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T Lymphocyte Characteristics and Immune Tolerance During Human Pregnancy

Gergely Toldi, András Treszl and Barna Vásárhelyi
*Research Group of Pediatrics and Nephrology, Hungarian Academy of Sciences
Hungary*

1. Introduction

To ensure a fruitful and healthy pregnancy, every maternal organ system needs to adapt to the novel physiological needs raised by the developing fetus. The maternal immune system is no exception. Since the conceptus is half of foreign origins, presenting paternal antigens, it is considered a semi-allograft to maternal immunity. Therefore, an immune tolerance must develop to avoid immunological rejection of the fetus. In the normal course of pregnancy, the mother extends her 'definition of self' for 40 weeks on the foreign antigens of the fetus, and the conceptus is accepted by the mother's immune system. The impairment of this tolerance and the development of an abnormal immune response directed at the fetus play a major role in adverse pregnancy outcomes, including spontaneous abortion, preterm labour and preeclampsia. In recurrent abortion and preeclampsia, abnormal maternal immune reactions have an autoimmune character, and the disorders resemble many features typically seen in autoimmune diseases, or in association with autoimmune reactions. Although this does not mean that recurrent abortion or preeclampsia should be considered autoimmune conditions, it still suggests that abnormal autoimmune processes play an important role in their pathogenesis. In this regard, preeclampsia mimics autoimmune responses observed in both allograft rejection and graft-versus-host disease.

Several aspects of the development of the pregnancy-specific immune tolerance have been described recently. Initially, the contact between maternal and fetal cells is taking place on a local level and is restricted to the decidua, but during the second trimester of pregnancy, it is extended to the entire body of the mother. Both the innate and adaptive arms of immunity are involved in these events. In this chapter we will focus on the role of T lymphocytes, the adaptive cellular elements of the immune system. We will discuss the characteristic alterations of T lymphocyte subsets in prevalence and functionality in healthy and pathologic development of the immune tolerance in human pregnancy.

2. Th1 and Th2 cells

T helper (Th) lymphocytes are traditionally classified into the Th1 and Th2 subsets based on their cytokine production pattern (Romagnani, 1991). The most important cytokines produced by Th1 cells are interleukin-2 (IL-2), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). Considered to be the main effectors of phagocyte-induced host defense, these cells are highly protective against infections sustained by intracellular agents. On the

other hand, Th2 cells produce IL-4, which stimulates IgE and IgG1 antibody production, IL-5, which promotes the growth and differentiation of eosinophils, and IL-10 and IL-13, which together with IL-4 inhibit macrophage functions. The Th2 subset is mainly responsible for phagocyte-independent host defense, for example against helminthic infections.

For many years, it was hypothesized that normal pregnancy induces a shift from Th1 immunity towards Th2 immunity. However, it has been demonstrated recently that the levels of particular Th1 cytokines are raised, instead of lowered in normal pregnancy compared with the non-pregnant state (Challis et al., 2009). Current findings indicate that gravidity is both a pro-inflammatory and an anti-inflammatory condition, depending upon the stage of gestation. Grossly, pregnancy has three distinct immunological phases. The events of implantation and the first trimester require a strong inflammatory response to ensure the adequate remodelling of the uterine epithelium and the removal of cellular debris following the implantation of the blastocyst. Therefore, healthy pregnancy cannot be regarded as merely a state of relative immunosuppression, as once thought. On the contrary, by means of various cytokines, successful implantation is dependent upon the active contribution of the maternal immune system to stimulate adequate invasion of the trophoblastic tissue into the maternal uterine wall. Thus, the first trimester of pregnancy is hallmarked by pro-inflammatory events. The second immunological phase of gravidity consists of the second and third trimesters. This is the period of fetal growth and development, when an anti-inflammatory state is established. Finally, delivery represents the third immunological phase of pregnancy, when pro-inflammatory events dominate again and promote uterine contractions to deliver the fetus and the placenta (Mor & Cardenas, 2010).

IFN- γ appears to be a key cytokine in the regulation of pregnancy related inflammatory events. Under pathologic conditions with insufficient immune tolerance such as in preeclampsia, IFN- γ production is significantly increased compared with healthy pregnancy (Piccinni, 2010). In mice, it was reported that IL-4, IL-5 and IL-10 are detectable at the fetomaternal interface during all periods of gestation, whereas the presence of IFN- γ is transient, being detectable only in the first period (Lin et al., 1993; Wegmann et al., 1993). Although decidual NK cells are able to produce IFN- γ (Ashkar et al., 1998), they do not have a central role in fetal allograft rejection, since they do not express receptors specific for antigens and thus are not sensitive for the presence of paternal alloantigens. The significance of local Th2-type cytokine production in the decidua has been observed in humans as well. Piccinni et al. measured cytokine production in decidual CD4 cells isolated from women with recurrent spontaneous abortions. Compared with women with a normal pregnancy, the decidual CD4 cells from women with abortion show a defect in IL-4 and IL-10 production (Piccinni et al., 1998).

The antigens of the developing fetus are present at two interfaces as pregnancy progresses. The first interface is found between the invasive extravillous cytotrophoblast and maternal immune cells in the decidua. This local, tissue interface is of importance for immune adaptation during implantation. The second interface is that between the syncytiotrophoblast and the immune cells in maternal blood. This systemic interface is established at about the 10th gestational week and becomes increasingly important in the second half of pregnancy (Sargent et al., 2006a). Two contrary requirements influence the extent of invasion by fetal extravillous cytotrophoblast cells in the maternal decidua: the anchorage of the placenta to ensure fetal nutrition and the protection of the uterine wall against over-invasion (von Rango, 2008). If, due to excessive immunological tolerance and

acceptance of trophoblast cells, the uterine wall is over-invaded, pathologic conditions, such as placenta accreta, increta or percreta might develop (Bulmer, 1992). If the adequate invasion of the uterine spiral arterioles by extravillous cytotrophoblasts does not occur, this sets up the conditions for placental hypoxia and oxidative stress that eventually triggers a maternal systemic inflammatory response, leading to clinical manifestations of preeclampsia (von Rango, 2008).

This disorder is characterized by hypertension, proteinuria, edema and endothelial dysfunction generally evolving in the third trimester of pregnancy; however, it may also occur earlier. Although preeclampsia is quite common (i.e. it affects about 5-8% of all pregnancies globally), its clear cause and the mechanisms leading to immune dysfunction remain to be elucidated. Preeclampsia is estimated to be responsible for about 70,000 maternal deaths each year worldwide (Walker, 2000). HELLP syndrome (consisting of hemolysis, elevated liver enzymes, low platelet count) and eclampsia are other manifestations of the same disorder. Although these conditions are generally coupled with a number of other symptoms (including headaches, abdominal pain, nausea, vomiting, abnormal vision, dyspnoea, anxiety, mental confusion, seizures), these manifestations are not necessarily more serious than preeclampsia. Besides a maternal systemic inflammatory response, signs of systemic vasoconstriction may also be observed in the mother in these pregnancy-associated disorders (Baumwell & Karumanchi, 2007).

In preeclampsia, the anti-inflammatory state during the second and third trimesters develops insufficiently (Saito et al., 2007). An excessive maternal systemic inflammation is considered to be a dominant component in the pathogenesis of this pregnancy-specific disorder, since its important feature is the absence of Th2 skewness and thus the predominance of pro-inflammatory cytokines. Saito et al. reported on their observations regarding higher prevalence of IFN- γ and lower prevalence of IL-4-producing CD4 lymphocytes among peripheral blood mononuclear cells (PBMCs) of preeclamptic women compared with healthy pregnant women. Furthermore, the percentage of Th1 and Th2 cells and the Th1/Th2 ratio correlated with IFN- γ and IL-4 secretion levels (Saito et al., 1999a). In another study, this group observed increased production of IL-2, IFN- γ and TNF- α by PBMCs in preeclampsia and, interestingly, a positive correlation between mean blood pressure and Th1 cytokines (Saito et al., 1999b). The shift to a predominant Th1-type immunity in preeclampsia is reinforced by other experiments on intracellular cytokine measurements in T and NK cells, as well as by the assessment of cytokine secretion levels of PBMCs isolated from preeclamptic patients. (Azizieh et al., 2005; Darmochwal-Kolarz et al., 2002; Rein et al., 2002).

3. The influence of galectin-1 on the Th1/Th2 cell ratio

Previous studies demonstrated that soluble factors may also play a role in the development of the Th2 shift characteristic for healthy pregnancy. Such a factor is galectin-1, also produced by peripheral lymphocytes. Galectin-1, a 14 kDa protein, is a β -galactoside-binding mammalian lectin. Within the immune system, it is expressed by activated T, B and NK cells as well as macrophages (Blaser et al., 1998; Koopman et al., 2003; Rabinovich et al., 1998; Zuniga et al., 2001). Galectin-1 exerts immunoregulatory effects through various mechanisms. By binding to the cell surface glycoproteins, it inhibits T cell proliferation and induces apoptosis of activated Th1, Th17 and CD8 cells (Blaser et al., 1998; Perillo et al., 1995; Toscano et al., 2007). Galectin-1 has been demonstrated *in vitro* to inhibit T cell

adhesion to the extracellular matrix and to abrogate the secretion of proinflammatory cytokines (Rabinovich et al., 1999a). Furthermore, *in vivo* administration of galectin-1 in experimental models of autoimmunity skewed the balance toward a Th2-dominant cytokine profile (Rabinovich et al., 1999b; Toscano et al., 2006). Recent data show that galectin-1 promotes fetomaternal tolerance, since treatment with recombinant galectin-1 prevented fetal loss in an abortion-prone mouse model. The protective effect of galectin-1 was abrogated in regulatory T cell (Treg) depleted mice (Blois et al., 2007). Garin et al. showed that Tregs selectively up-regulate galectin-1 expression (Garin et al., 2007). Experiments using galectin-1 homozygous null mutant mice showed a reduced regulatory activity in Tregs and the blockage of galectin-1 binding diminished the inhibitory effects of human and mouse Treg cells (Rabinovich et al., 1998). These findings suggest that Tregs expressing galectin-1 may also support the acceptance of the fetus by maternal immune cells.

In a recent study, we measured circulating galectin-1 and anti-galectin-1 autoantibody levels, as well as intracellular galectin-1 expression of unstimulated peripheral blood T and NK cells in healthy pregnancy and preeclampsia (Molvarec et al., 2011). Our findings indicate that the majority of CD4⁺ and CD8⁺ T cells and NK cells express intracellular galectin-1 in healthy pregnant women, while only a small fraction of them do so in healthy non-pregnant women. In preeclampsia, the proportion of galectin-1-expressing peripheral T and NK cells was markedly decreased compared with healthy pregnancy. However, circulating levels of galectin-1 and anti-galectin-1 autoantibodies were not altered in preeclamptic patients as compared to healthy pregnant women, nor were related to the proportions of galectin-1-expressing peripheral blood lymphocytes in any of the study groups.

While Th1 and Th17 cells are susceptible to galectin-1-induced cell death, Th2 cells are protected from galectin-1 due to the differential sialylation of their cell surface glycoproteins. Indeed, galectin-1-deficient mice were shown to develop greater Th1 and Th17 responses (Toscano et al., 2007). Therefore, it is tempting to speculate that decreased production of galectin-1 by circulating T and NK cells might contribute to the development of the pro-inflammatory Th1 and Th17 immune responses, which are characteristic features of preeclampsia (Saito et al., 1999b; Toldi et al., 2011a).

4. Regulatory T cells

The recent discovery of a distinct T helper lymphocyte subset, referred to as Th17 cells, led to the transformation of the Th1/Th2 paradigm of immunity into a four-component model. This novel viewpoint incorporates Th1, Th2, Th17 and regulatory T cells (Tregs) as elements of a complex and mutually interacting network in the establishment of pregnancy-specific immune tolerance. Indeed, besides the imbalance of Th1 and Th2 cells, alterations of the prevalence of Th17 and Treg cells have been suggested to be of importance in the pathogenesis of adverse pregnancy outcomes (Saito, 2010).

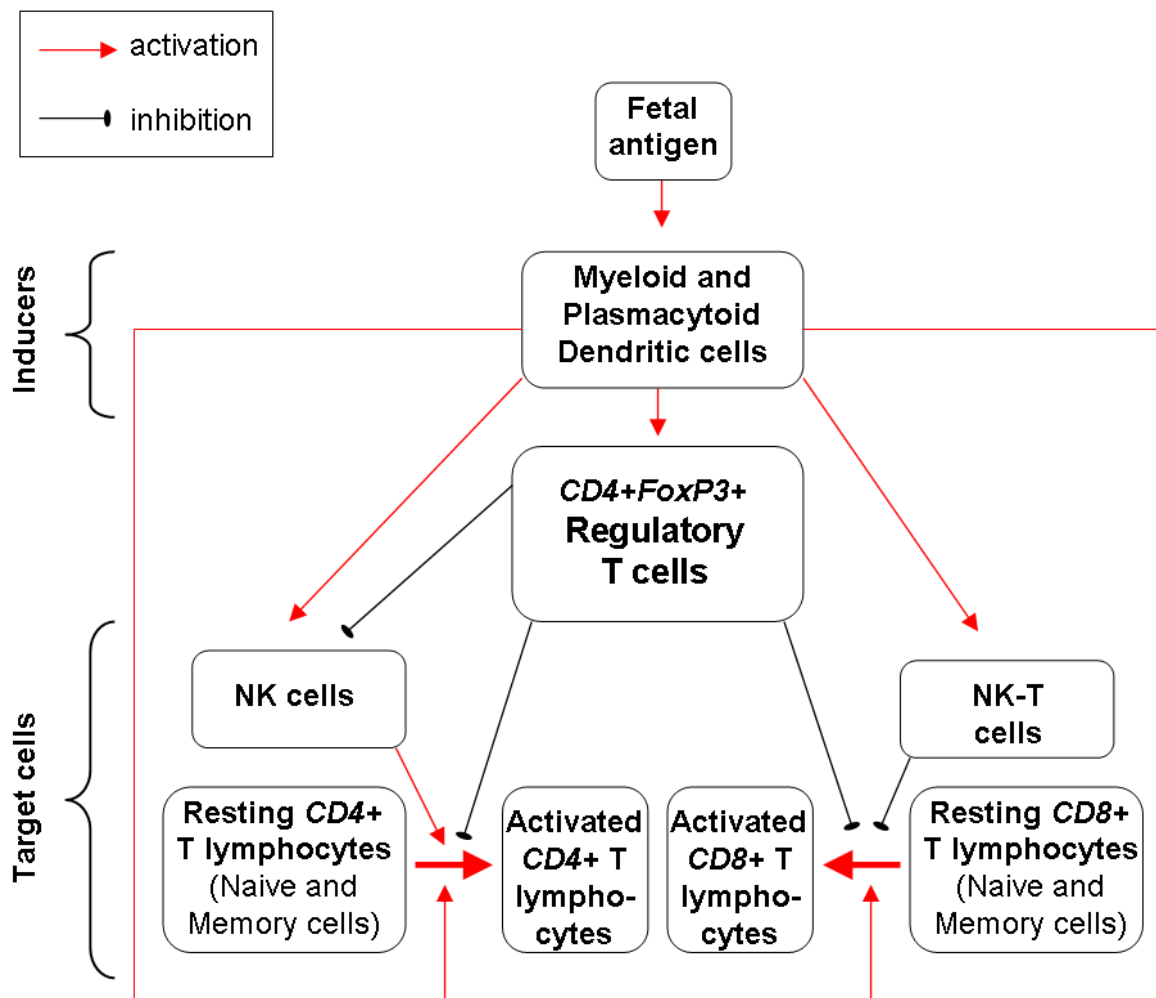
Tregs are important regulators of tolerance induction. During pregnancy, a systemic expansion of Tregs specific for paternally derived cells can be observed already at very early stages, indicating that their function is to protect paternally derived cells from immune rejection (Mjösberg et al., 2007). The prevalence of Tregs expands in the periphery and these cells are also present at significant numbers at the fetomaternal interface, preferentially in the maternal decidua. Sasaki et al. were the first to describe an increase in the CD4⁺ CD25⁺ Treg cell prevalence in decidual tissue in early human pregnancy (Sasaki et al., 2004). This

was supported by the works of Heikkinen et al. and Somerset et al. who observed an increase in the population of CD4⁺ CD25⁺ circulating Treg cells in early pregnancy and described a peak of this population during the second trimester and a subsequent gradual decrease to levels slightly higher than non-pregnant levels during the postpartum period (Heikkinen et al., 2004; Somerset et al., 2004). The tolerogenic impact of Tregs needs permanent antigen presentation without inflammatory co-stimulation. This explains the transient nature of the fetal tolerance and the fact that, especially during the first weeks of pregnancy, inflammatory infection of the mother may compromise pregnancy outcome. An expansion of Tregs in the decidua in healthy pregnant women accompanied by a low occurrence of Th17 cells was recently confirmed by Mjösberg et al. (Mjösberg et al., 2009). Furthermore, these authors propose that Tregs may be in charge of controlling the Th1 activity found locally in healthy early pregnancy.

Decreased Treg cell numbers during pregnancy are associated with immunological rejection of the fetus (Zenclussen, 2006). Sasaki et al. reported that spontaneous abortion cases are associated with lower systemic Treg levels when compared to normally developing pregnancies (Sasaki et al., 2004). A number of groups including ours demonstrated that the prevalence of peripheral Tregs is lower in preeclampsia compared with healthy pregnancy (Darmochwal-Kolarz et al., 2007; Sasaki et al., 2007; Steinborn et al., 2008; Toldi et al., 2008). Furthermore, Sasaki et al. reported that the prevalence of Tregs is lower not only in peripheral blood samples but also in deciduas of preeclamptic patients compared with healthy pregnant women (Sasaki et al., 2007).

Tregs function in a delicate cellular network that includes inducer (myeloid and lymphoid dendritic cells) and target cells (CD4 and CD8 cells, NK cells and NKT cells) of Tregs (Fig. 1). Besides the peripheral prevalence of Tregs, we also characterized the prevalence of inducers and cellular targets of this T cell subset in preeclampsia and healthy pregnancy in the third trimester. We made efforts to find out whether the alteration of the number of Treg inducers is associated with the decreased number of Tregs. According to a previous study, the ratio of these cells is skewed toward the myeloid dendritic cells in the third trimester of preeclamptic pregnancy (Darmochwal-Kolarz et al., 2003). Theoretically, this may contribute to low Treg prevalence due to the lower Treg inducer capacity of myeloid dendritic cells than that of lymphoid dendritic cells (Ito et al., 2007). In our patients, however, the prevalence and ratio of myeloid and lymphoid dendritic cells did not differ, possibly indicating that dendritic cells are not responsible for low Treg numbers, at least in this stage of pregnancy. The lack of association may support the contribution of non-cellular factors, including pro-inflammatory cytokines such as TNF- α which has a direct inhibitory effect on suppressive Treg function in vitro (Valencia et al., 2006). TNF- α and other pro-inflammatory cytokine levels (such as IFN- γ , IL-6 and IL-12) are reportedly increased in PE (Rusterholz et al., 2007). Lower Treg numbers are not reflected in the proportion of NK, NK-T and activated CD4 and CD8 cells, at least at this stage of pregnancy. This does not exclude, however, that the function (such as the cytokine production pattern) of these cells is modified due to altered Treg numbers.

Steinborn et al. further analyzed the prevalence of Tregs using various markers to identify this subset. Their analysis revealed two distinct Treg subsets that differed with regard to their FoxP3 and CD25 expression: CD4⁺ CD25⁺ FoxP3^{high} and CD4⁺ CD25^{high} FoxP3⁺ cells. When monitoring the two populations during healthy pregnancy and preeclampsia, they found a strong increase in the percentage of the CD4⁺ CD25⁺ FoxP3^{high} Treg



(Based on Toldi et al., 2008.)

Fig. 1. The cellular network of regulatory T cells (Tregs). Tregs are in connection with inducer and target cells through various mechanisms of activation and inhibition.

population during the first and second trimesters, while in the third trimester, this Treg subset decreased gradually until term. The prevalence of CD4⁺ CD25⁺ FoxP3^{high}⁺ Tregs correlated with suppressive capacity: Treg cells obtained from healthy pregnant women during the first and second trimester showed a two-fold higher suppressive activity than cells obtained in the third trimester or at term. The same correlation was true for patients affected by preeclampsia. The significantly diminished percentage of CD4⁺ CD25⁺ FoxP3^{high}⁺ Tregs correlated with low suppressive capacity. In contrast to healthy pregnancy, the percentage of CD4⁺ CD25^{high}⁺ FoxP3⁺ Treg cells was found to be increased in the circulation of preeclamptic women. In healthy pregnant women these cells expanded during the first trimester and reached maximum levels in the second trimester. Therefore, in preeclamptic women the population of CD4⁺ CD25^{high}⁺ FoxP3⁺ Treg cells was particularly apparent, while the population of CD4⁺ CD25⁺ FoxP3^{high}⁺ Tregs was significantly decreased. The authors proposed that CD4⁺ CD25⁺ FoxP3^{high}⁺ and CD4⁺ CD25^{high}⁺ FoxP3⁺ cell populations represent distinct Treg subsets, and that abnormalities in the balance of these subsets are associated with the presence of preeclampsia (Steinborn et al., 2008).

5. The role of regulatory T cells in the process of tolerance induction

Preeclampsia occurs more frequently in the first conception (Dekker & Sibai, 1998). However, preeclampsia appears to be a problem of primipaternity rather than primigravidity, since epidemiological data indicate that when the conception is with a new partner in multiparous mothers, the risk increases to the level seen in the first pregnancy (Robillard et al., 1993; Trupin et al., 1996). The symptoms of preeclampsia regress rapidly after delivery, suggesting that the exposure of the maternal immune system to the fetus and placenta, expressing paternal alloantigens, are of central importance in the pathogenesis.

In donated spermatozoa, semen exposure does not occur and the fetus is a semi-allograft to the mother. The risk of preeclampsia in donated spermatozoa is very high (18.2%) (Salha et al., 1999), suggesting that semen exposure reduces the risk of preeclampsia. Soluble MHC class I antigens in the seminal fluid are taken up by vaginal and uterine epithelial cells, and these antigens might induce tolerance to cells expressing paternally derived MHC class I antigens. Indeed, semen triggers an influx of antigen-presenting cells into the female reproductive tract (Robertson et al., 2003). Usually, the fetus is a semi-allograft to the maternal host. The risk of preeclampsia is also increased in complete allograft-pregnant cases. In ovum donation, the antigens of the fetus are derived from the husband and the donor woman. Exposure to the husband's semen is appropriately present. The risk of preeclampsia in ovum donation cases is high (16%), suggesting that the allografted fetus is a greater challenge for the maternal immune system, and is a risk factor for preeclampsia (Salha et al., 1999). In donated embryo transfer cases, the fetus is an allograft and semen exposure is not present. In this case, this risk of preeclampsia is even higher (33%), probably due to an additive effect of the allografted fetus and the absence of sperm exposure.

Regulatory T cells, which induce tolerance to paternal antigens, may explain these epidemiological findings. Darmochwal-Kolarz et al. demonstrated that the levels of CD4⁺ CD45RO⁺ and CD8⁺ CD25⁺ cells are increased in preeclampsia, suggesting the activation of CD4⁺ and CD8⁺ T lymphocytes. It seems possible that the activation of T lymphocytes is associated with the deficiency of Tregs (Darmochwal-Kolarz et al., 2007). Before the first pregnancy, Treg cell numbers may increase due to seminal priming. Koelman et al. reported that soluble MHC class I antigens are present in the seminal fluid. It is well known that continuous oral exposure to antigens induces tolerance, called 'oral tolerance'. Similarly, a continuous vaginal exposure to paternal soluble MHC class I antigens may induce tolerance to these antigens (Koelman et al., 2000). Robertson et al. suggest that insemination activates the maternal immune system and leads to hypo-responsiveness in T cells reactive with paternal alloantigens in mice (Robertson et al., 2003). This idea is supported by the epidemiological finding that condom users have a high risk for preeclampsia (Klonoff-Cohen et al., 1989).

The prevalence of Tregs is low in the mother before pregnancy (Fig. 2). This minor population of T cells, which reacts to paternal antigens and induces tolerance to them, expands after conception. When a threshold of prevalence is reached, sufficient immune tolerance to paternal antigens is achieved to ensure a healthy development of pregnancy. If the prevalence of Tregs does not reach the threshold, the risk for the development of preeclampsia is high. Subsequently, Tregs increase to a maximum in the second trimester of pregnancy and gradually decrease in the third trimester (Somerset et al., 2004). This finding is related to the clinical observation that the symptoms of preeclampsia generally appear in the third trimester, after 24 weeks of gestation. After delivery, the prevalence of Tregs may

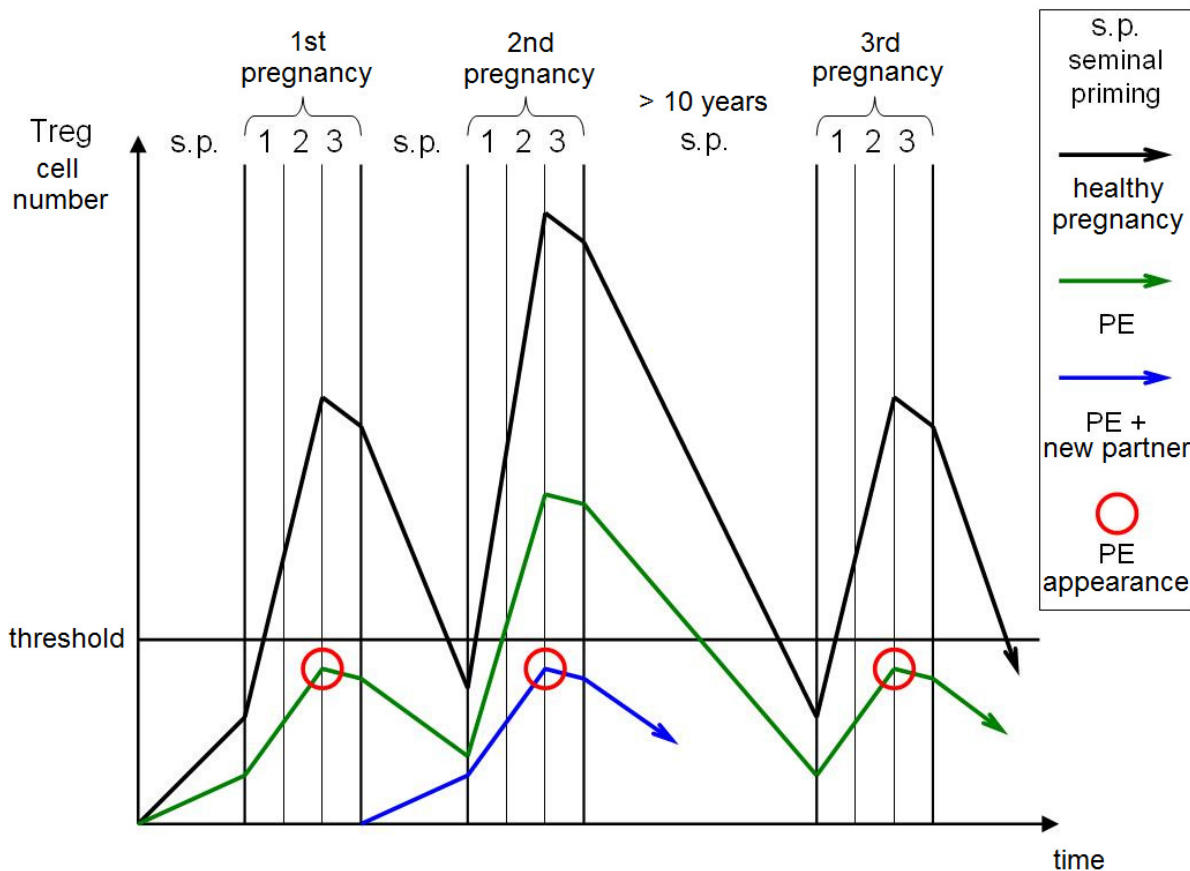


Fig. 2. Alterations of maternal regulatory T cell (Treg) numbers during pregnancy. The prevalence of Tregs is low before pregnancy. When a threshold of Treg prevalence is reached during healthy pregnancy (black curve), the symptoms of preeclampsia do not develop. This is aided by seminal priming. If the prevalence of Tregs does not reach the threshold, the risk of preeclampsia is high (green curve). After the conception of a second pregnancy with the same partner, the number of Tregs increases more rapidly compared with the first pregnancy, independently from the initial prevalence of Tregs during the first pregnancy. However, in a second pregnancy with a new partner, the risk for developing preeclampsia is similar to that in the first pregnancy (blue curve). An increasing interval (> 10 years) between the second and third deliveries was associated with an increasing risk of preeclampsia, and a lower prevalence of Tregs.

rapidly decrease and at the beginning of a second pregnancy, there may be only a low number of Tregs present. Seminal priming may keep the number of these cells to a certain level (Fig. 2). After the conception of a second pregnancy with the same partner, the number of Tregs increases more rapidly compared with the first pregnancy, independently from the initial prevalence of Tregs (and the presence or lack of preeclampsia) during the first pregnancy. However, in a second pregnancy with a new partner, Tregs which induce tolerance to the new partner's antigens may be very rare. Therefore, the risk for developing preeclampsia is similar to that in the first pregnancy.

Skjaerven et al. reported that preeclampsia occurred in 3.9% of first pregnancies, 1.7% of second pregnancies, and 1.8% of third pregnancies when mothers had the same partner. Furthermore, an increasing interval between the second and third deliveries was associated

with an increasing risk of preeclampsia. When more than 10 years had passed after the previous delivery, the frequency rose to 3.0%. They also showed that a paternal change was not associated with an increased risk of preeclampsia after adjustment for the interval between births (Skjaerven et al., 2002). These findings might be explained by the population of Tregs. Tregs may gradually decrease and reach very low levels when more than 10 years have passed after the last delivery. A low level of these tolerance-inducing T cells may be maintained by seminal priming. However, in a subsequent pregnancy, some pregnant women may not be able to achieve adequate tolerance, resulting in an increased risk of preeclampsia.

6. Th17 cells

CD4⁺ IL-17⁺ (Th17) cells, this recently identified subpopulation of CD4 lymphocytes, originate from a developmental lineage that is distinct from both Th1 and Th2 cells. Th17 cells produce IL-17 and other pro-inflammatory cytokines. IL-17 has an important role in the development of autoimmune disorders and in the induction and maintenance of chronic inflammation (Basso et al., 2009). The effect of Th17 cells on the inflammatory balance is opposed by Tregs. Th17 cells and Tregs originate from the same developmental lineage, distinct from both Th1 and Th2 cells. An exclusive dichotomy was observed in their generation: either Th17 or Treg cells develop from the ancestor cells depending on whether they are activated in the presence of transforming growth factor- β (TGF- β) or TGF- β plus inflammatory cytokines (Basso et al., 2009).

In addition to their distinct role in the regulation of the inflammatory status, Th1, Th2, Th17 and Treg cells mutually influence one another through the production of different cytokines. For instance, an increase in the Th17/Treg cell ratio may contribute to a shift towards the Th1 direction because IL-17 induces the production of other pro-inflammatory cytokines, such as that of IFN- γ (Afzali et al., 2007), while the inhibitory effect of Tregs on Th1 cells is decreased at the same time.

In a previous study, Santner-Nanan et al. found that, besides a higher prevalence of Tregs, the percentage of Th17 cells is significantly decreased in the third trimester of healthy pregnancy compared with non-pregnant controls and preeclamptic patients. Consequently, the Th17/Treg cell ratio was significantly decreased in healthy but not in preeclamptic pregnancies. Thus, preeclampsia is associated with the absence of normal systemic skewing away from IL-17 production towards FoxP3 expression. Additionally, preeclamptic women had significantly higher levels of soluble endoglin, an inhibitor of TGF- β receptor signaling, which may bias towards IL-17 production (Santner-Nanan et al., 2009).

In accordance with the above data, we also found that simultaneously with higher than normal Th17 numbers, the prevalence of Tregs is lower in PE, resulting in an elevated Th17/Treg ratio compared with uncomplicated pregnancy. The altered ratio of Th17/Treg cells may contribute to the shift towards the Th1 direction in preeclampsia. However, we could not detect a correlation between the prevalence of Th17 and Th1 cells (using the cell surface marker, CXCR3 for the identification of the latter subset). Thus, the effect of Th17 cells and IL-17 on the inflammatory status is more likely to be exerted in a direct manner rather than through the modulation of the Th1/Th2 balance in preeclampsia (Toldi et al., 2011a).

The alterations of Th17 cells have been observed in other pregnancy-related disorders as well, suggesting that the balance of Th17 cells and Tregs and not only that of Th1 and Th2

cells has crucial effects on the inflammatory status in human pregnancy. Ito et al. recently demonstrated the importance of IL-17-producing cells in the pathomechanism of preterm labour (Ito et al., 2010). Nakashima et al. found that the prevalence of decidual IL-17-producing cells is significantly higher in inevitable abortion cases (but not in missed abortion) than in normal pregnancy, indicating that these cells might be involved in the induction of inflammation in the late stage of abortion, but not in the early stage (Nakashima et al., 2010). In another study, Wang et al. showed that the prevalence of Th17 cells in the peripheral blood and decidua is increased in unexplained recurrent spontaneous abortion patients (Wang et al., 2010). They observed that the expression of a Th17-associated factor, RAR-related orphan receptor gamma (ROR- γ or RORc), is also higher in peripheral blood lymphocytes and decidua of these patients. In a recent study, Jianjun et al. found that the mRNA level of this factor in PBMCs and decidua is elevated in preeclamptic patients when compared with healthy pregnant women (Jianjun et al., 2010). Therefore, the increased expression of this transcription factor may partly be responsible for the increased prevalence of Th17 cells in peripheral blood of preeclamptic patients.

7. IL-17-producing CD8 and NK cells

Although IL-17 was first identified in CD4 cells, later on other immune cells, including CD8 and NK cells, were also shown to produce this cytokine (Passos et al., 2010; Shin et al., 1999). Emerging evidence suggests that these IL-17-producing lymphocyte subsets, especially NK cells, largely contribute to the inflammatory status during pregnancy. Sargent et al. proposed that the innate rather than the adaptive immune system controls immune regulation during human pregnancy, and that NK cells are of central importance to this process (Sargent et al., 2006b; Sargent et al., 2007). The aberrant activation of NK cells both systematically and locally in the placenta may be of major interest in the malfunction of immune tolerance and the pathogenesis of pregnancy-associated disorders.

In our previously unpublished investigation, we measured the peripheral prevalence of IL-17-producing cells in the CD4, CD8 and NK subsets and that of the IL-17 producing CD4, CD8 and NK cells in the overall lymphocyte population. We took peripheral blood samples from 24 healthy non-pregnant and 23 healthy pregnant age-matched women (with a median of 32 and 33 years, respectively), in the third trimester of pregnancy (with a median of 31 weeks of gestation). Non-pregnant women were in the early follicular phase of the menstrual cycle (between cycle days 3 and 5), and none of them received hormonal contraception. Informed consent was obtained from all participating subjects. We separated the mononuclear cells from the samples and performed cell surface marker and intracellular cytokine staining. Finally, samples were measured on a flow cytometer.

The prevalence of Th17 cells was lower among healthy pregnant compared with healthy non-pregnant women (2.8 [2.4-3.1] % vs. 3.2 [2.9-3.5] %). The prevalence of CD8+ IL-17+ (Tc17) cells among CD8 lymphocytes was higher in healthy pregnant than in healthy non-pregnant samples (4.8 [3.6-6.2] % vs. 2.4 [1.7-3.7] %). The frequency of CD56+ IL-17+ (IL-17-producing NK) cells was lower in healthy pregnant than in healthy non-pregnant samples (1.0 [0.6-1.2] % vs. 1.6 [1.1-2.5] %).

We further analyzed the frequency of IL-17-producing CD4, CD8 and NK lymphocytes. After the initial assessment of the Th17, Tc17 and IL-17-producing NK cell prevalence among CD4, CD8 and NK cells, respectively (Fig. 3, graphs a-c), we also determined the prevalence of these subsets among the overall lymphocyte population (Fig. 3, graphs d-f)

along with the frequency of CD4+ IL-17-, CD8+ IL-17- and CD56+ IL-17- cells among the overall lymphocyte population (Fig. 3, graphs g-i).

Our findings revealed two different mechanisms explaining the differing alterations observed in the prevalence of Th17 and Tc17 cells in the study groups. The alteration of CD4+ IL-17+ cell prevalence in the overall lymphocyte subset (Fig. 3, graph d) followed that seen among CD4 cells (Fig. 3, graph a), as the frequency of CD4+ IL-17- cells was comparable in both study groups (Fig. 3, graph g). Therefore, the prevalence of Th17 (CD4+ IL-17+) cells show absolute alterations, since Th17 cell numbers are altered not only in the CD4 subset, but also in the overall lymphocyte population. In contrast, Tc17 cell frequencies do not show significant difference if assessed among the overall lymphocyte population (Fig. 3, graph e); only the prevalence of CD8+ IL-17- cells differs between the study groups (Fig. 3, graph h); only the prevalence of CD8+ IL-17- cells differs between the study groups

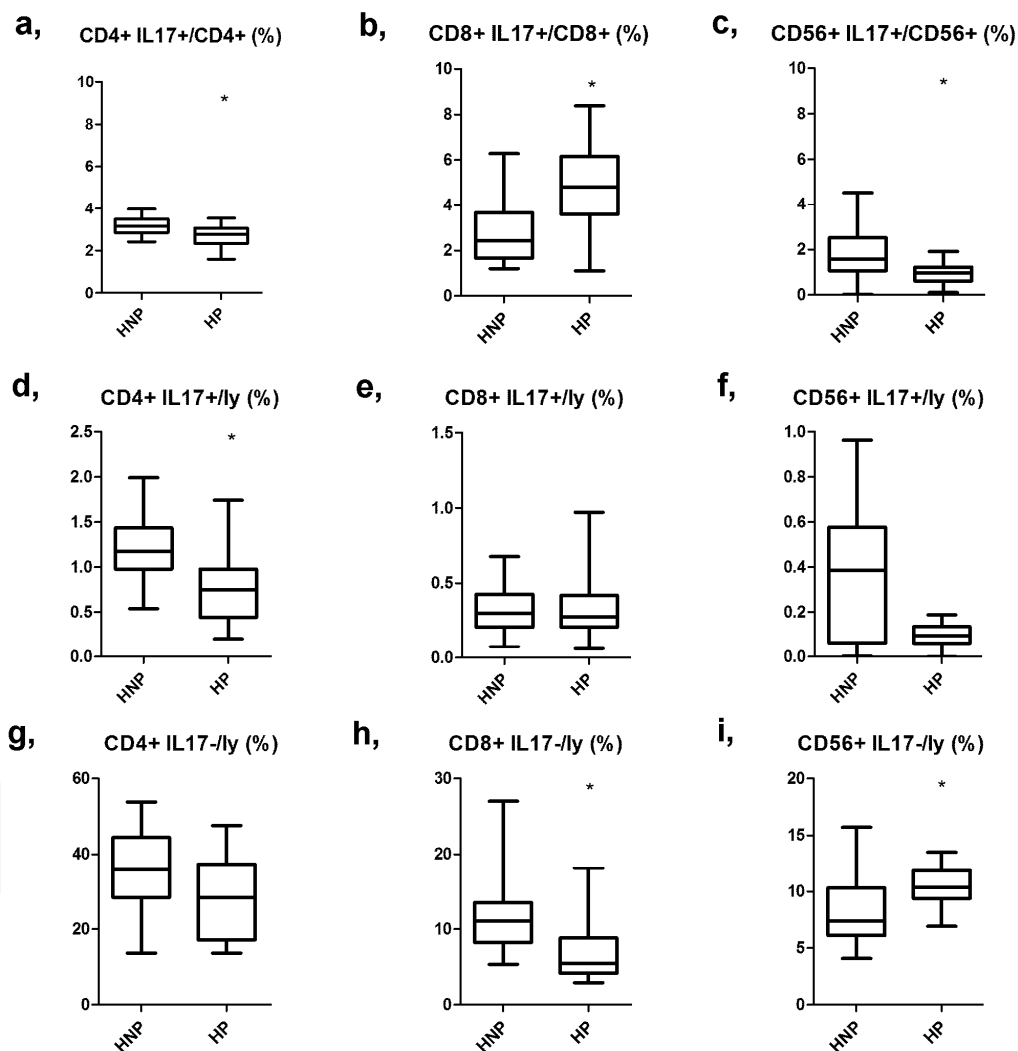


Fig. 3. Box-plots representing the prevalence of IL-17+ cells among CD4, CD8 and NK (CD56+) cells (graphs a-c) and in the overall lymphocyte population (ly) (graphs d-f), as well as the prevalence of IL-17- cells in the overall lymphocyte population (ly) (graphs g-i) in healthy non-pregnant (HNP) and healthy pregnant (HP) women. Horizontal line: median, box: interquartile range, whisker: range. * p values less than 0.05 were regarded significant.

(Fig. 3, graph h) and the direction of IL-17- cell alteration is the opposite of that seen at Tc17 cell prevalence in the CD8 subset (Fig. 3, graph b). Therefore, the prevalence of Tc17 cells does not show absolute alterations, only relative ones, as a consequence of different CD8+ IL-17- cell frequencies in the study groups. The alteration of IL-17-producing NK cell prevalences among parent populations showed similar tendencies to that observed in case of CD4 cells.

Of note, existing data are not consistent concerning the alterations of CD4 and CD8 cell frequencies during pregnancy. Tallon et al. found that CD4 cell numbers decrease in the second and third trimesters, while CD8 cells decrease during the third trimester (Tallon et al., 1984). Another study demonstrated that peripheral blood CD8 cells decreased during the first trimester, while CD4 cells decreased in the third trimester, with both populations increasing to non-pregnant values four months postpartum (Watanabe et al., 1997). The investigations by Kühnert et al. found no significant changes in the percentage CD4 and CD8 lymphocytes, nor in the CD4/CD8 ratio at any stage of pregnancy or postpartum.

Based on our results, the apparent increase in Tc17 cell prevalence in healthy pregnancy compared with non-pregnant controls appears to be the consequence of decreased CD8+ IL-17- cell frequency in this group, resulting in a higher proportion of Tc17 cells in the CD8 subset. Since Tc17 cells have been described to have a lower cytotoxic activity in comparison with CD8+ IL-17- cells (Huber et al., 2009), the decrease in the latter subset and a relative increase in Tc17 prevalence may be regarded as part of the immunosuppressive mechanisms characterizing healthy pregnancy.

In preeclampsia, our results show that in addition to CD4 cells, the prevalence of CD8 and NK cells that express IL-17 is also higher compared with healthy pregnant women. IL-17 production by these lymphocyte subsets might contribute to the development of a systemic pro-inflammatory environment in PE (Toldi et al., 2011a).

8. Lymphocyte activation kinetics

Previous studies demonstrated that not only the prevalence of T lymphocyte subsets, but also their functionality is altered during pregnancy. For instance, calcium handling of T cells differs in healthy pregnancy and in the non-pregnant state. Previous reports indicate a sustained increase of basal intracellular calcium level in lymphocytes of healthy pregnant and preeclamptic women compared with non-pregnant women, with the highest levels in preeclampsia (Hojo et al., 1999; Thway et al., 2004; von Dadelszen et al., 1999). The availability of cytoplasmic free calcium has an important role in controlling the level of lymphocyte activation. We hypothesized that the elements of calcium handling of activated T lymphocytes, including lymphocyte potassium channels, may be affected in healthy pregnancy and preeclampsia compared to the non-pregnant state. Voltage-gated Kv1.3 channels along with calcium-dependent IKCa1 channels play a key role in the regulation of intracellular calcium homeostasis as they counterbalance the increase of cytoplasmic free calcium content in the course of lymphocyte activation. These channels grant the efflux of potassium from the cytoplasm, thus maintaining an electrochemical potential gradient needed for further calcium entry. Specific inhibition of these channels results in a diminished calcium influx into lymphocytes and a lower level of lymphocyte activation (Panyi et al., 2006). Our group characterized the activation-elicited calcium influx in the Th1, Th2, CD4 and CD8 lymphocyte subsets in healthy pregnant, preeclamptic and non-pregnant women and tested its alteration upon the inhibition of Kv1.3 and IKCa1 potassium channels (Toldi et al., 2011b).

Until recently, primarily single-cell techniques were applied to study the process of lymphocyte activation, and no reliable high-throughput method was available to investigate lymphocyte activation kinetics in more than one lymphocyte subsets simultaneously. The use of single-cell techniques are limited by the fact that they are not suitable for the description of kinetic processes in a complex cellular milieu that contains different types of interacting immune cells. For this purpose, we developed a novel, flow cytometry-based approach that enabled us to monitor lymphocyte activation simultaneously in different lymphocyte subtypes.

For our measurements, we collected peripheral blood samples from healthy pregnant, preeclamptic and non-pregnant women. PBMCs were separated by a standard density gradient centrifugation. PBMCs were then incubated with conjugated anti-human surface marker monoclonal antibodies (CD4, CD8, CXCR3 for Th1 cells, CCR4 for Th2 cells) to identify lymphocyte subsets and were loaded with calcium sensitive Fluo-3 and Fura Red dyes to monitor alterations of the cytoplasmic free calcium level. PBMCs were divided into three vials. One vial was treated with margatoxin (MGTX), a selective blocker of the Kv1.3 channel. Another vial was treated with a triarylmethane compound (TRAM), a specific inhibitor of the IKCa1 channel. The third vial was used as control. Measurements were initiated directly after the addition of 20 μg of phytohemagglutinin (PHA) as an unspecific activating stimulus. Cell fluorescence data were measured and recorded for 10 minutes in a kinetic manner on flow cytometer.

Data acquired from the measurements were evaluated by fitting a double-logistic function for each recording (Kaposi et al., 2008). This function is used to describe measurements that have an increasing and a decreasing intensity as time passes. The software also calculates parameter values describing each function, such as the Maximum value (Max), the Time to reach maximum value (t_{max}), and the Area Under the Curve (AUC). These parameters represent different characteristics of lymphocyte calcium influx kinetics. The Maximum value represents the peak value of the calcium influx curve upon lymphocyte activation, thus it reflects the maximal amount of cytoplasmic free calcium in the course of activation. The Time to reach maximum value describes how soon the peak value of the calcium influx curve is reached. The Area Under the Curve describes the full amount of cytoplasmic free calcium during the whole period of lymphocyte activation and thus the magnitude of the elicited calcium response in general.

Our results indicate that calcium influx kinetics in activated T lymphocytes markedly differs in healthy pregnancy compared with the non-pregnant state: AUC values of the calcium response are lower in healthy pregnancy in the Th1, CD4 and CD8 lymphocyte subsets (Fig. 4). Based on this observation, it is reasonable to assume that the physiological immune tolerance towards fetal antigens in pregnancy is partly attributed to a lower calcium response. This hypothesis is further supported by the particular role of the impaired function of Th1 and CD8 lymphocyte subsets in maternal immune tolerance. On contrary to Th1 cells, the activation induced calcium response of the Th2 subset is not decreased compared with the non-pregnant state. The decreased activation of the Th1 subset (reflected by low AUC values in our study) and the lack of decrease in Th2 cells may partly be responsible for the well established Th2 skewness in healthy pregnancy.

Unlike in healthy pregnancy, we could not detect a difference in the AUC values of calcium influx kinetics of Th1 and CD8 cells in preeclampsia compared to non-pregnant women (Fig. 4). The absence of calcium influx characteristics specific for healthy pregnancy suggests that

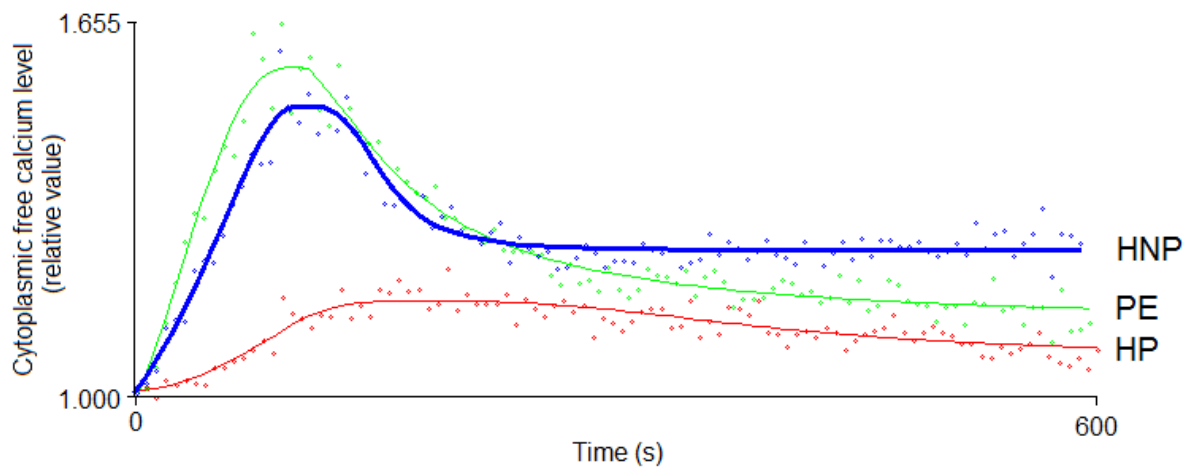


Fig. 4. Calcium influx kinetics of peripheral Th1 lymphocytes isolated from healthy non-pregnant (HNP), healthy pregnant (HP) and preeclamptic (PE) women (without lymphocyte potassium channel inhibitor treatment). Calcium influx is lower in healthy pregnancy compared with the non-pregnant state and preeclampsia.

this element may associate with the impaired maternal immune tolerance present in preeclampsia, since the calcium influx kinetics is comparable to that seen in non-pregnant samples. Indeed, the maintained activation properties of Th1 lymphocytes in preeclamptic patients may contribute to the lack of Th2 dominance associated with normal pregnancy. Similarly to the Th1 subset, CD8 cells in preeclampsia are also characterized by the lack of suppressed activation kinetics. Thus the decrease of cytotoxic activity observed in healthy pregnancy (Malinowski et al., 1994) is not present in preeclampsia. Interestingly, t_{max} values were decreased in Th2 and CD4 cells in preeclampsia compared with healthy pregnancy. This finding may indicate an increased reactivity of lymphocytes in preeclampsia, possibly reflecting an elevated responsiveness of T lymphocytes due to the ongoing maternal systemic inflammation.

Since Kv1.3 and IKCa1 potassium channels significantly influence the calcium response elicited upon lymphocyte activation, we tested their expression and function in healthy pregnancy and preeclampsia. According to comparable fluorescence values of the samples stained with specific antibodies against Kv1.3 channels, their expression is not altered in any of the investigated T lymphocyte subsets. Therefore, the differences detected between calcium influx of non-pregnant, healthy pregnant and preeclamptic lymphocytes upon treatment with specific inhibitors of the potassium channels is probably due to the altered function, and not to the altered expression of these channels.

Our results suggest that the overall lymphocyte population and particularly the CD4 subset are sensitive to MGTX and TRAM inhibition in each investigated study group, indicating that both Kv1.3 and IKCa1 channels play an important regulatory role in calcium influx. This is reflected by the decrease of the AUC and Max values compared with the respective samples where no inhibitors were applied. However, the sensitivity of calcium influx measured in other lymphocyte subsets shows clear variability. It is of particular interest that calcium influx of Th2 lymphocytes in healthy pregnancy was insensitive to potassium channel inhibition, while calcium influx decreased significantly in non-pregnant samples upon treatment with the specific channel blockers. Of note, Th2 lymphocytes in

preeclampsia presented with non-pregnant-like characteristics, and were also sensitive to MGTX and TRAM treatment. Since the regulatory function of Kv1.3 and IKCa1 channels on calcium influx appears to be limited in healthy pregnant samples (as the inhibition of these channels did not result in a decrease of the AUC and Max values), it is tempting to speculate that this may be an element contributing to the Th2 shift present in healthy pregnancy, but absent in preeclampsia. This hypothesis may be supported by reports suggesting that the shape of calcium influx influenced by potassium channel functions may determine the cytokine production profile of helper T lymphocytes (Dolmetsch et al., 1998; Fanger et al., 2000).

Interestingly, other differences were also observed between healthy pregnancy and preeclampsia. While calcium influx in CD8 and Th1 lymphocytes was resistant to potassium channel inhibition in preeclamptic samples, that of healthy pregnant lymphocytes was sensitive. Similarly to Th2 cells, while it is unclear whether the resistance of Th1 lymphocytes to potassium channel inhibition is reflected in their function, the insensitivity of the Th1 subset to the inhibition of regulatory lymphocyte potassium channels in preeclampsia may be linked to the Th1 skewness.

Our findings suggest that there is a characteristic pattern of calcium influx in T lymphocytes and its sensitivity to potassium channel inhibition in normal pregnancy that is missing in preeclampsia. This raises the notion that T lymphocyte calcium handling may have a role in the development of the pregnancy-specific immune tolerance.

9. The association between preeclampsia and autoimmunity

Considering the immunological alterations described in preeclampsia, one may notice characteristic similarities in the etiology shared with autoimmune disorders. As a result of the impaired immune tolerance, preeclampsia is distinguished by many features typically seen in autoimmune diseases, or in association with autoimmune reactions. This does not mean that preeclampsia should be considered an autoimmune condition. However, it does suggest that abnormal autoimmune processes play an important part in the pathogenesis of preeclampsia. An interpretation of preeclampsia can be found in analogies to organ rejection after allograft transplantation and in graft-versus-host disease (GVHD); like preeclampsia, these conditions are also characterized by a multitude of systemic symptoms. The similarities with acute organ rejection and GVHD are paralleled by the notion that if a 100% allograft can elicit autoimmune responses during organ transplantation, one should not be surprised that a 50:50 autograft-allograft can do the same thing. This recognition may lead to better clinical approaches to preeclampsia and thereby to better diagnosis and treatment (Gleicher, 2007).

The clinical relationship between autoimmune diseases and pregnancy is unique. No other diseases are characterized by an exacerbation pattern that is particularly pronounced in the peripartum and postpartum periods (Gleicher et al., 1993). A recently recognized example is peripartum cardiomyopathy, with disease flares from late pregnancy up to approximately three months postpartum (Ansari et al., 2002). Preeclampsia is also characterized by a peripartum exacerbation pattern, with the majority of cases developing after 36 weeks of gestation.

Besides the cellular abnormalities already discussed, autoantibody abnormalities have also been reported in association with preeclampsia. So far, a number of autoantibodies have been implicated in the pathogenesis (Branch et al., 1994; Milliez et al., 1990; Rappaport et al.,

1990; Yamamoto et al., 1993). The most important one of them appears to be an agonistic autoantibody against the angiotensin II type 1 receptor (Wallukat et al., 1999). Moreover, as in classical autoimmune diseases, more severe preeclampsia appears to result in more autoantibody abnormalities (El-Roeiy et al., 1991). Evidence suggests that classical, nonorgan-specific autoantibodies, such as antiphospholipid antibodies, are characteristic of preeclampsia, and especially in its more severe clinical expression (El-Roeiy et al., 1991; Yamamoto et al., 1993). Dekker et al. therefore recommended active laboratory surveillance for patients at risk (Dekker et al., 1995).

Both GVHD-related and classical autoimmune conditions often improve upon treatments that have been found successful also in preeclampsia and HELLP syndrome. Three examples are the treatment with corticosteroids, removal of autoantibody abnormalities via plasmapheresis, and the competitive binding of autoantibodies with intravenous immunoglobulin (Katz et al., 1990; Martin et al., 1990; Martin et al., 2006).

Considering the significant degree of bidirectional cell traffic during pregnancy, one can speculate that, in analogy to GVHD, the autoimmune phenomena, seen in association with preeclampsia, may be immune responses by fetal lymphocytes to epitopes shared by mother and fetus. Alternatively, the autoimmune response in preeclampsia could be distinct from that in GVHD, and purely autoimmune in nature. This would then represent an immune response solely against maternal self-epitopes on fetal cells that have entered the maternal circulation. A number of observations indicate that the paternal genotype is of importance in that regard. For instance, the more similar the paternal histocompatibility complex is to that of the mother, the more likely a miscarriage will occur due to increased autoantigenicity (Kishore et al., 1996). The fetus is not only an allograft but also an autograft. Since one half of the fetus is maternally derived in its antigenicity, the maternal immune system also faces an unprecedented autoimmune load. At no other period in life needs the maternal immune system to be prepared for autoimmune challenges of this extent. The immunological adjustment to pregnancy, therefore, does not only involve the development of tolerance to the paternal allogeneic, but also to the maternal autoimmunogenic components of the fetus.

Autoimmune phenomena are mostly seen in two periods of pregnancy: during early conception and the peripartum period. In early conception, abnormal immune activation may be coupled with pregnancy loss. Later stage presentation appears to be associated with preeclampsia. Indeed, women with early autoimmune activation, who (with treatment) do not miscarry, demonstrate a greatly increased risk for preeclampsia (Gleicher et al., 1993). This observation supports the idea of a common alloimmune or autoimmune etiology for both of these conditions. It has also been suggested that an analogy might exist not only between chronic graft rejection and preeclampsia, but also between acute graft rejection and recurrent abortion (Wilczyński, 2006).

Accepting the concept that preeclampsia is characterized by autoimmune phenomena may have benefits for better diagnosis and treatment. For example, autoimmune phenomena usually go through pre- or subclinical stages before their clinical manifestation. Laboratory markers are often already detectable at those early stages (Davidson & Diamond, 2001). If the autoimmune phenomena of preeclampsia were to follow a similar pattern, earlier diagnosis and specific treatment might become possible.

10. Conclusion

T lymphocytes play a central role in the development of the maternal immune tolerance during pregnancy. Pathologic alterations of T cells in prevalence and functionality

contribute to the onset of pregnancy-related disorders, and might represent a possible future target for therapeutic intervention.

It has been recognized for many years that the prevalence of distinct T cell subpopulations are subject to a characteristic adjustment during pregnancy in order to facilitate the specific needs raised by the developing fetus. The course of this adjustment may be insufficient in pregnancy-related disorders. Recently, it has been demonstrated that not only cell numbers, but also the functionality of cells needs to go through specific alterations to help the maternal immune system in acquisition of the pregnancy-specific tolerance. As an example, we have demonstrated that there is a characteristic pattern of calcium influx in T lymphocytes and its sensitivity to lymphocyte potassium channel inhibition in healthy pregnancy that is missing in preeclampsia, raising the notion that T lymphocyte calcium handling may have a role in the distinctive immune status of uncomplicated pregnancy. Further research needs to be carried out applying different methods to identify additional factors involved in these alterations. This approach might lead to better strategies for the prevention and treatment of adverse pregnancy outcomes, facilitating the development and maintenance of immune tolerance needed for healthy pregnancy.

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12. References

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