THE INHIBITORY EFFECT OF PROGESTERONE DEPENDENT IMMUNOMODULATION ON NK ACTIVITY IS MANIFESTED VIA ALTERING CYTOKINE PRODUCTION AND ARACHIDONIC ACID METABOLISM

Ph.D. Thesis

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I. LIST OF ABBREVIATIONS

AA	arachidonic acid
ASA	acetylsalicylic acid
BSA	bovine serum albumin
CD	cluster of differentiation
ConA	concanavalin A
COX	cyclooxygenase
CTL	cytotoxic T lymphocyte
ELISA	emzime linked immunosorbert assay
HLA	human leukocyte antigen
IFN-γ	interferon γ
IL	interleukin
INDO	indomethacine
IVF	in vitro fertilization
KAR	killer activator receptor
KIR	killer inhibitory receptor
LAK	lymphokine activated killer
LPS	lipopolysacharide
MAb	monoclonal antibody
MHC	major histocompatibility complex
NC	natural cytotoxic
NK	natural killer
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PG	prostaglandin
PHA	phytohaemagglutinin
PIBF	progesterone-induced blocking factor
PLA2	phospholipase A2
RSA	recurrent spontaneous abortion
TBS	tris buffered saline
TCR	T cell receptor

Th	T helper
TNF-α	tumor necrosis factor α

II. INTRODUCTION

1. Immunological relationship between the mother and fetus. The role of the trophoblast

Medawar[1] was the first to formulate the basic problem of human pregnancy : " How does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is antigenically foreign body ? " . Fifty percent of fetal antigens are of paternal origin, thus the fetus should be seen as an allograft and rejected via T cell mediated, major histocompatibility complex restricted mechanisms.

It would seem plausible that non-recognition of fetal antigens by the maternal immune system favors a normal pregnancy outcome. However, this is not true. Antibodies with anti-parental specificity have been detected in sera of multiparous women [2] , clearly showing that maternal recognition of fetal antigens does not compromise pregnancy. Inadequate recognition of fetal antigens might result in failed pregnancy. Activation of the immune system seems to be necessary for a normal pregnancy outcome. Non-specific immunstimulation of the pregnant females reduces the originally high resorption rates in abortion prone murine strain combination [3] and a similar effect is achieved by immunization of the mothers with paternal strain type spleen cells [4]. These findings obtained in murine model, can be extended to human system. In women suffering recurrent spontaneous abortions (RSA) the induction of recognition of paternally derived human leukocyte antigen by lymphocyte immunization improves pregnancy outcome [5,6,7]. Komlos et al.[8] suggested that HLA matching between the parents is associated with spontaneous abortion and in a recently published 10 year prospective study Ober et al.[9] confirmed these data.

Taken together these data suggest that immunologic recognition of pregnancy is needed for the success of gestation.

The fetus itself does not come into direct contact with maternal tissue. It is the trophoblast, which forms the interface between the maternal and fetal compartments. The trophoblast is a tissue of fetal origin and it is in intimate and continuous contact with maternal immunocompetent cells throughout gestation. Thus, the trophoblast should be the interface

where fetal antigens are presented to the maternal immune system, and also the target of maternal anti-fetal effector mechanisms. Since placental cells are devoid of HLA class II antigens, interest has focused on the expression of HLA class I molecules. Different classes of the trophoblast (ec. syncyctiotrophoblast, villous cytotrophoblast) are devoid of HLA antigens. Extravillous cytotrophoblast cells form the only trophoblast subpopulation which do express HLA molecules [10,11]. The HLA class Ia or classical gene family include the three highly polymorphic molecules HLA-A, -B and -C, which are expressed in most somatic tissues and able to present intracellular peptides to cytotoxic T cells [12]. Three additional class I genes, the less polymorphic HLA-E, -F and -G antigens form the class Ib or non-classical group. These molecules show homology to classical class I molecules but generally have limited polymorphism, low level cell surface expression and more restricted tissue distribution [13]. Immunohistochemically HLA-G [14], -E and a small amount of HLA-C were the only HLA class I molecules that could be detected in extravillous chorionic cytotrophoblast cells [15,16,17].

The polymorphism of HLA-G is low, only a few allels have been described. Since paternally inherited HLA-G is present on the trophoblast cells, it would be possible to be recognized as foreign by the maternal immune system. The limited HLA-G polymorphism ensures that paternal and maternal HLA-G are extremely similar or identical and hereby does not induce a maternal alloresponse.

The trophoblast does not induce transplantation immunity and resists NK as well as CTL mediated lysis in vitro [18]. Transfection with HLA-G renders cells that had been originally CTL or NK sensitive, resistant to lysis by these effectors. In addition HLA-G may cause anergy and apoptosis of allogeneic cytotoxic CD8+ T cells [19]. This suggest that resistance of trophoblast cells to cytotoxic effectors is due to the presence of HLA-G. Antigen recognition by cytotoxic T lymphocytes is MHC restricted, whereas activation of NK cells depends on the lack of MHC expression on the target cells. Since trophoblast is readily killed by lymphokine activated killer cells, the former phenomenon can be explained by a disturbed antigen recognition. HLA-G might induce resistance to lysis by decidual NK cells expressing KIR (ec. LIR1/ILT2, ILT4, p49, BY55) that recognize HLA-G [20, 21,22]. Ligand binding of these receptors conveys a negative signal, thus the presence of HLA-G might defend the trophoblast from NK mediated lysis.

The next potential function that HLA-G could exert, upon ligation to KIR receptors, would be the release of particular cytokines by these NK cells [23]. In vitro experiments have

shown that co-culture of human peripheral mononuclear blood cells with HLA-G expressing cells resulted in a Th2 response [24].

Another possible role of HLA-G is antigen presentation. According to our present knowledge HLA-G presents antigens for γ/δ T cells. HLA-G is likely to exert anti-viral function as well. There is evidence that HLA-G is capable of binding peptides of viral origin and play a critical role in the presentation of viral peptides to cytotoxic T cells in the placenta [14].

The other important MHC class Ib molecule is HLA-E. However, the importance and function of HLA-E during pregnancy is still under investigation. NK cells can interact with HLA-E complexed with specific peptides on target cells and it is mediated by the CD94/NKG2 receptor [25]. Recent studies have demonstrated that the ligand of CD94/NKG2A was in fact HLA-E and not HLA-G [26,27]. There is evidence now that HLA-G plays a role in the regulation of HLA-E expression [28]. All these data indicate that HLA-G presents antigens for γ/δ T cells and at the same time defends the trophoblast from cytotoxic effector mechanisms.

2. Progesterone dependent immunmodulation

a. The effects of progesterone

Progesterone is essential for the maintenance of pregnancy in a number of mammalian species. It inhibits the contractions of myometrial smooth muscle [29], blocks the activity of uterine collagenase [30] and modifies the activity of proteolytic enzymes in blastocyst as well as in the uterus [31]. It is produced first by corpus luteum and later by placenta. The serum concentrations of progesterone range from 100 to 500 nM during pregnancy. High concentrations of progesterone prolong the survival of xenogenic and allogeneic grafts [32,33] and this hormone affects various phases of the immune response in vitro. Many publications reported that progesterone blocks T cell activation in concentrations of 5 to 20 μg/ml [34]. Stites et al.[35] reported on different mechanisms resulting in T cell activation blocking by progesterone and cortisol. In most investigations dealing with in vitro effects of progesterone on lymphocytes reactivity, only supraphysiological (0.5 –20 μg/ml) doses were found to be effective. Thus, it was concluded that progesterone might have a role as a natural immunosuppressant during pregnancy, although its action was assumed to be restricted to the materno-fetal interface where progesterone concentrations reaches the high level required for

in vitro blocking [36]. NK activity of healthy pregnant women's lymphocytes can be suppressed by relatively low (100-400 nM) concentration of progesterone, whereas 100 times higher concentrations are required for reducing the natural cytotoxic activity of non-pregnancy lymphocytes [37] and this effect was shown to be inhibited by equimolar concentrations of progesterone receptor blocker RU486 [28]. In lymphocytes from pregnant women progesterone at physiological concentrations inhibited natural cytotoxic (NC) activity in a dose-related manner, and in vitro an inverse relationship was found between progesterone concentration and cytotoxic activity of these lymphocytes. Preincubation of lymphocytes with progesterone depleted pregnancy serum did not result in significant inhibition on cytotoxic activity. In fact, absorption with anti-progesterone antibody caused an 80 % decrease on cytotoxic activity of pregnant sera [38,39]. This findings led to the hypothesis that progesterone sensitive pregnancy lymphocytes might express specific progesterone binding sites.

b. Progesterone receptor expression on lymphocytes

Lymphocytes of healthy pregnant women are more sensitive to the natural cytotoxicity blocking effect of progesterone because they have significantly higher progesterone binding capacities than those from nonpregnant individuals or pregnant women at risk for premature pregnancy termination [40]. By immuncytochemistry with different progesterone receptor specific monoclonal antibodies our laboratory demonstrated progesterone-receptors in peripheral pregnancy lymphocytes [41]. The incidence of progesterone receptor expressing lymphocytes was neglectable in peripheral blood of non-pregnant individuals. The reactivity was mainly localized in the CD8+ population, and the majority of progesterone receptor bearing peripheral lymphocytes expressed γ/δ TCR [42,43]. Lymphocyte progesterone receptors with a molecular weight of 40 kDa do not seem to be identical with the classical progesterone binding sites (110 kDa). The percentage of progesterone receptor positive lymphocytes in peripheral blood increased throughout gestation. Progesterone receptors appear in peripheral blood lymphocytes as early as the tenth day of gestation and disappear during term labor. Recurrent abortion, spontaneous abortion and threatened pre-term delivery were associated with lower number of receptor positive cells [44]. Therefore, it is conceivable, that lack of lymphocytic progesterone receptors during pregnancy might have functional consequences. The regulation of lymphocyte progesterone receptor expression is activation dependent, but hormone independent. In resting human lymphocytes progesterone receptors are induced by

mitogenic or alloantigenic stimulation and the mRNA of progesterone receptor is present in activated, but not in resting human lymphocytes [42]. Furthermore, due to a chronic stimulation, lymphocytes of transplanted patients express progesterone receptors also [45]. Recurrent spontaneous abortions are claimed by some authors to be associated with the lack of maternal allorecognition of the conceptus. A high rate of HLA matching between the parents has been implied by some groups [8,9]. In pregnant women with spontaneous abortions of unexplained etiology the rate of receptor positive cells was significantly lower than that in healthy pregnant women of corresponding gestational ages [41]. It is likely, that in these cases, owing to unusually coincident HLA matching or to holes in the T cell repertoire for placental antigens, fetal cells fail to stimulate maternal lymphocytes, which might account for the failure of the latter to develop progesterone receptors. Immunotherapy with paternal lymphocytes for unexplained recurrent abortion induced a higher expression of progesterone receptors on lymphocytes. Increase of progesterone receptor expression correlated with the success or failure of gestation [46]. These data suggest that in pregnancy a chronic alloantigeneic stimulation due to the presence of fetus might be responsible for induction of progesterone receptors.

In the decidua γ/δ TCR positive cells significantly increase in number [47,48]. The number of these cells in the uterus is higher in allogeneic than in syngeneic pregnancy and the expression of the γ/δ TCR in the pregnant uterus has been shown to be hormonally controlled [49]. Therefore, it can not be ruled out that this population might play a role in recognition of fetal antigens. The majority of decidual γ/δ T cells are in activated form [47,48,50]. In peripheral blood of pregnant women there is an increased ratio of γ/δ TCR positive lymphocytes and more than 90% of these cells express progesterone receptor suggesting a state of activation [43]. These data allow the assumption that decidual γ/δ cells play a major role in progesterone dependent immunomodulation.

c. Progesterone-induced blocking factor

In the presence of progesterone, progesterone receptor positive lymphocytes produce a 34 kD protein [51] , named the progesterone-induced blocking factor (PIBF). PIBF appears on the lymphocytes of healthy pregnant women [52], but not on those from pathological pregnancies. The percentage of positive cells is significantly lower among peripheral blood lymphocytes of women showing clinical symptoms of threatened preterm delivery and also from women with miscarriages and preterm deliveries. Similar results were obtained by ELISA. PIBF can be

detected in sera of pregnant women and its concentration is higher than that of non-pregnant individuals or pregnant women with symptoms of threatened abortion. Furthermore, the majority of sera from term deliveries was characterized by lower than normal concentrations of PIBF, suggesting an association between the serum level of this protein and the termination of pregnancy [53]. Thus, the presence or absence of PIBF correlates with the failure or success of gestation.

NK activity of peripheral blood lymphocytes is inversely correlated to their PIBF positivity. Van den Heuven et al.[54] have demonstrated the presence of progesterone receptor positive NK cells in the decidua. Decidual CD56+ cells inspite of their high perforin content show a low rate of cytotoxicity. All decidual CD56+ cells produce PIBF [55]. PIBF blocks NK activity by inhibiting degranulation of peripheral lymphocytes [55] therefore, it can not be excluded, that low NK activity of decidual NK cells is due to the high presence of PIBF in the decidua.

Progesterone-treated murine pregnancy lymphocytes release a similar factor. Treatment of Balb/c mice that were 8 days pregnant with progesterone receptor blocker (RU486) resulted in 100 % resorption of the fetuses. Simultaneous administration of the supernatant from progesterone-treated murine pregnancy spleen cells restored the resorption rate to the original 6 % [56]. These data suggest that functional lymphocytic progesterone binding sites are needed for the maintenance of normal murine pregnancy as well. In pregnant mice PIBF exerts a strong anti-NK activity and it has an anti-abortive effect, since PIBF prevents resorptions induced by transfer of high NK activity spleen cells [57].

In women with recurrent spontaneous abortions paternal lymphocyte immunization not only induces a higher expression of progesterone receptor expression but suppresses NK cell activity as well [58].

Studies on IVF patients showed, that PIBF appears on the lymphocytes of pregnant women early after implantation. The percentage of PIBF expressing cells increases as a result of pregnancy and the stimulus for PIBF induction occurs in an early period of periimplantation event, and may thus help with early escape from maternal immune surveillance [59]. These data support the concept that PIBF may play an important role in early implantation possibly by inhibiting destructive function of natural killer lymphocytes.

Another immunmodulatory effect of PIBF is influencing the rate of antibody synthesis and the quality of antibody production. PIBF enhances asymmetric antibody production of hybridoma cells [60]. Asymmetric antibodies have a mannose-rich olygosaccharide linked to one

of the Fab arms of the molecule and though they bind the antigen with the same specificity as this conventional counterparts, these Ig molecules are generally unable to activate effector functions, such as complement fixation, phagocytosis and cytotoxicity, however, they can block the antigen and thus might play a role in the protection of the fetus. The sera of pregnant mice treated with anti-PIBF antibody contained four times less asymmetric antibodies than those of normal pregnant animals [61] suggesting the role of progesterone in the regulation of nonprecipitating antibody production as well.

3. Altered cytokine production during pregnancy

Cytokines play a major role both in the establishment and in the maintenance of normal, human pregnancy and may have beneficial or negative influence on pregnancy outcome depending on the cytokine level present.

CD4+ cells can be subdivided into different subsets on the kind of lymphokines they produce [62]. T helper 1 (Th1) cells secrete IFN-γ and TNF, whereas T helper 2 (Th2) cells secrete IL-4, IL-5 and IL-13. Th1 cells induce cellular-mediated inflammation and tissue injury, whereas Th2 cells are prominent in the pathogenesis of allergic diseases [63,64]. Cytokines present during the initiation of a T cell response can determine the development of a particular Th subset. For example Th2 cells develop when naive T cells are stimulated in the presence of IL-4, while IL-12 is a critical factor driving the development of Th1 cells [65,66,67,68].

Gestation is associated with a transient depression of maternal cell-mediated immunity to protect the semi-allogeneic embryo from rejection. The hallmark of this immune tolerance is a profound modulation of T cell responses, best characterized by a sift from Th1 (IL-2, IFN-γ) to a Th2 (IL-4, IL-10, IL-13) type cytokine response [69,70,71,72] It was demonstrated that a failure of the generation of Th2-type cytokine responses is associated with recurrent abortions [73], complications [74], and poor pregnancy outcome. Th2 cytokines with anti-inflammatory characteristics has been suggested to be compatible with a successful pregnancy [75]. Abortion-prone matings (CBAxDBA/2) in mice associated with the predominant production of Th1 cytokines, including IFN-γ, IL-2 and TNF−α, culminate in fetal loss, which can be reversed by administration of the Th2 cytokine IL-10 during pregnancy [76,77,78,79]. Although both pro- and

anti-inflammatory cytokines, including IL-2, IL-4, IL-10 and IFN-γ, are expressed in activated PBMCs throughout pregnancy in contrast to their placental production, there is a significant up-regulation of IL-10 during early pregnancy, which may be sufficient to exert moderate Th2 predominance [80]. On the other hand there is a more profound systemic Th1 balance in pathologic pregnancies [81]. These studies suggest that systemic or placental presence of Th2 cytokines, particularly IL-10, would be supportive of normal pregnancy.

a. The protective role of IL-10 in pregnancy

Although human IL-10 does not fit in the classical Th2 cytokine profile because it can be produced by both Th1 and Th2 cells as well as non-T cells, this 18-kDa polypeptid exhibits predominantly inhibitory effects on inflammatory reactions [82]. One of the major roles of IL-10 is down-regulation of chemokine and cytokine production by Th1 cells and macrophages [83,84,85]. IL-10 also interferes with antigen presentation and directly or indirectly inhibits CD8+ T cell or NK cell responses [86,87,88,89].

IL-10 may also act as the mediator of several other intrauterine regulators [90,91]. Progesterone, catecholamines and prostaglandins have been shown to induce production of IL-10 [92,93,94]. It has been demonstrated that IL-10 is a key cytokine produced by human placenta, the expression of which was significantly down-regulated at term before the onset of labor [80]. Several lines of evidence suggest that IL-10 may play a major role in influencing the activity of the placental trophoblast, which has been proposed as a key cell type in regulating fetal immunoprotection [91,95,96]. The placenta produces proinflammatory cytokines, which are thought to be associated with trophoblast apoptosis, protease production, and stimulation of several uterotonins (prostaglandins, etc.) which are produced in increased levels at the time of spontaneous or preterm labor [97,98,99]. IL-10 displays a potent bioactivity in down-regulating the expression and activities of proinflammatory cytokines and uterotonins [82,100]. Importantly, IL-10 modifies the activity and expression of prostaglandin dehydrogenase in cultured term human villous trophoblast and chorion trophoblast cells [100]. Furthermore IL-10 is a potent inhibitor of cell-mediated immunity, which has been shown to be immunologically incompatible with establishment of the feto-placental unit in mice [70]. The down-regulation of IL-10 at term may serve as one of the initial signals in a complex regulatory scheme necessary to ensure up-regulation of proinflammatory cytokines (such as TNF-α, IL-1β) and uterotonins at parturition [80].

The regulatory role of IL-10 is supported by the observations that this cytokine successfully blocks LPS-induced preterm delivery in mice [101]. In the resorption prone CBA x DBA/2 murine mating combinations the placentas are quantitatively or qualitatively deficient in their production of the anti-inflammatory Th2-type cytokines IL-4 and IL-10 compared with the non resorption-prone CBA x BALB/c mating combination. Wastage in the previous mating combination is accompanied by increased levels of local inflammatory cytokines. Alloimmunization enhances the placental production of IL-4 and IL-10 in CBA x DBA/2 matings [79]. The re-expression of IL-10 mRNA after labor has been also described [80] which might be a part of delayed negative feedback mechanism. It has been suggested that TNF-α, IL-12 and prostaglandin E2 induce IL-10 to autoregulate their own production [102,103]. IL-10 has been shown to reverse experimental fetal growth restriction and demise [104] , while human placental tissues or isolated cytotrophoblasts from 26-wk and 33-wk preterm labor deliveries lack expression of IL-10 [80]. The lack of IL-10 results in pregnancy failure. IL-10 gene knock out mice are born with significantly lower birth weight than their heterozygous siblings. This can be prevented by rIL-10 or anti-TNF treatment [105]. Thus IL-10 may be critical in normal fetal development and down-regulation of inflammatory responses in the placental microenvironment. These results indicate that the placentally produced anti-inflammatory cytokines can play a key role in the survival to term of the fetal allograft, by counteracting deleterious inflammatory cytokines.

b. Th-1 type cytokines in pregnancy

Several lines of evidence from studies using ex vivo cultures or gene-knockout mutations mice, suggest that multiple cytokines are involved at various distinct steps in NK-cell development and in the regulation of the activity of these cells.. IL-15 plays important role in an early differentiation step of NK cells and their subsequent expansion [106,107]. IL-12 and IL-18 can induce IFN-γ production by immune cells and enhance NK-cell cytotoxicity [108,109]. IL-12 is produced by myelomonocytic cells as a heterodimer composed of two disulfide-linked chains, p35 and p40, encoded by separate genes [110,111]. Simultaneous expression of the two genes is required for the production of biologically active IL-12 heterodimer. In mice deficient in IL-12 p40 or IL-18, production of IFN-γ and NK-cell cytotoxicity decrease significantly [112,113]. The reduced cytotoxicity of NK cells can be corrected to normal levels by treatment with IL-2 in vitro or with IL-12 and IL-18 in vivo. Based on these results it is

tempting to postulate that IL-12 and/or IL-18 are needed for the terminal differentation of NK cells into fully functioning cells with the ability to produce IFN-γ[114].

Several reports have now confirmed that unbalanced presence of Th1 cytokines IL-2, IFN-γ, TNF–α during murine pregnancy results in fetal ablation. In human studies significantly lower mRNA levels of the Th1-type cytokines (IL-2 and IFN-γ) were observed during pregnancy compared with non-pregnant female controls. The mRNA levels of IL-18 were also significantly reduced during gestation. These effects were already prominent in women during the first trimester and continued throughout pregnancy. IL-2 and IFN-γ levels tended to increase towards late pregnancy but decreased again immediately after delivery. The IL-4/IFN-γ ratio (as an indicator of TH1/Th2 balance) was significantly higher during first and second trimesters compared with early post-partum or with non-pregnant women. This dominant Th2 cytokine profile was even more pronounced if the IL-4/IL-2 ratio was calculated [72]. Th1-type cytokines clearly have adverse effects on the conceptus in vitro and in vivo. IL-2 and TNF-α are abortificent in mice [115]. Excessive TNF and IFN-γ have also been demonstrated in the placenta and decidua of aborting CBAxDBA/2 mice [77], on the other hand anti-TNF antibodies or TNF antagonists, e.g. pentoxifillin normalize the high resorption rates in CBA/J x DBA/2 matings[116]. IFN-γ inhibits trophoblast outgrowth and causes the degeneration of attached blastocysts[117]. IFN-γ also activates cytotoxic T cells and NK cells, which, in a lymphokine activated state may damage trophoblast. In mice low doses of IFN-γ slow down intrauterine developement, whereas administration of high doses result in abortion [116]. TNF-α inhibit mouse embryonic and fetal developement and also the proliferation of human trophoblastic cell lines in vitro. Yui et al. have shown that TNF–α causes apoptosis of the human cytotrophoblast cells which are known to express receptors for TNF-a, and they suggested that TNF-α augmented by IFN-γ may bring about a premature depletion of progenitor trophoblast cells resulting in intrauterine damage [97]. In vivo inflammatory cytokines such as IL-2, TNF-α and IFN-γ can terminate normal pregnancy when injected into pregnant mice [118]. In supernatants of trophoblast activated peripheral lymphocytes of recurrent spontaneous aborters elevated levels of TNF-α and β have been demonstrated, suggesting that these cytokines might act as a mediators in the developement of RSA. These data above nevertheless led to the concept that succesful allopregnancy is a Th2 phenomenon [71].

These observations may explain the beneficial effect of pregnancy on various T cell-mediated, organ specific autoimmune diseases. It has been described that the activity of multiple sclerosis, rheumatoid arthritis or Crohn's disease is reduced during pregnancy but

flares up in the post-partum period [119,120,121]. As disease activity in these disorders is often associated with increased Th1-type cytokine responses in the blood, it was suggested that a cytokine shift during pregnancy may be protective process. Systemic lupus erythematosis, in which the principal pathology is mediated by excessive autoantibody production, tends to flare up during pregnancy, especially in women with recently active disease before contraception [122]. There are also a number of infectious diseases caused by intracellular pathogens (ec. HIV-associated infections [123], malaria [124] and toxoplasmosis [125]) which appear to be exacerbated by pregnancy.

Thus, a shift in the balance of these two cytokines appears to be an important element in the generation or correction of immune dysfunctions. During pregnancy materal immune response is biased towards humoral immunity and away from cell-mediated immunity which could be harmful to the fetus.

Taken together, these data suggest that normal intrauterine developement largely depends on the cytokine balance. A Th2 biased cytokine pattern favors a normal outcome, whereas, a Th1 dominant response is deleterious.

4. The role of NK cells in the feto-maternal relationship

Natural killer (NK) cells belong to the system of innate immunity and for cytolytic activity do not require former antigenic stimulation. NK cells are defined as large granular lymphocytes, which usually express CD16 and/or CD56 in human and do not express CD3 or any known T-cell receptors[126]. NK cells may kill target cells by utilizing both secretory mechanism (perforin/granzyme-mediated) and non-secretory (cell membrane-bound Fas ligand-mediated) mechanisms, where Fas ligand is expressed on the cell surface of NK cells and responsible for Fas-mediated cytotoxicity against Fas-expressing target cells [127,128].

NK cells are able to kill MHC negative target cells (a common consequence of virus infection or malignant transformation) without prior sensitization. A recent model of NK activity, the so-called „two-receptor model" [129] suggest the role of two different receptors at the same time. The NK cell activation receptor (so called killer activating receptor or KAR) that binds to target cell carbohydrate structures, and the killer inhibitory receptor (or KIR) specific for MHC class I molecules. The specific recognition of self-MHC class I molecules on target cells upon NK cell receptors may repress cytotoxic function of NK cells. Cytotoxicity may occur if target cells lose MCH class I expression, if NK cells lack the

appropriate killer inhibitory receptor or if there are changes in the structure of the peptide-MHC molecule that impair recognition [130]. Engagement of the MHC class I specific receptor transmits negative signals that override the action of KAR, probably downstream of early activation events. Multiple forms of each receptor, with potentially different specifities may be expressed by a single NK cell.

NK activity plays an important role during pregnancy : it was shown to display deleterious effects on fetal development, resulting in spontaneous abortion in mice [131]. On the other hand NK cells function is a first line of defence against infectious agents and malignant processes. Normal human pregnancy is characterized by low peripheral NK activity [132,133]. In humans increased NK activity seems to play a role in spontaneous abortions of unknown etiology, since it is well known that there is an increase in NK activity preceding all forms of spontaneous pregnancy termination [37,134]. Cytotoxic mechanisms exerted by NK cells can induce ablation of placenta, on the other hand , TNF-α produced by NK cells via facilitating prostaglandin synthesis induces uterine contractions and initiates the induction of labor. NK cells also play a physiological role in the regulation of haematopoesis, where their effects are exerted by cytokine production. In early human pregnancy the majority of uterine lymphocytes resembles phenotypically fetal NK cells. They are $CD56^{bright}$ granulated NK cells, which do not express CD16 or CD3 [135]. Only 10 % of peripheral NK cells shows similar characteristics. These NK cells show a low spontaneous cytotoxic activity in spite of their high perforin content, but when activated by IL-2 they kill trophoblast cells. Since in early pregnancy these cells are enriched at the implantation sites (where trophoblast infiltrates the decidua) it suggests that one of the functions of these cells is control of placentation.

In mice there is a direct evidence for the involvement of high NK activity in abortion. NK cell infiltration was demonstrated in damaged mouse fetuses and placentae. Adoptive transfer of high NK activity spleen cells to pregnant mice induces abortion[136].

The lack of harmful NK activity to the trophoblast during normal pregnancy is due to several mechanisms. The presence of HLA-G and HLA-E on the trophoblast provides protection againts NK activity partly due to recognition of these self MHC class I molecules via killer inhibitory receptors[137], partly due to inhibitory effect of HLA-G on transendothelial migration of NK cells, by which HLA-G may inhibit NK cell trafic across the placenta[138]. On the other hand the generalized shift of systemic T-cell responses to Th2, as well as the effect of PIBF are also responsible for the inhibiton of NK activity.

5. The immunologic effects of prostaglandins

The biochemical signals that initiate human parturition are not completely understood. There is a evidence that prostaglandins, in particular PGE2 and PGF2α, are important mediators in the onset of human labor by inducing myometrial contractions [139], ripening of the cervix [140] and membrane rupture [141]. Prostaglandins are produced by amnion, chorion, decidua, myometrium and placenta [142]. Many factors can increase prostaglandin production, including cytokines and growth factors [143,144,145,146].

The first step in the synthesis of prostaglandins is the hydrolysis of arachidonic acid from cell membrane phospholipids, predominantly by the action of phospholipase A2 (PLA2). There are multiple forms of PLA2 which include the cytosolic group IV (cPLA2) [147], the secretory group (sPLA2) [148] and a cytosolic Ca^{2+}-independent PLA2 (iPLA2) [149]. The free arachidonic acid can then be converted to the intermediates PGG2 and PGH2 by the action of prostaglandin H synthase (alternatively known as cyclo-oxygenase (COX) and then be further metabolised to prostaglandins, prostacyclin or tromboxanes. There are two isoforms of COX[150] : COX-1 is constitutively expressed, and COX-2, which can be upregulated in response to stimuli such as cytokines and growth factors [144,151]. Fetal membrane PLA2 activity increases throughout gestation and total cellular cPLA2 has also been found to be high before the onset of labor [152]. Futhermore there is evidence for increased amnion COX enzyme activity and mRNA expression of COX-2 at term [153].

Since inflammatory mediators such as IL-1β can rapidly induce cPLA2 and COX-2 mRNA and protein expression and activity, with a parallel increase in PGE2 synthesis in amnion-derived WISH cells[154] and also in villous and chorion trophoblast, it is possible that they are involved in the biochemical mechanisms of parturition. This hypothesis is supported by the data that IL-1β is found in increased levels in the aminotic fluid of women in preterm labor associated with infection [155] as well as spontanous labor at term [156]. IL-1ra has been found to prevent IL-1 induced preterm labor in mice [157]. Other pro-inflammatory cytokines such as TNF-α or IL-6 cause similar changes in COX-2 expression and prostaglandin output when added to fetal membranes in vitro [143,145]. Anti-inflammatory cytokines may oppose these effects. For example IL-10 inhibited the output of PGE2 from intact fetal membranes under

basal and LPS-stimulated conditions, and there was a parallel decrease in the expression of mRNA for COX-2 [158]. IL-4 also inhibits COX-2 mRNA and protein production in cytokine-stimulated WISH cells [159]. These data suggest that in inflammatory conditions, proinflammatory cytokines (such as IL-1, IL-6 and TNF−α) can influence PG output through effects on PG synthesis and metabolism and that these effects may be opposed by an antiinflammatory cytokine. These interactions may be important in the progression of preterm labor [100].

The stimulating effect of proinflammatory cytokines in prostaglandin synthesis can be inhibited by glucocorticoids and progesterone, since they downregulate cPLA2 and COX2 mRNA expression [160,161]. Other inhibitors of PLA2 catalysis, such as quinacrine inhibit IL-1 and TNF release from LPS-stimualted cells [162], while the activatos of PLA2, such as melittin, can cause cells to increase cytokine synthesis in the absence of LPS. Taken together, these data indicate a pivotal role for PLA2 in the regulation of cytokine production [163,164].

These findings suggest that COX-2 inhibitors may be useful tocolytics in the setting of preterm labor with occult chorio-decidual infection. Selective COX-2 inhibitors have been shown to block spontaneous uterine contractions in the rat and prevent preterm birth in humans [165,166]. Some authors described that PGE_2 inhibits lymphocyte function in vitro and it has been proposed to be important in suppressing maternal rejection of the implanted intrauterine conceptus [167]. Other observations gave no support to this concept. Studies by Hilkens et al have shown that the net modulatory effect of PGE2 on the cytokine secretion profile of T cells critically depends on the mode of T cell activation and consequently the availability of IL-2. Since this parameter varies with the experimental conditions and the T cell population studied, these findings may explain why certain immune responses may be either up- or down-regulated by PGE2 under different conditions [168]. On the other hand the effects of PGE2 on human peripheral blood lymphocyte responses to PHA are concentration-dependent. PBL response to PHA is stimulated at low concentrations of PGE series (10-7 M), whereas high concentrations (10-5 M) markedly inhibit the response [169]. High concentrations (10^{-6}–10^{-7} M) of PGE2 inhibit the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and activated killers from precursor cells. As with PGF2, lower concentrations of the E series (10^{-8}–10^{-9} M) enhance the generation of antigen-specific CTL but inhibit the generation of lymphokine and spontaneously-activated CTL [170]. Though PGE2 is the most studied prostaglandin, it is not the only one produced, and certainly not the only one endowed with an immunomodulatory potential. Ching et al. demonstrated a significant correlation between NK

cell cytotoxicity and the plasma prostaglandin F series concentration in cord blood [171]. Mice with ablated gene for the PGF2α receptor experience normal gestation but fail to initiate labour and delivery [172]. Our earlier studies reveled an increased PGF2α sensitivity of human peripheral lymphocytes together with a decreased PGE2 sensitivity during labor [173].

Both in human and rat decidua, the constitutive level of cyclooxygenase appears to be down-regulated. Smith and Kelly showed [174] that PGE_2 synthesis is normally suppressed in the first trimester decidua by a progesterone-dependent mechanism, and others found that PGE_2 is present at inceased concentrations in decidua of human and mouse spontaneous abortions [175]. Gerdon et al. [176] reported on increased PGE_2 levels in the decidua of DBA/2-mated CBA/J mice prior to the onset of resorption.

These results above questioned the in vivo relevance of PGE2 data obtained in vitro, gave no support to the view that PGE_2 represents an important intrauerine molecular blocking process and concluded that inhibition of PGE_2 synthesis by indomethacine (INDO) does not generally lead to maternal rejection of the conceptus [177]. Women who take INDO or acetylsalicylic acid (ASA) have not been reported to suffer a high abortion rate (even though clinically used doses of INDO and ASA appear to reduce PGE_2 production in vivo) as reflected in clinical improvement in conditions such as threatened abortion, polyhydramnions, and arthritic inflammation [178]. ASA treatment starting before implantation may reduce the rate of abortion in some types of patients suffering from recurrent miscarriages [179] furthermore, the frequency of post-maturity and the length of gestation were significantly increased in women who regularly took large doses of prostaglandin synthesis inhibitors [180]. As a site of action, the uterine smooth muscle has generally been accepted. Data above suggest that immunologic actions of prostaglandin synthesis inhibitors provide additional benefit to their known effects on the uterine musculature and blood supply.

III. AIMS OF THE STUDY AND RESULTS

1. The mechanims participating in the anti-abortive effect of PIBF in mice (paper 1.)

Immunologic effects of progesterone are mediated by a protein named the progesterone-induced blocking factor (PIBF) [51]. Lymphocytes of healthy pregnant women are able to produce PIBF and the percentage of PIBF-positive cells is significantly reduced in peripheral blood lymphocytes of recurrent aborters [52]. Among other effects this protein inhibits NK activity [38] and displays an antiabortive effect in mice [57]. Earlier data show that adoptive transfer of high NK activity spleen cells into pregnant mice induces abortion [136]. Simultaneous PIBF treatment of pregnant mice corrects the abortive effect of NK activity [57]. The above data provide indirect evidence for the importance of PIBF in maintaining normal gestation. Direct evidence for the biological significance of PIBF would be the induction of pregnancy loss by neutralization of endogenous PIBF. NK activity is one major component of natural cell-mediated cytotoxicity with natural cytotoxic (NC) activity being the second component [181]. Limited data indicate that NC activity is present in the decidua of pregnant mice [182].

Since PIBF is antiabortive in mice and inhibits NK activity in vitro, the present study was aimed at investigating wheter neutralization of endogenous PIBF activity in vivo results in pregnancy termination and if it does, what are the mechanisms that lead to this event.

We have shown that neutralization of endogenous PIBF activity results in pregnancy loss in mice. Both progesterone receptor block and anti-PIBF treatment induced an increased rate of resorptions, and in the former case pregnancy loss was due to the inability of spleen cells to produce PIBF. However, anti-PIBF treatment did not in all cases result in pregnancy termination, 23 % of mice did not respond. In non-responders, NK activity was significantly lower than that in good responders. It is conceivable that in these mice abortion did not occur because the spleen cells of the animals produced more than normal levels of PIBF, which was not all neutralized by the addition of anti-PIBF and abortion was not induced.

NK and NC activities are probably the most important effector pathways in the fetomaternal immunological relationship. NK activity has been shown to play role in spontaneous pregnancy termination [131,132,134]. Previously we showed that PIBF exerted a marked inhibitory effect on NK activity in vitro [38]. NK activity is the major factor in spontaneous abortions in mice. In murine systems increased NK activity results in pregnancy termination [136], which can be corrected by simultaneous PIBF administration [57].

Our results show that treatment of anti-PIBF-treated mice with an anti-NK antibody (anti-NK-1.1 monoclonal antibody, PK136) corrected the resorption rates and the mean number of implantation sites. Anti-NC (1C4) treatment that neutralize cytotoxic cells also completely corrected the anti-PIBF induced resorptions. The third monoclonal antibody (2B6-F2) reduces both NK and NC activity in Balb/c mice [183], and corrected both high resorption rates and low implantation rates due to the lack of PIBF. *Our findings suggest that both NC and NK activity are involved in pregnancy termination in Balb/c mice, and that PIBF exerts its antiabortive effect via inhibition of nonspecific effector mechanisms.*

2. The in-vivo effect of PIBF on cytokine production, and the relationship between cytokine production, NK activity and pregnancy loss in mice (paper 2.)

A long line of evidence supports the role of nonspecific immunologic mechanisms in pregnancy loss both in mice and humans. Natural killer (NK) activity is decreased in human pregnancy, whereas spontaneous pregnancy termination is associated with increased NK activity [173]. There is direct evidence for the role of high NK activity in pregnancy termination in mice. Modulation of NK activity influences resorption rates [131]. NK activity in the decidua of pregnant mice is mediated in part by NC cells [182].

Normal pregnancy is characterized by decreased cell-mediated responses [184, 185] and an increased rate of antibody production, indicating a Th2-biased immune response. In experimental conditions the outcome of pregnancy can be influenced by modulating the cytokine balance. The administration of tumor necrosis factor alpha (TNF-α), interferon (IFN-γ) or interleukin-2 (IL-2) to normal pregnant mice causes abortions [186,187.] Th2-type cytokines IL-4, IL-5 and IL-10 are detectable in murine feto-placental units in all three trimesters of pregnancy [188]. It has been shown that PIBF alters the profile of cytokine secretion by activated lymphocytes, since IL-3, IL-4 and IL-10 production of ConA-activated murine spleen cells was significantly increased in the presence of PIBF [189]. Progesterone has been shown to exert a positive effect on the induction of IL-5 gene expression in T cell lines [190]. Among other effects PIBF inhibits NK activity and displays an anti-abortive effect in mice [38,183] . Our previous data revealed an association of pregnancy termination and the lack of lymphocytic PIBF positivity, as well as a negative correlation between PIBF production and NK activity in

humans. Thus, PIBF determines the relationship between pregnancy termination and increased NK activity.

The mechanism through which PIBF influences NK activity is manifested via multiple systems, and the relationship between the different pathways has not been clarified yet. Since PIBF affects cytokine secretion of in vitro activated lymphocytes, its action on NK activity might involve cytokine effect, because though the trophoblast resists NK-mediated lysis in vitro, it is susceptible to lysis by lymphokine-activated killer (LAK) cells [191].

Our study was aimed at investigating the in vivo effect of PIBF on cytokine production as well as the relationship between altered cytokine production, NK activity, and pregnancy loss in anti-PIBF treated mice.

In pregnant mice, the neutralization of endogenous PIBF activity, resulted in a high resorption rate and NK activity, together with decreased splenic IL-10 production. Because IL-10 inhibits cytokine production by Th1-type cells as well as CD8+ T cells, the lack of IL-10 production might be one of the factors responsible for high NK activity. The treatment resulted in an increased percentage of IFN-γ-positive spleen cells as well. We found a positive relationship between NK activity and the percentage of IFN-γ positive spleen cells. Futhermore in animals with a high resorption rate the rate of splenic IFN-γ expression was significantly higher than in those with a low resorption rate. Splenic IL-10 production, on the other hand, was inversely related to resorption rates.

Earlier (paper1) we have shown that treatment of anti-PIBF-treated mice with anti-NK or anti-NC monoclonal antibodies (PK136, 1C4, 2B6F2) corrected the high resorption rates and the low mean implantation sites. The PK136 antibody reacts with LGL-1-cells. This functional subset of NK-1.1$^+$ cells that contains the majority of LAK cell progenitors [192]. The trophoblast is resistant to lysis by NK cells, but it is lysed by LAK cells [191], thus the latter cell type may be involved in trophoblast damage. The antibody 1C4 reacts with natural cytotoxic cells, and the third monoclonal antibody (2B6F2) reduces both NK and NC activity in Balb/c mice.

In the present experiments injection of anti-PIBF-treated mice with anti-NK and anti-NC monoclonal antibodies corrected the high splenic IFN-γ, and decreased IL-10 production. The highest inhibiton in the ratio of IFN-γ positive spleen cells was obtained using the antibody 2B6F2, which neutralizes both NK and NC activity. Since neutralization of NK and NC activity in anti-PIBF treated mice corrected not only resorption rates but also cytokine values, this implies that NK cells are not simply targets of cytokines but these cells are

producers of the cytokines themselves. In mouse IL-10 is produced by Th2 cells as well as by macrophages and B cells [193,194]. IFN-γ is produced by NK cells. Our findings are in accordance with the results of Clark and Chaouat [192] showing that not NK activity per se, but cytokines produced by the NK cells are responsible for the high resorption rates in mice.
Our data suggest that PIBF contributes to the success of gestation via cytokine-mediated inhibition of NK activity.

Fig. 1. The putative mechanism of progesterone dependent immunomodulation and the effects of RU486, anti-PIBF and anti-NK treatment in murine pregnancy

3. The relationship between the effect of PIBF on arachidonic acid metabolism and the IL-12 expression in humans (paper 3. and paper 4.)

PIBF exerts a strong anti-natural killer activity [51]. The mechanism through which PIBF influences NK activity is manifested via multiple systems, and the relationship between the

different pathways has not been clarified yet. We have shown that PIBF affects cytokine secretion, via increasing IL-10 and decreasing IFN-γ and IL-12 production, PIBF inhibits NK cell cytotoxicity [189].

Earlier data from this laboratory show that PIBF inhibits arachidonic acid (AA) release from mononuclear cells. In vitro AA increased the cytotoxicity by peripheral blood lymphocytes. Although the phospholipase inhibitory potential of our substance has not been tested, it is likely that PIBF acts before the level of the cyclooxygenase and lipooxygenase enzymes, since its blocking effect on cytotoxic activity was voided in the presence of exogenous AA [195]. Our previous observations revealed a relationship between $PGF_{2\alpha}$ levels, progesterone binding capacity and cytotoxic activity of the lymphocytes [196]. Earlier we showed that LPS selectively stimulated prostaglandin synthesis and enhanced cytotoxicity [196].

IL-12 stimulates cytotoxic NK activity, therefore in this study we investigated the effect of LPS on IL-12 production by peripheral blood mononuclear cells. LPS treatment significantly increased the percentage of IL-12 positive cells compared to the control. In a previous experiment we demonstrated a significant reduction of cytotoxicity as a result of treatment with the specific cyclooxygenase inhibitor, indomethacin (INDO). Therefore we investigated the effect of INDO on LPS induced IL-12 production. In our hands inhibition of prostaglandin synthesis by either blocking the phospholipase A2 enzyme or the cyclooxygenase enzyme counteracted the effect of LPS on IL-12 expression, thus LPS possibly acts on IL-12 production by increasing prostaglandin synthesis.

We also investigated the relationship between the effect of PIBF on AA metabolism and IL-12 production. Neutralization of endogenously produced PIBF by anti-PIBF antibody stimulated IL-12 production, while control antibody (preimmune polyclonal rabbit IgG) and INDO did not cause any change in the IL-12 expression. PIBF blocks cytotoxicity by inhibiting arachidonic acid liberation from phospholipids. Since anti-PIBF treatment significantly increased IL-12 production, we investigated whether a simultaneous treatment with quinacrine (that blocks phospholipase A2 enzyme) or INDO (a known inhibitor of cyclooxygenase) could reduce the effect of anti-PIBF on IL-12 production. Both quinacrine and INDO treatment corrected anti-PIBF induced increased IL-12 production, suggesting that *PIBF inhibits IL-12 production via an action on arachidonic acid metabolism.*

Prostaglandin synthesis inhibitors were reported to be effective in the treatment of threatened abortion and preterm labor in humans, although the value of indomethacin treatment for threatened abortion has been questioned. As a site of action, the uterine smooth

muscle has generally been accepted. The present data suggest that immunological actions of prostaglandin synthesis inhibitors provide additional benefit to their known effects on the uterine musculature and blood supply.

Earlier we reported that progesterone binding capacity in lymphocytes of patients at risk for premature pregnancy termination is impaired in comparison with those from healthy pregnant women [40]. Lymphocytes from the former patients are unable to bind a sufficient amount of progesterone, therefore no PIBF is released, thus arachidonic acid metabolism proceeds normally and results in high IL-12 production and consequently higher cytotoxic activity. This concept is supported by clinical observations of Varga et al. [197] which revealed a beneficial effect of low dose aspirin treatment of recurrent aborters, selected on the basis of repeatedly high NK activity and reduced PIBF producing capacity of their lymphocytes. Aspirin treatment reduced NK activity and resulted in an 82 % success rate in contrast to the untreated group, where the success rate was 44 %.

On the basis of these findings we suggest the following mechanism: progesterone binding of the lymphocytes is followed by the release of PIBF that affects arachidonic acid release. The subsequent block of prostaglandin synthesis reduces IL-12 production and result in a lowered NK activity which favors a normal pregnancy outcome.

IV. THESES

1. The lack of PIBF effect results in pregnancy termination in mice.

Neutralisation of endogenous PIBF activity results in pregnancy loss in mice. Both progesterone receptor block (with RU486) and anti-PIBF treatment induced an increased rate of resorptions, and in the former case pregnancy loss was due to the inability of spleen cells to produce PIBF.

2. The lack of PIBF effect results in increased NK and NC activity in mice.

Both anti-PIBF and RU 486 treatments resulted in increased NK activity of the spleen cells. There was a positive relationship between NK activity and the rate of resorptions.

3. The anti-abortive effect of PIBF is mediated via controlling NK activity in mice.

Anti-PIBF treated mice showed a significantly increased resorption rate and reduced number of implantation sites. Mice simultaneously treated with anti-PIBF and anti-NK1.1 (PK136), anti-NC 1.1 (1C4) or anti-Ly-6c (2B6F2) did not show significantly altered resorption rates and the mean numbers of implantation sites were also comparable to untreated controls.

4. In vivo inhibition of progesterone-dependent immunomodulation results in altered cytokine production in mice.

Anti-PIBF treatment resulted in an increased percentage of IFN-γ positive spleen cells, and decreased splenic IL-10 production. There is a positive relationship between NK activity and the percentage of IFN-γ positive spleen cells. In animals with a high resorption rate the rate of splenic IFN-γ expression was significantly higher than in those with a low resorption rates. Splenic IL-10 production, on the other hand, was inversely related to resorption rates.

5. Cytokines produced by the NK cells are responsible for the high resorption rates in anti-PIBF treated mice.

Neutralization of NK and NC activity in anti-PIBF treated mice corrected not only resorption rates but also cytokine values, suggesting that NK cells are not simply targets of cytokines, but these cells are producers of the cytokines themselves.

6. Lipopolysaccharide stimulates IL-12 production by increasing prostaglandin synthesis in human pregnancy lymphocytes.

Lipopolysaccharide treatment of pregnant lymphocytes significantly increased the percentage of IL-12-positive cells compared with the control cells. Inhibiton of arachidonic acid release (by blocking of the phospholipase A2 enzyme) or prostaglandin synthesis (by blocking of the cyclooxygenase enzyme) counteracted the effect of lipopolysaccharide on IL-12 production. The inhibitory effect of quinacrine or indomethacin on lipopolysaccharide induced IL-12 production was concentration dependent.

7. PIBF inhibits IL-12 production via an action on arachidonic acid metabolism.

Neutralization of endogenously produced progesterone-induced blocking factor by anti-PIBF antibody increased IL-12 production. Simultaneous treatment with the phospholipase A2 inhibitor quinacrine or the cyclooxygenase inhibitor indomethacin could reduce the stimulating effect of anti-PIBF on IL-12 production.

CONCLUSIONS

In pregnancy chronic alloantigeneic stimulation due to the presence of the fetus activates decidual γ/δ T cells. These activated γ/δ T cells express progesterone receptor. In the presence of progesterone, progesterone receptor positive lymphocytes produce a 34 kDa protein, named the progesterone-induced blocking factor (PIBF). PIBF appears on the lymphocytes of healthy pregnant women, but not on those from pathological pregnancies. The percentage of positive cells is significantly lower among PBL of women showing clinical symptoms of treatened preterm deliveries. NK activity of PBL is inversely correlated to PIBF positivity of the lymphocytes. Thus, PIBF determines the relationship between pregnancy termination and increased NK activity. The mechanism through which PIBF influences NK activity is manifested via multiple systems.

- PIBF interferes with arachidonic acid metabolism. Via inhibiting the release of arachidonic acid, PIBF reduces the rate of prostaglandin production, which has a major role in the initiation of labor. The effect of PIBF on NK activity is mediated by cytokines. In anti-PIBF treated pregnancy lymphocytes we observed a significantly increased IL-12 production, which was corrected by quinacrine or indomethacin. These data suggest, that the phospholipase A2 and cyclooxygenase pathway is involved in the action of PIBF on IL-12 production, and in return on its suppressive effect on NK activity.

- PIBF alters the Th1/Th2 balance by stimulating the secretion of Th2-type cytokines (ec. IL-3, IL-4, IL-10) as well. This altered cytokine ratio contributes to decreased cell-mediated responses and increased antibody production. Besides influencing the rate of antibody synthesis, PIBF acts on the quality of antibody production.
- In the presence of PIBF, B cells produce hypermannosylated (asymmetric) antibodies. These antibodies although bind the antigen with the same specificity as conventional conterparts, these Ig molecules are generally unable to activate effector functions and thus might play a role in the protection of the fetus.
- Recent data revealed that PIBF blocks NK activity by inhibiting degranulation and perforin release of CD56+ lymphocytes, therefore it cannot be excluded, that low NK activity of decidual NK cells is due to the high presence of PIBF in the decidua.

In conclusion via these multiple systems PIBF reduce NK cytotoxic activity that favours a normal pregnancy outcome.

Fig. 2. The role of PIBF in normal pregnancy

V. MATERIALS AND METHODS USED IN THIS STUDY

1. Separation of peripheral blood lymphocytes

Heparinized venous blood was obtained from healthy pregnant women between the 16^{th} and 26^{th} week of gestation. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-plaque gradient (Pharmacia, Uppsala, Sweden) and washed in Roswell Park Memorial Institute (RPMI) 1640 medium (GibcoBRL, Life Technologies, Paisley, Scotland). Lymphocytes were resuspended in RPMI 1640 containing 10 % fetal calf serum (GibcoBRL, Life Technologies, Paisley, Scotland) and adjusted to a cell count of $1x10^6$/ml.

2. Treatment of peripheral blood lymphocytes

Lipopolysaccharide (prepared from second phase Shigella sonnei in this Institute) was diluted μg/ml in RPMI 1640 medium. Indomethacin (Sigma Chemical Co., St. Louis , MO) was dissolved in ethanol and further diluted in RPMI medium from 0.01 μg/ml up to 5 μg/ml concentrations. Stock solutions were always freshly prepared before the incubations. PIBF specific polyclonal antibody was prepared in this laboratory as described earlier (17) and used at a concentration of 400 μg/ml. One million lymphocytes were incubated for 3 h at 37 C^0, 5 % CO_2 with the following : a, medium (RPMI medium plus 10 % fetal calf serum); b, medium containing 10 μg/ml LPS; c-f, medium containing LPS and 0.01μγ/ml, 0.1 μg/ml, 1 μg/ml and 5 μg/ml indomethacin; g, medium containing 400μg/ml anti-PIBF; h, medium containing anti-PIBF and 5 μg/ml indomethacin; i, medium containing 5 μg/ml indomethacin; j, 400μg/ml preimmune rabbit polyclonal IgG antibody was used as control.
At the end of the incubation the cells were washed twice in RPMI 1640 medium and centrifuged on glass microscope slides.

3. Immuncytochemistry

Human peripheral blood mononuclear cells or mouse spleen cells were washed twice in RPMI 1640 medium and centrifuged on glass microscope slides. The slides were dried at room temperature, the cells were fixed for 5 min. in ice cold acetone and washed in tris buffered saline (TBS). All incubations were carried out at room temperature in humid

chamber. After blocking endogenous peroxidase activity with 1 % H_2O_2 the cells were further incubated in TBS containing 1 % bovine serum albumin (BSA, Sigma Chemical Co., St. Lois, MO) for blocking nonspecific protein binding. The following Ab-s were used as primary antibodies: mouse anti-human IL-12 Mab (purchased from R&D Systems, Abingdon, Oxon, UK), anti-mouse IL-10- and IFN-g Mab (both from Endogen, Cambridge, MA), rabbit anti-mouse anti-PIBF IgG. Anti-human and anti-mouse IgG1 ab and IgG from nonimmunized rabbits gave the isotype controls. The monoclonal antibodies were diluted 1 : 50 in TBS supplemented with 0.5 % BSA. Anti-PIBF was added at 10 μg/ml. The cells were incubated for 1 h at room temperature in a humidified atmosphere. Secondary antibodies (horseradish peroxidase-labeled-anti-mouse-, anti-goat- and anti-rabbit IgG) purchased from Dako (Denmark), were applied at dilution of 1 :100 or 1:200 for an additional 30 or 45 minutes. For the detection of cytokines the reaction was developed by diaminobenzidine (Sigma Chemical Co., St. Lois, MO) followed by silver intensification. Anti-PIBF positive cells were visualized by aminoethylcarbasol. The nuclei were counterstained with haematoxylin and the slides were mounted with gelatin-glycerol. The slides were read in a blinded fashion and the percentage of positive cells was determined after at least 500 lymphocytes were counted in the microscope at high power magnification.

Fig. 3. Figure of a IL-12 positive lymphocyte

4. Determination of cytokines by ELISA

Spleen cells from treated and untreated pregnant Balb/c mice were washed and, the cell count was adjusted to $1x10^6$/ml. The cells were activated with 1 μg/ml ConA (Sigma Chemical Co., St. Lois, MO) for 48 hr. After 48 hr the supernatants were collected and tested for IL-10. For IL-10 determination we used a Biotrak (Amersham. Little Chalfont, UK) kit. The assay was preformed following the steps suggested by the manufacturer.

5. Production of human and murine PIBF

Lymphocytes from healthy blood donors or spleen cells of 10-week-old Balb/c mice (Lati, Godollo, Hungary) were adjusted to a cell count of $1x10^6$/ml in RPMI supplemented with 10 % fetal calf serum (Gibco, Grand Island, NY) and were stimulated by 1 μg/ml of ConA (Sigma Chemical Co., St. Lois, MO) for 48 hr at 37^0C in CO_2 incubator. The cell number was then adjusted to $10x10^6$/ml and the cells were further incubated with 20 μg/ml of progesterone for 16 hr. At the end of the incubation period supernatants were collected. Progesterone was removed by dialysis. The supernatants were then 2000-fold concentrated on Amicon filters and used as the source of the human or murine PIBF.

6. Production of PIBF-specific IgG

The PIBF containing supernatants were subjected to SDS-PAGE on 12 % polyacrylamide gels. The separated bands were blotted to nitrocellulose filters, the 34-kDa band was cut out, dissolved in DMSO, and injected into rabbits weighting 4 kg each, together with complete Freund's adjuvant. Boosters with incomplete Freund's adjuvant were given at two weekly intervals. IgG was purified on protein A columns. The PIBF-specific antibody content was tested by ELISA.

7. Treatment of pregnant mice by RU486, anti-PIBF, anti-NK and anti-NC antibodies

Fourteen-week-old Balb/c mice (LATI, Godollo, Hungary) were kept under standard conditions (4 animals per cage). Female mice were caged overnight with the males and checked for the presence of vaginal plugs the following morning. The day on which the plug

was observed is considered to be Day 0.5 of pregnancy. Pregnancy was later verified by scoring corpora lutea. Various treatments were administered on Day 8.5 of pregnancy.

a. Females were injected intraperitoneally with 0,3 mg/kg RU 486 or with 0.5 mg of rabbit anti-PIBF IgG. Mice treated with the same amount of normal rabbit serum or untreated mice of similar gestational age were used as controls.

b. A group of anti-PIBF-treated mice was at the same time injected with monoclonal antibodies to cells mediating natural killer (NK), natural cytotoxic (NC), or natural T cell (NT) activity. The monoclonal antibodies were:

   1. PK136 (anti-NK-1.1) recognizes a 76- to 80 kDa type II integral membrane C-type lectin protein encoded by a member of the mouse NKR-PI gene family [198,199].

   2. 1C4 (anti-NC-1.1) which recognizes a 45-kDa surface receptor and blocks spleenic natural cytotoxic (NC) activity approximately 70% both in vitro and in vivo [200,201].

   3. 2B6-F2 (anti-Ly-6c) identifies a subpopulation of murine Ly-$6c^+$ $NK1.1^+$ natural T (NT) cells [202,203]. In the presence of complement 2B6-F2 reduces splenic NK activity by approximately 50% in Balb/c mice.

The monoclonal 2B6-F2 antibody (kind gift from the laboratory of Smart YC (University of Newcastle, N.S.W., Australia) and PK136 antibody (obtained from American Tissue Type Collection, USA) were used as serum-free supernatants and administered at a dose of 50 μg/mouse, whereas 1C4 (kind gift from the laboratory of Smart YC YC (University of Newcastle, N.S.W., Australia) was affinity-purified IgG and administered at a dose of 25 μg/mouse.

The animals were sacrificed on Day 10.5 and their uteri were inspected. The ratio of living versus resorbed embryos was determined. Spleens were removed under aseptic conditions, cell suspension was prepared, and cytotoxic activity was determined in a 16-hr test against human embryonic fibroblast targets. NK activity was also tested using the 4- hr single-cell cytotoxicity assay against YAC targets. Smears were prepared and the expression of PIBF, IL-10 and IFN-γ on the lymphocytes was determined by immuncytochemistry.

8. Cytotoxic assays

1. ***16-hr Cytotoxicity Assay***

   Human embryonic fibroblast derived from 10- to 12-week embryos were used as targets. Cells were seeded on 96-well Nuclon tissue culture plates at a density of 5000 target cells/well in 0.2 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (GibcoBRL, Life Technologies, Paisley, Scotland). The target cells were allowed to attach by overnight incubation. The following day the medium was replaced by 0.2 ml of lymphocyte suspension containing $5x10^5$ lymphocytes. After 16 hr of incubation, the plates were washed with PBS three times in order to remove lymphocytes and damaged target cells. This was followed by addition of alkaline phosphatase substrate (Sigma tablets No. 104.) in diethanolamine buffer at a concentration of 1 mg/ml. The plates were incubated for 10 min at 37^0C in the dark and the resulting yellow reaction product was quantified photometrically at 405 nm. The percentage reduction in enzyme activity relative to the target cell control was considered as a measure of cytotoxicity.

2. ***4-hr Single Cell Cytotoxicty Assay for NK activity***

   We used the assay originally described by Grimm and Bonavida [204]. Briefly, 100 μl of lymphocytes and the same amount of YAC target cells (2 x 10^6 cells/ml each) were centrifuged at 500 rpm for 5 min and incubated at 37^o C, in 5% CO_2 for 10 min. The pellets were then resuspended and 200 ul of 1% agarose (Serva, Heidelberg, Germany) in RPMI-1640 medium (GibcoBRL, Life Technologies, Paisley, Scotland) was added to the mixture. One hundred μl of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and submerged in RPMI-1640 medium. The slides were incubated for 4 hr at 37^o C in 5% CO_2. Then the gels were stained with 0.5% Trypan blue for 1 min. After 2 min washes with phosphate buffered saline, the gels were fixed in 2% formaldehyde for 5 min and desalted in distilled water. The slides were read using a light microscope with x 400 magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 100 lymphocytes. Results are expressed as a

percentage of target binding cells. Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates and results are expressed as a percentage of dead conjugates (cytotoxic binding cells). The percentage of NK cell activity was calculated according to the formula NK%= (target binding cells% x cytotoxic binding cells %)/100. All results for cytotoxic target binding cell % were corrected for the proportion of target cells that died spontaneously in control plates.

Fig. 4. Methods used in the murine experiments

9. Statistics

The two tailed Student t test and the χ^2 test as well as one-way analysis of variance with the Bonferroni correction were used for statistical evaluation of the data. Mean +/- SEM are indicated in the table and figures. Differences were considered significant if P value was equal or less than 0.05.

VI. PAPERS

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