, were examined for PLAP by ELISA. The figure represents an examination of STBM preparations from three different placentae.

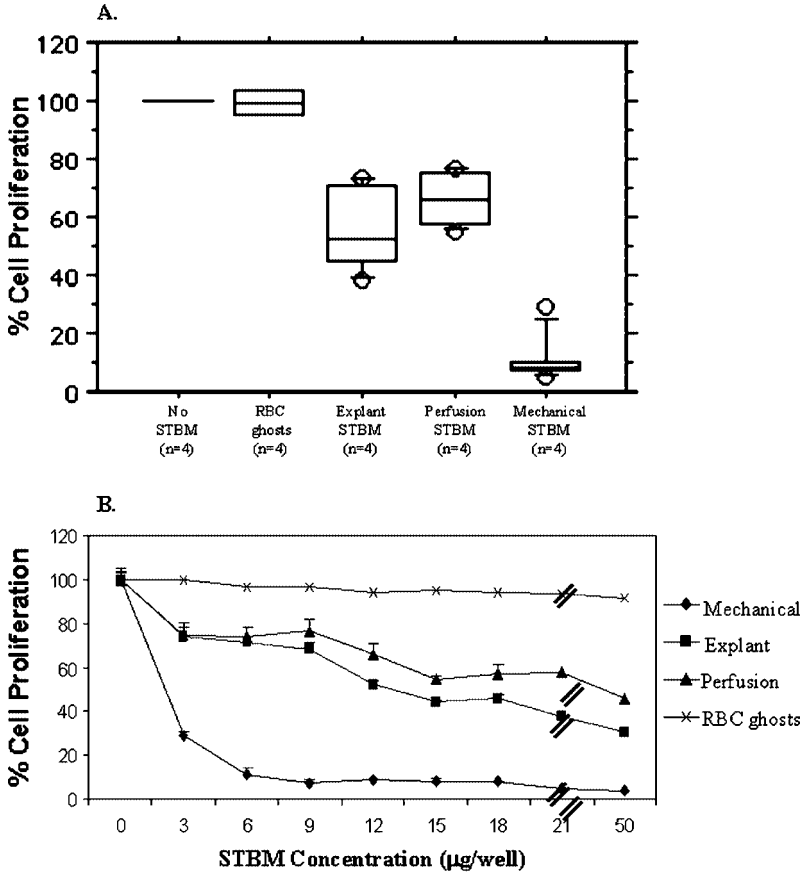


Figure 3. Inhibition of endothelial cell proliferation by STBM. (A) Sub-confluent HUVEC cells were incubated for 40 h with 12 µg/well STBM from different STBM preparations in DMEM:F12 complete medium. Cell proliferation is represented as the percentage of cell proliferation relative to untreated control as 100% cell proliferation. A representative analysis of STBM preparations from four placentae is illustrated. (B) Dose-dependent effect of various STBM preparations on inhibition of HUVEC proliferation. A representative analysis of STBMs prepared from one placenta is shown.

Inhibition of HUVEC proliferation by STBM preparations

In order to functionally characterize the three different STBM preparations, we used HUVEC, a well-characterized endothelial cell model. These cells have been previously used to assess the growth inhibition properties of mechanically prepared STBM [15]. The effects of equal amounts of STBM, as determined by protein content, were compared on HUVEC growth. Particles prepared from red blood cells were used as a control. Mechanically derived STBM inhibited HUVEC proliferation up to 90% at 12 μg STBM per well while the other two STBM preparations at a similar dose showed only a 30–50% inhibition of endothelial cell proliferation (Figure 3A). All STBM preparations inhibited HUVEC proliferation in a dose-dependent manner (Figure 3B). When the dose of STBM derived from explants or from placental perfusion was increased to 50 μg/well, a further reduction in HUVEC proliferation could be observed. However, the high degree of inhibition of proliferation achieved with the mechanically produced STBM could not be obtained with the other preparations. To investigate the specificity of this suppressive effect on cell proliferation, all three STBM preparations were tested on U-937 (nonadherent) and A431, HELA, NIH-3T3, LN-18 (adherent) cell types (Figure 4A–4C). Our study showed that cell proliferation was not significantly reduced by any of the STBM preparations in any of these cell lines, unlike the effect we had observed with HUVECs.

Mechanically derived STBM disrupt HUVEC monolayer

When HUVEC cells were incubated together with the three different STBM preparations at a dose of 12 μg/well, only the mechanically derived STBM caused disruption of the cell monolayer, leading to cell detachment (Figure 5). The kinetics of detachment was dose dependent. With a 12 μg dose of mechanical STBM, the disruption of the HUVEC monolayer started after 12 h of incubation, resulting in the detachment of almost 90% cells within the next 12 h. At low concentrations (3 and 6 μg), detachment started after treatment periods exceeding 12 h but eventually resulted in 80–90% HUVEC detachment at 24 h. In contrast, the other two STBM preparations, even at higher concentration (50 μg/well) neither disrupted the continuity of the HUVEC monolayer nor caused any cell detachment (data not shown). The detachment caused by the mechanically prepared particles was similar to that described by Smarason and colleagues [15].

Mechanically derived STBM induce apoptosis

As described above, all three STBM preparations inhibited HUVEC proliferation, albeit not to the same extent. Furthermore, only mechanically derived STBM caused detachment and disruption of the HUVEC monolayer. For this reason, we next examined whether any of the STBM preparations triggered HUVEC apoptosis. Apoptosis was

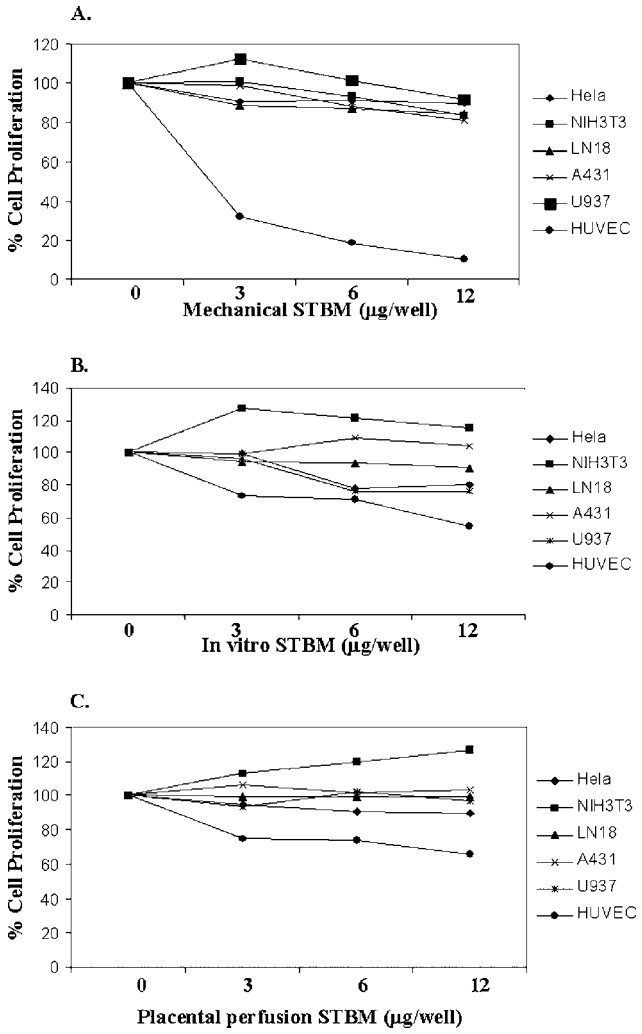


Figure 4. Effects of different STBM preparations on cell proliferation comparing HUVEC and other cell types. Each point is mean of three replicates and expressed as the percentage of cell proliferation relative to untreated control as 100% cell proliferation. (A) Treatment with mechanically prepared STBM, (B) treatment with in vitro STBM, (C) treatment with placental perfusion STBM. A representative experiment from one placenta preparation is shown.

investigated by measuring the release of DNA fragments into the cytoplasm during cell death using a commercial ELISA kit. HUVEC treated with 5 μM staurosporine for 3 h were used as a positive control. The analysis indicated that mechanically derived STBM caused approximately 3–5-fold higher levels of HUVEC apoptosis than the untreated control cultures (Figure 6). No induction of apoptosis was evident in the cultures treated with the two other STBM preparations, even at high concentration (50 μg/well), or RBC ghost particles.

DISCUSSION

In this study, we have produced STBM micro-particles from normal term placentae according to three different preparatory methods and have investigated their effects on human umbilical cord endothelial cells proliferation and viability in

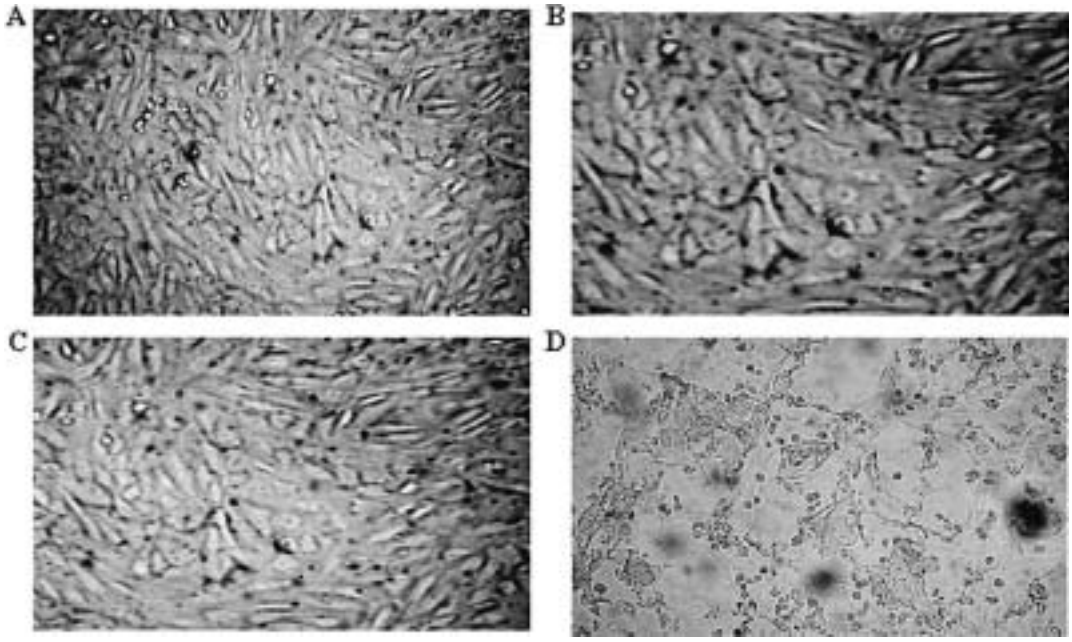


Figure 5. Effects of different STBM preparations on HUVEC monolayer integrity. (A) Intact confluent HUVEC monolayer incubated 24 h without STBM, (B) with 12 μg explant-derived STBM, (C) with 12 μg STBM from placental perfusion. (D) Disrupted HUVEC monolayer following incubation with 12 μg of mechanically derived STBM. Magnification is 400×.

vitro. We show that all three STBM preparations produced micro-particles of similar sizes, which all contained the syncytiotrophoblast-specific membrane protein PLAP, thereby confirming their origin as bona fide STBM.

Our data indicate that all three preparations altered HUVEC cell proliferation in a dose-dependent manner, with the mechanically derived STBM showing the greatest degree of growth inhibition. While it has previously been reported that mechanically derived STBM can inhibit HUVEC proliferation [11,15,16], this is the first study to our knowledge that has examined the potential effects of two other forms of in vitro generated STBM on endothelial cell growth. The inhibition of cell proliferation by all three STBM preparations was specific to HUVEC only. Although we had observed a slight effect on some of the cell lines we had examined, these results were very similar to the observations made previously by Smarason et al. [15]. In this regard our findings complement and extend upon previous reports made by this group [15].

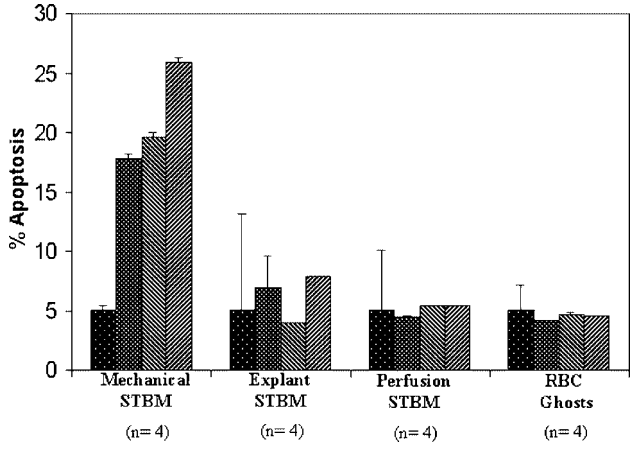


Figure 6. Mechanically prepared STBM induce HUVEC apoptosis. Confluent HUVEC were incubated with various concentrations of the different STBM preparations and apoptosis was measured. The bars in each preparation represent 0 μg (- -), 3 μg (- -), 6 μg (- -), 12 μg (- -) STBM/well, respectively. 5 μM staurosporine was used as positive control. Apoptosis is expressed as percentage relative to staurosporine-induced apoptosis, which was designated as 100%. The results of four different STBM preparations are illustrated.

The finding that the concentration of PLAP is about 4–5-fold higher in the mechanically derived STBM compared to the other two preparations suggests that the mechanical preparation contains a higher amount of syncytiotrophoblast-derived material. Nevertheless, the observation that increased amounts of STBM (up to 50 μg/well) from the other preparations do not achieve the high degree of growth inhibition triggered by mechanical STBM strongly suggests that the quality of the micro-particles produced rather than their quantity is determining for altering HUVEC proliferation and induction of cell death.

Although the molecular mechanisms through which HUVEC cell proliferation is affected have not been addressed here, it appears that they differ amongst the three STBM populations. Whereas STBM prepared either from explant cultures or by placental perfusion affect cell growth without causing a loss of endothelial cell integrity, mechanically prepared STBM massively disrupted the HUVEC cell monolayer and triggered endothelial cell death. Although Smarason and colleagues [15] have previously described that the mechanically produced STBM do cause cell death of HUVEC cell cultures, they used a chrome release assay to ensure this. The problem with the chrome release assay is that it only

demonstrates loss of membrane integrity, and does not distinguish between apoptotic or necrotic cell death. Furthermore, it can exhibit very different kinetics to the onset of truly apoptotic features such as DNA fragmentation [28]. Our data, therefore, now clearly demonstrate that this event is triggered by apoptosis. From our study it is however unclear whether the onset of apoptosis preceded detachment of the endothelial cells from the culture dish or occurred once the monolayer had become disrupted. Since a previous study has reported that the anti-proliferative activity of mechanically derived STBM might be due to the interaction of adhesion molecules on STBM with endothelial cells [29], it is tempting to propose that apoptosis follows cell detachment induced by competitive binding to the gelatin-bound ligands.

Our comparative analysis, therefore, indicates that the three STBM preparations differ functionally with regard to their effect on HUVEC cultures. Consequently, our findings raise the issue of which of the different STBM preparations is closest to normal physiology.

In vivo, the shedding of placental particles is a continuous feature of normal pregnancy, which amplifies with the advance of pregnancy as the placental size increases. This shedding has been proposed to be part of the normal renewal of the syncytiotrophoblast, involving apoptosis of aged nuclei and de novo cytotrophoblast fusion [8]. In pre-eclampsia, apoptosis rates are significantly increased in the syncytiotrophoblast [30]. Therefore, it is most probable that the release of STBM in large amount in pre-eclampsia is the product of increased syncytial apoptosis [2]. STBM prepared either from explant cultures or by placental perfusion may more closely mimic the physiological status of apoptotic release, as the micro-particles released by these methods are more likely to result from normal syncytiotrophoblast turnover [18] than the STBM isolated by physical disruption of villous integrity like the mechanical preparation, which may lead to necrotic particle release.

A remarkable feature of STBM prepared by explant culture or perfusion is that they do inhibit HUVEC proliferation without the induction of a significant degree of endothelial cell apoptosis. As the degree of inhibition occurred in a dose-dependent manner, this suggests that these STBM might alter endothelial cell function rather than cell integrity. This is in good agreement with the in vivo findings, where evidence of endothelial cell activation in pre-eclampsia is observed from increased plasma levels of von Willebrand factor, fibronectin, sVCAM-1 and sE-selectin [31,32]. Furthermore, in pre-eclampsia, endothelial function is altered as myometrial arteries from women suffering with the disorder exhibit an attenuated endothelium-dependent vasodilatory response [33]. As none of these observations suggest an increased endothelial cell turnover and/or massive endothelial cell apoptosis, the possible physiological role of particles having properties similar to those exhibited by mechanically prepared STBM will have to be further addressed.

Therefore, we conclude that investigations studying in vitro effect of placental STBM on endothelial cells will have to consider the mode in which these micro-particles are prepared.

ACKNOWLEDGEMENTS

We thank Dr. Ian Sargent (Oxford) for helpful discussions. We would also like to thank Mr. Daniel Mathys (ZMB, University of Basel) for his excellent technical assistance with scanning electron microscopy and Mrs. Vivian Kiefer-Vargas for her kind help in the preparation of specimens for SEM. We also thank Mrs. Acar Gülsolmaz (University Women's Hospital Basel) for her invaluable help in collecting placentae.

REFERENCES

[1] Witlin AG, Sibai BM. Hypertension in pregnancy: current concepts of preeclampsia. Annu Rev Med 1997;48:115–27.
[2] Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response. Placenta 2003;(Suppl A)S21–7.
[3] Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, Assche V. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. Br J Obstet Gynaecol 1994;101: 669–74.
[4] Leung DN, Smith SC, To KF, Sahota DS, Baker PN. Increased placental apoptosis in pregnancies complicated by pre-eclampsia. Am J Obstet Gynecol 2001;184:1249–50.
[5] Redman CWG, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. Placenta 2000;21:597–602.
[6] Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia, an endothelial cell disorder. Am J Obstet Gynecol 1989;161:1200–4.
[7] Chua S, Wilkins T, Sargent I, Redman C. Trophoblast deportation in pre-eclamptic pregnancy. Br J Obstet Gynaecol 1991;98:973–9.
[8] Huppertz B, Frank HG, Kingdom JC, Reister F, Kaufmann P. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. Histochem Cell Biol 1998;110:495–508.
[9] Johansen M, Redman CWG, Wilkins T, Sargent IL. Trophoblast deportation in human pregnancy—its relevance for pre-eclampsia. Placenta 1999;20:531–9.
[10] Redman CWG, Sacks GP, Sargent IL. Preeclampsia, an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol 1999;180:499–506.
[11] Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet Gynaecol 1998;105:632–40.
[12] Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, et al. Quantitative abnormalities of fetal DNA in maternal serum in pre-eclampsia. Clin Chem 1999;45:184–8.
[13] Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001;184:414–9.
[14] Hahn S, Holzgreve W. Fetal cells and cell-free fetal DNA in maternal blood: new insights into pre-eclampsia. Hum Reprod Update 2002;8: 501–8.
[15] Smarason AK, Sargent IL, Starkey PM, Redman CWG. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. Br J Obstet Gynaecol 1993;100:943–9.
[16] Cockell AP, Learmont JG, Smarason AK, Redman CWG, Sargent IL, Poston L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br J Obstet Gynaecol 1997;104:235–40.
[17] Smith NC, Brush MG. Preparation of human placental surface membrane. Nature 1974;252:302–3.

[18] Huppertz B, Kingdom J, Caniggia I, Desoye G, Black S, Korr H, et al. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta 2003;24: 181–90.

[19] Schneider H, Panigel M, Dancis J. Transport across the perfused human placenta of antipyrine, sodium and leucine. Am J Obstet Gynecol 1972; 114:822–8.

[20] Di Santo S, Malek A, Sager R, Andres AC, Schneider H. Trophoblast viability in perfused term placental tissue and explant cultures limited to 7–24 hours. Placenta 2003;24:882–94.

[21] Khalfoun B, Degenne D, Reyenes GC, Bardos P. Effect of human syncytiotrophoblast plasma membrane-soluble extracts on in vitro mitogen-induced lymphocyte proliferation. A possible inhibition mechanism involving the transferrin receptor. J Immunol 1986;137: 1187–93.

[22] Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical vein. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745–56.

[23] Sundstorm C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565–77.

[24] Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973;51: 1417–23.

[25] Scherer WF, Syverton JT, Gey GO. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J Exp Med 1953;97:695–710.

[26] Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J Virol 1969;4:549–53.

[27] Diserens AC, de Tribolet N, Martin-Achard A, Gaide AC, Schnegg JF, Carrel S. Characterization of an established human malignant glioma cell line: LN-18. Acta Neuropathol 1981;53:21–8.

[28] Grogg D, Hahn S, Erb P. CD4+ T cell-mediated killing of major histocompatibility complex class II-positive antigen-presenting cells (APC). III. CD4+ cytotoxic T cells induce apoptosis of APC. Eur J Immunol 1992;22:267–72.

[29] Kertesz Z, Linton EA, Redman CWG. Adhesion molecules of syncytiotrophoblast microvillous membranes inhibit proliferation of human umbilical vein endothelial cells. Placenta 2000;21:150–9.

[30] Ishihara N, Matsuo H, Murakoshi H, Fernandez JBL, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol 2002;186:158–66.

[31] Deng L, Bremme K, Hansson LO, Blomback M. Plasma levels of von Willebrand factor and fibronectin as markers of persisting endothelial damage in preeclampsia. Obstet Gynecol 1994;84:941–5.

[32] Heyl W, Handt S, Reister F, Gehlen J, Schröder W, Mittermayer C, et al. Elevated soluble adhesion molecules in women with pre-eclampsia: do cytokines like tumour necrosis factor-α and interleukin-1β cause endothelial activation? Eur J Obstet Gynaecol Reprod Biol 1999;86: 35–41.

[33] Ashworth JR, Warren AY, Baker PN, Johnson IR. Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. Br J Obstet Gynaecol 1997;104:1152–8.

bind to *P. falciparum (15, 16)*, reduced affinity for LFA-1, and no apparent affinity for fibrinogen *(16)*. Important insights into the function of ICAM-1 may be obtained from future investigations of relationships in additional individuals homozygous and heterozygous for K29M ICAM-1.

In summary, previous studies exploring sICAM-1 as a marker for cardiovascular and other diseases may need to be reevaluated in light of the demonstration that commercial sICAM-1 ELISAs vary markedly in their ability to recognize this ICAM-1 variant, which is common (20–35% allele frequency) in African-American populations.

This study was supported in part by the General Clinical Research Center of the Wake Forest University School of Medicine (Grant M01 RR07122), and National Heart, Lung, and Blood Institute Grants R01 HL67348 (to D.W.B.) and R01 AR48797 (to J.J.C.). K.P.B. was supported by an American Diabetes Association Mentor-based Fellowship.

References

1. Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM Jr, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997;96:4219–25.
2. Blann AD, Seigneur M, Steiner M, Miller JP, McCollum CN. Circulating ICAM-1 and VCAM-1 in peripheral artery disease and hypercholesterolaemia: relationship to the location of atherosclerotic disease, smoking, and in the prediction of adverse events. Thromb Haemost 1998;79:1080–5.
3. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998;351:88–92.
4. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-Reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 2000;342:836–43.
5. Greenland P, Smith SC Jr, Grundy SM. Improving coronary heart disease risk assessment in asymptomatic people: role of traditional risk factors and noninvasive cardiovascular tests. Circulation 2001;104:1863–7.
6. Pradhan AD, Rifai N, Ridker PM. Soluble intercellular adhesion molecule-1, soluble vascular adhesion molecule-1, and the development of symptomatic peripheral arterial disease in men. Circulation 2002;106:820–5.
7. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 2002;347:1557–65.
8. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation 2003;107:363–9.
9. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO 3rd, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: a statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003;107:499–511.
10. Wagenknecht LE, Bowden DW, Carr JJ, Langefeld CD, Freedman BI, Rich SS. Familial aggregation of coronary artery calcium in families with type 2 diabetes. Diabetes 2001;50:861–6.
11. Lenchik L, Register TC, Hsu FC, Lohman K, Nicklas BJ, Freedman BI, et al. Adiponectin as a novel determinant of bone mineral density and visceral fat. Bone 2003;33:646–51.
12. Fernandez-Reyes D, Craig AG, Kyes SA, Peshu N, Snow RW, Berendt AR, et al. A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. Hum Mol Genet 1997;6:1357–60.
13. Zimmerman PA, Wieseman M, Spalding T, Boatin BA, Nutman TB. A new intercellular adhesion molecule-1 allele identified in West Africans is prevalent in African-Americans in contrast to other North American racial groups. Tissue Antigens 1997;50:654–6.
14. Bellamy R, Kwiatkowski D, Hill AV. Absence of an association between intercellular adhesion molecule 1, complement receptor 1 and interleukin 1 receptor antagonist gene polymorphisms and severe malaria in a West African population. Trans R Soc Trop Med Hyg 1998;92:312–6.
15. Adams S, Turner GD, Nash GB, Micklem K, Newbold CI, Craig AG. Differential binding of clonal variants of *Plasmodium falciparum* to allelic forms of intracellular adhesion molecule 1 determined by flow adhesion assay. Infect Immun 2000;68:264–9.
16. Craig A, Fernandez-Reyes D, Mesri M, McDowall A, Altieri DC, Hogg N, et al. A functional analysis of a natural variant of intercellular adhesion molecule-1 (ICAM-1Kilifi). Hum Mol Genet 2000;9:525–30.
17. Ohashi J, Naka I, Patarapotikul J, Hananantachai H, Looareesuwan S, Tokunaga K. Absence of association between the allele coding methionine at position 29 in the N-terminal domain of ICAM-1 (ICAM-1^{Kilifi}) and severe malaria in the northwest of Thailand. Jpn J Infect Dis 2001;54:114–6.
18. Vijgen L, Van Essche M, Van Ranst M. Absence of the Kilifi mutation in the rhinovirus-binding domain of ICAM-1 in a Caucasian population. Genet Test 2003;7:159–61.

DOI: 10.1373/clinchem.2004.036806

Detection of Fetal DNA and RNA in Placenta-Derived Syncytiotrophoblast Microparticles Generated in Vitro,

Anurag Kumar Gupta,[1] Wolfgang Holzgreve,[1] Berthold Huppertz,[2] Antoine Malek,[3] Henning Schneider,[3] and Sinuhe Hahn[1]* ([1] Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, University of Basel, Basel, Switzerland; [2] Department of Anatomy, University Hospital, RWTH, Aachen, Germany; [3] University Women's Hospital, Inselspital, Bern, Switzerland; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, Spitalstrasse 21, CH4031 Basel, Switzerland; fax 41-61-265-9399, e-mail shahn@uhbs.ch)

Fetal DNA and RNA can be readily detected in maternal plasma samples *(1–4)*. Most of this material appears to be of placental origin *(5)*, and it appears to be in a predominantly cell-free form *(2)*, whereas circulatory mRNA is membrane-encapsulated *(6)*.

Pregnancy is associated with the release of microparticles by the syncytiotrophoblast membrane into the maternal circulation *(7)*. These particles, frequently termed STBM, are released by turnover of the syncytiotrophoblast monolayer covering the entire villous tree *(8–11)*. This process of normal physiologic syncytiotrophoblast turnover involves the release of apoptotic material into the maternal circulation by the extrusion of syncytial knots and the associated release of STBM *(8–11)*. The amount of material that is released by apoptotic shedding of syncytial knots (and STBM) is several grams per day *(9)*, and the circulating concentrations are increased significantly in preeclampsia *(11)*.

STBM particles have been suggested to evoke the mild maternal inflammatory response accompanying normal pregnancies *(12)*, and increased release has been proposed to play a role in the etiology of preeclampsia by triggering maternal endothelial cell damage *(13, 14)*.

As these particles are difficult to detect and prepare from maternal blood samples, use is frequently made of in vitro-prepared particles to study their physiologic activity *(13)*. In this context, we have recently extensively examined three different modes of STBM preparation: mechanical dissection of fresh placental villous tissues; in vitro

cultures of villous explants; and perfusion of single placental cotyledons *(15)*.

All three preparations lead to the production of STBM as confirmed by the presence of the syncytiotrophoblast-specific protein placental alkaline phosphatase, physiologic activity on human endothelial cell cultures, and their morphology, as seen by scanning electron microscopy *(15)*.

Intrigued by the seemingly parallel increased release of STBM and circulatory fetal nucleic acids in preeclampsia *(2, 7, 14, 16, 17)* and its potential relationship to the placental distress associated with the disorder, we examined whether these two events may be more intimately associated. For this reason, we examined whether fetal nucleic acids are physically associated with STBM.

In our study, after the receipt of informed consent and Institutional Review Board approval, we prepared, by the three previously described methods *(15)*), STBM from placentas from normal full-term pregnancies in which healthy males were delivered. In brief, villous explants were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (Gibco Invitrogen Life Technologies) supplemented with 10 g/L antimycotics and antibiotics (Gibco Invitrogen Life Technologies), 100 mL/L fetal calf serum, 25 kIU/L heparin (Roche Diagnostics), 50 kIU/L aprotinin (Fluka Chemicals), and 2 mmol/L $MgSO_4$ for 72 h at 37 °C in 5% CO_2, after which the culture supernatant was collected and stored at −70 °C. Mechanically dissected STBM were prepared by washing villous tissue three times in phosphate-buffered saline (PBS) containing 100 mmol/L $CaCl_2$, after which the tissue was manually dissected and rinsed overnight at 4 °C in 100 mL of 0.15 mol/L NaCl supplemented with 10 g/L antimycotics and antibiotics. After rinsing, the tissues were discarded, and the supernatant was collected and stored at −70 °C. For the collection of STBM from placental perfusion, the intervillous space (maternal compartment) of a single cotyledon was perfused with an in vitro system, using a medium composed of NCTC-135 tissue culture medium diluted with Earle's buffer (1:1) with added glucose (1.33 g/L), dextran 40 (10 g/L), 40 g/L bovine serum albumin, heparin (2.5 kIU/L), and clamoxyl (250 mg/L). The perfusates from the intervillous space were collected and stored at −70 °C.

STBM from these three preparations were harvested by a three-step centrifugation procedure at 4 °C: 1000*g* for 10 min, 10 000*g* for 10 min, and 70 000*g* for 90 min. The final pellet, containing the STBM, was washed once with PBS, resuspended in 1 mL of sterile PBS containing 50 g/L sucrose, and stored at −70 °C until use.

We examined the presence of fetal DNA and RNA in these STBM. The amount of fetal DNA was measured by a TaqMan® real-time PCR assay for a Y-chromosome-specific sequence (*SRY*) *(17)*, whereas the presence of fetal mRNA was quantified by a similar quantitative reverse transcription-PCR (RT-PCR) assay for the corticotropin-releasing hormone (*CRH*) gene, which is known to be expressed in the placenta *(18)*.

The protein content in each STBM preparation was quantified with the advanced protein assay reagent (Cytoskeleton). DNA was extracted from STBM by use of the High Pure PCR Template Preparation Kit (Roche Diagnostics). Total RNA was isolated using High Pure RNA Isolation Kit (Roche Diagnostics) and eluted in 50 μL of elution buffer. cDNA was reverse-transcribed from 500 ng of total RNA by use of a commercial reverse transcription system (Promega).

Table 1. Concentrations of fetal DNA and *CRH* mRNA in STBM prepared by villous explant culture, mechanical dissection, and placental perfusion.[a]

STBM preparation method	Copies/mg of STBM	
	DNA (*SRY*)	mRNA (*CRH*)
Villous explant culture		
Median	94 530.5	4986
Range	9444–158 135	1232–10 500
Mechanical dissection		
Median	2593	121 144.5
Range	307–7745	17 868–331 607
Placental perfusion		
Median	620	222 352.5
Range	268–2975	98 190–275 981

[a] Six placentas were used for each STBM preparation. Fetal DNA (*SRY* locus) and mRNA (*CRH*) concentrations were determined by real-time PCR and real-time RT-PCR, respectively.

Real-time quantitative PCR and real-time quantitative RT-PCR were used for all DNA and mRNA quantifications as described previously *(17, 19, 20)*. The real-time PCR and real-time RT-PCR reactions were set up according to the manufacturer's instructions (Applied Biosystems) in a reaction volume of 25 μL. Each sample was analyzed in duplicate, and the corresponding calibration curve was run in parallel with each analysis. Absolute concentrations of *CRH* mRNA and *SRY* DNA were expressed as copies/mg of STBM.

Our analysis showed that all STBM preparations contained both fetal DNA and mRNA, although the concentrations of each of these fetal analytes differed in the three preparations (Table 1). In this regard, the highest concentration of fetal DNA was detected in STBM prepared by in vitro villous explant cultures (Fig. 1A), whereas the highest concentration of fetal CRH RNA was present in STBM obtained by perfusion of a placental cotyledon (Fig. 1B).

Although we took great care to harvest as many of the STBM as possible by the use of high-speed ultracentrifugation, we were still able to detect considerable amounts of fetal DNA in the STBM-free supernatant of villous explant preparations. The amounts of fetal DNA in the supernatants cleared by ultracentrifugation were approximately fourfold higher than those in the matching STBM preparations. Provided that these results can be extrapolated to the release of fetal DNA into maternal plasma, then it is possible that the major proportion of circulatory fetal DNA may exist in a completely particle-free form. On the other hand, very little CRH mRNA was detected in the STBM-free villous explant supernatants (~10% of that

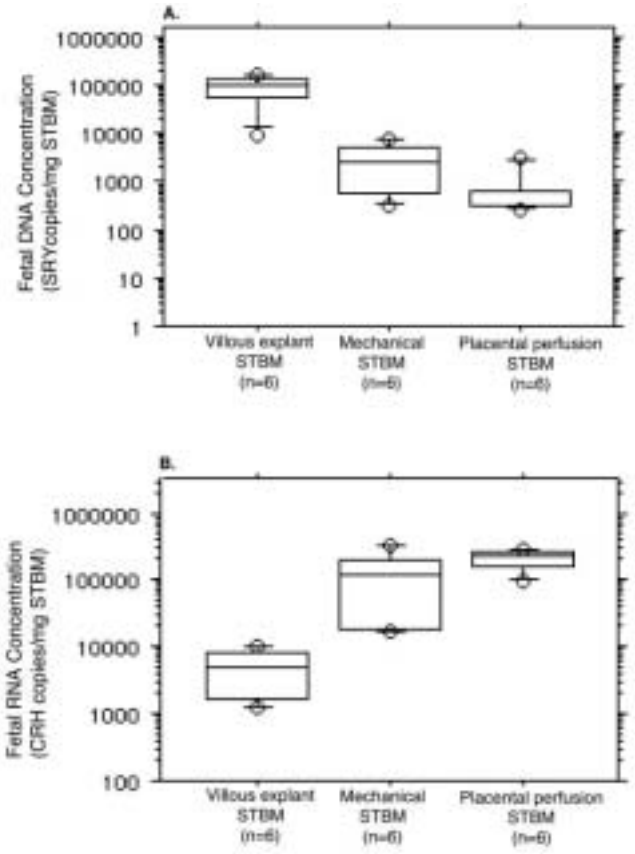


Fig. 1. Box-plots of fetal DNA and mRNA concentrations in STBM prepared by villous explant culture, mechanical dissection, and placental perfusion.

Fetal DNA (*SRY* locus; *A*) and mRNA (*CRH*; *B*) concentrations were determined by real-time PCR and real-time RT-PCR, respectively, and are represented as copies/mg of STBM. Six placentas were used for each STBM preparation. The *line inside each box* represents the median value; the *limits of the boxes* represent the 75th and 25th percentiles; the *error bars* indicate the 10th and 90th percentiles; and ○ indicate outliers.

present in the STBM preparation). Again, provided that the observations we have made with in vitro-generated STBM correspond to the in vivo situation, then it is possible that fetal mRNA species may be largely associated with membrane particles, as has been reported previously *(6)*. It is also likely, that these few mRNA species present in the cleared culture supernatants are associated with very small microparticles that are not effectively harvested by high-speed ultracentrifugation.

In our study, STBM prepared by placental perfusion may be regarded as being the closest representatives of those generated under normal physiologic conditions in that here STBM are collected directly from the intervillous space, the site where they would typically enter the maternal circulation. The presence of fetal DNA and mRNA species in all three STBM preparations, particularly in those obtained by perfusion of the maternal compartment of the placenta under near-physiologic conditions, implies that cell-free fetal nucleic acids may similarly be associated with STBM in vivo. This facet, however, needs to be confirmed by the analysis of STBM isolated from maternal blood samples, currently a technically demanding undertaking.

The difference we observed in fetal DNA and mRNA content in the three STBM preparations may be attributable to the manner in which these particles are generated, in that those obtained by perfusion or in vitro culture are generated predominantly by apoptotic cell turnover, in contrast to STBM isolated by mechanical disruption, in which release of STBM may involve necrotic pathways *(15)*.

In this context it is worth noting that the release of STBM differs in normal pregnancy compared with preeclampsia *(9, 21)*. In normal pregnancy, the shedding of placental particles occurs continuously as part of the self-renewal of the syncytiotrophoblast monolayer, a process that involves apoptosis of the aged nuclei and fusion of cytotrophoblast cells *(9)*. In preeclampsia, this process is altered in that syncytiotrophoblast apoptosis rates are dramatically increased, which has been suggested to contribute to the increased release of STBM, possibly by apo-necrotic pathways *(21)*.

Therefore, provided that circulatory fetal nucleic acids are indeed associated with STBM in vivo, then it is possible that the analysis of the fetal DNA and RNA content of STBM in the maternal circulation in normal and pathologic pregnancies may yield new insights into the underlying mechanisms leading to their release by the syncytiotrophoblast. Furthermore, if this proviso concerning the presence of fetal nucleic acids with STBM in vivo is true, then it may also provide a new strategy for the enrichment of these fetal analytes from maternal blood samples.

We thank Drs. Corinne Rusterholz and Bernhard Zimmermann for helpful discussions. We would like to thank the staff of Women's Hospitals in Aachen, Basel, and Bern for invaluable help in collecting placentas.

References

1. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Hahn S, Holzgreve W. Fetal cells and cell-free fetal DNA in maternal blood: new insights into pre-eclampsia. Hum Reprod Update 2002;8:501–8.
3. Poon LLM, Leung TN, Lau TK, Lo YMD. Presence of fetal RNA in maternal plasma. Clin Chem 2000;46:1832–4.
4. Oudejans CBM, Go ATJJ, Visser A, Mulders MAM, Westerman BA, Blankenstein MA, et al. Detection of chromosome 21-encoded mRNA of placental origin in maternal plasma. Clin Chem 2003;49:1445–9.
5. Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, et al. mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;100:4748–53.
6. Ng EKO, Tsui NBY, Lam NY, Chiu RW, Yu SC, Wong SC, et al. Presence of filterable and non-filterable mRNA in the plasma of cancer patients and healthy individuals. Clin Chem 2002;48:1212–7.
7. Redman CWG, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. Placenta 2000;21:597–602.
8. Chua S, Wilkins T, Sargent I, Redman C. Trophoblast deportation in pre-eclamptic pregnancy. Br J Obstet Gynaecol 1991;98:973–9.
9. Huppertz B, Frank HG, Kingdom JC, Reister F, Kaufmann P. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. Histochem Cell Biol 1998;110:495–508.
10. Johansen M, Redman CWG, Wilkins T, Sargent IL. Trophoblast deportation in human pregnancy—its relevance for pre-eclampsia. Placenta 1999;20:531–9.

11. Ishihara N, Matsuo H, Murakoshi H, Fernandez JBL, Samoto T, Maruo T. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol 2002;186:158–66.
12. Redman CWG, Sacks GP, Sargent IL. Preeclampsia, an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol 1999;180:499–506.
13. Smarason AK, Sargent IL, Starkey PM, Redman CWG. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. Br J Obstet Gynaecol 1993;100:943–9.
14. Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet Gynaecol 1998;105:632–40.
15. Gupta AK, Rusterholz C, Huppertz B, Malek A, Schneider H, Holzgreve W, et al. A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. Placenta 2004; in press.
16. Lo YMD, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999;45:184–8.
17. Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001;184:414–9.
18. Shibasaki T, Odagiri E, Shizume K, Ling N. Corticotropin-releasing factor-like activity in human placental extracts. J Clin Endocrinol Metab 1982;55:384–6.
19. Ng EK, Leung TN, Tsui NB, Lau TK, Panesar NS, Chiu RW, et al. The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin Chem 2003;49:727–31.
20. Li Y, Zhong XY, Kang A, Troeger C, Holzgreve W, Hahn S. Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome. J Soc Gynecol Investig 2003;10:503–8.
21. Huppertz B, Kingdom J, Caniggia I, Desoye G, Black S, Korr H, et al. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta 2003;24:181–90.

Previously published online at DOI: 10.1373/clinchem.2004.040196

High Ischemia-Modified Albumin Concentration Reflects Oxidative Stress But Not Myocardial Involvement in Systemic Sclerosis, *Didier Borderie,[1†*] Yannick Allanore,[2†] Christophe Meune,[3] Jean Y. Devaux,[3] Ohvanesse G. Ekindjian,[1] and André Kahan[2]* (Departments of [1] Biochemistry A, [2] Rheumatology A, and [3] Nuclear Medicine, Paris V University, Assistance Publique-Hôpitaux de Paris, Cochin Hospital, Paris, France; † these authors contributed equally to this work; * address correspondence to this author at: Service de Biochimie A, 27 rue du faubourg Saint-Jacques, 75014 Paris, France; fax 33-1-5841-1585, e-mail didier.borderie@cch.ap-hop-paris.fr)

Systemic sclerosis (SSc) is a connective tissue disease characterized by widespread vascular lesions and fibrosis of the skin and internal organs. In SSc, vasospasm causes frequent episodes of reperfusion injury and free-radical-mediated endothelial disruption. Primary myocardial involvement is far more common than initially suspected on clinical grounds *(1–5)* and affects survival rates because it is associated with a poor prognosis *(6, 7)*. Myocardial fibrosis is thought to occur secondarily to repeated focal ischemia in the coronary microcirculation as a result of abnormal vasoreactivity, with or without associated structural vascular disease *(4, 5)*. The early and accurate identification of cardiac involvement is therefore of paramount clinical importance.

The concentration of ischemia-modified albumin (IMA), as measured by the albumin cobalt binding test (Ischemia Technologies, Inc.), is a new marker to rule out transient myocardial ischemia *(8, 9)*. This test measures the binding of exogenous cobalt to the NH_2 terminus of human albumin. In the presence of myocardial ischemia, structural changes occur in the NH_2 terminus of albumin, rapidly reducing its capacity to bind transition metal ions after an ischemic event *(10)*.

We assessed the accuracy of the albumin cobalt binding test for detecting ischemia in SSc patients and investigated the roles of myocardial ischemia and peripheral oxidative stress in this condition. We also considered carbonyl residues and advanced oxidation protein products (AOPP) as factors indicative of protein oxidation.

We included consecutive patients hospitalized for systematic follow-up who fulfilled the American Rheumatism Association preliminary criteria for SSc. The exclusion criteria were pregnancy; symptoms of heart failure, including class III or IV dyspnea (New York Heart Association); venous distension and recent major lower limb edema; pulmonary arterial hypertension (systolic arterial pressure >40 mmHg and/or mean artery pressure >25 mmHg, determined by echocardiography); severe pulmonary involvement (forced vital capacity or carbon monoxide diffusing capacity <50% of the predicted normal value); renal involvement (creatinine concentration > 106 μmol/L); or severe disease complications such as cancer or gangrene. At the time of the study, none of the patients was taking medication for cardiac or vascular disease. If previously treated with vasodilators, patients were asked to stop taking these drugs 3 days before admission. This interruption period corresponds to five times the half-life of calcium channel blockers and angiotensin-converting enzyme. All patients gave informed consent for all procedures, and the study was approved by the local ethics committee (Paris, Cochin).

We assessed the following in all patients: blood cell count, Westergren erythrocyte sedimentation rate, serum creatinine concentration, and anti-centromere and anti-topoisomerase I antibody concentrations. The concentration of high-sensitivity C-reactive protein was measured by immunoturbidimetry on a Roche modular PP instrument using the CRP latex Tina-quant® assay (Roche Diagnostics). Pulmonary involvement was assessed by computed tomography scan, forced vital capacity, and the ratio of carbon monoxide diffusion capacity to hemoglobin concentration. Pulmonary arterial systolic pressure was determined by Doppler echocardiography at rest. The thickness of the skin was quantified on a scale of 0–3, by use of the modified Rodman skin scoring technique, for each of 17 body surface areas *(11, 12)*.

All patients underwent thallium-201 myocardial single-photon-emission computerized tomography at rest, using a gamma camera (Starport 400AT; General Electric) interfaced with an ADAC computer (DPS 3300). Myocardial perfusion was assessed semiquantitatively by two expe-

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2005.02758.x

Constant IFNγ mRNA to protein ratios in cord and adult blood T cells suggests regulation of IFNγ expression in cord blood T cells occurs at the transcriptional level

A. Kumar Gupta, C. Rusterholz, W. Holzgreve and S. Hahn
Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, University of Basel, Basel, Switzerland

Accepted for publication 5 January 2005
Correspondence: Dr Sinuhe Hahn Laboratory for Prenatal Medicine University Women's Hospital/Department of Research, Spitalstrasse, 21 CH 4031 Basel Switzerland.
E-mail: shahn@uhbs.ch

Summary

Low levels of IFNγ produced by umbilical cord blood (UCB) T lymphocytes upon activation may be due to the need for a high threshold of activation or to intrinsic blocking transcription/translation. We examined IFNγ mRNA accumulation and protein expression in pharmacologically stimulated human UCB and adult blood (AB) T cells. Our data indicate that both IFNγ mRNA accumulation and protein synthesis were significantly lower in stimulated UCB T cells than the AB T cells. Since the RNA dependent kinase PKR, an inhibitor of translation, can be activated by low levels of IFNγ mRNA, we measured its involvement. Treatment with 2-amino-purine, an inhibitor of PKR, did not enhance IFNγ protein expression in UCB T cells. Furthermore, our studies indicated that IFNγ promoter hypermethylation does not appear to regulate IFNγ expression either, as treatment with the demethylating agent, 5-aza-2′-deoxycytidine, did not lead to a significant increase in IFNγ mRNA accumulation in UCB T cells. What is readily evident from our studies is that the IFNγ mRNA to protein ratio was similar in UCB and AB T cells and it was not altered by any of the treatments used. These results therefore suggests that IFNγ expression in UCB T cells is suppressed at the transcriptional level by an unknown mechanism(s).

Keywords: T lymphocytes, cytokines, Th1/ Th2, gene regulation, cord blood, IFNγ

Introduction

Neonatal T cells are generally considered to be immature due to their inability to mount an efficient immune response against pathogens [1–3]. Many reports have indeed shown that both cytotoxic and helper neonate T lymphocytes differ qualitatively and quantitatively from adult T lymphocytes in their response to produce effector cytokines, for example IFNγ, *in vivo* and *in vitro* [4–7]. The basis for this difference, however, remains unclear.

It has been proposed that the neonatal T cells obtained from cord blood require a high threshold of activation for producing mature effector functions. The proliferation of low cytokine producing neonatal cells can be enhanced with high concentrations of pharmacological agents PMA and ionomycin. In this regard, studies have shown that in the presence of strong adjuvants or costimulatory signals, neonatal $CD4^+$ and $CD8^+$ T cells are capable of mature-level responses [8–11]. It has also been suggested that UCB T lymphocytes have an intrinsic defect, the nature of which is unclear, for producing Th1 specific cytokines, and preferentially favour Th2 responses [12,13]. Previous findings from our laboratory have described that upon stimulation with the high concentrations of PMA and ionomycin both $CD4^+$ and $CD8^+$ UCB T lymphocytes expressed mature levels of the activation marker CD69 but did not produce Th1 cytokine such as IFNγ [14]. Therefore, activation of UCB T cells per se does not appear to be blocked, but rather production of Th1 cytokines, like IFNγ, is hindered.

The pleiotropic cytokine IFNγ is known to be principally produced by NK cells, cytotoxic $CD8^+$ T cells and the Th1 subset of $CD4^+$ T cells. IFNγ is essential for both innate and adaptive immunity, generally amplifying Th1 immune responses and acting through binding to the IFNγ receptors on the cell surface that trigger Jak-STAT signalling pathway [15,16]. Neonatal lymphocytes, in comparison to adult

lymphocytes, produce 10–15 fold less IFNγ protein upon anti-CD3 crosslinking or stimulation with phorbol ester and calcium ionophore [17–21].

The poor IFNγ expression by neonatal T cells might involve mechanisms regulating gene transcription and/or translation. In this regard it has been proposed that differential methylation of the IFNγ promoter is closely associated with low IFNγ expression in the UCB T lymphocytes. In $CD4^+$ UCB T cells IFNγ promoter is hypermethylated at CpG and non CpG sites within and adjacent to the promoter [22–24], which could reduce IFNγ transcription. In addition to the above reports describing transcriptional regulation of IFNγ expression, Ben Asouli *et al.* [25] have shown that the human IFNγ mRNA (at low concentration) auto regulates its own translation through a pseudoknot that activates the RNA dependent protein kinase PKR. When activated, PKR blocks translation through the phosphorylation of the eIF2 α subunit of the RNA polymerase II dependent elongation complex [26]. In addition to the mechanisms described above, reduced constitutive NFATc2 (nuclear factor of activated T cells) expression has been reported in UCB T lymphocytes during primary stimulation, which may be one underlying molecular mechanism for the low IFNγ expression in UCB T lymphocytes [27].

Therefore, in order to further explore the regulation of IFNγ expression in neonatal T cells, we analysed both umbilical cord blood (UCB) and adult blood (AB) T cells for IFNγ mRNA accumulation, using real time taqman® PCR, and IFNγ protein expression, either by FACS or ELISA. In order to obtain maximal T cell activation we used two pharmacological agents: PMA and ionomycin. In this study we show that the regulation of IFNγ expression in neonatal T cells occurs only at the transcriptional level, and an intrinsic program hinders the induction of IFNγ expression in these cells.

Materials and methods

The Cantonal Institutional Review Board of Basel, Switzerland approved this study. UCB samples were collected from healthy term babies at the University Women's Hospital, Basel and AB samples were obtained from healthy donors at the blood donation centre, University Hospital, Basel. Written informed consent was obatined in all instances.

Isolation and stimulation of T lymphocytes

PBMC and cord blood mononuclear cells were isolated from whole blood by centrifugation over a ficoll paque plus gradient (Amersham Biosciences, Uppsala, Sweden). Then $CD4^+$ and $CD8^+$ T cells were enriched with $CD4^+$ and $CD8^+$ T cell specific microbeads (Miltenyi Biotech, Gladbach, Germany) either together or separately using MACS mini columns (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. The purity of the enriched T lymphocytes routinely reached >90%, as confirmed by FACS analysis.

$0{\cdot}5 \times 10^6$ cells/ml T cells were stimulated in 12 well plates (Nunc, Denmark) for varying time periods ranging from 3 to 48 h at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 5% FCS, 2 mM L-glutamine (Gibco Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Invitrogen Life Technologies). 50 ng/ml PMA (Sigma Chemicals, St. Louis, MO, USA) and 1 μM ionomycin (Sigma Chemicals) were used as stimuli. To analyse intracellular IFNγ expression golgistop (BD Biosciences, Basel, Switzerland) was added to the cell culture 3 h prior to FACS analysis [28]. Further treatments included the use of 2-amino-purine (2-AP, Sigma Chemicals) and 5-aza-2′-deoxycytidine (Sigma Chemicals) as indicated in the figures or figures legends. Our analysis using the tryptan blue dye exclusion test indicated that at ≥90% of the T cells were viable after each drug treatment.

Intracellular cytokine staining and flow cytometry

Following stimulation, cultured T cells were washed twice in PBS containing 0·5% FCS and incubated with FITC labelled mAb (BD Biosciences) against CD3, CD4 or CD8 surface antigens for 15 min at 4°C. The cells were again washed twice with PBS containing 0·5% FCS then fixed and permeabilized using cytofix/cytoperm plus kit (BD Biosciences) according to the manufacturer's instructions. Cells were then incubated with PE labelled mAb (BD Biosciences) against IFNγ or IL-2 cytokines. Phenotypic analysis of naïve and mature T cells was performed by two colour flow cytometry using a Becton Dickinson FACScan flow cytometer (BD Biosciences). A minimum of 10 000 gated events was acquired and analysed with the Cell Quest Pro software (BD Biosciences).

IFNγ ELISA

IFNγ levels were measured using a commercial human IFNγ ELISA kit (Ebioscience Inc, San Diego, CA, USA) according to the manufacturer's instructions. ELISA plate was read at 450 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) and the data was analysed using Soft Max Pro Software.

Total RNA isolation and real time RT-PCR

Total RNA was isolated using High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). cDNA was reverse transcribed from 1 μg of total RNA using a reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions. For the real time RT-PCR analysis, 25 μl reaction mixture consisted of 1 μl of cDNA, 1× taqman universal master mix (Applied

Biosystems, Foster City, CA, USA) and 1 × predeveloped taqman assay reagents (PDAR, Applied Biosystems), containing gene specific probes and primers for IFNγ or 18 s RNA. For these experiments the ABI Prism 7000 system (Applied Biosystems) was used. 18 s RNA was used as an internal control to correct unequal sample loading. It has previously been shown that the amount of 18sRNA per cell does not vary with cell activation [29]. Thermal cycler conditions comprised of 2 min at 50°C, 10 min at 95°C followed by 45 cycles for 15 s at 95°C and at 60°C for 1 min.

The change in IFNγ mRNA expression was assayed by normalization to the 18sRNA internal control. In order to obtain the fold difference the data was analysed using the $\Delta\Delta C_T$ method described previously by Livak and Schmittgen, where $\Delta\Delta C_T = (\text{IFN}\gamma\ C_T - 18\text{sRNA}\ C_T)_{\text{stimulated}} - (\text{IFN}\gamma\ C_T - 18\text{sRNA}\ C_T)_{\text{unstimulated}}$ [30].

Results

UCB T lymphocyte synthesize very low amount of IFNγ protein and mRNA

UCB and adult T lymphocytes were stimulated with the high concentrations of PMA and ionomycin and the frequency of IFNγ positive cells was measured by FACS. This analysis indicated that following 3 h of stimulation the frequencies of IFNγ positive UCB T cells were 100 fold less than the AB T cells (Fig. 1 a,b). In contrast the frequencies of the IL-2 positive T cells were similar in both UCB and AB (Fig. 1c,d). Although the frequencies of IFNγ positive UCB T cells further increased about 10 fold (3·39%) and 20 fold (5·17%) after 24 h and 48 h of stimulation, respectively (Fig. 1e,f), it never reached the equivalent levels attained by AB T cells.

To examine whether low IFNγ expression by UCB T cells was a consequence of low IFNγ gene transcription, IFNγ mRNA accumulation was quantified by real time PCR. The amount of IFNγ mRNA was normalized against the quantity of 18 sRNA [29]. Following 24 h of stimulation with PMA and ionomycin, 12-fold less IFNγ mRNA was accumulated by UCB T lymphocytes than the AB T lymphocytes (Table 1). IFNγ mRNA accumulation, measured at 3 h after stimula-

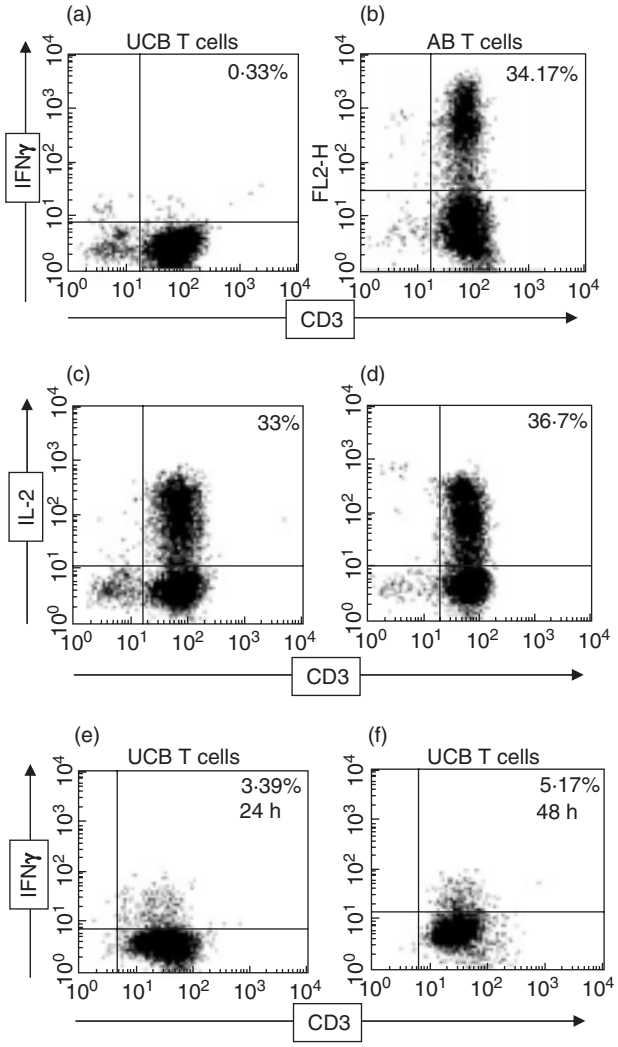


Fig. 1. IFNγ and IL-2 production by UCB and AB T cells. Enriched UCB and AB T cells were stimulated for 3 h to 48 h with PMA and ionomycin. The frequencies of the IFNγ and IL-2 positive cells were measured by FACS. 10 000 gated events were acquired. The frequencies of (a) the IFNγ positive UCB T cells were 100 fold less than (b) the IFNγ positive AB T cells, while the IL-2 positive T cell population was similar in both groups (c, d). Frequencies of IFNγ positive UCB T cells were increased approximately (e) 10-fold and (f) 20-fold upon 24 h and 48 h stimulation, respectively, in comparison to 3 h stimulation.

Table 1. Real time quantitative PCR analysis of IFNγ gene expression using $2^{-\Delta\Delta CT}$ method. Cord and adult blood T lymphocytes were stimulated for 24 h using PMA and ionomycin. The fold change in IFNγ gene expression was normalized to an internal control (18sRNA gene). 10 samples of each, cord and adult blood, were analysed. The fold change in the IFNγ gene was calculated using $2^{-\Delta\Delta CT}$ method as described in the materials and methods. Following 24 h stimulation UCB T lymphocytes accumulated 12 fold less IFNγ mRNA.

Samples	IFNγ C_T	18 s RNA C_T	Δ C_T*	ΔΔ C_T**	$2^{-\Delta\Delta CT}$†
Unstimulated CB T cells	35·47 ± 0·78	15·93 ± 0·41	19·54 ± 0·82	0·0 ± 0·0	1
Stimulated CB T cells	25·98 ± 0·77	15·37 ± 0·34	10·61 ± 0·78	− 8·93 ± 0·15	489·10 ± 0·50
Unstimulated AB T cells	32·80 ± 0·94	15·78 ± 0·32	17·01 ± 0·76	0·0 ± 0·0	1
Stimulated AB T cells	20·00 ± 0·69	15·51 ± 0·40	4·49 ± 0·56	− 12·51 ± 0·80	5865·3 ± 0·57

*Δ C_T (IFNγ C_T.18 s RNA C_T); **ΔΔ C_T (Δ C_T $^{\text{stimulated}}$ − Δ C_T $^{\text{unstimulated}}$); †Normalized IFNγ amount relative to unstimulated T cells

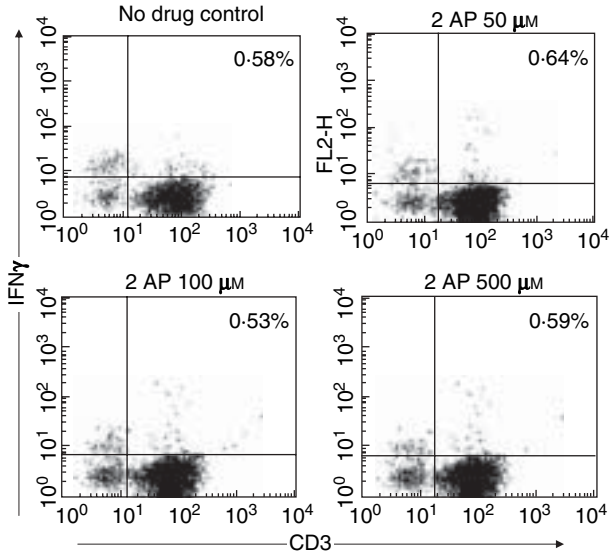


Fig. 2. Effect of 2-AP on IFNγ expression in UCB T cells. UCB T cells were stimulated for 3 h with PMA and ionomycin in the presence of varying concentrations of PKR activation inhibitor, 2-AP, which was added during the last 30 min of the incubation. Frequencies of IFNγ positive were measured using FACS. 10 000 gated events were acquired. The treatment of 2-AP did not show any increase in the frequency of IFNγ positive UCB T cells in comparison to non treated UCB T cells.

tion, by UCB T cells was 30 fold less than the AB T cells (data not shown).

IFNγ production in UCB T cells is not regulated by PKR

It has been reported earlier that when present in low amount, IFNγ mRNA negatively autoregulates its own translation by activating the IFNγ dependant protein kinase PKR [25]. Therefore, we hypothesized that this low IFNγ protein produced by UCB T cells could be due to PKR activation. To check this hypothesis, we stimulated UCB T cells in the presence of increasing concentrations of the PKR activation inhibitor, 2-amino-purine (2-AP), and measured frequencies of the IFNγ positive cells using FACS. FACS analysis indicated that 2-AP treatment did not enhance the frequency of IFNγ positive UCB T cells (Fig. 2).

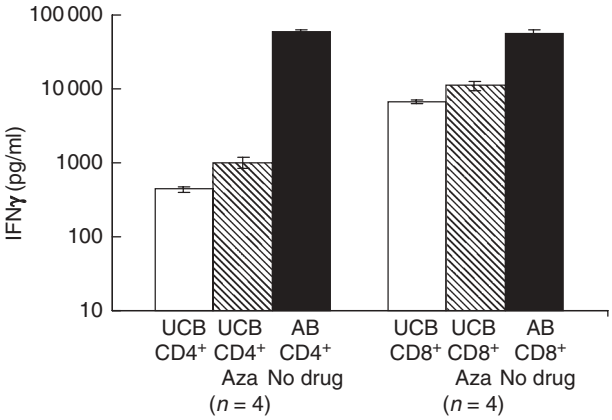


Fig. 3. Effect of 5-aza-2′-deoxycytidine on IFNγ protein production. Enriched UCB T cells were stimulated for 24 h in the presence of 5 μM 5-aza-2′-deoxycytidine (Aza). Following treatment supernatants from the cultures were collected and IFNγ levels were quantified using an commercial ELISA as described in the materials and methods. Each bar represent mean ± SD.

Promoter demethylation is not sufficient to induce IFNγ expression in UCB T lymphocytes

Recent findings have indicated that the hypermethylation of IFNγ promoter might contribute to the mechanisms preventing IFNγ gene expression in neonatal vs. adult blood T cells. We therefore examined whether treatment with 5-aza-2′-deoxycytidine (aza); a potent promoter demethylating agent [22,23,31] would relieve the transcriptional block, thereby resulting in optimal IFNγ mRNA accumulation in UCB T cell subsets. For this study, UCB $CD4^+$ and $CD8^+$ T cells were stimulated with PMA and ionomycin for 24 h in the presence of 5-aza-2′-deoxycytidine and IFNγ mRNA accumulation was quantified using real time- quantitative RT-PCR. It has been shown earlier that the treatment with 5-aza-2′-deoxycytidine cause demethylation of the IFNγ promoter without affecting T cell viability and their proliferation [22,23,31]. Our results show that neonate $CD4^+$ and $CD8^+$ T cells accumulated approximately 2 fold more IFNγ mRNA after 5-aza-2′-deoxycytidine treatment in comparison to nontreated controls (see Table 2). This increase in IFNγ mRNA also translated into a corresponding increase in IFNγ secretion (Fig. 3). Thus, demethylation of IFNγ gene

Table 2. Real time PCR analysis of the IFNγ mRNA accumulation UCB T cell after 5-aza-2′-deoxycytidine (Aza) treatment. Enriched UCB T cell subsets were stimulated with PMA and ionomycin in the presence of 5 μM aza for 24 h. The mean fold change in the IFNγ gene expression was calculated using equation $^{\Delta\Delta CT} = (\text{IFN}\gamma\ C_T - 18\text{sRNA}\ C_T)_{+Aza} - (\text{IFN}\gamma\ C_T - 18\text{sRNA}\ C_T)_{-Aza}$. The fold change was calculated by $2^{-\Delta\Delta CT}$. The aza treatment resulted in a two fold increase in IFNγ mRNA accumulation.

Samples	IFNγ C_T	18 s RNA C_T	Δ C_T*	ΔΔ C_T**	$2^{-\Delta\Delta\ CT}$†
UCB $CD4^+$T cells – Aza	26·35 ± 0·27	12·13 ± 0·11	14·22 ± 0·16	0·0 ± 0·0	1
UCB $CD4^+$T cells + Aza	25·39 ± 0·25	12·23 ± 0·16	13·16 ± 0·09	− 1·06 ± 0·07	2·08 ± 0·18
UCB $CD8^+$T cells – Aza	25·42 ± 0·49	12·58 ± 0·32	12·84 ± 0·17	0·0 ± 0·0	1
UCB $CD8^+$T cells + Aza	24·48 ± 0·53	12·83 ± 0·38	11·65 ± 0·15	− 1·18 ± 0·02	2·26 ± 0·32

*Δ C_T (IFNγ C_T-18 s RNA C_T); **ΔΔ C_T (Δ C_T $^{+Aza}$ – Δ C_T $^{-Aza}$); † Normalized IFNγ amount relative to untreated T cells

promoter is not sufficient to establish mature-like levels of IFNγ expression. This observation indicates that the tight regulation of IFNγ expression in UCB T cells most likely involves several mechanisms.

IFNγ mRNA to protein ratio is constant in UCB and AB T cells

We were able to precisely measure IFNγ mRNA and protein levels in the same samples, by the use of real-time RT-PCR and sensitive ELISA assays. This feature, enabled us to determine that the ratio between these two parameters. The IFNγ mRNA to protein ratio was constant in both UCB and AB T cell subsets (Table 3). Furthermore, we determined that this ratio was not altered by treatment with 5-aza-2′-deoxycytidine, which lead to small 2-fold increments in mRNA accumulation (see Table 2), as it was paralleled by a similar increase in IFNγ protein production (Fig. 3). Our results therefore imply that IFNγ expression in UCB T cells is not regulated at the translational level and that the main regulatory modes reside at the transcription level.

Discussion

The complex mechanism regulating IFNγ expression in neonatal T cells is still a puzzle, and it remains to be resolved whether this is regulated at the transcription or at translational level. In order to address this issue in more detail we have used real-time RT-PCR assays to accurately assess IFNγ mRNA levels, and FACS or ELISA analysis to examine IFNγ protein levels in the same samples. We used high doses of PMA and ionomycin to synergistically activate T cells in a receptor and APC-independent manner. PMA activates protein kinase C (PKC), but does not elevate Ca^{2+}. Ionomycin elevates Ca^{2+}, but Ca^{2+} alone does not activate PKC in the absence of PMA, addition of the Ca^{2+}-mobilizing agent ionomycin along with PMA synergistically activates T cell proliferation [32,33]. Our data indicate that the induction of IFNγ mRNA accumulation in neonatal T cells stimulated with PMA and ionomycin is 30 fold less than similarly stimulated adult T cells. In a similar manner the level of IFNγ protein was found to be much lower in stimulated neonatal T cells than in adult T cells.

IFNγ mRNA has been shown to activate the RNA-dependent protein kinase PKR, a stress kinase that is also activated by double-stranded RNA. The *cis*-acting RNA elements within IFNγ transcripts function as sensors of intracellular PKR levels and regulate IFNγ mRNA splicing and translation [34,35]. Since low levels of IFNγ mRNA has been shown to activate PKR [25] and we could observe very low IFNγ mRNA accumulation by cord blood T cell therefore we examined the effect of PKR inhibitor drug 2-AP on stimulated UCB T cells. Our data indicate that treatment with 2-AP did not increase IFNγ protein expression in PMA and ionomycin stimulated UCB T cells, a finding that is in agreement with reports indicating low levels of PKR activation in T lymphocytes [36].

It is well established that the methylation of DNA is an epigenetic mechanism for the modulation of gene expression in mammalian cells [37]. DNA methylation changes chromatin structure and may help the recruitment of transcription factors to the target genes [38]. Many of the studies have reported that the differential CpG methylation of IFNγ promoter is responsible for the low IFNγ expression [22,23,31].

Table 3. IFNγ mRNA to protein ratio in the UCB and AB T cell subsets. Enriched UCB and AB T cell subsets were stimulated for 24 h with PMA and ionomycin. IFNγ mRNA accumulation was measured using real-time PCR and calculated according to $2^{-\Delta\Delta CT}$ method. IFNγ protein was measured by ELISA and the IFNγ mRNA to protein ratio was calculated. The ratio between these two parameters are fairly constant in the T cell subsets obtained from UCB and AB.

Samples	IFNγ mRNA	IFNγ protein (pg/ml)	mRNA/protein ratio	Mean ± SD (mRNA/protein ratio)
UCB $CD4^+$T cells	576	883	0·652	0·72 ± 0·08
	739	982	0·752	
	3666	4368	0·839	
	1370	2068	0·662	
AB $CD4^+$T cells	31872	47945	0·664	0·68 ± 0·03
	99334	150347	0·660	
	41189	55264	0·745	
	33456	49264	0·679	
UCB $CD8^+$T cells	8135	7066	1·151	1·07 ± 0·08
	2721	2384	1·141	
	8481	8159	1·039	
	4482	4564	0·982	
AB $CD8^+$T cells	49324	40311	1·223	1·08 ± 0·13
	187682	94708	0·925	
	66451	57811	1·149	
	70728	68811	1.027	

Here, we examined whether IFNγ promoter hypermethylation could be reversed by treatment with a demethylating agent, 5-aza-2′-deoxycytidine. Our results show that this treatment only lead to a 2 fold increase in IFNγ mRNA accumulation in PMA and ionomycin stimulated neonatal T cells. Of interest was that this 2 fold increase in the mRNA accumulation was paralleled by an equivalent increase in IFNγ protein production.

This finding lead us to assess the IFNγ mRNA: protein ratios in our various study groups. This analysis indicated that this ratio was fairly constant in all the groups examined, whether they be activated neonatal or adult T cells. This analysis strongly suggest that in UCB T cells IFNγ expression is not regulated on a post-transcriptional level but rather on transcriptional level, at least, partially involves promoter methylation.

The exact mechanism regulating efficient induction of IFNγ mRNA accumulation is still unclear but may involve several other mechanisms. The dynamic changes in the histone tail acetylation have been shown to play an important role in the effector functions of T cells [37,39]. Avni *et al.* [39] have suggested that TCR stimulation activates a histone tail modification mediated change in chromatin structure in the immature T cells, which allows the binding of TCR-induced transcription factors to the promoter regions of IFNγ gene. In the absence of polarizing cytokines, both early histone hyperacetylation and early cytokine gene expression are reduced to the low basal amounts as observed in UCB T cells. In UCB T cells certain cytokine genes, such as that for IFNγ, are positioned in such a manner that only limited gene transcription is possible upon TCR stimulation. The difference between neonatal and adult T cells is that inactive cytokine genes in neonatal T cells are located in euchromatin regions, whereas a large fraction of the silenced genes in differentiated T cells are repositioned to centromeric heterochromatin regions due to inhibitory modifications, previously established by the polarizing cytokines, that down-regulate the expression of inappropriate genes. It is likely therefore that in immature T cells, TCR stimulation initiates permissive chromatin modifications that facilitate early gene expression. These findings also provide a possible reason for the delayed response against stimulus that UCB T cells display before they are able to produce efficient effector functions upon TCR stimulation, as a period of time is required for this repositioning of key effector genes in maturation of naïve to memory T cells [40]. Our data provide some support for this hypothesis, in that longer periods of PMA and ionomycin stimulation (24 h and 48 h) enhanced the frequency of IFNγ positive cells in the UCB T cell population.

To summarize, we propose here that the regulation of IFNγ in UCB T lymphocytes occurs at the transcriptional level and not post-transcriptional or translational level, as we observed fairly constant mRNA to protein ratios in all of the T cell groups examined. The nature of this transcriptional block, however, still remains to be resolved or may involve several factors such as deficient regulatory proteins, RNA or transcription factors rendering IFNγ up-regulation slower and lower than that of adult T cells.

References

1 Lewis DB, Yu CC, Meyer J, English BK, Kahn SJ, Wilson CB. Cellular and molecular mechanisms for reduced interleukin 4 and interferon-gamma production by neonatal T cells. J Clin Invest 1991; **87**:194–202.

2 Delespesse G, Yang LP, Ohshima Y, Demeure C, Byun DG, Sarfati M. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 1998; **16**:1415–9.

3 Kovarik J, Siegrist CA. Immunity in early life. Immunol Today 1998; **19**:150–2.

4 Takahashi N, Imanishi K, Nishida H, Uchiyama T. Evidence for immunologic immaturity of cord blood T cells. Cord blood T cells are susceptible to tolerance induction to in vitro stimulation with a superantigen. J Immunol 1995; **155**:5213–9.

5 Sautois B, Fillet G, Beguin Y. Comparative cytokine production by in vitro stimulated mononucleated cells from cord blood and adult blood. Exp Hematol 1997; **25**:103–8.

6 Adkins B. T-cell function in newborn mice and humans. Immunol Today 1999; **20**:330–5.

7 Berard M, Tough DF. Qualitative differences between naive and memory T cells. Immunology 2002; **106**:127–38.

8 Miscia S, Di Baldassarre A, Sabatino G, Bonvini E, Rana RA, Vitale M, Di Valerio V, Manzoli FA. Inefficient phospholipase C activation and reduced Lck expression characterize the signaling defect of umbilical cord T lymphocytes. J Immunol 1999; **163**:2416–24.

9 Bogunia-Kubik K, Perez-Cruz I, Fallen PR, Madrigal JA, Cohen SB. Cord blood lymphocytes have a low frequency of cytokine producing T cells due to a high threshold for activation. Immunol Lett 2000; **72**:145–6.

10 Hermann E, Truyens C, Alonso-Vega C *et al.* Human fetuses are able to mount an adultlike CD8 T-cell response. Blood 2002; **100**:2153–8.

11 Zhang J, Silvestri N, Whitton JL. Hassett DE. Neonates mount robust and protective adult-like CD8(+)-T-cell responses to DNA vaccines. J Virol 2002; **76**:11911–9.

12 Chalmers IM, Janossy G, Contreras M, Navarrete C. Intracellular cytokine profile of cord and adult blood lymphocytes. Blood 1998; **92**:11–8.

13 Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J Clin Invest 1986; **77**:860–7.

14 Schatt S, Holzgreve W, Hahn S. Stimulated cord blood lymphocytes have a low percentage of Th1 and Th2 cytokine secreting T cells although their activation is similar to adult controls. Immunol Lett 2001; **77**:1–2.

15 Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu Rev Immunol 1997; **15**:563–91.

16 Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997; **15**:749–95.

17 Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996; **271**:1723–6.

18 Hassan J, Reen DJ. Cord blood CD4+ CD45RA+ T cells achieve a lower magnitude of activation when compared with their adult counterparts. Immunology 1997; **90**:397–401.
19 Reen DJ. Activation and functional capacity of human neonatal CD4 T-cells. Vaccine 1998; **16**:1401–8.
20 Perez-Cruz I, Fallen P, Madrigal JA, Cohen SB. Naive T cells from cord blood have the capacity to make Types 1 and 2 cytokines. Immunol Lett 2000; **75**:85–8.
21 Cerbulo-Vazquez A, Valdes-Ramos R, Santos-Argumedo L. Activated umbilical cord blood cells from pre-term and term neonates express CD69 and synthesize IL-2 but are unable to produce IFN-gamma. Arch Med 2003; **34**:100–5.
22 Melvin AJ, McGurn ME, Bort SJ, Gibson C, Lewis DB. Hypomethylation of the interferon-gamma gene correlates with its expression by primary T-lineage cells. Eur J Immunol 1995; **25**:426–30.
23 White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD. J Immunol 2002; **168**:2820–7.
24 Yano S, Ghosh P, Kusaba H, Buchholz M, Longo DL. Effect of promoter methylation on the regulation of IFN-gamma gene during in vitro differentiation of human peripheral blood T cells into a Th2 population. J Immunol 2003; **171**:2510–6.
25 Ben Asouli Y, Banai Y, Pel-Or Y, Shir A, Kaempfer R. Human interferon-gamma mRNA autoregulates its translation through a pseudoknot that activates the interferon-inducible protein kinase PKR. Cell 2002; **108**:221–32.
26 Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998; **67**:227–64.
27 Kadereit S, Mohammad SF, Miller RE *et al.* Reduced NFAT1 protein expression in human umbilical cord blood T lymphocytes. Blood 1999; **94**:3101–7.
28 Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Meth 1993; **159**:197–207.
29 Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Meth 2000; **46**:69–81.
30 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T) Method. Methods 2001; **25**:402–8.
31 Katamura K, Fukui T, Kiyomasu T *et al.* IL-4 and prostaglandin E2 inhibit hypomethylation of the 5′ regulatory region of IFN-gamma gene during differentiation of naive CD4+ T cells. Mol Immunol 1998; **35**:39–45.
32 Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994; **76**:263–74.
33 Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 1985; **313**:318–20.
34 Kaempfer R. RNA sensors: novel regulators of gene expression. EMBO Report 2003; **4**:1043–7.
35 Clemens MJ. Targets and mechanisms for the regulation of translation in malignant transformation. Oncogene 2004; **23**:3180–8.
36 Li S, Nagai K, Koromilas AE. A diminished activation capacity of the interferon-inducible protein kinase PKR in human T lymphocytes. Eur J Biochem 2000; **267**:1598–606.
37 Agarwal S, Rao A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 1998; **9**:765–75.
38 Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997; **389**:349–52.
39 Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao A. T (H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. Nat Immunol 2002; **3**:643–51.
40 Grogan JL, Mohrs M, Harmon B. Lacy DA, Sedat JW, Locksley RM. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity 2001; **14**:205–15.

***C*urriculum –*V*itae**

Anurag **K**umar Gupta
Laboratory for Prenatal Medicine,
University women's Hospital/Department of Research,
University of Basel, Spitalstrasse, 21,
CH-4031 Basel, Switzerland.
Tel. 0041-61-265 9223, Fax. 0041-61-265 9399
E-mail: Anurag.Gupta@unibas.ch, guptaag@yahoo.com

Academics:

Post Doctoral Fellow, 1st May 2005- till date
Laboratory for Prenatal Medicine/ Department of Research, University of Basel, Switzerland.

PhD, Dec. 2001- April 2005
Laboratory for Prenatal Medicine/ Department of Research, University of Basel, Switzerland.
Finished PhD study in April 2005. The title of the thesis was " Immune-modulation by the placenta and its dysregulation in preeclampsia: role of syncytiotrophoblast microparticles and cytokines".

JRF, Jan.1999-Dec. 2000
School of Life Sciences, Jawaharlal Nehru University (JNU), New Delhi. India
Worked as a Junior Research Fellow (JRF) with Prof. Asis Datta in a Hindustan Lever Limited sponsored project – "Genetic modification of Castor oil".

Master of Science (Life Sciences), 1996-1998
K.N. Kaul Institute of Life Sciences (Faculty of Life Sciences) C.S.J.M. University, Kanpur, U.P., INDIA.

Advance Diploma in Computer Application, 1995-1996.
UPTEC Computer Consultancy, Software Technology Park, Kanpur, U.P., INDIA**.**

Bachelor of Science, 1992-1995
Dayanand Anglo Vedic (D.A.V.) College, Kanpur University, Kanpur, U.P., INDIA.

XII Std (10+2), 1989-1991
S.R.K. Inter College, Firozabad, U.P., INDIA.

XStd (10th), 1987-1989
S.R.K. Inter College, Firozabad, U.P., INDIA.

Technical e**x**pertise:

- ***General Molecular Biology Techniques -*** such as Cloning, Plasmid preparations, Transformation, Southern Blotting, Northern Blotting, SDS-PAGE, Native-PAGE, DNA sequencing, Polymerase Chain Reaction, **Real time quantitative PCR Taqman®**.

- ***Biochemical Techniques -*** Macromolecules estimation using colorimeter, fluorometer and UV-spectrophotometry, Thin layer chromatography (TLC), working knowledge with instruments and techniques used in human pathological diagnosis and, various kinds of X-rays used for diagnostic purposes.

- ***Plant Tissue Culture and Plant Molecular Biology -*** Media standardisation, micropropogation of different explants such as internode, leaf, mesocotyl, hypocotyl, embryo, agrobacterium and Particle bombardment mediated transformation.
- ***Immunological techniques:*** - Fluorescence Activated cell sorting (FACS), MACS, ELISA, Immuno-histochemistry, Cell proliferation assays and apoptosis assays like caspase activation, DNA fragmentation and annexin V assay.

- ***Cell culture***: - Tissue explant culture. Isolation, enrichment and culture of T cells, neutrophils and Human umbilical vein endothelial cells (HUVEC).

- Working knowledge of DOS-7, Windows, Word Processor, Spread Sheets, DBMS, Programming in BASIC & 'C' Unix Networking, Multimedia, Corel Draw. Also adept in using Internet tools for research purposes like BLAST, clustal W.

- Specimen preparation for Electron microscopy.

- Working experience of handling ^{35}S, ^{3}H and ^{32}P.

Other Accomplishments:

- Qualified at **UGC- CSIR NET** examination conducted by Council of Scientific and Industrial Research, Govt. of India for Junior Research Fellowship and lifetime lectureship in any Indian University.
- Recipient of the research grant from **Roche Research foundation, Basel, Switzerland**, for the period of one year (Jan.2002-Dec.2002).

Work E**x**perience:

- **January'1999- Dec.'2000:** I joined School of Life Sciences, JNU as Junior Research Fellow in the above mentioned project and learned all possible tools of molecular biology to carryout research.
- **January'2001-April'2001:** I was honorary teaching and instructing M.Sc. students of Life Sciences and Biochemistry at faculty of Life Sciences, CSJM university, Kanpur, UP, India.

Interests:

I enjoy my leisure by indulging myself in Wildlife Conservation, Reading books, playing Cricket, Filling crosswords, Singing.

Pursuing my interest in wild life, I designed a project on conservation of tigers during my Masters. The objective of the project was to make observations of eating, living and mating habits of tigers in captivity of zoological parks. These observations were analyzed along with pedigree of all the individual tigers in order to find conducive environment for propagation of this endangered species in the captivity.

Fulfilling my school time interest of looking into scientific aspects of day-to-day domestic life. I joined a short-term course in cosmetic manufacturing during a break before starting Masters.

Poster presentation and workshops:

1. Presented poster at **Experimental biology, 2005 April 2-6, 2005. San Diego, CA, USA.** The title of the poster was: Presence of neutrophil NETs in preeclampsia, generated by placentally derived IL-8 and syncytiotrophoblast micro-particles.

2. Presented poster at **Society for Gynecologic Investigation, March 23-26, 2005. Los Angeles, CA, USA.** The title of the poster was: Trophoblast derived micro particles (STBM) do not induce T lymphocyte activation and apoptosis.

3. Presented poster at **Society for Gynecologic Investigation, March 23-26, 2005. Los Angeles, CA, USA.** The title of the poster was: Fetal DNA and RNA are associated with placentally derived syncytiotrophoblast micro-particles (STBM).

4. Presented poster at Annual Congress of the **British Society for Immunology (BSI), 2003, 2-5 December 2003, Harrogate, UK.** The title of the poster was: "Interaction of placental micro-particles with the endothelial cells".

5. Presented poster at **American Society of Reproductive Immunology (ASRI) 23rd annual meeting, June18-21, 2003, Yale School of Medicine, Yale University New Haven, CT, USA.** The title of the poster was: Role of PKR and promoter methylation in the regulation of IFN gamma in naïve T cells.

6. Attended workshop on "**New methods for studying immune properties of cells in mucosal tissues**" at 23rd ASRI meeting, June 16-17, 2003, Yale University, New Haven CT, USA.

Publications:

1. **Gupta AK,** Holzgreve W, Hahn S. Microparticle-free placentally derived soluble factors down-modulate the response of activated T cells. **Human Immunology; In press.**

2. **Gupta AK,** Rusterholz C, Holzgreve W, Hahn S. Syncytiotrophoblast micro-particles do not induce apoptosis in peripheral T lymphocytes, but differ in their activity depending on the mode of preparation. **J. Repro. Immunol; In press.**

3. **Gupta AK,** Rusterholz C, Holzgreve W, Hahn S. Constant IFNγ mRNA to protein ratios in cord and adult blood T cells suggests regulation of IFNγ expression in cord blood T cells occurs at the transcriptional level. **Clin. Exp. Immunol. 2005;** 140: 282-288**.**

4. **Gupta AK,** Rusterholz C, Huppertz B, Malek A, Schneider H, Holzgreve W, Hahn S. A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. ***Placenta*** **2005;** 26: 59-65**.**

5. Rusterholz C, **Gupta AK,** Huppertz B, Holzgreve W, Hahn S. Soluble factors released by placental villous tissue: interleukin-1 is a potential mediator of endothelial dysfunction. **Am. J. Gyn. Obstet, 2005; 192: 618-624.**

6. **Gupta AK,** Huppertz B, Malek A, Schneider H, Holzgreve W, Hahn S. Detection of fetal DNA and RNA in the plcentally derived syncytiotrophoblast micro particles. **Clin Chem. 2004** Nov; 50(11): 2187-2190.

7. Li Y, Zimmermann B, Zhong XY, **Gupta AK**, Holzgreve W, Hahn S. Determination of RHD zygosity using real-time quantitative PCR. **Swiss med weekly 2003**; 133:442-445.

8. **Gupta AK,** Hasler P, Holzgreve W, Gebhardt S, Hahn S. Massive presence of neutrophil NETs directly in the intervillous space of preeclamptic placentae. **Submitted.**

Date of birth:	05th October 1975.
Birthplace:	Firozabad, India.
Citizenship:	Indian.
Sex:	Male
Private Address:	Johanniterstrasse, 13 CH-4056 Basel, Switzerland Tel. 0041-76-332 1575

References:

1. Dr. Sinuhe Hahn
University Women's Hospital/ Dept. of Research
University of Basel,
Basel, Switzerland
Ph.No. – 0041-61- 265 9224
E-mail: Shahn@uhbs.ch

2. Prof. Wolfgang C. Holzgreve
Dept. of Gynaecology and Obstetrics,
University of Basel,
Basel, Switzerland
Ph.No. – 0041-61- 265 9 012
E-mail: wholzgreve@uhbs.ch

3. Dr. Berthold Huppertz
Dept. of anatomy,
University Hospital, RWTH,
Aachen, Germany
Ph.No. – 0049-241- 808 9975
E-mail: bhuppertz@ukaachen.de

Date:21.7.2005
Place: Basel **Anurag Kumar Gupta**