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Apoptosis and Th1/Th2 balance in pregnancy: a molecular study.

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*“**Ich habe keine besondere Begabung, sondern bin nur leidenschaftlich neugierig**[1]“.*

Albert Einstein

[1] *“I do not have any particular talent, but I’m only passionately curious”.*

Ai miei genitori

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Abbreviations

Abs	antibodies
Bag-1	Bcl-2 associated athanogene-1
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma-2
BgVV	Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin
Caspases	cysteine-aspartic-acid-proteases CD cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHAPS	3,3–cholamidopropyldimethyl ammonio–1-propanesulfonate
Cy5	carbocyanine 5
DAB	diaminobenzidin
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleosidtriphosphate
e.g.	for example
FACS	flow activating cell sorting
FC	flow cytometry
FITC	fluorescein-isothiocyanate
h	hour
HEPES	4,2–hydroxyethylpiperazine–1- ethanesulfonic acid
HLA-G	human leukocyte antigen G
HO	heme-oxygenase
hs	hours
i.e.	that is
IFN	interferon
IL	interleukin
KO	knock-out
LIF	leukaemia inhibitory factor
mRNA	messenger RNA
NTC	no template control
OD	optical density

ON	overnight
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
RNA	ribonucleic acid
RNAse	ribonuclease
RSA	recurrent spontaneous abortion
RT	room temperature
RT–PCR	reverse transcriptase-polymerase chain reaction
TBS	Tris buffered saline
TGF	transforming growth factor
TNF	tumor necrosis factor

1. Introduction

In 1953, the Brazilian-born British biologist Sir Medawar first discussed the immunological problem of pregnancy, where the mother contrive to nourish within herself, a fetus that is an antigenically foreign body. Later, several theories were proposed in order to explain why the fetus is not rejected from its mother. The placental trophoblast cells have been defined as a barrier between mother and fetus (Chaouat *et al.*, 1983; Wegmann *et al.*, 1988), therefore immunological rejection cannot be induced since there is a lack of paternal major histocompatibility complex (MHC) alloantigen (class I and II) expression (Hunt *et al.*, 1988). Studies disproved these theories. Mother and fetus interact dynamically with each other: maternal lymphocytes pass into the fetus and vice versa (Piotrowsky and Croy, 1996; Bonney and Matzinger, 1997), and murine trophoblastic cells express fetal class I MHC molecules (Chatterjee-Hasrouni and Lala 1981; Zuckerman and Head 1986). Moreover, Clark and colleagues (1991) demonstrated that the fetus is rejected as an allograft if removed from its place and transplanted in muscle or kidney of the mother, thus underlying the ability of the fetus to express paternal MHC antigens. Other studies proposed that trophoblast cells are able to induce apoptosis of maternal Th1 type lymphocyte by releasing FAS ligand (FASL) at the feto-maternal interface (Zhang *et al.,* 1989; Runic *et al.,* 1996; Hunt *et al.,* 1997; Kauma *et al.,* 1999), in order to protect the fetus from rejection. However, an *lpr* mutation - defective in the Fas function - had no effect on pregnancy outcome in mice, whereas with *gld* mutation – lacking functional FasL – the mice displayed extensive leukocyte infiltrates and cell death at the feto-maternal interface, delivering small litters (Hunt *et al.*, 1997). Recently, two novel mechanisms involved in pregnancy protection were introduced in th reproductive field: heme oxygenase (HO) enzymes system and regulatory T cells (Treg). HO enzyme is responsible for the heme degradation into bilirubin and CO. Studies proposed HO allowing the acceptance of mouse allograft (Soares *et al.,* 1998; Woo *et al.,* 1998; Araujo *et al.,* 2003). Recently, diminished expression of both HO isoforms (HO-1 and HO-2) at the feto-maternal interface of mice undergoing Th1-mediated abortion compared to normal pregnant mice have been reported, suggesting a protective effect of these enzymes in allogenic murine pregnancy maintenance (Zenclussen *et al.*, 2002b, 2005a). Moreover, in humans,

a significant reduction of both HO-1 and HO-2 expression has been also revealed in placenta samples from patients with pregnancy complications compared to normal pregnant women (Barber *et al.*, 2001; Zenclussen *et al.*, 2003b). On the other hand, Treg $CD4^+CD25^+$ cells, a unique subpopulation of T cells, induce a local immune privilege, allowing graft acceptance (Waldmann *et al.*, 2006). During pregnancy, a diminished number of Treg cells have been observed in mice undergoing abortion compared to normal pregnancy (Zenclussen *et al.*, 2005b; 2006), and, moreover, during human gestation elevated levels of circulating Tregs were associated with successful pregnancy (Somerset *et al.*, 2004).

A burning point in pregnancy-related research is represented by the role of pro-(Th1 such as TNF-α or IFN-γ) and anti-inflammatory cytokines (Th2/Th3, such as IL-10, TGF-β) in determining successful pregnancy or abortion. Studies indicated that the Th1/Th2 cytokine ratio might not be the overall explanation for successful reproductive outcome, but a simplification (Svensson *et al.*, 2001; Zenclussen *et al.*, 2002a; Chaouat *et al.*, 2003). In mice, production of type 1 cytokines at implantation sites has been found essential for normal implantation, whereas in human normal pregnancy is associated with a systemic inflammatory response. In general, it is accepted that local changes in the balance of Th1/Th2 cytokine profiles occurring during pregnancy within the maternal uterus and at the feto-maternal interface contributes to implantation, placental development and fetal survival. Augmented Th1 cytokines production is involved in immunological rejection of the fetus, and, particularly TNF-α, triggers apoptosis (Sidoti-de-Fraisse *et al.*, 1998; Baud and Katrin, 2001).

This work is aimed to investigate the incidence of apoptosis in a well-known experimental model of Th1-induced abortion, characterized by increased local TNF-α levels. Moreover, considering the protective effect of heme oxygenase (HO) proposed during allogeneic pregnancy, we studied whether this effect is due to the anti-apoptotic properties of HO since the mechanisms involved in its cytoprotective effect are still unclear.

1.1. Abortion

The common term *abortion* defines the premature termination of pregnancy and expulsion from the human maternal uterus of an embryo or a fetus weighing 500 g or less, incapable of survival (Stirrat, 1990). The weight criterion, associated with 20-22 weeks of gestation, is considered as the minimum for viability and to discriminate between pathological causes (Stirrat, 1990). In humans, miscarriage very often occurs during the first trimester, prior to week 13^{th} of pregnancy, whereas in mice at the 8^{th} day of pregnancy (Suzuki *et al*., 1997). In mice the term *abortion* has been substituted with *resorption*, in order to describe sites identified by their small size and necrotic hemorrhagic appearance, compared to normal embryos and placentas, (Suzuki *et al*., 1997).

1.2. Recurrent Spontaneous Abortion (RSA)

Recurrent spontaneous abortion (RSA) is usually defined as the loss of three or more consecutive pregnancies (Stirrat, 1990; Crosignani *et al.,* 1991, Coulam *et al*., 1996; Pandey *et al.,* 2005). “Primary recurrent spontaneous aborters” are considered those who have lost all previous pregnancies and have no live birth, while “secondary recurrent spontaneous aborters” have at least one successful gestation irrespective of the numbers of pregnancy losses (Stirrat, 1990). Epidemiological studies suggest that the risk of subsequent pregnancy losses is approximately 24% after two clinical abortions, 30% after three and 40% after four consecutive spontaneous pregnancy losses (Stirrat, 1990; Regan, 1991). The reasons associated with repeated abortion are multifactorial and can be divided into embryological (mainly due to an abnormal karyotype, 3-6%; Coulam *et al*., 1996) and maternal driven causes, like anatomical (e.g. uterus malformation, 1-16%), endocrinological (5-20%), placental anomalies, infections (0.5%, Pandey *et al.,* 2005), stress factors (Arck *et al.,* 2001), autoimmune factors (20%, Pandey *et al.,* 2005). Since women experiencing RSA represent a heterogeneous population, specific markers are necessary to identify those who will respond to various treatments. Diagnostic tests have been used to identify putative immunological causes associated with RSA including autoantibodies such as anti-phospholipid antibodies (APA), anti-nuclear antibodies (ANA) but not anti-thyroid antibodies (ATA, Coulam *et al*., 1997). Several treatments have also been adopted to improve pregnancy outcome in women with RSA such as aspirin/heparin therapy,

intravenous immunoglobulin (IV Ig) therapy, lymphocyte immunotherapy or 1α, 25-dihydroxy-vitamin-D3 (VD3) therapy depending on the diagnosis (Pandey *et al.,* 2005). Unfortunately, in these treatments used for improving pregnancy outcome in women with RSA, several risks and side effects have been reported.

1.3. The placenta

1.3.1. General features

Fetal growth and survival are critically dependent on the placenta, an organ resulting by the coexistence of two different components, the decidual tissue and the trophoblastic cells, which have maternal and fetal origin, respectively (Mossmann, 1937). The placental specific tissue is involved in regulating the physiologic contacts between mother and its fetus, providing the embryo with nourishment, eliminating its wastes and exchanging respiratory gases, in order to allow fetal optimal development. In addiction, the placenta directs maternal endocrine, immune, and metabolic functions to the embryo's advantage (Handwerger and Freemark, 2000). Placenta could be defined as an endocrine organ since it is an important source of hormones, such as human chorionic gonadotrophin, produced by the trophoblast before implantation (Handwerger and Freemark, 2000) and human placental lactogen, a protein hormone similar to growth hormone, influencing the preparations of the breasts for lactation and the lipid or carbohydrate metabolism (Handwerger and Freemark, 2000).

1.3.2. The human and murine placenta

The development of human as well as murine placentas takes place in three phases: implantation, decidualization and placentation. Implantation is represented by a complex sequence of events, describing the process of blastocyst attachment to the maternal endometrium before its invasion into the tissue (Aplin *et al.,* 1991; Abrahamsohn and Zorn, 1993). During early pregnancy, the uterus changes from an irregular shape to a lumen with crypts, where blastocysts become positioned before starting to implant. In humans, implantation takes place between days 6 and 7 post coitum (Bernische and Kaufmann, 2001), whereas in mice between days 4 and 5 after vaginal plug detection (Reinius, 1967). The contact between the embryo and the surface of the uterine epithelium increases gradually at the beginning of implantation and this is probably caused by a combination of

blastocyst swelling and closure of the uterine lumen (Bernische and Kaufmann, 2001). In order for implantation to be successful, the embryo must reach a proper stage of development and the endometrium must exhibit a state of receptiveness toward the embryo. After implantation, the maternal endometrium progressively transforms into decidua (decidualization process), and then placental formation starts until development is complete (placentation process, Bernische and Kaufmann, 2001).

1.3.2.1. The human placental structure

The human placenta is a discoid organ about 25 cm in diameter and 3 cm in thickness. Placenta is formed by the chorionic plate facing the fetus, where the umbilical cord is attached, and the basal plate which abuts the maternal endometrium (Fig.1; Bernische and Kaufmann, 2001). Internally human placenta consists of a vast array of finger-like processes, the villi. These arise from the chorionic plate as stem villi, and branch repeatedly to give rise to intermediate and finally terminal villi (Georgiades *et al.*, 2002). Terminal villi are the most important sites for maternal-fetal exchange, bathing directly by maternal blood. The outer surface of the villus is formed by terminally differentiated, multinucleated syncytiotrophoblast cells and a stem cell population called cytotrophoblasts, which differentiate and fuse to the syncytiotrophoblasts (Georgiades *et al.*, 2002).

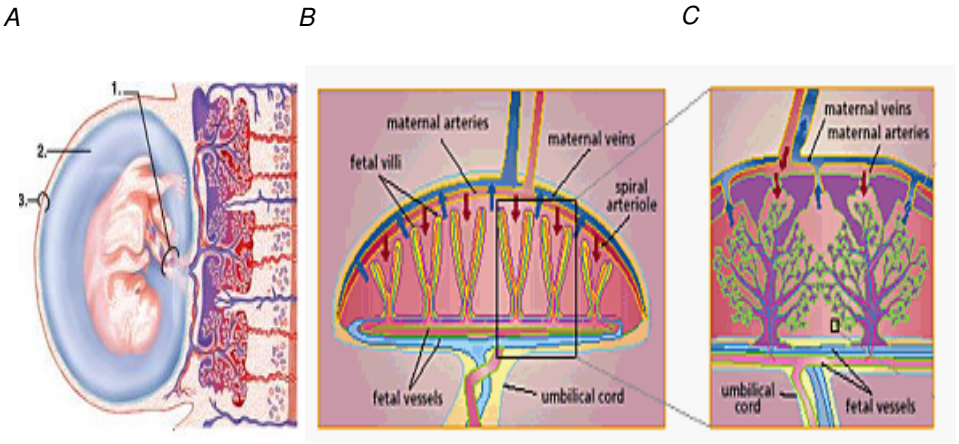

Fig. 1 *Structure of the human placenta. In A, a general view of fetus with its placenta is shown. The fetal blood vessels attach to the placenta through the umbilical cord. The mother's blood supply runs through the placenta, which supplies the fetus with nutrients and removes waste products from the fetal blood (1. umbilical cord; 2. amniotic fluid; 3. amniochorionic membrane, from www.mhhe.com). In B, human placenta with fetal villi containing fetal vessels and the*

umbilical cord are depicted. The villi are bathed in maternal blood. In C, two fetal villous trees illustrate the complex branching pattern (from Rinkenberger and Werb, Nature Genetics 2000).

The physiologically important exchange of substances between maternal and fetal blood takes place through the feto-maternal interface situated between the two circulations, where the following cell types are situated: **a**) the villous trophoblasts; **b**) a basement membrane closed to the syncytiotrophoblasts; **c**) a villous core; **d**) basement membrane; **e**) fetal capillary endothelial cells (Bernische and Kaufmann, 2001). Moreover, decidual tissue, representing the maternal part, is characterized by polygonal- or round-shaped cells together with a highly vascularized area rich in extravillous cytotrophoblasts. Other cells involved in immune response are usually populating the decidua such as uterine natural killer cells (uNK) that represent the majority of leukocytes infiltrated at the time of implantation (Aplin *et al.*, 1991; Bernische and Kaufmann, 2001), macrophages, T cells, B cells, granulocytes, plasma cells (Bernische and Kaufmann, 2001).

1.3.2.2. The murine placental structure

The murine placenta develops until the 14th day of gestation, whereas the fetus continues growing until delivery. This feature could be associated with the observation that ten or more fetuses may coexist in the uterus. Suzuki and colleagues (1997) nicely reported as murine placental growth is correlated to the formation of a more complex labyrinth rather than to any increase in size after twelve days of pregnancy (Fig. 2).

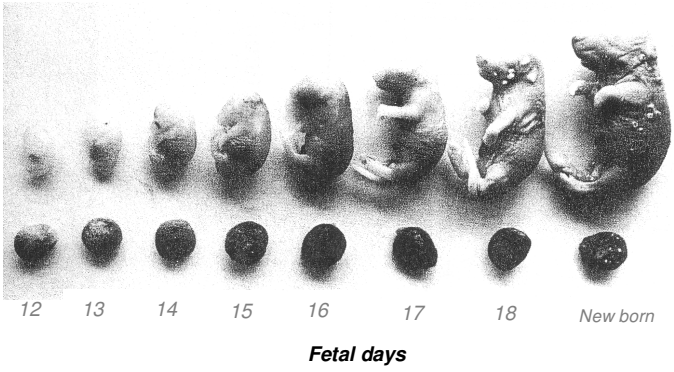

Fig. 2 *Comparison of size development between murine placentas (bottom) and fetuses (top) from fetal day 12 to just after birth (Suzuki et al., Trophoblast Res. 1997).*

The murine placenta be could divided into four areas such as 1) the chorionic plate; 2) the labyrinthic zone; 3) the junctional zone formed by spongiotrophoblast cells and giant cells; 4) the decidua basalis, whose function is the nourishment of the embryo, the contribution of formation of individual embryonic vascular systems during placental development, and restriction of trophoblast invasion (Abrahamsohn and Zorn, 1993). In the murine placenta, four differentiated trophoblast cell phenotypes can be readily defined (Georgiades *et al.*, 2002), such as **a**) The trophoblast giant cells, morphologically characterized by a big nucleus continuously producing DNA without cell division though a process called endoreduplication, and eosinophilic material in the cytoplasm. These cells exhibit invasive characteristics and posses significant endocrine activities. They are in contact with the maternal part, represented by round or polygonal decidual cells. **b**) The spongiotrophoblast cells, morphologically distinct from giant cells but with the same endocrine properties. **c**) The trophoblasts rich in glycogen, called glycogen cells. **d**) The labyrinthic cells forming a region containing fetal and maternal blood channels that circulate independently, and allowing physiological exchanges such as nutrients and oxygen to the fetus or waste products to the mother. Murine placenta and its internal organization are illustrated in Figs. 3 A and B.

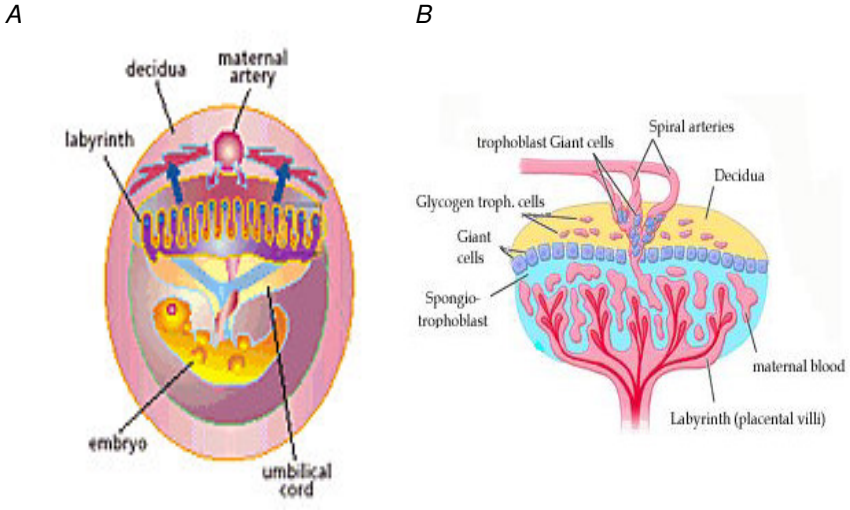

Fig. 3 *In A, general view of mouse fetus and its placenta in contact with the maternal part (deciduas; from Rinkenberger and Werb, Nature Genetics 2000). In B, the mature murine placenta with its cellular populations, decidual cells, giant cells, spongiotrophoblast cells, glycogen cells and labyrinthic cells (from Watson and Cross, Physiology 2005).*

1.4. Mice and reproduction

Female mice have an estrous cycle (periodic state of sexual excitement, that immediately precedes ovulation and during which the female is most receptive to mating) which is 4-6 days long (Lee Silver, 2001). Following copulation, female mice will normally develop a vaginal plug, which prevents further copulation. This plug (ejaculate from the male) stays in place for about 24 hours. The gestation period is about 19-21 days. The litter size is between 3 and 14 (average 6-8). The newborn are blind and furless. Fur starts to grow some three days after birth and the young open their eyes one to two weeks after birth. Females reach sexual maturity at about 6 weeks, whereas males after 8 weeks, but both can breed as early as 35 days (Lee Silver, 2001).

1.4.1. The murine combination CBA x DBA/2: a model to study spontaneous abortion

The mouse is extensively used as a model system to understand the genetic control of human development and several diseases. Beyond the readily available mouse mutations, this animal continues to represent the mammal of choice in research for a lot of compelling reasons such as low cost, a very short generation time and their docile and easy handling. Moreover, they are small enough (15-19 cm including the tail) so that thousands can be housed in relatively small rooms and they breed readily in captivity. Mice are mainly used as a model for human diseases in order to develop new drugs, and to test the safety of proposed drugs.

In pregnancy-related research, Clark and colleagues (1980) first discovered an increased rate of resorption (about 20-40%) when mating CBA/J (H-2k) female mice with DBA/2J (H-2d) males, which did not occur if mated with other H-2d bearing males like BALB/c. The abbreviation “H-2” refers to the mouse histocompatibility complex (MHC), which has a high degree of homology with the human leukocyte antigen (HLA) complex (the human MHC). The increased resorption rate, calculated as number of abortions in relation to the total number of implantations (Clark *et al.*, 1980, Chaouat *et al.*, 1986), occurs in certified strains of CBA/J female mice originating from Jackson Laboratory’s line (Bar Harbor, Main U.S.A.), therefore represented by a “J”. Over 20 generations of sister x brother inbreeding, all are genetically as alike as possible, being homozygous at more than 98% of their loci (Staats, 1985). Increased abortion or resorption rates

observed in CBA/J females previously mated with DBA/2J males have been associated with a high amount of Th1 cytokines at the feto-maternal interface (Raghupathy, 1997, 1999; Clark *et al.*, 1999; Chaouat *et al.*, 2003; Zenclussen *et al.*, 2002a, 2003), therefore the combination CBA/J x DBA/2J is defined as an immunological abortion model for human pregnancy failure (Dealtry *et al.*, 2000). The role of Th2-type cytokines in reducing fetal resorption was demonstrated by Chaouat and colleagues (1995), as they observed that placentas from resorption-prone CBA/J x DBA/2J mating produced less IL-4 and IL-10 than the control combination, CBA/J x BALB/c. Moreover, injection of neutralizing anti-IL-10 antibody in DBA/2J-mated CBA/J females increased the resorption rate, while anti-IFN-γ antibody partially reduced the number of rejected feti (Chaouat *et al.*, 1995). Thus, successful pregnancy was thought to be a Th2 phenomenon (Wegmann *et al.*, 1993; Chaouat *et al.*, 1995, 2003; Clark *et al.*, 1999), resulting from both down-regulation of Th1 type cytokine production, and the limitation of any resulting inflammatory responses. (Dealtry *et al.*, 2000).

1.5. Reproductive Immunology

1.5.1. Hypothesis on mechanisms involved in fetal rejection

Why does the fetus survive within the maternal uterus despite its paternal inheritance? Unfortunately, nowadays, this represents an unanswered question. Several studies have concentrated on the mechanisms allowing the fetus to survive without being rejected. Initially, in the 1953 Sir Medawar first recognized the paradoxical nature of the immunological relationship between the pregnant mother and her antigenically foreign child (a work which earned him a Nobel prize). He proposed several reasons why the fetus does not habitually provoke an immunological reaction from its mother, namely (1) the existence of an anatomical separation of the embryo from its mother, a barrier considered at that time impermeable to the maternal cells; (2) the antigenic immaturity of the fetus associated with the (3) immunological inertness of its mother. Years later, Chaouat and colleagues (1983) and Wegmann (1988) revisited the concept of the anatomical separation and proposed the “trophoblast barrier-immunotrophism mechanism”, where the trophoblast cells play a protective role for the rejectable fetus within the maternal immune system. Later, several studies described as maternal lymphocytes pass into the fetus and vice versa, pointing to a cellular

interaction between mother and fetus (Piotrowsky and Croy, 1996; Bonney and Matzinger, 1997). Another hypothesis proposed was the lack of expression of paternal major histocompatibility complex (MHC) alloantigens (classes I and II) on the fetal trophoblast (cells having paternal origin) forming the feto-maternal interface, considered a possible protection from maternal immune rejection (Hunt *et al*., 1988), although previously studies agreed that some murine trophoblast cells express fetal class I MHC molecules (Chatterjee-Hasrouni and Lala 1981; Zuckerman and Head 1986). Moreover, Clark and colleagues (1991) demonstrated that the fetus is rejected as an allograft if removed from its place and transplanted in muscle or kidney of the mother, thus underlying the ability of the fetus to express paternal MHC antigens.

The fetus has also been defined as a parasite taking all nutrients required from its mother and giving her back the wastes, and the placental trophoblast cells have been resembled to a successful tumor protecting the fetus and growing undisturbed within the maternal uterus until it reaches the vessels (Clark *et al*., 1999). From the end of the ´80s other concepts were suggested in the reproductive immunology field, such as the ability of the trophoblast to induce apoptosis of maternal Th1 type lymphocyte by releasing FAS ligand (FASL) at the feto-maternal interface (Zhang *et al.,* 1989; Runic *et al.,* 1996; Hunt *et al.,* 1997; Kauma *et al.,* 1999). As matter of fact, the local production of FasL represented a mechanism through which trophoblast cells may induce tolerance and self-regulate survival during invasion and subsequent placentation. However, *lpr* mutation in mice – resulting in loss of Fas function- had no effect on pregnancy outcome (Hunt *et al*., 1997). Recent studies proposed no evidence for apoptosis of decidual leukocytes in normal and abnormal pregnancy in humans (Pongcharoen *et al.,* 2004), showing that trophoblast survival is not depending upon FasL-mediated apoptosis of maternal leukocytes at the feto-maternal interface. Moreover, the specialized fetal tissue in contact with maternal uterus could contribute to tolerance by mechanisms such as inactivation of natural killer (NK) cells through HLA-G expression (Rouas-Freiss *et al*., 1997) or depleting tryptophan (Munn *et al*., 1998). A point of continued heated debate in pregnancy research is the involvement of pro- and anti-inflammatory cytokines in determining either successful pregnancy or abortion. In fact, it was proposed that pathologic pregnancies leading to abortion are characterized by cell-mediated (Th1)

immunity, and that successful pregnancies are biased towards a T helper type 2 (Th2) response. This concept was often defined as the "Th1/Th2 paradigm in pregnancy", now considered too simplistic because the production of Th1 cytokines is essential for implantation and a correct placental development (Joswig *et al.*, 2003). Novel studies suggested the existence of alternative pathways inducing tolerance during pregnancy. Cells expressing indolamine 2,3-dioxygenase (IDO) were proposed to represent an immunosuppressive barrier protecting the fetus from maternal T cell immunity (Munn *et al.*, 1998; Mellor and Munn, 2001), suggesting IDO as an alternative pathway leading to fetal tolerance. However, new data re-questioned the importance of IDO due to the normality of pregnancy outcome in mice $IDO^{-/-}$ (Baban *et al.*, 2004). $CD25^{+}CD4^{+}$ regulatory T cells (Treg) became evident for their potential protective ability to suppress activation of the immune system, therefore in homeostasis and tolerance to self (Sakaguchi *et al.*, 1995; 2004). Aluvihare and colleagues suggested an important role of Treg in a model of normal murine pregnancy (2004). Our group recently reported the essential role of Treg in pregnancy, which consists in protecting the fetus from attacks by maternal immune system (Zenclussen *et al.*, 2005b, 2006). In fact, the transfer of Treg cells isolated from normal pregnant mouse and injected into DBA-mated CBA/J female mice completely prevented fetal rejection (Zenclussen *et al.*, 2005b). These and other studies suggest that during normal pregnancy a systemic expansion of maternal $CD25^{+}$ T cells is induced and is capable of suppressing alloresponses, whereas absence of regulatory T cells is associated to an aggressive response against the fetus by the maternal immune system, leading to abortion (Aluvihare *et al.*, 2004; Zenclussen *et al.*, 2005b, 2006). An emerging mechanism involved in inducing fetal survival is represented by the heme oxygenase (HO) enzyme pathway. HO proteins play an important role in allowing graft acceptance (Soares *et al.*, 1998); its up-regulation is further associated with successful pregnancy, whereas diminished levels of HO lead to abortion and acute graft rejection (Soares *et al.*, 1998; Zenclussen *et al.*, 2003b; 2005a; Sollwedel *et al.*, 2005; Zenclussen M.L. *et al.*, 2006).

Very recently, Mincheva-Nilsson and colleagues (2006) proposed that proteins A and B (MICA and MICB) appear to suppress immune activity by binding to the NK cell receptor (NKGG 2D). In this work, they found that human placentas express mRNA for MICA and MICB, and that MIC protein expression was found in the

outermost fetal component of the placenta – the syncytiotrophoblast cells -. They observed a reduced expression of NKG2D in white blood cells of pregnant women (approximately 55%) compared to non-pregnant ones , pointing to a possible novel immune escape mechanism for fetal survival.

Despite years of research in reproductive immunology, the main mechanisms involved in fetal tolerance remain unclear, although new interesting pathways are emerging which might open novel therapeutic opportunities.

1.5.2. Cytokines network at the feto-maternal interface

1.5.2.1. Cytokines: general features

Cytokines are peptides with a molecular weight < 100 kDa, with pleiotropic regulatory effects on haematopoietic and many other cells, which participate in host defense and repair processes (Vilcek, 1998). The term “cytokine” includes lymphocyte-derived factors such as “lymphokines” (Interleukins, IL, Interferons, IFN, Tumor necrosis factors, TNF), monocyte-derived factors called “monokines”, haematopoietic “colony stimulating factors”, “growth factors (GF)” of the connective tissue and chemoattractant cytokines (chemokines, CC; Vilcek, 1998). Cytokines are produced by cell-mediated as well as innate immunity. An important source of cytokines is represented by helper T lymphocytes (Th lymphocytes). Cytokines bind to high-affinity receptors on target cells (most act on the cells that produce them, so called *autocrine action*, or in adjacent cells, *paracrine action*) to promote cytokines gene transcriptions mainly through signal transducers and activation of transcription (Stat) pathways (Stat-3, -4 or -6 pathways are activated after binding of Th1 or Th2 cytokines) (Jacobson *et al.*, 1995; Kaplan *et al.,* 1996; O`Garra and Naoko, 2000).

1.5.2.2. Cytokines: role during pregnancy

During pregnancy, the cross talk among maternal and fetal tissues could contribute to the maintenance of gestation, and its disturbance (i.e. implantation failure, preeclampsia) might result in abortion. During gestation, cytokines are mainly involved in (1) the stimulation of the growth, differentiation and function of maternal epithelial cells from uterus and tubes; (2) stimulation of the growth, differentiation of the embryo before and after trophoblast implantation; (3)

coordination and synchronization during embryo development, and they are involved in the relationship at the feto-maternal interface (Tabibzadeh and Babaknia 1995; Dealtry *et al*., 2000). Placental tissue produces cytokines and hormones, which are essential in the regulation at the feto-maternal interface. Saito (2000) nicely depicted a hypothetical scenario showing cross talk between ovary, placenta and decidua necessary to ensure successful pregnancy. As illustrated in Figure 4, the ovary releases progesterone hormone resulting in stimulation of Th2 cells to produce cytokines. Th2 cytokines such as IL-4 and leukemia inhibitory factor (LIF) promote the secretion of human chorionic gonadotropin (hCG) from placental trophoblasts (Saito *et al.,* 1997), which further induces progesterone production from the ovary, resulting again in Th2 cell induction. Moreover, Th2 cytokines such as IL-4 and IL-10 are known as factors able to induce differentiation of Th2 lymphocytes from Th0 cells (Mosmann and Sad, 1996), and may control intrauterine natural killer (NK) cells by inhibiting their augmentation through IL-2Rα IL-2Rβ, IL2Rγ control, therefore preventing possible attacks on the trophoblasts (Saito *et al.,* 1996). Placental cells (trophoblasts, Tc) and decidual macrophages (MΦ), both of which have tryptophan catabolic enzymes (such as indolamine 2,3-dioxygenase, IDO) involved in controlling the activation of maternal cytotoxic T cell, collaborate in order to protect the placental tissue against attacks by maternal T cells (Munn *et al*., 1998). IDO expression at the feto-maternal interface was proposed to be necessary to prevent immunological rejection of fetal allograft as the inhibition of tryptophan catabolism during pregnancy allows maternal lymphocytes to mediate abortion (Munn *et al*., 1998). Munn and colleagues (1998) hypothesized that T cells possess a specific cell-cycle regulatory checkpoint sensitive to the level of tryptophan, suggesting that IDO suppresses T cell activation and proliferation in local microenvironments by nutrient depletion. Since the exact mechanism by which IDO-expressing trophoblast cells protect the allogeneic fetus from its rejection remains to be elucidated and considering that recently studies (Baban *et al*., 2004) describe as mice $IDO^{-/-}$ have normal gestation, the importance of IDO in determining successful pregnancy needs to be revised.

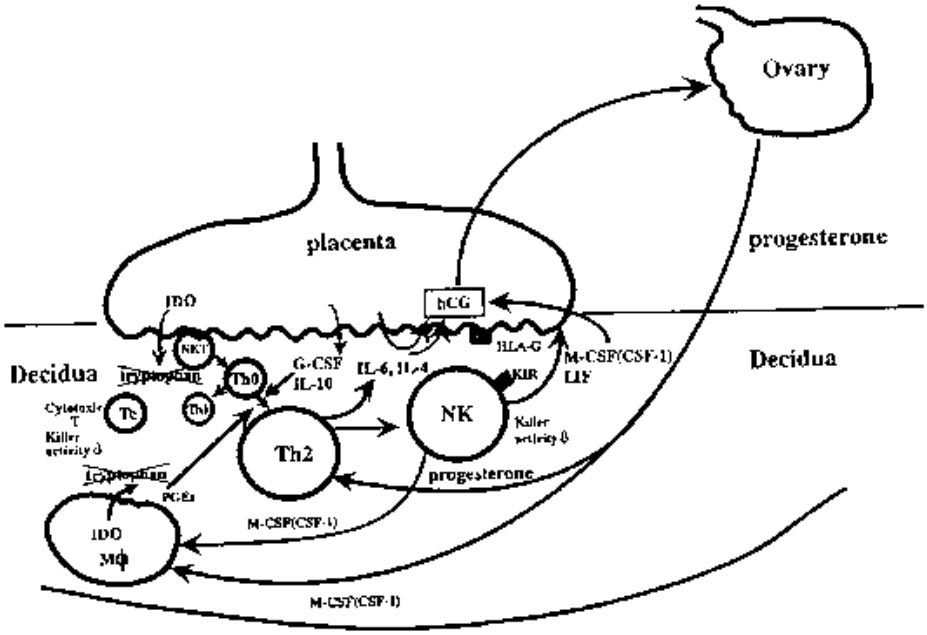

Fig. 4 *Cytokine and endocrine networks at the feto-maternal interface (Saito, J. Reprod. Immunol., 2000).*

1.5.2.3. The multifunctional cytokine TNF-α during pregnancy

TNF-alpha is a pleiotropic pro-inflammatory cytokine with a wide variety of functions in many cell types, such as immune and host defense responses, angiogenesis stimulation, tissue remodeling influence (Baud and Karin, 2001), and apoptosis promotion via its two receptors, TNF-RI and TNF-RII (Hengartner *et al.*, 2000). TNF-α is representative of a still growing family of cytokines and cell surface proteins such as lymphotoxin-α(LT-α), Fas ligand (FasL), receptor-activator of NF-κB (nuclear factor-kappa B) ligand (RANKL), CD40 ligand (CD40L) and TNF-related apoptosis-inducing ligand (TRAIL), which display 25-30% sequence similarity, mostly in residues responsible for their trimerization (Baud and Karin, 2001). Studies revealed the location of FasL receptor in syncytiotrophoblasts, whereas FasL has been reported in villous cytotrophoblast cells in the first trimester placentas (Huppertz *et al.*, 1998). Other studies proposed FasL in both cellular layers of trophoblasts through gestation (Runic *et al.*, 1996; Zorzi *et al.*, 1998). Interestingly, mice with a mutated FasL gene express a dysfunctional protein. During pregnancy, namely on day 10, the placentas of these

mice exhibited leukocyte infiltration and necrosis at the feto-maternal interface, suggesting how a functional FasL became very important at midgestation (Hunt *et al.*, 1997). Controversial is the cause and effect relationship. Although the pregnancy outcome in these animals is always associated to death because of placental disintegration, it is still not clear if this is due to immune cells attack or if it could stimulate leukocytes infiltration. In pregnancy-related studies, TNF-α was identified in the ovary, oviduct, uterus and placenta (Terranova *et al.*, 1995), and seems to be expressed also in embryonic tissues at all stages of development (Kohchi *et al.*, 1994). Moreover, TNF-α, together with TNF-α-like factors, represents a key factor in normal processes such as cell renewal or tissue homeostasis (Hunt *et al.*, 1992, 1996), and during implantation, this multifunctional cytokine ensures a correct attachment of the blastocyst to the uterine wall by creating the right inflammatory environment (Sharkey, 1998; Ashkar *et al.*, 1999, 2000). In fact, during early pregnancy, the uterine epithelium undergoes apoptosis, and regression of decidual cells coordinates trophoblast invasion. The apoptotic degeneration of the maternal tissue seems to be mediated by TNF-receptor 1 (Joswig *et al.*, 2003). Several investigations proposed TNF-α as being involved in pregnancy failure. High levels of TNF-α in amniotic fluid are associated with infection or preterm labour (Romero *et al.*, 1989). Studies showed that administration of lipopolysaccharide (LPS) to pregnant animals causes abortion, since LPS, an endotoxin and component of the cell-wall of gram-negative bacteria, binds to its receptors and sends a powerful stimulus for secretion of cytokines, such as TNF-α, by macrophages which induces pregnancy failure (Silver *et al.*, 1994). Moreover, in the abortion murine combination CBA/J x DBA/2J, increased level of TNF-α as well as IFN-γ cytokines were observed when compared to the controls (Tangri and Raghupathy, 1993; Clark *et al.*, 1999; Raghupathy, 2001, Fest *et al.*, 2006) and proposed as a cause of abortion. TNF-α is a potent factor, which exerts positive and negative effects during pregnancy. This factor´s intriguing dichotomy together with its powerful ability in triggering apoptosis via its two receptors makes this molecule a pivotal component during pregnancy.

1.5.3 Successful or failed pregnancy outcome: the Th1/Th2/Th3 paradigm

T helper lymphocytes (Th lymphocytes) $CD4^+$ can be divided into subsets of cells, Th1 and Th2 lymphocytes, differing in pattern of cytokine production and in the role during immune response (Raghupathy, 2001). Th1 cells are involved in the regulation of cellular immunity by secreting Th1 or pro-inflammatory cytokines such as interleukin –1, IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, interferon gamma, IFN-γ or tumor necrosis factor, TNF-α, whereas Th2 cells play a role in humoral response activation by secreting Th2 or anti-inflammatory cytokines like IL-4, -5, -10 and IL-13 (Mosmann *et al.,* 1989, 2005). The existence of a third group, denominated Th3, involved in the secretion of TGF-β with suppressive activity toward Th1 response, was proposed by Chen and colleagues (1994). During pregnancy, an additional source of cytokines is represented by those cytokines secreted by decidual epithelium and stroma, cyto- and syncytiotrophoblast cells, chorion, amnion and uterine natural killer cells (uNK). The cytokines secreted by these cells are also defined as type 1 or type 2 reactivity (Mosmann and Sad, 1996), although it is usual to describe them as Th1/Th2. Cytokines originating from these tissues take part in induction of maternal tolerance towards fetal allograft, regulate local immunity against infective factors, modulate placental hormonal production and tissue remodeling during trophoblast invasion (Guilbert *et al*., 1993; Robertson *et al*., 1994; Bennet *et al*., 1998). An appropriate cytokine balance at the feto-maternal interface during different stages of gestation is thought to be fundamental to avoid fetal rejection (Wegmann *et al*., 1993; Clark *et al*., 1999; Raghupathy *et al*., 1997, 1999). The imbalance between pro- and anti-inflammatory cytokines towards an inflammatory profile is thought to be involved in reproductive failures in humans as well as in mice (Clark *et al*., 1999; Raghupathy *et al*., 1997, 1999). In this sense, it has been proposed under the definition of "Th1/Th2/Th3 paradigm" that a Th2-Th3 type response could be associated with successful pregnancy (Lin *et al*. 1993, Chaouat *et al*., 1995; Raghupathy, 2001) while the prevalence of an inflammatory response, i.e. a Th1-type response, would not allow the fetus to survive (Lin *et al*., 1993; Krishnan *et al*., 1996a; Zenclussen *et al*., 2003a). The possible positive role of a switch to Th2 cytokines during pregnancy was proposed after observing that injection of Th1 cytokines (such as TNF-α, IFN-γ and IL-2) into pregnant mice causes abortion (Clark *et al*., 1999),

whereas up-regulation of IL-10 could prevent naturally occurring fetal loss in CBA/J x DBA/2J combination (Chaoaut *et al*., 1995). Moreover, gestional tissues harvested from mice undergoing spontaneous abortion showed a Th1 cytokine bias (Clark *et al*., 1999), whereas placental IL-10 production is low in abortion-prone crossing of mice (CBA/J x DBA/2J; Clark *et al*., 1999). Nevertheless, recently published data indicated that the Th1/Th2 cytokine ratio might not be the overall explanation for successful reproductive outcome, but a way to simplify (Svensson *et al*., 2001; Zenclussen *et al*., 2002a; Chaouat *et al*., 2003). Moreover, mice lacking both functional genes for IL-4 and IL-10 have normal litter sizes confirming that the Th1/Th2 ratio may not be as important as it was thought in the ´90s (Svensson *et al*., 2001). Recently, Barber and colleagues (2005) reported that Th1 cytokines such as TNF-α and IFN-γ are synthesized in the placenta in response to *Lysteria monocytogenes* infection, in order to eradicate this pathogen. Their data showed a requirement for Th1 cytokines during pregnancy to combat infections. Generally, it is accepted that local changes in the balance of Th1/Th2-cytokine profiles occurring during pregnancy within the maternal uterus and at the feto-maternal interface, contributes to implantation, placental development, fetal survival and immunity.

1.5.3.1. Th1 activity during pregnancy

Th1 cytokines, although harmful during mid gestation, are necessary at the early stages of pregnancy (Ashkar *et al*., 1999, 2000; Saito *et al*., 1999). After attachment of the blastocyst to the uterine epithelium (implantation), embryonic signals initiate the process of decidualization, in order to ensure placental development. Implantation itself is totally dependent on the presence of pro-inflammatory cytokines such as IL-1, TNF-α and IFN-γ (Sharkey, 1998; Ashkar *et al*., 1999, 2000) as well as the leukemia inhibitory factor (LIF), a pleiotropic cytokine first identified by Hilton (1988), which is up-regulated in mice on day 4 of pregnancy, just before implantation (Bhatt *et al.* 1991). LIF deletion in mice causes abnormal decidualization, uterine stroma remodeling and implantation defects (Stewart *et al.*,1992). Despite fundamental in the early stage of fetal development, pro-inflammatory cytokines seem to be involved in pregnancy failure (Wegmann *et al.,* 1993; Raghupathy, 1997, Clark *et al*., 1999). Tangri and Raghupathy (1993)

showed that high TNF-α and IFN-γ levels were present in the uteri of aborting CBA/J x DBA/2J mice compared to normal pregnancy controls, and Wegmann (1993) realized that these two cytokines (TNF-α and IFN-γ) are the more representative involved in the onset of abortion. Recently, Fest and colleagues reported TNF-α as a crucial cytokine mediating abortion after stress application or Substance P injection (Fest *et al.*, 2006). Chaouat and colleagues (1995) described a significant decrease in Th2 cytokines by decidual cells in the CBA/J x DBA/2J abortion combination compared to pregnant CBA/J females previously mated with BALB/c mice. Additionally, IL-6 is augmented in abortion-prone mice as compared to normal pregnant mice (Zenclussen *et al.*, 2003a). Despite several works pointing to the relevance of Th1 cytokines in causing abortion if elevated, novel data suggest that Th1 augmentation alone does not explain abortion as Th1 cytokines seem to be essential for placental immunity, such as against *L.monocytogenes* infection (Barber *et al.*, 2005). Further, Zenclussen and colleagues (2002a) described augmented IL-12 in blood from normal pregnant women as compared to non-pregnant age-matched women. Patients prone to miscarriage, on the contrary, have lower IL-12 levels compared to normal pregnant individuals. Chaouat later reported increased levels of Th1 cytokine IL-18 in abortion-prone mice as compared to normal pregnant mice (Chaouat *et al.*, 2003).

Nowadays, it is well known that Th1 activity is necessary for implantation to occur as well as for the regulation of placentation and defense against infection. However, if for some reason augmented they may compromise pregnancy.

1.5.3.2. Th2 activity during pregnancy

Trophoblast, decidua, chorionic and amniotic membranes are all the source of Th2 cytokines and TGF-β (Roth *et al.*, 1996; Bennett *et al.*, 1998). Wegmann and colleagues (1993) proposed for the first time that during pregnancy Th1/Th2 activity balance is strongly shifted towards Th2 activity (the so called “Th2 phenomenon”). Accordingly, an increased Th1 activity is incompatible with successful pregnancy. The role of Th2-type cytokines in reducing abortion was demonstrated by Chaouat (1995), who showed that placentas from resorption-prone CBA/J x DBA/2J mice produced less IL-4 and IL-10 compared to placentas

from normal pregnant mice. Moreover, this study revealed that administration of Th1 activity inhibitors, such as antibodies against IFN-γ or exogenous Th2 cytokines (IL-3 or IL-10) protect the embryos from rejection. Krishnan and colleagues (1996 a,b) infected pregnant and non-pregnant C57BL/6 mice with the parasite *Leishmania major.* Only pregnant mice, in which the Th1 response was physiologically suppressed although not totally abrogated, could not clear the infection. On the contrary, non-pregnant mice were fully recovered by a strong Th1 type cells response. These studies supported the concept of a maternal Th2 immune response during successful pregnancy. On the other hand, other studies showed that IL-4 and IL–10 knock-out mice have normal pregnancy, pointing out that these cytokines, despite their proven protective role during pregnancy, are not essential for successful completion of gestation, thus re-questioning their importance (Svensson *et al*., 2001).

1.6. Pregnancy-protective molecules: heme oxygenase enzymes (HO)

1.6.1. Overview

The heme oxygenase (HO) enzymes were originally characterized in 1969 (Tenhunen *et al*.) as responsible for the heme degradation into bilirubin and CO. HO has been proposed to have tissue-protective properties by avoiding toxic accumulation of free heme from damaged red blood cells. Studies described HO being involved in the control of vascular tone (Christova *et al*., 2000), regulation of anti-inflammatory processes (Sass *et al*., 2003; Song *et al*., 2003) and anti-apoptotic responses (Soares *et al*., 1998; Brouard *et al*., 2000; Ke *et al*., 2002; Song *et al*., 2003). HO is also responsible for the reduction of oxidative stress and subsequent tissue damage in several organ systems (Siow *et al*., 1999; Masini *et al.,* 2003). Three forms of HO such as HO-1, HO-2 and HO-3 were identified (reviewed in Montellano, 2000). HO-1 is a 32 KDa, highly induced by several factors, like some pro-inflammatory Th1-type cytokines (Terry *et al.,* 1998), ultra violet radiations (Keyse *et al.,* 1989), oxidative stress (Applegate *et al.,* 1991), and hypoxia (Maines, 1997), therefore coined as a “stress protein”. On the contrary, HO-2, a protein of 36 KDa, does not seem to be inducible (Montellano, 2000). However, it is known that HO-2 is inducible in reproductive tissues such as placenta and decidua (Lyall et al., 2000; Zenclussen *et al.* 2003b, 2005a). HO-2 is found in numerous tissues throughout the body, and appears to be involved in the

maintenance of basal heme metabolism (Elbirt and Bonkovsky, 1999). HO-3 represents a less characteristic and active of the three forms (Maines 1997; McCoubrey *et al.,* 1997), which might have a regulatory role in heme-dependent cellular processes (McCoubrey *et al.,* 1997). HO-1 is ubiquitously distributed in mammalian tissues but expressed in high concentrations in spleen and liver, areas having an increased erythrocyte turnover (Elbirt and Bonkovsky, 1999). Interestingly, heme oxygenase is expressed at high concentrations in the placenta (Ihara *et al.* 1998, Zenclussen *et al.* 2005a). Free heme can undergo auto-oxidation to produce superoxide (O^{-}_{2}) and hydrogen peroxide (H_2O_2), which may promote the formation of highly toxic reactive oxygen species (ROS), therefore HO is recognized as anti-oxidant by removing free heme (Montellano, 2000). In addiction, biliverdin and Fe^{2+} (second and third catalytic products of heme degradation) contribute to the HO oxidant effect. Inadequate HO-1 expression represents a common event in the pathogenesis of inflammatory diseases like endotoxic and septic shock (Cohen, 2002) as well as atherosclerosis (Libby, 2002), coronary artery disease, abdominal aortic aneurysm, myocardial infarction, chronic rejection of transplanted organs (Bach *et al.,* 2005) or autoimmune diseases such as arthritis or multiple sclerosis (Nathan, 2002). Moreover, there is convincing evidence that the up-regulation of HO-1 allows the acceptance of mouse allograft (Soares *et al.,* 1998; Woo *et al.,* 1998; Araujo *et al.,* 2003), and protects tissues from oxidative injury (Agarwal *et al.,* 1995; Lee *et al.,* 1996; Poss *et al.,* 1997).

1.6.2. Heme oxygenases: protective effects during pregnancy

Taking into consideration the role of heme oxygenases in transplantation, and the fact that the fetus could be considered a semi-allograft within the maternal uterus (Medawar, 1953), our group has hypothesized that in pregnancy, HO enzymes may be involved in tolerance towards the semiallogeneic fetus. Considering that placenta transports a huge amount of oxygen, it is very probable that HO plays a determining role during pregnancy (Zenclussen *et al.* 2003b, 2005a; Sollwedel *et al.*, 2005; Zenclussen M.L. *et al.*, 2006). This would ensure the correct development of the fetus within maternal uterus. Recent studies reported diminished expression of both isoforms, HO-1 and HO-2, at the feto-maternal interface from mice undergoing Th1-mediated abortion compared to normal

pregnant mice, suggesting a protective effect of heme oxygenases in allogenic murine pregnancy maintenance (Zenclussen *et al.*, 2002b, 2005). Moreover in humans, a significant reduction of both HO-1 and HO-2 expression has been also revealed in placenta samples from patients with pregnancy complications as compared to normal pregnant women (Barber *et al.*, 2001; Zenclussen *et al.*, 2003b). A hypothetical scenario nicely proposed by Zenclussen and colleagues (Fig. 5; 2002b) compares the different regulations of HO in the case of normal pregnancy or abortion. Here, in the situation of pregnancy failure, diminished levels of HO-1 in decidual cells, trophoblast and/or endothelial cells would lead to augmented levels of free heme, which is per se toxic, further allowing pro-inflammatory lymphocytes to reach the feto-maternal interface.

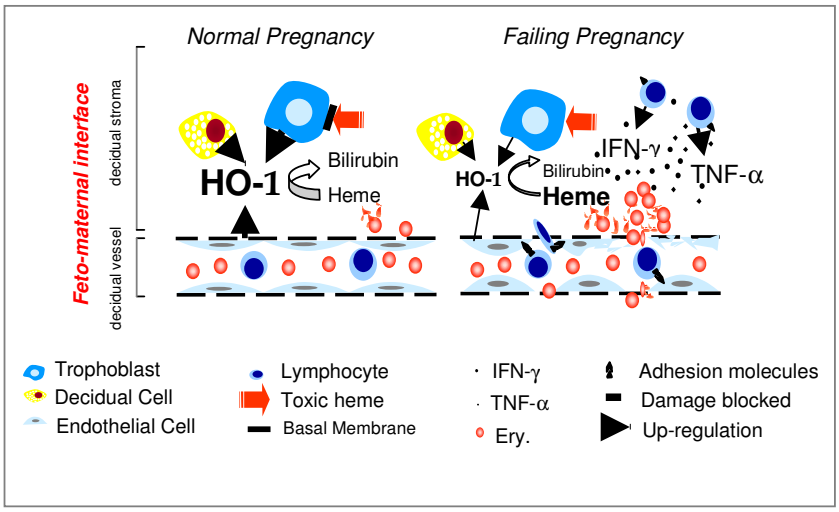

Fig. 5 *Hypothetical scenario showing how down-regulated levels of HO could be implicated in the onset of abortion, from Zenclussen et al. Scand.J.Immunol.2002b.*

1.7. Apoptosis

1.7.1. Cell death: history and types

A cell can – and often does - kill itself through a process known as *apoptosis* or *programmed cell death* (PCD). This capacity is essential during development, defense as well as in maintaining of tissue homeostasis. PCD is defined as cell death that occurs at a specific point in development (and is, therefore, "programmed", Zhang *et al.*, 2003). The interest in cell death began in the middle of the 19th century with some morphological observations. Then, in 1972, the Australian pathologist J.F.R Kerr and his Scottish colleagues A.H. Wyllie together with A.R. Curie described a similar death in mammals, affecting individual cells within a tissue. They observed how certain dying cells share a number of common morphologic features and how apoptosis observed during development is the same process happening in mature organisms and continuing throughout life. They coined the term *apoptosis* derived from the Greek "apoptein" (αποπτειν), meaning "dropping off", as in the dropping off of flower petals or falling leaves (Kerr *et al.*, 1972; Wyllie *et al.*, 1980), to distinguish it from *necrosis* (νεκροσις, meaning in Greek "make dead"). *Apoptosis* and *necrosis* are two forms of cell death, clearly distinct from each other by different morphological as well as biochemical events (Table 1; Wyllie *et al.*, 1980). Necrosis is induced accidentally by injury and exhibits early lysis of the plasma membrane before any significant alterations in nuclear morphology, whereas apoptosis is a genetically controlled event defined by a specific pathway pattern leading to characteristic cell shrinkage together with nuclear and cytoplasmatic changes (Wyllie *et al.*, 1980; Liu *et al.*, 1997; Kothakota *et al.*, 1997; McPherson and Goldberg, 1998). Apoptosis and necrosis can take place simultaneously in tissues or cells exposed to the same stimulus (Shimizu *et al.*, 1996), although often the intensity of the initial insult decides which event will prevail over the other (Bonfoco *et al.*, 1995). Interestingly, apoptosis in response to lack of adhesion or inappropriate adhesion has been termed *anoikis* (a Greek word meaning "homelessness"); it represents the programmed cell death of anchorage-dependent cells that detach from the extracellular matrix (ECM, Frisch and Francis, 1994). Usually cells stay close to each other in order to constitute the tissue, which they belong to. The communication between proximal cells as well as between cells and ECM provides an essential signal for growth or survival, therefore, when cells are

detached from the ECM, they undergo apoptosis. All the features characterizing apoptosis, including nuclear fragmentation and membrane blebbing, are observed during anoikis. Chipuk and Green (2005) described another type of cell death, called *caspase-independent cell death* (CICD), occurring when a signal that normally engages apoptosis fails to activate caspases, but the cell, nevertheless, dies. This kind of death is induced as defined for apoptosis, but in absence of caspase activity to dissemble the cell. Operationally, this is usually seen when caspase inhibitors are present or when the pathway is genetically disrupted. CICD does not resemble apoptosis; in particular there is no chromatin condensation, DNA fragmentation or cellular blebbing. In the following table the main features of three types of cell death (apoptosis, necrosis and caspase-independent cell death) have been summarized.

Target	APOPTOSIS	NECROSIS	CASPASE-INDEPENDENT CELL DEATH
Nucleus	*Cleavage of caspase targets* *DNA laddering* *Nuclear envelop alterations* *Fragmentation of nuclei* *Chromatin condensation*	*DNA random fragmented*	*Partial chromatin condensation* *Marginalization of chromatin around nuclear membrane* *No DNA laddering* *Shrinkage of nucleus* *Dilatation of perinuclear cisternae*
Mitochondrion	*Outer-membrane permeabilization* *Cleavage of caspase targets* *Loss of membrane potential*	*Mitochondrial swelling*	*Outer membrane swollen or absent* *Membrane potential present*
Cytoplasm	*Cleavage of caspase targets* *Condensation* *Increase of [Ca^{2+}]* *Protein cross-linking*	*Dilatation of endoplasmic reticulum* *Decrease of [Ca^{2+}]*	*Vacuolated* *No controlled organelles break-down* *Darkened cytoplasm* *Aggregation of ribosome*
Plasma membrane	*Cleavage of caspase targets* *Loss of membrane-phospholipid asymmetry* *Annexin V positive*	*Fall down of ionic transport* *Swelling* *Membrane lysis* *Propidium Iodide positive*	*Ragged cell surface* *Membrane blebbing* *Annexin-V negative*
Cell	*Loss of proliferation capacity* *Cell detachment from matrix* *Apoptotic bodies formation* *Phagocytosis of dying cell* *Any immune response activation* *Slow event (max 24 hours)* *Active process requiring ATP*	*Release of cytoplasmic content in the extracellular space* *More cells involved* *Immune response activation* *Fast event (minutes)* *Passive process not requiring ATP*	*Loss of proliferation capacity* *No apoptotic bodies present* *Cells often remain attach to the matrix* *Cells are removed by unknown mechanisms*

Table 1 *Summary of the features distinguishing and characterizing the three cell death types, from Chipuk and Green, Nature 2005.*

1.7.2. Features

1.7.2.1. Phases characterizing apoptosis

Programmed cell death plays a determining role in many biological mechanisms such as that involved in construction, maintenance and repair of tissues. In animal development, apoptosis is essential for establishing tissue architecture (Meier *et al.,* 2000; Yuan and Yankner, 2000), whereas in the immune system it is the most common form of death, regulating lymphocyte maturation, receptor repertoire selection and homeostasis (Green, 2003). Apoptosis derailment is involved in severe diseases such as autoimmunity or cancer, where immune cells fail to die due to an over-expression of bcl-2 (an anti-apoptotic gene) and/or under-production of Fas (death receptor; Krammer, 2000). On the other hand, acquired immunodeficiency syndrome (AIDS) is characterized by too much apoptosis leading to a depletion of $CD4^+$ T helper cells, called immunodeficiency (Krammer, 2000). Apoptosis exists as a mechanism to remove cells. This process could be result of infected or damaged cells, or when there are excessive quantities of cells or they have failed a developmental “test”, or simply because their presence is no longer required for physiological processes. There are several inductors involved in apoptotic activation such as glucocorticoids (Wyllie *et al*., 1980), DNA-damaging agents (ionizing, radiations, chemo-therapeutic or methylating agents), reactive oxygen species (ROS) and lack of survival signals (Hengartner, 2000). This first phase is called *induction phase* and is followed by an *effector phase*, representing a point of no-return, since the numerous apoptotic stimuli converge into two main pathways (intrinsic or extrinsic, death cell receptor or mitochondrial respectively) involving proteases called caspases (cysteine proteases with specificity for aspartate residues), are no longer reversible. *Degradation phase* occurs whereby the cellular functions and structures begin to get destroyed, giving rise to a full-blown phenotype (apoptotic bodies) which will be further completely engulfed by macrophages or neighbor cells (Hengartner, 2000).

1.7.2.2. Pathways inducing apoptosis

Depending upon the stimuli, apoptosis can be initiated by one of two pathways: the death receptor mediated (*extrinsic*) or mitochondrial pathways (*intrinsic*). It was suggested that these two were independent of each other, then it has been demonstrated that Bid, a member of Bcl-2 family proteins, serves as a link

between the two for completing apoptosis (Li *et al*., 1998, Luo *et al*., 1998). The nematode *Caenorhabditis elegans* has been a good model organism for deeply investigating the core components of cell death machinery (Hengartner *et al*., 2000).

✓ *Death receptor-mediated pathway*

The death receptor-mediated pathway - or extrinsic pathway - is triggered by members of the death-receptor super-family (e.g. CD95, also called Fas or Apo-1), and tumor necrosis factor receptor I or II (TNF-RI or TNF-RII, respectively; Ashkenazi and Dixit, 1998). The signal is provided by interaction between specific ligands (e.g. FasL/CD95L and TNF-α) and their death receptors on the cellular membrane, leading to receptor clustering induction and formation of the death-inducing signaling complex (DISC). The members of the death receptor family contain five cysteine-rich repeats in their extracellular domain and a death domain (DD) in their cytoplasmic tail. The DD is essential to initiate the apoptotic signal (Danial and Korsmeyer, 2004). After ligand-receptor binding, the death receptor oligomerizes and DISC recruits, via the adaptor proteins FADD (Fas-associated death domain) and TRADD (TNFR-associated death domain), inactivated uncleaved pro-caspase-8 molecules, resulting in its activation (Danial and Korsmeyer, 2004). Once cleaved, this initiator caspase is able to generate active effector caspases (-3, -6, and -7), which cleave a number of target substrates. In particular, caspase-3 plays a major role in disassembly of the nucleus by processing several nuclear substrates, being actively transported to the nucleus through the nuclear pores (Faleiro and Lazebnik, 2000). The death receptors and their pathways are summarized in the following schematic picture (Fig. 6).

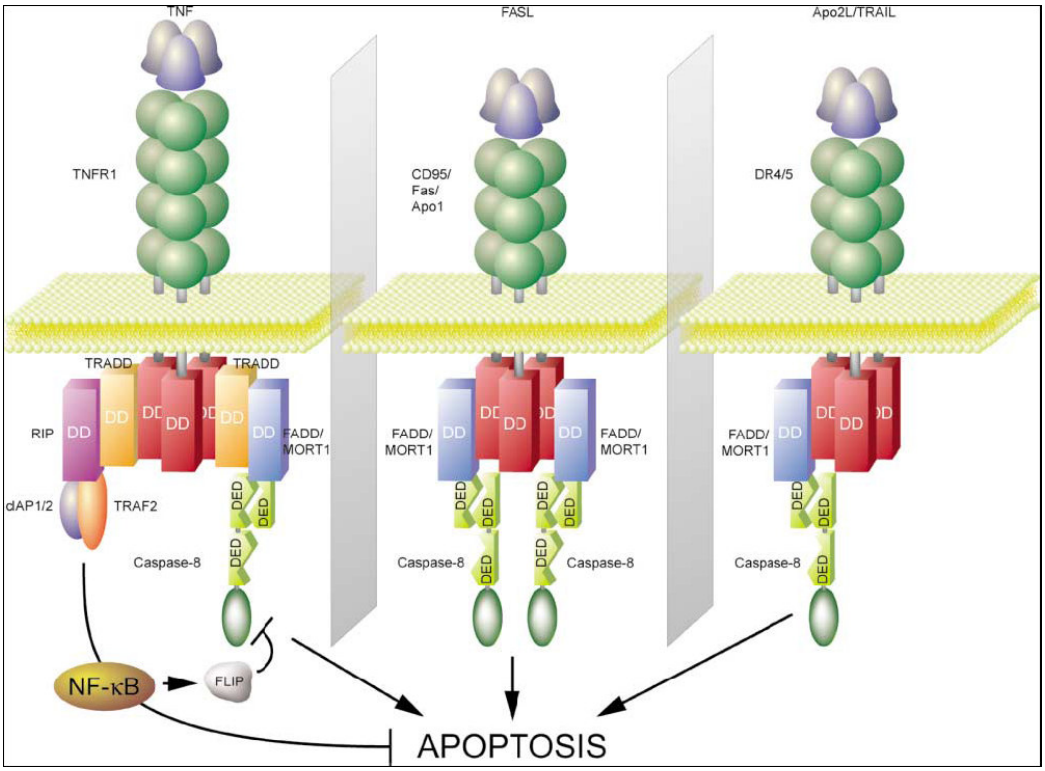

Fig. 6 *The distinct composition of the Death-Inducing-Signaling Complex (DISC) downstream of the various death receptors TNFR1, CD95, and DR4/5 (from Danial and Korsmeyer, Cell, 2004).*

✓ *Mitochondrial pathway*

A number of stimuli, including chemotherapeutic agents, UV radiations, so-called stress molecules (i.e. reactive oxygen and reactive nitrogen species) and growth factor withdrawal appear to mediate apoptosis via the mitochondrial pathway, a death receptor-independent way (Hengartner, 2000). The inner membrane of mitochondria contains ATP synthase, electron transport chain and adenine nucleotide translocator (ANT). Under physiological conditions these molecules allow the respiratory chain to create an electronic gradient (membrane potential). On the other side, the outer membrane contains a voltage-dependent anion channel and its permeabilization results in the release of the molecules stored in the intermembrane space such as cytochrome c, procaspases, kinases, Smac/DIABLO (second mitochondrial derived activator of caspase/direct IAP binding protein with low pI), which blocks the function of IAP (inhibitor of apoptosis protein) and apoptosis-inducing factor, AIF. The release of cytochrome c in the cytoplasm is one of the major steps in the mitochondrial pathway, associated with

permeabilization of the outer membrane, since it promotes the apoptosome formation, constituted by cytochrome c, apoptotic protease-activating factor, Apaf-1 (Cain *et al*., 1999), and procaspase-9, which in turn activates executioner caspases to orchestrate apoptosis. Mitochondria are an important component of the endoplasmic reticulum (ER) stress-induced apoptotic pathway. ER regulates apoptosis by sensitizing mitochondria (i.e. via stress agent releasing) to a variety of extrinsic and intrinsic death stimuli or by initiating cell demise signals of its own (Breckenridge *et al*., 2003). The mechanism by which ER stress is coupled to activation of caspases is still unclear, but it is known that caspase-12 (characterized by Nakagawa and Yuan, 2000), localized at the cytosolic face of ER, is remarkably specific to insult that elicits ER stress and is not proteolytically activated by other death stimuli (Nakagawa *et al.,* 2000). As a matter of fact, caspase-12 null mice or cells are partially resistant to apoptosis induced by ER stress but not by other apoptotic stimuli, pointing to the specificity of caspase-12 for ER (Nakagawa *et al.,* 2000). In Fig. 7 the two pathways (death receptor mediated and mitochondrial) leading to apoptosis have been summarized in order to give a better picture of this cellular process.

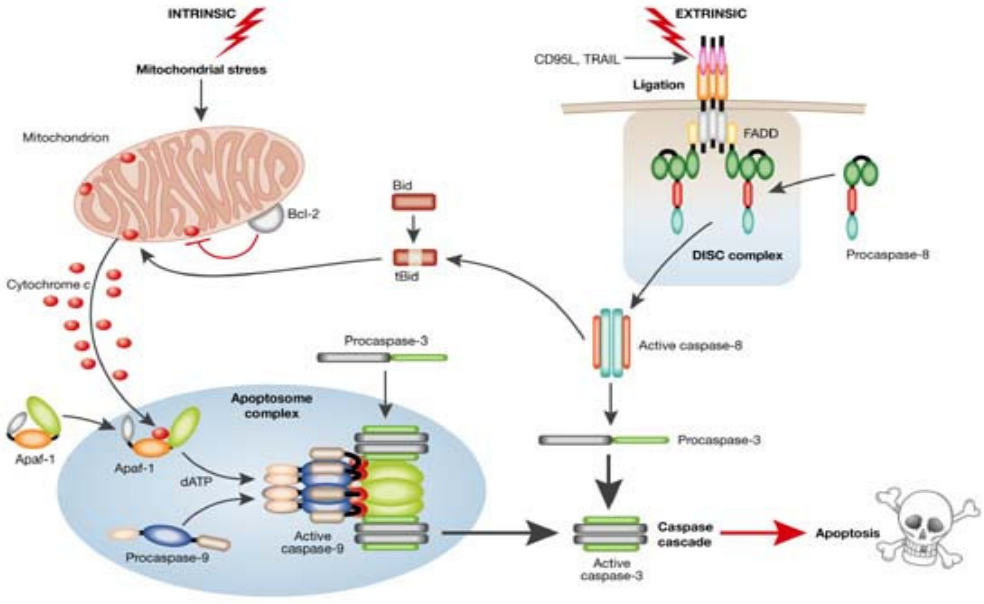

Fig. 7 *Two major apoptotic pathways are illustrated: one activated via death receptor activation ('extrinsic') and the other by stress-inducing stimuli ('intrinsic'). Triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL)-R1/-R2, results in rapid activation of the initiator caspase*

8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space. The release of cytochrome c, from mitochondria is regulated in part by Bcl2 family members, with anti-apoptotic (Bcl2/ Bcl-X_L/Mcl1) and pro-apoptotic (Bax, Bak and tBid) members inhibiting or promoting the release, respectively. Once released, cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1–caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 then activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype (from MacFarlane and Williams, EMBO Reports, 2004).

1.7.2.3. Caspases: natural born killers

Caspases are cysteine proteases, sharing similarities in amino acid sequence, structure and substrate specificity, with a critical cysteine residue in their active site, which, if mutated, is consequentially connected with the loss of the activity (Cohen *et al.*, 1997; Nicholson, 1999). Caspases can be divided into two main classes: initiator and effector caspases (Thornberry and Lazebnick, 1998). Initiator caspases (such as caspase 8, if activated by death receptors, or 9, if the death is induced by toxic agent) are upstream of effector caspases (like caspases 3, 6, 7) defined as the executioners in the cell because, cleaving other proteins, they lead to morphological features as membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies (Hengartner, 2000). The caspase cascade leads to irreversible cell suicide. The proteolytic activity of caspases targets several subsets of proteins involved in homeostasis, repair and structure of cells (Hengartner *et al.*, 2000). These proteins can be summarized in the following groups: ***a***.) Enzymes involved in genome repair, i.e. poly (ADP-ribose) polymerase (PARP), which was the first protein identified as a substrate for caspases, cleaved by caspase-3 prior to the degradation of nuclear DNA into fragments (Kaufmann *et al.*, 1993); ***b***.) Enzymes involved in cell replication and cell cycle progression, i.e. the inactivation of DNA topoisomerase II by caspases interferes with DNA function (McPherson and Goldberg, 1998); ***c***.) DNA. The characteristic internucleosomal fragmentation of DNA associated with apoptosis (Wyllie, 1980) is triggered by caspase-activated deoxyribonuclease

(CAD) enzyme (Liu *et al.*, 1997); ***d***.) Nuclear and cytoskeletal proteins. Caspase-6 degrades laminins and this results in chromatin condensation and nuclear fragmentation; caspase-3 cleaves gelsoline in the cytoplasm which disrupts actin filaments leading to an altered cytoplasmic architecture (Kothakota *et al.*, 1997); ***e***.) Cell membranes and matrix attachment. Caspases-3 and -6 cleave focal adhesion kinase (FAK), disrupting cell adhesion irreversibly and interfering with transmission of matrix-derived cell survival signals (Wen *et al.*, 1998).

1.7.2.4. The Bcl-2 family

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis. The mammalian Bcl-2 family consists of both, pro- and anti-apoptotic proteins, which show sequence and structural similarity in the Bcl-2 homology (BH) regions. The proteins of the Bcl-2 family inhibit or promote the death process by their ability to modulate mitochondrial homeostasis (Gupta, 2003). Several family members were identified (Cory and Adams, 2002), which were divided into three subfamilies:

a. Bcl-2 subfamily (anti-apoptotic): Bcl-2, Bag-1, Bcl-xL, Bcl-w, Mic and A1;
b. Bax subfamily (pro-apoptotic): Bax, Bak and Bok;
c. BH3 subfamily (pro-apoptotic): Bad, Bak, Bcl-xS, Bid, Bik, Hrk, BNIP3 and BimL;

The Bcl-2 family can be defined by the presence of conserved motifs known as Bcl-2 homology domains (BH1 to BH4). While Bcl-2 and its homologs (Bcl-xL, Bcl-w, Mic and A1) contain all four BH domains, the Bax subfamily is constituted at least by BH1 and BH2. The BH3 subfamily members possess only the central short (9-16 residue) BH3 domain and are unrelated to any known protein. Some members of the Bcl-2 family are also characterized by dimerizing to further modulate apoptosis; Bag 1, for example, has been found to form a heterodimer with Bcl-2 resulting in the enhancement of the anti-apoptotic effect of Bcl-2 (Hengartner, 2000; Cory and Adams, 2002). Bax and Bak have been shown to play a critical role in cytochrome c release from mitochondria and thus initiating apoptosis (Hengartner, 2000; Cory and Adams, 2002). Bad plays a critical role in the Bax-mediated apoptosis pathway by dimerizing with Bcl-xL, causing the displacement of Bax, which allows apoptosis to proceed. Bcl-xS, a shorter version

of Bcl-x (lacking amino acids 126-188), apparently utilizes a different pathway than Bax to induce cell death (Cory and Adams, 2002). Pro-apoptotic members, including Bax, Bak, Bid, Bim, promote activation of death-inducing proteins, such as cytochrome c from mitochondria, while anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, inhibit this release (Grinberg *et al.*, 2002; Gross *et al.*, 2002). Following activation, these death-inducing proteins promote apoptotic cell destruction through multiple pathways including caspase activation and nuclear DNA fragmentation (Hengartner, 2000). It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome c and apoptosis-inducing factor (AIF; Hengartner, 2000). It is thought that Bcl-2 and Bcl-xL act preventing this pore formation. Heterodimerisation of Bax or Bad with Bcl-2 or Bcl-xL is thought to inhibit their protective effects (Cory and Adams, 2002).

1.7.3. Apoptosis and pregnancy

Programmed cell death, along with cell proliferation and differentiation, plays crucial roles during normal embryogenesis (Joswig *et al.*, 2003). Pregnancy loss and the occurrence of inborn structural anomalies are often preceeded by excessive apoptosis in targeted embryonic and extra-embryonic tissues (Toder *et al.*, 2002). Apoptosis has also been demonstrated in normal placenta throughout pregnancy as responsible for its growth and remodelling, in balance with the cell proliferation (Smith *et al.*, 1997). Studies have shown the expression of Bcl-2 protein in syncytiotrophoblast cells, an expression that is abundant in very early placenta, less abundant in midterm placenta and least abundant in term placenta (Maruo *et al.*, 2001). These observations indicate that very early placenta is characterized by highly proliferative activity of syncytiotrophoblast cells associated with increased occurrence of apoptosis. During the initial steps of mouse implantation, the uterine epithelium of the implantation chamber undergoes apoptosis, which is thought to be a response to the blastocyst (Hardy, 1999). With progressing implantation, regression of the decidual cells via apoptosis allows a restricted and co-ordinated invasion of trophoblast cells into the maternal compartment: this invasion represents an extremely important process for pregnancy progression. The analysis of apoptosis in murine uterine epithelium and decidua during early pregnancy (days 4.5 – 7.0) revealed an important function for

apoptosis, as Bax (pro-apoptosis) and Bcl-2 (anti-apoptosis) show a complementary expression pattern with Bax in the primary and Bcl-2 in the adjacent decidual zone (Gu *et al.*, 1994).

In this work we investigated whether miscarriage was preceded by augmented apoptotic rates. Therefore, the incidence of apoptosis has been studied in a well-known experimental model of immunological abortion, such as the murine combination CBA/J x DBA/2J, which presents increased local TNF-α levels at the feto-maternal interface (Tangri and Raghupathy, 1993; Clark *et al.*, 1999; Raghupathy, 2001).

On the other hand, we focused our attention on heme oxygenase-1 (HO-1). Considering HO-1 anti-apoptotic properties, we examined if the protective effects of up-regulated HO-1 leading to successful pregnancy could be related to changes in the apoptotic rate and in the expression of apoptotic-related molecules.

2. Aims of the study

2.1. To investigate whether the apoptotic rate was different in abortion-prone mice compared to normal pregnant animals

Based on the evidence that murine abortion is characterized by excessive type-1 cytokine production (Wegmann *et al.,* 1993; Raghupathy, 1997) and diminished Th2 cytokine levels (Chaouat *et al.,* 1995), and taking into account that TNF-α is an important apoptosis inductor (Gupta, 2002), we aimed to investigate whether abortion was preceded by augmentation in the apoptotic rates. To carry out this study, we analysed the activity of a key enzyme of programmed cell death, namely the caspase-3, which activation represents a point of no return in apoptosis induction. We also studied another important hallmark of programmed cell death such as the DNA fragmentation. Additionally, several anti- and pro- apoptotic molecules involved in cell death pathways were identified at the feto-maternal interface and quantified to study their participation in the onset of pregnancy failure.

Thus, in abortion-prone mice compared to normal pregnancy controls we analysed:

I. **The ability of immune cells isolated from decidua to produce Th1 or Th2 cytokines after PMA/Ionomycin**

Local changes to the balance of Th1/Th2-type cytokines occur during pregnancy within the maternal uterus and at the feto-placental unit. The changes in cytokines profiles contribute to implantation (Sharkey, 1998), placental development, and fetal survival until birth. In normal pregnancy, the predominant cytokines at each stage of gestation are involved in limiting maternal immune rejection of the semi-allogeneic embryo/fetus, especially at the feto-maternal interface (Clark *et al.*, 1999; Chaouat *et al.*, 2003). As described in several studies, excessive production of pro-inflammatory cytokines (i.e. TNF-α, IFN-γ, IL-6) is strictly correlated to the onset of abortion (Wegmann *et al.*, 1993; Clark *et al.*, 1999; Chaouat *et al.*, 2003, Zenclussen *et al.*, 2003a). Additionally, Th2 cytokines were reported to be protective (Lin *et al.*, 1993; Raghupathy, 1997, 1999; Dealtry *et al.*, 2000).

Consequently, the Th1:Th2 ratio plays an important role in pregnancy outcome. We measured Th1 and Th2 cytokine secretion (i.e. TNF-α and IL-10) by decidual immune cells from abortion-prone and normal pregnant mice using flow cytometry. This was performed in order to confirm that fetal rejection is associated to the up-regulation of pro-inflammatory cytokines also in this experimental setting as previously proposed (Raghupathy, 1997; Zenclussen *et al.*, 2003a; Chaouat *et al.*, 2003).

II. **The mRNA levels of TNF-α in placenta as well as in decidua**

A wide range of cytokines involved in the dialogue between mother and its fetus are produced during the first period of pregnancy in order to ensure successful blastocyst implantation and placental development (Sharkey, 1998; Dealtry *et al.*, 2000). TNF-α has a potential effect upon implantation and trophoblast invasion by regulating integrin expression and function via the TNF-RII (Bowen *et al.*, 2000). On the other hand, studies revealed that up-regulation of Th1-type cytokine pattern such as TNF-α, IFN-γ or IL-6, is deleterious for a correct fetal development (Raghupathy, 1997; Zenclussen *et al.*, 2003a; Chaouat *et al.*, 2003). Knowing that TNF-α represents an important signaling molecule in apoptosis via its two receptors, TNF-RI and TNF-RII (Bowen *et al.*, 2000), and that its up-regulation is associated with fetal rejection (Chaouat *et al.*, 2003), we investigated the TNF-α mRNA levels in placentas as well as in decidua from abortion-prone and normal pregnancy mice in order to confirm more augmented levels of this pro-inflammatory cytokine in abortion than in normal pregnant mice as already reported in the literature (Clark *et al.*, 1980; Chaouat *et al.*, 1988, 2003; Zenclussen *et al.*, 2003a, 2005).

III. **The incidence of apoptosis (early and late) in decidual and in spleen lymphocytes**

The apoptotic process plays an important role in the development of the immune system (Opferman and Korsmeyer, 2003; Green, 2003). Although apoptosis participates in the development of a cell lineage, its aberration in immune cell could be deleterious, since it is implicated in the initiation of conditions such as immune deficiency, autoimmunity and cancer (Vaux and Korsmeyer, 1999). In this

work, we investigated the *ex-vivo* apoptotic rate (early and late) of spleen and decidual lymphocytes immune cells by flow cytometry, in order to analyze whether resorption is associated with a possible alteration in immune cell apoptosis.

IV. **Activity of caspase-3 enzyme in placental homogenates**

Caspases exist as inactive zymogens with an amino-terminal prodomin and two subsequently cleaved fragments of about 10 KDa and 20 KDa (p10 and p20) that form an active p10-p20 heterotetrameric enzyme (Nicholson, 1999; Cohen *et al.*, 1997). Initiator caspases such as caspases-8, -9 are involved in transducing receptor-mediated and stress-induced death signals, and activate effector caspases, such as caspases-3, -6, -7, are responsible for cleavage of many cellular proteins during the execution phase of apoptosis (Thornberry and Lazebnick, 1998). Moreover, caspase-3 activation represents one of the most important signs of whether cells or tissue are undergoing apoptosis (Cohen, 1997). We analysed caspase-3 activity in placental homogenates from abortion and normal pregnant mice, in order to investigate whether apoptotic activity might be modified in whole tissue (immune + non-immune cells) in the pathological situation of immunological abortion as compared to normal pregnancy.

V. **The DNA fragmentation in placental tissues**

Apoptosis is a multistage process dependent upon a tightly regulated and well-defined program for its initiation and execution. The terminal morphological and biochemical events of programmed cell death are characterized by specific changes in cell surface and nuclear morphology (Hengartner, 2000). Nuclear morphology changes characteristic of apoptosis appear within the cell together with a distinctive biochemical event: the endonuclease-mediated cleavage of nuclear DNA, therefore formation of DNA fragments of oligonucleosomal size (180-200 bp). The fragmented DNA can be detected by TUNEL (terminal deoxynuclotidyltransferase-mediated UTP end labelling) procedure. We analysed DNA fragmentation in placental tissues from abortion and normal pregnant mice by this method as an additional apoptosis indicator.

VI. **Pro- and anti-apoptotic molecules in placental and decidual tissues**

In this study we decided to investigate the role of anti- and pro-apoptotic molecules when our analysis revealed no significant incidence of apoptosis in abortion-prone mice as compared to the controls. Considering the huge amount of molecules involved in programmed cell death, we decided to analysed some of the most representative. Bcl-2 acts for one of the bigger players to avoid apoptosis. Moreover, its over-expression was proposed to block the apoptosis in pro-B-lymphocyte cell line (Hockenbery *et al.*, 1990). Another protein with anti-apoptotic activity is the BCL-2-associated athanogene-1 protein (Bag-1), whereas Bcl-x, whose alternative splicing resulted in a shorter (Bcl-x short, Bcl-xS) or a larger (Bcl-xL) mRNA, has antagonist functions: pro- (Bcl-xS) or anti-apoptotic (Bcl-x-L). On the other hand, Bax forms homodimers and heterodimers with Bcl-2 *in vivo*, so that when Bax predominates, programmed cell death is accelerated, and the death repressor activity of Bcl-2 is countered. Taking these facts into consideration, we analysed:

a. The cellular localization of pro- and anti-apoptotic proteins (Bcl-2, Bag-1, Bcl-xS and caspase-3) in placental as well as decidual tissues

b. The protein expression of pro- and anti-apoptotic molecules (caspase-3, Bcl-xS and Bcl-2, Bag-1) in placental as well as decidual tissues

c. The mRNA levels of mentioned pro- and anti-apoptotic genes in placental as well as decidual tissue

2.2. To investigate whether the anti-apoptotic properties of HO-1 participate in its protective effect during pregnancy

HSP-32, also known as heme oxygenase-1 (HO-1) is induced following exposure to several stressful stimuli, like lipopolysaccharide (Camhi *et al.*, 1995), ultraviolet radiation (Keyse *et al.*, 1989), hydrogen peroxide, heavy metal and organic chemicals (Keyse *et al.*, 1989). It is known that HO-1 over-expression is involved in cardiac xenograft survival (Soares *et al.*, 1998; Araujo *et al.,* 2003). HO-1 and HO-2 are down-regulated at the feto-maternal interface in abortion-prone mice when compared to normal pregnant mice (Zenclussen *et al.*, 2005a), as well as in patients suffering spontaneous abortion during the first trimester, as compared with age-matched women undergoing normal pregnancies (Zenclussen *et al.*, 2003b). Moreover, over-expression of HO-1 by cobalt-protoporphyrin (Co-PP, Rosenberg, 1993) injection (Zenclussen *et al.*, 2005; Sollwedel *et al.*, 2005) or by adenoviral gene transfer (Zenclussen *et al.*, 2006) improved pregnancy outcome in mice undergoing abortion compared to the controls. HO-1 induction is thought to be anti-apoptotic in several cell types. Therefore, we aimed to investigate whether the HO-1 protective effects for pregnancy were related to changes in the apoptotic rate and in the expression of apoptotic-related molecules.

The animals were divided in four groups as previously described (see Methods), and thus we analysed:

I. **The activity of caspase-3 enzyme in placental homogenates.**

II. **The DNA fragmentation in placental tissue.**

III. **The cellular localization of pro- and anti-apoptotic proteins (caspase-3, Bcl-xS and Bcl-2, Bag-1) in placental as well as in decidual tissue.**

IV. **The protein expression of pro- and anti-apoptotic molecules (caspase-3, Bcl-xS and Bcl-2, Bag-1) in placental as well as in decidual tissue.**

V. **The m-RNA levels of mentioned pro- and anti-apoptotic genes in placental as well as in decidual tissue.**

3. Materials

3.1. Chemicals, Media and Kit.

AEC, 3-amino-9-ethylcarbazol	DAKO Cytomation, Glostrup, Denmark
Ampli-Taq DNA polymerase	Eurogentec, Berlin, Germany
Annexin V FITC conjugated	Sigma, Taufenkirchen, Germany
aqua ad injiectabilia	Braun, Melsungen, Germany
Bovine serum albumin (BSA)	Sigma Chemie GmbH, Steinheim, Germany
Caspase-3 colorimetric assay	Sigma, Taufenkirchen, Germany
Chloroform	Sigma, Steinheim, Germany
Diamino benzidine (DAB)	Vector Laboratories, Peterborough, England
DNAse	Stratagene, Amsterdam, Niederland
dNTPs, desoxy–nucleotidtriphosphate	Amersham Biosciences, Freiburg, Germany
DTT, 1,4-Dithio-DL-threitol	FLUKA Biochemika, Sigma, Seelze, Germany
Ethanol absolute	Fluka, Sigma, Seelze, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Taufenkirchen, Germany
FBS, fetal bovine serum	Biochrom AG, Berlin, Germany
Lympholyte-M	Linaris, Bettingen am Main, Germany
Hank´s Balance Salt Solution (HBSS)	Gibco, Invitrogen, Karlsruhe, Germany
Hematoxilin	Roth, Karlsruhe, Germany
Histo-Kit	Roth, Karlsruhe, Germany
H_2O_2 30%	Merck, Darmstadt, Germany
HRP Horseradish peroxidase	DAKO, Glostrup, Denemark
In Situ Cell Death Detection Kit, POD	Roche, Mannheim, Germany
Ionomycin	Sigma, Taufenkirchen, Germany
L-Glutamine	Gibco, Invitrogen, Karlsruhe, Germany
Methanol	Baker, Deventer, Holland
2-Mercaptoethanol	Sigma, Taufenkirchen, Germany
Monensin	Sigma, Taufenkirchen, Germany

NaH_4Cl	Merck, Darmstadt, Germany
Oligo dthymidine-nucleotide (odT)	Amersham Biosciences, Freiburg, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
Penicillin/Streptomycin	Gibco, Invitrogen, Karlsruhe, Germany
Phorbol-12,13-mystirate (PMA)	Sigma, Steinheim, Germany
Rainbow marker	New Englands, BioLabs, Frankfurt am Main, Germany
Reverse Transcriptase	Amersham Biosciences, Freiburg, Germany
RNAse-Inhibitor	Protegé, Mannheim, Germany
RPMI 1640 w L-Glue	Gibson, Invitrogen, Karlsruhe, Germany
Saponin	Riedel-de Haen, Seelze, Germany
Sodium azide (NaN_3)	Merck, Darmstadt, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium pyruvate	Sigma, Taufenkirchen, Germany
Triphosphate buffered saline (TBS)	DULBECCO´S, PAA Laboratories GmbH
Trizol	Invitrogen, Karlsruhe, Germany
Trypsin/EDTA+HBSS	Gibco, Invitrogen, Karlsruhe, Germany
Xylol	Mallinckrodt Baker, Deventer, Holland

3.2. Antibodies (Abs)

- *Flow cytometry* (all antibodies are monoclonal anti-mouse Abs)

CD4-FITC (L3T4)	clone: H129.19	BD Pharmingen, Heidelberg, Germany
CD8a-Cy5 (Ly-2)	clone: 53-67	BD Pharmingen, Heidelberg, Germany
TNF-α-PE	clone: MP6-XT22	BD Pharmingen, Heidelberg, Germany
IL-10-PE	clone: JES5-16E3	BD Pharmingen, Heidelberg, Germany

- *Immunohistochemistry and western blot*

PRIMARY Abs

- *rabbit anti-mouse Abs*

Bag-1 (C-16)	Santa Cruz Biotechnology, Heidelberg, Germany
Bcl-2 (N-19)	Santa Cruz Biotechnology, Heidelberg, Germany
Bcl-xS (L-19)	BD Pharmingen, Heidelberg, Germany

- *goat anti-mouse Ab*

Caspase-3 p11 (K-19)	BD Pharmingen, Heidelberg, Germany

The antibodies against bcl-xS (L-19) and caspase-3 p11 (K-19) for staining in paraffin sections were kindly provided by Dr. Deteyen from Med.Klinik m.S.Hepathologie/Gastroenterologie, Virchow Klinikum, Berlin

SECONDARY Abs

biotinylated antibody goat anti-rabbit	Vector, Peterborough, UK
biotinylated antibody rabbit anti-goat	Vector, Peterborough, UK

3.3. Solutions.

Assay buffer 10 x (casp.3 activity)	20 mM HEPES pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT (1 x in mq water);
Binding buffer (Annexin-V)	25 mM $CaCl_2$, 1.4 M NaCl, 0.1 M Hepes/NaOH pH 7.4
Blocking-buffer (WB)	5 % milk powder in TBS
Caspase-3 substrate (Ac-DEVD-pNA)	Dilute the 20 mM stock to 2 mM with 1 x assay buffer
Caspase-3 inhibitor (Ac-DEVD.CHO)	Dilute the 2 mM stock solution to 200 µM with 1 x assay buffer
Caspase-3 positive control	Dilute an aliquot to 5µg/ml in 1 x assay buffer containing 1 mg/ml BSA
Citrate buffer (10 mM)	from 0.1 M $C_6H_8O_7$ x H_2O, 0.1M $C_6H_5O_7Na_3$ x 2 H_2O

Coomassie staining solution	10 % acetic acid, 20 % ethanol, R250Coomassie in dH_2O
Coomassie de-staining solution	10 % acetic acid, 20 % ethanol in dH_2O
DAB-solution	5 ml dH_2O, 2 drops buffer stock solution, 4 drops DAB stock solution and 2 drops H_2O_2-solution
ECL Analysis System	Reagent 1 (1 ml), reagent 2 (1 ml)
Ethanol	95 %, 75 % with dH_2O
Flow Cytometry buffer	1 % BSA, 0.1 % NaN_3 in PBS
FBS–RPMI medium	10 % FBS in RPMI 1640 (FBS is inactivated before using by 56℃ in the bath for 30 min).
H_2O_2 peroxid water	3 % in methanol
HRP-solution (Kit)	5 ml TBS (pH 7.6) + 1 drop. reag. A and 1 drop reag. B together
Lysis buffer (casp.3 activity)	50 mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT. Dilute 5 x lysis buffer in mq water.
Lysis buffer (protein isolation; casp-3 act.)	200 mM HEPES pH 7.40, 1 % CHAPS, 50 mM DTT, 20 mM EDTA
Lysis buffer (spleen lymph. isolation)	1.5 M NH_4Cl, 10 mM $KHCO_3$, 100 µM EDTA
PFA solution (FACS)	1 % PFA in PBS
p-Nitroaniline (casp.3 activity)	Dilute the stock solution in 1 x assay buffer and determine absorbance at 405 nm using a quartz cuvette.
Pounceau Red (10x) WB	2 g Ponceau S, 30 g trichloroacetic acid 30 g sulfosalicylic acid, dH_2O up to 100 ml
Pounceau Red (1x) WB	with dH_2O
Saponin solution (FACS)	0.1 % saponin in PBS
SDS-running buffer (WB)	0.025 M Tris pH 8.3, 0.192 M glycerine; 0.1 % SDS dH_2O to 1L
TBS, Tris buffered saline solution (IHC)	pH 7.4, 100 mM Tris base,0.9 % NaCl
TBS (WB)	100 mM TRIS pH 7.5, 0.9 % NaCl, dH_2O up to 1L
5x Transfer buffer (WB)	15.15 g Tris pH 8.3, 72 g glycine, dH_2O up to 1L
1xTransfer buffer (WB)	200 ml 5 x Transfer buffer 20 % methanol (200 ml) dH_2O up to1L

Washing solution (WB)	0.1% Tween, 5 % milk powder in PBS

3.4. Laboratory instruments, materials and PC programs.

LABORATORY INSTRUMENTS

ABI Prism7700 Sequence Detection System	Perkin Elmer Apply Biosystems
Balance LC 6200 D	Sartorius, Goettingen, Germany
Bath	Typ SW 20 Julabo GmbH, Seelbach, Germany
Centrifuge	Rotixa/RP Hettich, Tuttlingen, Germany
Cryostate	HM 500 OM, Micron GmbH, Walldorf, Germany
FACS-Calibur	Becton Dickinson, San Jose, USA
Gloves powder free	Kimberly-Clark Coorporation, Zaventem, Belgium
Homogenisator Ultra Turrax T8	IKA Labortechnik, Staufen, Germany
Incubator	Heraeus, Hanau, Germany
Laminar flow, steril	Heraeus, Hanau, Germany
Light microscope	Zeiss Axiophot, Jena, Germany
Mastercycler personal	Eppendorf, Hamburg, Germany
Miomax MR Imaging film	Kodak, Stuttgart, Germany
Spectrophotometer	Hewlett Packard, Waldbronn, Germany
Western blot chambers	BioRad, Munich, Germany

MATERIALS

1.5 ml Eppendorf tubes	Eppendorf, Hamburg, Germany
15/50 mls Falcon tubes	Falcon, Becton Dickinson Labware, Franklin Lakes, USA
Transfer pipetes 3.5 ml	Sarstedt, Nürmbrecht, Germany
T75 and T25 flasks	BD Pharmingen, Heidelberg, Germany
100 µm net	BD Pharmingen, Heidelberg, Germany
1000/200/10 µl pipetes	BD Pharmingen, Heidelberg, Germany
slides/cover slides	Menzel Glasbearbeitungswerk GmbH, Braunschweig, Germany

PC PROGRAMS

Cell Quest Program	Becton Dickinson, San Jose, USA
ImageQuantTL	Amersham

"Statistical Package for the Social Sciences" – SPSS -, Version 11.5

3.5. Animal care.

The present study was authorised by the German ministry and carried out accordingly to institutional guidelines (LaGetSi, Berlin, Germany Reg.0070/03) as well as the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, USA. DBA/2J male and CBA/J female mice were purchased from Charles River, Germany, while BALB/c males were obtained from BgVV, Germany. The animals were maintained in the barrier animal facility of the Biomedical Research Center of Charité, Campus Virchow Klinikum, Medical University of Berlin, with a 12-hour light/dark cycle and water and food *ad libitum*.

4. Methods

4.1 CBA/J x DBA/2J murine mating and experimental design

As proposed by Clark (1980), the combination CBA/J x BALB/c represents the normal pregnancy group (0 % abortion, control), whereas the combination CBA/J x DBA/2J presents spontaneous miscarriage, with an abortion rate between 15 and 30 % (Clark 1980; Chaouat 1988; Zenclussen 2002a,b; 2003a; 2005). Two months old CBA/J females were paired with 3-4 months old BALB/c or DBA/2J males. In every cage, two to three females were placed with one male.

Several studies proposed increased Th1 cytokine levels in abortion-prone mice compared to the CBA/J x BALB/c control combination (Lin *et al.*, 1993; Krishnan *et al.*, 1996a, Zenclussen *et al.*, 2003a). In this work we aimed to analyse if apoptosis might play a determining role in the onset of miscarriage, therefore the animals were divided in the following groups:

1. group CBA/J x BALB/c normal pregnancy (control; n = 11)
2. group CBA/J x DBA/2J abortion-prone combination (n = 14)

Recent investigations revealed that up-regulation of HO-1 enzyme by cobaltum-protoporphyrin (Co-PP; Rosenberg, 1993) application (Zenclussen *et al.,* 2005; Sollwedel *et al.*, 2005) or by adenoviral gene transfer (Zenclussen *et al.* 2006) could prevent feti from being rejected, thus improving pregnancy outcome in abortion-prone mice. In this work, we investigated whether the protective effect of HO-1 in preventing fetal rejection could be associated with its anti-apoptotic properties. For testing our hypothesis, HO-1 was up- or down-regulated by injecting intraperitoneally (i.p.) abortion-prone mice with a single dose of Co-PP or zinc-protoporphyrin (Zn-PP) respectively, on day 4 of pregnancy. The animals were grouped as follows:

1. group CBA/J x BALB/c + PBS i.p. normal pregnancy (control; n = 17)
2. group CBA/J x DBA/2J + PBS i.p. (n = 10)
3. group CBA/J x DBA/2J + 5 mg/kg Co-PP i.p. (n = 6)
4. group CBA/J x DBA/2J + 40 mg/kg Zn-PP i.p. (n = 5)

The females were checked twice (8:00 AM and 8:00 PM) for vaginal plugs. The day, at which the vaginal plug was detected, was considered as day 0 of pregnancy. Then, the females were separated from the males. On day 14 of

pregnancy, the females were sacrificed by cervical dislocation. We have chosen this time point because the placenta is completely formed while the fetus is still growing. The uteri were removed and the implantation sites were documented. The abortion rate was calculated as number of abortions per total number of implantations for every animal. The number of implantations has to be always comparable between the groups. In the following pictures the uteri from non-pregnant (Fig. 8 A), normal pregnant (Fig. 8 B) and abortion-prone DBA/2J-mated CBA/J females (Fig. 8 C) are shown. More in detail, Fig. 8 D depicts the different development between a fetus from a mouse after 14 days of pregnancy and a resorbed fetus from the same time point, which is characterized by its necrotic appearance and small size.

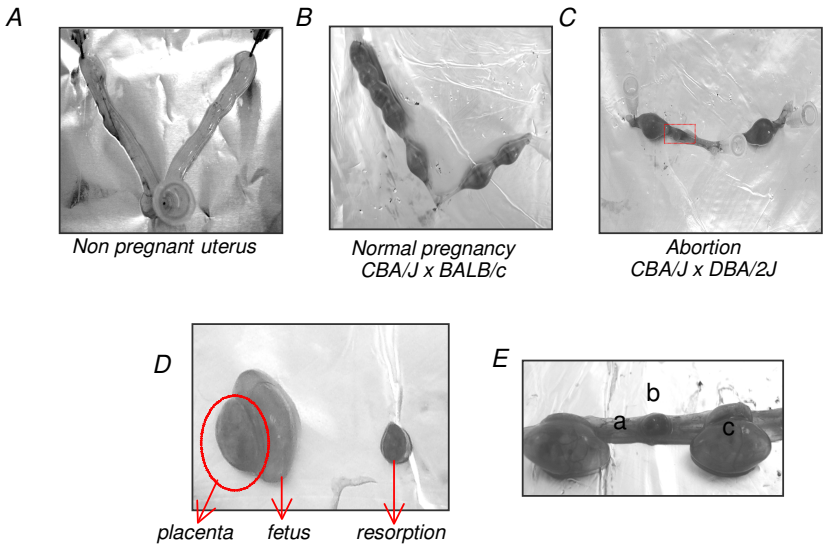

Fig. 8 *Representative pictures showing uteri from non pregnant CBA/J female (A), from CBA/J female having a successful pregnancy (B) and from DBA/2J-mated CBA/J female (C) showing resorption. In D, 14-day-old fetus (together with its placenta) is compared to a resorption, in which fetus and placenta cannot be distinguished. Resorption tissue is characterized by is necrotic and hemorrhagic appearance. General view (E) of maternal decidual tissue (a), resorption (b), and healthy fetus with its placenta (c).*

Resorption samples have been excluded from this study, since proteins and RNA isolations were often unsuccessful due to the degree of destruction of the tissue. Besides, as can be observed in Fig.9 B, in histology samples some placental cell types (i.e. spongiotrophoblast and labyrinthic cells) are missing.

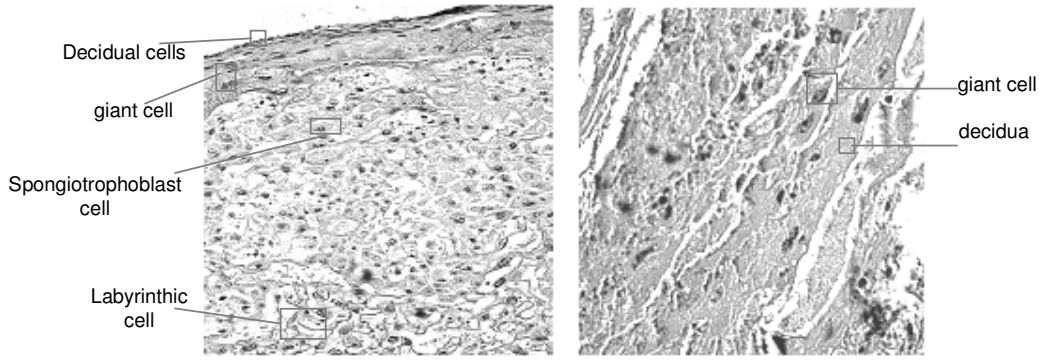

Fig. 9 *General view of healthy placental (A) or resorption (B) tissues.*

4.2. Sample collection

On day 14 of pregnancy, CBA/J females previously mated with BALB/C or DBA/2J males, were sacrificed by cervical dislocation, placentas were extracted and washed in PBS. The samples for RNA or protein isolation were snap frozen in liquid nitrogen and kept at – 80 °C until use, whereas those for flow cytometry analysis were placed on ice in tubes containing 5 ml RPMI medium. Placentas for IHC or TUNEL assay were fixed and kept in 96% alcohol for 24 hs at 4°C. The following day the samples were dehydrated in cold ethanol 100% for 1 to 2 hs at 4°C with 4 changes in total, then put in cold xylol for 1-2 hs at 4°C with 2 changes in total. As final step, the samples were immerged in xylol and left at RT for 1-2 hs with 1 change in total (Saint Marie, 1962). The placentas were then embedded in paraffin at 56 °C for 1-2 hs. The samples were kept at 4 °C until use. Paraffin sections from each placental sample were cut at 5 µm thickness using a microtome, kept at 37 °C ON and then conserved at RT. Before use, the samples were placed on a slide pre-treated with 0.2 % aminosylane. Then, paraffin-embedded sections were de-waxed as follows: 10 min in 100 %, 95 %, 75 % Ethanol , then 5 min in TBS.

4.3. Protein isolation

Frozen placental and decidual tissues from abortion-prone and normal pregnant mice were left in ice to permit thawing and then disaggregated using a glass homogenizer in lysis buffer (PBS and 0.1 % Triton-X containing 100 mM Phenylmethylsulfonylfluoride, 100 mM Benzamidin and 100 mM E-Aminocaproic

acid as protease inhibitors). After squeezing, the homogenates were collected in 1.5 ml eppendorf and left on ice. The determination of the total protein concentration was performed by Bio-Rad protein Assay Dye Reagent using BSA at a standard concentration of 0.1 mg/ml to 1 mg/ml. The protein determination was performed in triplicate, after diluting the samples 1:10 in a 96-well plate, and then read at 405 nm using a spectrophotometer. The whole procedure was carried out at 4 °C. After their quantification, the proteins were aliquoted and kept in –80 °C until use.

4.4. RNA isolation

RNA isolation from placenta or decidua tissue was performed by adding 1 ml of Trizol to 100 mg of tissue and squeezing using a homogeniser (Ultra Turrax T8). The RNA was then extracted with chloroform, precipitated with absolute ethanol, washed, and finally resuspended in RNase-free water. The quantification was performed by reading ultraviolet absorbance at 260 nm. Then, the samples were aliquoted in a concentration of 1 µg/µl, and kept at –80 ° C until use.

4.5. Ex-vivo isolation of lymphocytes from decidual tissue

Decidual tissue from normal pregnant or abortion-prone mice was washed in PBS, cut into small pieces in 50 ml tubes with 5 ml of Hanks´ Balance solution (HBSS) and left on ice until use. Decidual tissue pieces were then filled up with HBSS containing 1 mM DTT and incubated for 20 min with occasional shaking at 37 °C in the bath. The supernatant containing detached decidual cells was filtered through a 100 µm net and placed into a new 50 ml falcon tube on ice. The remaining decidual tissue in the tube was filled up with new HBSS but without DTT and again incubated for 20 min with occasional shaking at 37 °C in the bath. This procedure was repeated twice. On the other hand, the supernatant containing the cells was every time collected in the same tube after centrifuging at 1500 rpm, and left on ice. Immune cells were then isolated by a Lympholyte-M gradient as indicated by the manufacturer and washed with RPMI medium containing 10 % fetal bovine serum (FBS). The cells were centrifuged at 1500 rpm at 4 °C for 10 min, and then the pellet was re-suspended in 1 ml of RPMI + 10 % FBS and left on ice until used.

4.6. Ex-vivo isolation of lymphocytes from spleen tissue

Spleen cells were isolated in a 6-well plate containing 5 ml of RPMI by crushing the organs on a 100 µm net. The total amount of isolated cells (6 ml) were collected in a 50 ml Falcon, then 1X lysis buffer was added up to 50 ml and incubated for 10 min at RT to permit the erythrocyte lysis. The cells were centrifuged at 1500 rpm at 4°C 10 min, then the pellet re-suspended in 15 ml RPMI containing 10 % FBS and centrifuged again at 1500 rpm at 4°C for 10 min. Finally, splenocytes were re-suspended in 1 ml RPMI + 10% FBS and left on ice until used.

4.7. Flow cytometry

4.7.1. Flow cytometry principle

Flow cytometers (FC) or fluorescence-activated cell sorting (FACS) machines were developed in the 1960s (Van Dilla *et al.,* 1969) as analytical instruments offering an advanced technology for rapidly characterizing or separating cells based on their physical, biochemical and functional properties within a heterogeneous population. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells at a concentration of 0.5–20 x10^6 cells/ml. Measurements include fluorescence from fluorescent probes bound to cellular constituent such as DNA or cell-surface antigen as well as intracellular components (i.e. proteins like cytokines). The cells are illuminated at a specific wavelength from two or more lasers. The cells stained with fluorochromes like FITC as Fluorescein-Isothiocyanate, PE as Phycoerythrine or Cy5 as Carbocyanine, are measured using an argon laser at a wavelenght (λ) from 488 nm to 666 nm (FITC. $\lambda =$ 525 nm; PE $\lambda = 578$ nm; Cy5 $\lambda =$ 666 nm). The fluorescence intensity is proportional to the amount of antibodies bonded to the cells. Forward scatter (FSC) is defined as the measure of the cell size, since the greater the scatter, the bigger will be the cell size. The side scatter (SSC) defined the intracellular granularity of the cells and its thickness. In the following picture (Figs. 10 A and B) are represented the blood cell populations detected (Fig. 10 A) and, once localized, gated. Only lymphocyte cells have been considered for the analysis.

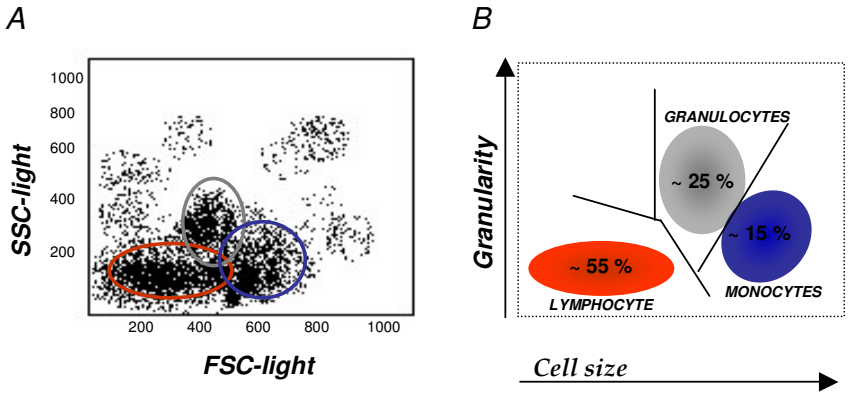

Fig. 10 *The SSC (side scatter) and FSC (forward scatter) signals resulting from blood cells (FSC-SSC-Dotplot). SSC is referred to as cellular granularity, e.g. granulocyte blood cells with their irregularity nuclei have a much more intense SSC than do the more irregular lymphocytes or erythrocytes. FSC is related to the size or cell volume signal. It is also determined by other factors such as the refractive index of the particles, therefore, a large particle will bend more light than a small one. In B the subdivision of the cellular population is associated with its percentage. Modified from Stefan Fest´s MD thesis.*

Cytometers are provided with an electronic circuit, a compensation network that measures the intensity of the signal from the signal on the other photodetector. As represented in the Figures 11 (A and B), compensation allows correcting for the overlap between the fluorescence spectra of different fluorochromes. In our study, the compensation was always performed before starting the measurements.

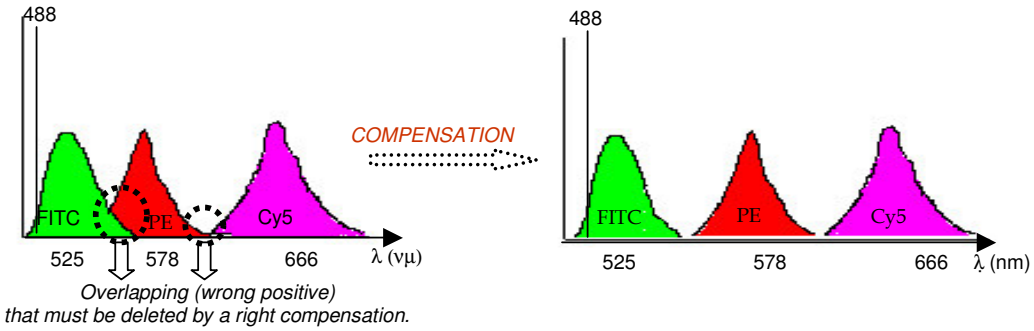

Fig. 11 *Compensation (right) allows correcting the fluorescence spectra crossover of different fluorochromes (left).*

4.7.2. Analysis of the data

The FC data were analysed by using the Cell Quest Program for three parameters, such as Cy5-, PE- and FITC-conjugated antibodies against each other. After measurement of the negative control, the lower window (gate) on the left side was created with at least the 99.9% of the entire unstained cell population. The negative control or the isotype control staining is representative of the correct staining due to the absence of auto-fluorescence or unspecific bindings of the antibodies used. The cell populations are defined by quadrants, which are described by location (LL, lower-left represents cells negative for the descriptors on both the x- and y-axes; UR, upper-right represents cells dual-positive for the descriptors on both the x- and y-axes; UL, upper-left represents cells positive for the y-axis descriptor, but negative for the x-axes descriptor; LR, lower-right represents cells positive for the x-axis descriptor, but negative for the y-axis descriptor). The results are expressed as percentages of cells in specific quadrants compared to all cells represented in the plot.

4.7.3. Stimulation of cytokine production and Golgi blockade

Isolated spleen or decidual lymphocytes was left on ice, each in 1 ml RPMI + 10 % FBS, and then re-suspended, transferred into a 6-well plate (1.5 x 10^6 cells/ml) and stimulated using 1 ml RPMI medium and 10% FBS with 50 ng/ml PMA and 1 µg/ml ionomycin in a humidified incubator at 37°C with 5% CO_2. After the first hour, 2 µM monensin was added in each well to the medium for blocking the Golgi in order to stop the intracellular transport, and the incubation continued for 3 more hours.

4.7.4. Staining with extracellular antibodies

After incubation, the 2 ml cell re-suspension was divided into FACS tubes and washed by adding FC buffer. Then, the cells were centrifuged at 1500 rpm for 10 min at 4°C, the supernatant was removed and the pellet was re-suspended in 100 µl FC buffer containing the diluted extracellular antibodies (1:100, CD4-FITC- and CD8-Cy-5-labelled). The cells were incubated for 10 min at 4°C in darkness.

4.7.5. Fixation

After incubation, the cells were washed by adding FC buffer, then centrifuged at 1500 rpm at 4°C for 10 min, the supernatant was removed and the pellet was re-suspended in 100 µl 1% PFA. The cells were incubated ON at 4°C, to permit the cellular fixation.

4.7.6. Permeabilization and intracellular staining

The following day, cells were washed with FC buffer, then centrifuged at 1500 rpm at 4 °C for 10 min, and the supernatant was removed. The pellet was re-suspended in 100 µl of a solution containing the intracellular antibodies (PE-labeled anti-TNF-α or anti-IL-10-PE), both diluted 1:200 in 0.1 % saponin. The cells were incubated for 20 min at 4°C in darkness. After incubation, the cells were washed with 0.1 % saponin, centrifuged at 1500 rpm at 4°C for 10 min. The supernatant was removed and the pellet was re-suspended in 100µl in FC buffer to be read in a FACS-calibur. Negative controls were performed by using the respective isotype controls and unstained cells. Table 2 summarized the schema of the staining.

FACS-tubes	*1*	*2*	*3*
Type of sample	Control	anti-	anti-
FITC	/	CD4	CD4
PE	/	TNFα	IL-10
Cy5	/	CD8	CD8

Table 2 *Scheme of pipetting for staining decidual lymphocytes. One cell tube can be simultaneously stained with three fluorochrome-antibodies. For every sample, unstained cells representing the negative control were in each case measured.*

4.8. Apoptosis detection by annexin-V/Propidium Iodide labeling

4.8.1. Principle

An event characterizing early-programmed cell death is the loss of membrane phospholipid asymmetry, with the translocation of phosphatidylserine (PS) from the inner leaflet of the phospholipid bilayer to the cells surface (Hengarter, 2000). Exposure of PS provides a simple means for detecting cells undergoing apoptosis. Annexin–V (35 KDa), a member of annexin family of calcium-dependent phospholipid binding proteins, has a high affinity for PS-containing phospholipid

bilayers (Fig. 12 A and B). Therefore, annexin-V-FITC conjugated represents a convenient tool in detecting early apoptosis (Vermes *et al.,* 1995).

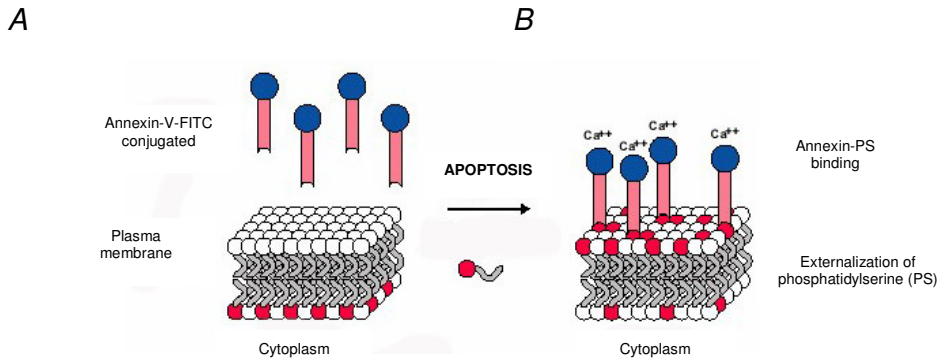

Fig. 12 *Schematic representation of the Annexin V assay. The picture shows the inversion of phosphatidylserine (PS) after induction of apoptosis, and subsequent binding of annexin-V-FITC to the cell surface under well-defined calcium concentration (A and B). From www.bdbiosciences.com.*

4.8.2. The assay

The assay employs a fluorescein-labeled annexin-V (annexin-V-FITC) together with propidium iodide (PI). As already described above, during the early stage of programmed cell death, the PS are exposed on the cell membrane, and annexin-V binds them in a calcium-dependent manner. PI can readily move across the plasmatic membrane binding the DNA, therefore staining late apoptotic cells, whose membranes are already permeable or dead cells. Therefore, this assay allowed the detection of apoptosis as well as the differentiation between early apoptotic cells (annexin-V^+) and late apoptotic cells (annexin-V^+/PI^+) from viable (annexin-V^-/PI^-) or necrotic (PI^+) cells (Fig. 13).

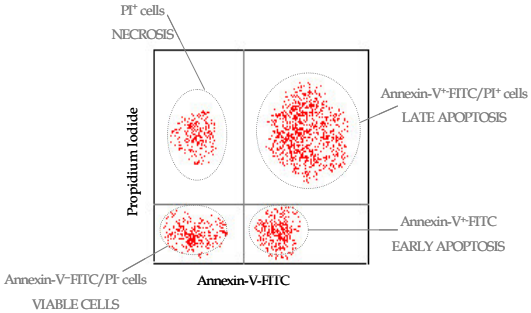

Fig. 13 *Representative dot plots of FITC-annexin-V/PI two parameter flow cytometry. In the picture it is possible to detect and distinguish early apoptotic cells (annexin-V^+) and late apoptotic cells (annexin-V^+/PI^+) from viable (annexin-V^-/P^-) or necrotic (PI^+) cells.*

4.8 3. Experimental procedures

The *ex-vivo* early and late apoptotic rates in decidual and spleen lymphocytes from normal or abortion-prone mice were analysed by detecting annexin-V-FITC conjugated and PI positive cells using flow cytometry. After isolation, spleen and decidual cells were collected and placed into tubes, then washed by adding 1 ml of cold PBS. The cells were then centrifuged at 1500 rpm at 4 ℃ for 10 min, the supernatant was removed and the pellet re-suspended in 100 µl 1X binding buffer containing 2 µl/sample of annexin-V-FITC as well as 2 µl/sample PI. This was incubated for 15 min in darkness at RT. After incubation, the cells were washed with 1 ml cold PBS, then centrifuged 1500 rpm at 4℃ for 10 min and the supernatant was removed. The pellet was re-suspended in 100 µl/tube of 1X binding buffer and the cells were analysed by flow cytometry within 1 hour. Negative controls for each sample were performed by omitting annexin-V-FITC conjugated or PI.

4.8.4. Data analysis

The analysis of the data was performed by using the Cell Quest Program for the parameters FITC-annexin-V conjugated and propidium iodide. After the measurement of the negative control, the lower window (gate) on the left side was created with at least the 99% of the entire unstained cell population. The populations of cells are defined by quadrants as already mentioned.

4.9. Apoptosis in placenta: measurement of caspase-3 activity

4.9.1. Principle and assay

The effector protein, caspase-3, is the most studied caspase of all mammalian caspases. This enzyme can process caspases 2, 6, 7 and 9 and specifically cleaves many key proteins, leading to apoptosis induction. In addition caspase-3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as blebbling (Hengartner, 2000). The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Asp-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety, as depicted in the Fig. 14 (Sass *et al*., 2003).

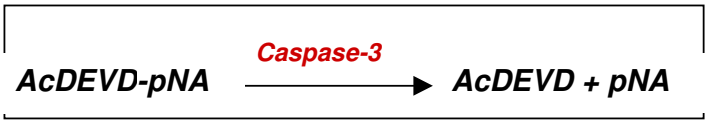

Fig. 14 *Illustrative representation of caspase-3 hydrolysis of AcDEVD-pNA substrate.*

P-nitroaniline has a high absorbance at 405 nm. The concentration of the pNA, directly proportional to the caspase-3 activity, can be calculated in µmol pNA released per min per ml of placental protein homogenate. The formula is: $\mu mol\ pNA \times d / t \times v$, where "*v*" is the volume of sample in ml, "*d*" is the dilution factor and "*t*" is the reaction time in minutes.

4.9.2. Experimental procedure

4.9.2.1. 96 well plate disposition

	Placental homogenates	***Caspase-3 5µg/ml***	***1x assay buffer***	***Caspase-3 inhibitor AcDEVD-CHO 200 µM***	***Caspase-3 substrate AcDEVD-pNA 2 µM***
Reagent blank	--	--	90 µl	--	10 µl
Placental homogenates	5 µl	--	85 µl	--	10 µl
Placental homogenates + inhibitor	5 µl	--	75 µl	10 µl	10 µl
Caspase-3 positive control	--	5 µl	85 µl	--	10 µl
Caspase-3 positive control + inhibitor	--	5 µl	75 µl	10 µl	10 µl

Table 3 *Reaction schema of pipetting for 96-well plate.*

4.9.2.2. Steps of the experiment

Before starting the experiment, a series of p-nitroaniline solutions at the concentration range of 10 to 200 µM (Table 4) have been prepared. 100 µl of each dilution was added to a well of the 96-well plate. Assay buffer (100 µl) was included as blank. The absorbance was read at 405 nm using a spectrophotometer. A calibration curve of the absorbance was carried out by plotting the OD_{405} values versus the concentration of p-nitroaniline per well, in mmol, as indicated in Table 4.

µM p-Nitroaniline	µmol p-Nitroaniline per 100 µl
0	*0.001*
20	*0.002*
50	*0.005*
100	*0.01*
200	*0.02*

Table 4 *Scheme of p-nitroaniline standard concentrations, prepared by diluting the p-nitroaniline stock solution in 1x assay buffer.*

After isolation, 50 µg of placental homogenates (5 µl) or caspase-3 positive control (5 µl) was put in the specific wells, as illustrated in the Table 3. The order of addition on the well plate was made as follows:

- 1x assay buffer.
- caspase-3 inhibitor.
- caspase-3 substrate.

The reaction started immediately after addition of caspase-3 substrate. The plate was gently mixed by shaking while avoiding bubbles, covered and incubated at 37°C for 90 minutes. When the signal was too low, the incubation continued ON, as suggested by the manufacturer. The results were calculated normalizing for the p-nitroaniline standard concentrations described in Table 4.

4.10. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)

4.10.1. Principle

Extensive DNA fragmentation is a characteristic event of late apoptosis (Hengartner, 2000). The assay TdT-mediated dUTP nick end labelling (TUNEL) represents a fast and very sensitive method to detect the cleavage of nuclear DNA into nucleosome-sized fragments (approximately 180 bp unit size) using an enzymatic labelling of the 3´-OH terminal with modified nucleotides (X-dUTP, X= fluorescein; Fig. 15). During the reaction, the enzyme terminal deoxynucleotidyl transferase (TdT) catalyses polymerisation of fluorescein labelled nucleotides to free 3´-OH DNA ends of double-stranded DNA breaks independent of a template. Incorporated fluorescein is then conjugated with anti-fluorescein antibody

conjugated with horse-radish-peroxidase (POD). After reaction, stained cells can be analysed with a light microscope.

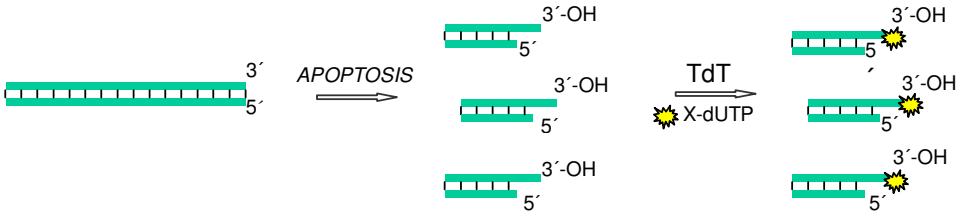

Fig. 15 *Schematic illustration of TdT-mediated dUTP nick end labelling (TUNEL) method.*

4.10.2. Steps of the experiment

The detection of apoptotic cell death at single cell level was performed using the *In Situ* Cell Death Detection Kit, POD. The sections were treated with 10 mM citrate buffer pH 6 for 5 min at 600 W in microwave to unmask possible unspecific binding in the tissue, then refilled with TBS. The samples were then washed in TBS twice (5 min each). 25 µl/section of TdT enzyme + nucleotides mix were added and incubated for 60 min in a humidified chamber, modifying the incubation temperature from the original protocol (RT instead of 37 °C). The samples were then washed in TBS twice (5 min each), and the endogenous peroxidases were blocked by adding 3 % H_2O_2 on methanol for 30 min. After washing, the samples were incubated in POD (25 µl/sample) for 30 min at RT, and washed again. The sections were developed with 3-amino-9-ethylcarbazole (AEC) + substrate-chromogen, counterstained with haemalum for 2 min and mounted in Aquatex. Negative controls were performed by replacing the TdT enzyme with the label solution.

4.10.3. Data analysis

The number of $TUNEL^+$ cells/mm^2 of placental tissue was evaluated using a light microscope with a scaled-eye piece, pre-calibrated with a slide micrometer using a magnification of 200 X (20 X objective and 10 X ocular). The counting was performed without knowledge of the sample's outcome.

4.11. Cellular localization of pro- and anti-apoptotic molecules at the fetal--maternal interface

4.11.1. Principle

The term “immunohistochemistry” (abbr. IHC) refers to techniques applicable to light as well as fluorescence microscopy, using antibodies (Abs) specific for identifying antigens located in normal or pathologic tissues. The IHC method we used, is called “Labelled (Strept)Avidin-Biotin Method”(LSAB), since the basic reaction is based on the strong affinity (K_m= 10^{-15} M) of the glycoprotein streptavidin/avidin for biotin (Gonzalez *et al*., 1999). Biotin is easily conjugated to antibodies and enzyme markers. In the system used in the context of this work, after binding of the antigen with the first antibody, this complex recognizes and binds the biotinylated-secondary antibody (bridge antibody, Fig. 16). Then, the secondary biotinylated antibody, through biotin, recognizes the biotin-binding hydrophobic pockets in avidin or streptavidin already labeled with peroxidases (Horse radish-peroxidase, HRP, solution). The use of chromogens (e.g. 3,3-Diamino benzidine, DAB, or 3-Amino-9-Ethylcarbazol, AEC, complex) permits the staining pattern to be visualized through the specific color (brown or red). Non-specific background staining is avoided by blocking endogenous peroxidase via pre-incubation of the sections in absolute methanol containing hydrogen peroxide. Polyclonal antibodies usually contain antibodies specific for several antigenic determinants on the antigen resulting in “false positives” (Bancroft and Gamble, 2002). Therefore, the negative control, performed without the primary antibody by adding only BSA or serum, represents an essential test for the non-specificity of the secondary antibody. Moreover, in order to exclude the possibility of the primary antibody having unspecific bindings, thus creating “false positives”, the primary Ab could be incubated with its blocking peptide, a synthetic molecule that mimics the antigen by binding the primary antibody two hours before use, in a ratio 5:1.

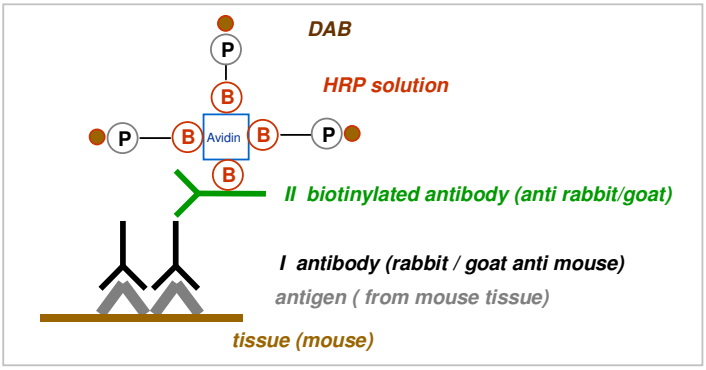

Fig. 16 *Representative schema of Labelled StreptAvidin Biotin (LSAB) method.*

4.11.2. Steps of the experiments.

After de-waxing, in order to block the endogenous peroxidase activity, the sections were treated with 3 % hydrogen peroxide in methanol (50 µl/ section) for 20 min at RT in a humidified chamber. Then, the tissues were again washed twice in TBS and blocked with 5 % BSA in TBS (50 µl/ section) for 20 min at RT in a humidified chamber. The samples were incubated ON at 4 °C with the primary antibody (the dilutions used are described in Table 5) in 5 % BSA in TBS (30 µl/ section) in a humidified chamber. On the following day, after washing twice in TBS, the samples were stained with the secondary biotinylated antibody diluted in 5 % BSA for 1h at RT (50 µl/section). Then 1 drop/section of HRP-conjugated solution was added and incubated for 30 min at RT in a humidified chamber after washing twice in TBS. Finally, the sections were developed with DAB (50 µl/section), incubating for 7 min RT and then washed twice in TBS. To counterstain, the sections were incubated with haemalum (50 µl/section) for 2 min at RT and then washed in tap water until a limpid solution was obtained. Then, the sections were quickly washed sequentially in alcohol 75 %, 95 %, 100 % and left 2 x 5 min in xylol. The cover slide was mounted using Roti Histo-kit. Samples treated with AEC+substrate-chromogen (1 drop/section, instead of DAB), were washed twice in TBS, counterstained with Hemalaun, washed in tap water and immediately mounted with Aquatex mounting media. Negative controls were performed by replacing the primary antibody with 5 % BSA in TBS or by incubating the antibodies previously with a commercial blocking peptide for two hours.

I .Antibody	Rabbit–anti–mouse- -**Bcl-2** -**Bcl-xS** -**Bag-1** polyclonal	Goat-anti-mouse- -**caspase-3** polyclonal
Dilution	*1:100*	*1:100*
II. Biotinilated antibody	Goat–anti-rabbit	Rabbit–anti-goat
Dilution	*1:200*	*1:200*

Table 5 *List of the primary and secondary antibodies used and their dilutions.*

4.11.3. Light microscope analysis.

The pattern and intensity of staining in the different cell types of placenta and decidual samples was evaluated by two independent observers using a light microscope at a magnification of 200 x (20 x objective and 10 x ocular). The degree of staining in each placental cell type, as well as in decidua, was graduated semi-quantitatively as: negative (-), weak (+), moderate (++), high (+++) or intense (++++), and these marks were later converted into numerical scores (0, 1, 2, 3 and 4, respectively) for a semi-quantitative analysis as previously described (Zenclussen *et al.*, 2003 a).

4.12. SDS Page and Western Blot (WB)

4.12.1. Principle

Western blotting assay detects the protein amount in cells as well as in tissue extracts. First, the proteins are separated by their size using a SDS-polyacrylamide gel electrophoresis (known also as SDS-PAGE electrophoresis). The sodium duodecylsulphate sulfate (SDS), a reductor reagent, completely denatures the proteins by breaking their covalent bindings responsible for the tertiary or quaternary structure. When the proteins are separated on the gel, they can be transferred in a nitrocellulose membrane and then labelled by incubating with a labelled antibody or by using a primary antibody followed by a secondary enzyme-conjugated antibody. The bands can be visualized e.g. on X-ray film, and then quantified. The ImageQuant TL program can analyse 8- to 16-bit greyscale

Tiff images or gel files by comparing the relative darkness, or intensity, of different bands on the scanned image, evaluating the relative density of bands. Considering that the computer image is composed of individual pixels, each pixel represents a numerical value corresponding to the optical density of the gel at that point.

4.12.2. Experimental procedure and analysis

10 µg of placental proteins were separated in a 10 % sodium duodecylsulphate (SDS)-polyacrylamide gel electrophoresis at 100 V for 1 to 2 hours at RT and then transferred to a nitrocellulose membrane for immunoblotting ON at 10 V. Membranes were blocked for two hours at RT in a 5% milk solution/TBS and thereafter incubated ON at 4 °C with antibodies against Bcl-xS, caspase-3, Bag-1, Bcl-2 diluted 1:200 in 5 % milk/TBS. After washing twice with 0.5 % Tween and 5 % milk in TBS, a secondary biotinylated antibody HRP-conjugated diluted 1:5000 in 5 % milk/TBS was added to the system for 1 h at RT. Bands were revealed by chemiluminescence using a commercial kit under exposure onto Miomax MR Imaging film. As loading control, we used a β–actin antibody in a dilution 1:1000. Positive bands were quantified using ImageQuant TL program from Amersham. Data are expressed as X/β-actin, X being the analysed protein.

4.13. Real time RT-polymerase chain reaction (PCR)

4.13.1. Traditional PCR versus Real Time RT-PCR

A powerful technique for directly amplifying short segments of the genome is provided by the polymerase chain reaction (PCR). PCR assay allows the amplification of a region between two defined sites. The steps to perform one PCR cycle are summarized in Fig. 17. Briefly, double stranded DNA is denatured, and the single strand is annealed with two short primer sequences (~ 20 bases each for real time PCR and bigger for traditional PCR), which are complementary to the sites on the opposite strands on either side of the target region. The DNA polymerase enzyme is used to synthesize a single strand from the 3´-OH end of each primer. The entire cycle can then be repeated by denaturing the preparation and starting again. The number of copies of the target sequence grows exponentially until reaching a plateau at which more template accumulates than the enzyme can extend; then the increase in target DNA becomes linear. The

traditional PCR method uses agarose gel for detection of PCR amplification at the final phase or end point of the PCR reaction. There are several disadvantages related to analysis at the end point, like low resolution, the fact that the results are not expressed as numbers, poor precision and low sensitivity. Moreover, at that time the reaction is already stopped, no more products are formed and they begin to degrade.

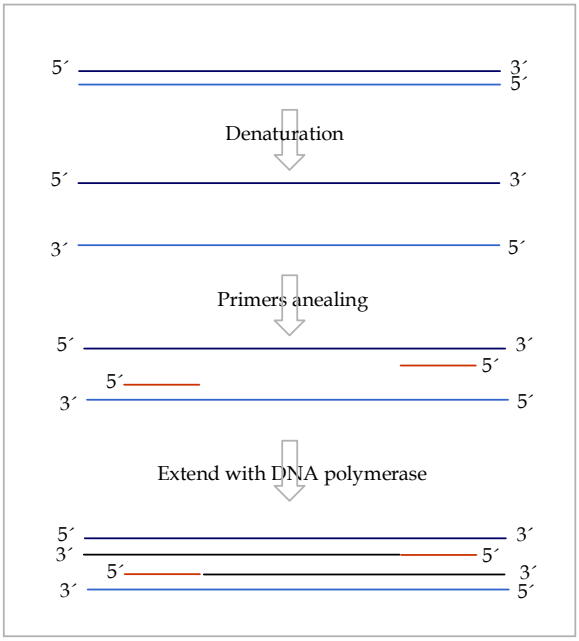

Fig. 17 Representation of PCR method.

4.13.2. Principle of Real Time RT-PCR

Real Time PCR is a very sensitive and accurate assay, which represents a novel technique for measuring the ”real time” accumulation of products during the exponential phase of the PCR reaction using a dual-labeled fluorogenic probe. This probe is an oligonucleotide, dually labeled with a reporter dye (e.g. FAM, 6-carboxyfluorescein) covalently attached to the 5´end and to a quencher dye (e.g. TAMRA, 6-carboxytetramethylrhodamine) covalently attached to the 3´end. The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe is intact. During the extension phase of the PCR reaction, the hybridized probe is hydrolyzed by 5´-nuclease activity of the Taq-polymerase, separating the quencher from the reporter. This results in an increase in

fluorescence emission of the reporter dye, which is proportional to the initial amount of template (Overberg *et al*. 1999). Real time RT-PCR detects PCR amplification products using a start material cDNA in an earlier phase of the reaction, when there is the 100 % of reaction efficiency. A schematic representation of the real time-RT-PCR principle and the graphic analysis are depicted in Figs. 18 A, B, C.

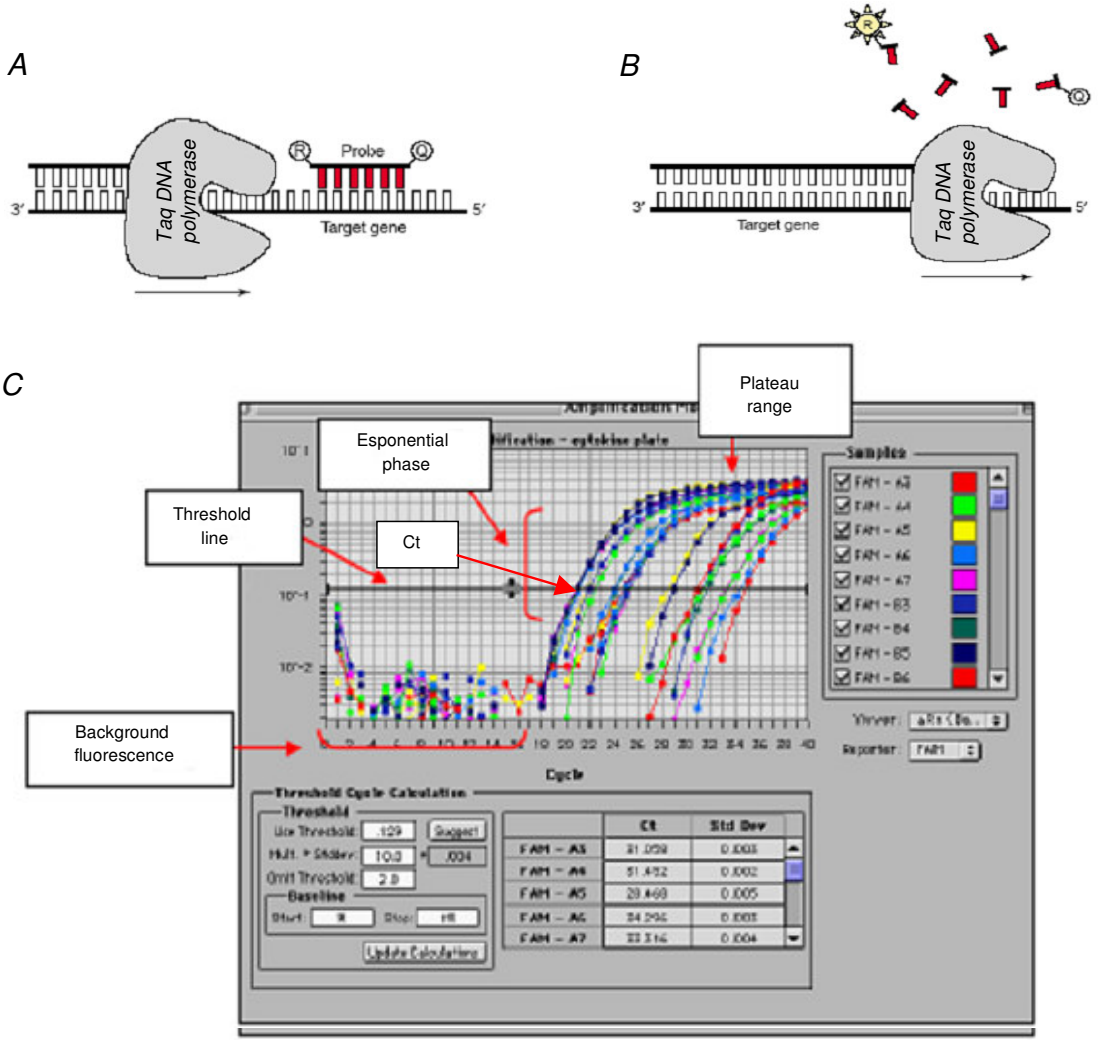

Fig. 18 *Schematic representation of the real time-PCR principle (A and B) and graphic analysis (C). Upon amplification of the target gene, the probe is displaced and subsequently hydrolyzed by the Taq DNA polymerase. The probe is formed by a reporter (R) and a quencher (Q), which are attached to the 5' and 3' ends to the target gene. The quencher absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. The Taq polymerase enzyme separates the reporter from the quencher fluorochrome so that the fluorescence of the reporter fluorochrome becomes detectable at 488nm. (A, B from Mocellin et al., Trends Mol Med 2003). During each consecutive PCR cycle this fluorescence increases because of the progressive and exponential*

accumulation of free reporter fluorochromes. The cycle at which the fluorescence reaches a threshold value is called threshold cycle (C_T value) which is inversely proportional to the starting amount of the target DNA. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle, in real time (Threshold value is the level of detection or the point at which a reaction reaches a fluorescent intensity above background.

4.13.3. cDNA synthesis

For cDNA synthesis, samples containing 2 µg of total RNA were placed for 2 min on ice and 2.5 mM dNTPs, 2 U/ml DNAse I and 40 U/ml RNA-se inhibitor were mixed and added in a reaction buffer. After incubation for 30 min at 37 °C, the mix was heated to 75 °C for 5 min. 200 U/ml reverse transcriptase and RNA-se inhibitor were prepared in RNA-se free water and incubated at 42 °C for 60 min followed by incubation at 94 °C for 5 min. The samples were left at –20 °C until used.

4.13.4. Experimental procedure

Amplification reactions consisted of 1 µl of cDNA (for Bag-1, Bcl-xL, Bcl-2, Bax) or 2 µl of cDNA (for TNF-α), 6.25 µl Mastermix containing PCR buffer, dNTPs, $MgCl_2$ and Ampli-Taq DNA polymerase, 3 µl of the primer mix, 0.5µl of the fluorescent probes and RNA-se free water until 13 µl. PCR reactions were performed as follows: 2 min at 50 °C followed by an initial denaturation step of 10 min at 95 °C, followed by 15 s at 95 °C and 1 min at the appropriate annealing temperature (usually 60 °C) for 40 cycles. β-actin was used as housekeeping gene. All reactions were performed on the ABI Prism 7700 Sequence Detection System. Bag-1, Bcl-2, Bax, Bcl-xL and TNF-α were tested in placenta and decidua samples.

Primers	*Nucleotide sequences*
Bcl-2	
forward primer	TGAACCGGCATCTGCACA
reverse primer	CAG AGGTCG CAT GCT GGG
probe (FAM labelled)	AACGGAGGCTGGGATGCCTT GTG
Bag-1	
forward primer	GCTAAC CAC CTG CAAGAATTG AAT
reverse primer	GTTTGCAGAGAG CCT CCG C
probe (FAM labelled)	TTCTGACAT CCAGCAGGGTTTTCTGGC
Bcl-xL	
forward primer	GGTGAGTCGGATTGCAAGTTG
reverse primer	GTAGAGATCCACAAAAGTGTCCCAG
probe (FAM labelled)	CCTGAATGACCACCTAGAGCCTTGGATCC
Bax	
forward primer	GCGTGGTTGCCCTCTTCTACTT
reverse primer	AGCAGCCGCTCACGGAG
probe (FAM labelled)	CAAACTGGTGCTCAAGGCCCTGTGC
TNF-α	
forward primer	TCGAGTGACAAGCCCGTAGC
reverse primer	CTCAGCCACTCCAGCTGCTC
probe (FAM labelled)	CGTCGTAGCAAACCAAGCGGA
β–actin (*housekeeping gene*)	
forward primer	GCTTCTTTGCAGCTCCTTCGTT‘
reverse primer	GTTGTCGACGACCAGCGC
probe (FAM labelled)	CAGCCTTCCTTCTTGGGTATGGAATCCT

Table 6 *Primer and probe sequences employed in the present study.*

4.13.5. Analysis

The real time RT-PCR results were obtained as exportable computer data. For each probe the amplification plot was obtained and analysed in duplicate. “Ct” represents the cycle number at which the amplification reaction begins. Each sample was normalized to β-actin by calculating the difference between the Ct for β-actin and the cT for the molecules studied (anti- and pro-apoptotic molecules), as: ΔcT= cT β-actin- cT each molecule. As lower ΔcT means more cDNA, which was obtained from mRNA, and considering the exponential increase of cDNA in PCR, the mRNA quantity has been calculated using the following formula: mRNA= $2^{-\Delta cT}$

4.14. Statistical analysis.

The data were analysed using Microsoft Excel, Version 2002, together with the software “Statistical Package for the Social Sciences“ – SPSS -, version 11.5. The data are expressed as medians or medians ± 75 % quartiles (graphics include box plots) due to their non-parametric distribution and the heterogeneity of the group. The box plots include all the data, while showing the distribution of the samples.

Besides, the total number of samples employed for each experiment is indicated at the bottom of each box plot. The differences between abortion-prone and normal pregnancy groups were calculated using the non-parametric Mann-Whitney-*U* test, whereas when analyzing more then one sample (e.g. in Co-PP and Zn-PP treated animals) the non-parametric Kruskal Wallis test was used. In all cases *p<0.05; **p<0.01; ***p<0.001 was considered statistically significant.

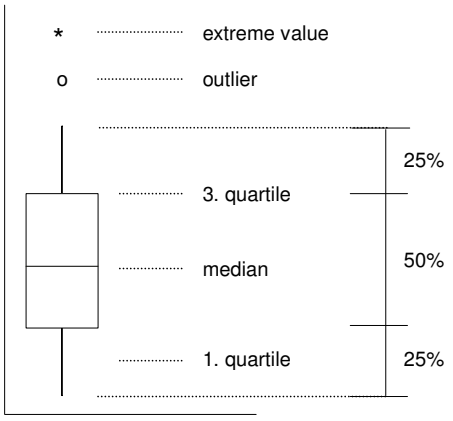

Fig. 19 *Representation of a typical box-plot-graphic including all the data.*

5. Results

5.1 Apoptosis in the abortion-prone murine combination CBA/J x DBA/2J as compared to the normal combination CBA/J x BALB/c

5.1.1. DBA/2J-mated CBA/J females showed increased abortion rate as compared to BALB/c mated CBA/J

As previously reported (Clark *et al.,* 1980; Chaouat *et al.,* 1988; Zenclussen *et al.,* 2003a), CBA/J females previously mated with DBA/2J male showed significantly up-regulated abortion rate compared to BALB/c-mated CBA/J females (Fig. 20 A). We did not observe differences among both groups concerning the number of implantations (Fig. 20 B).

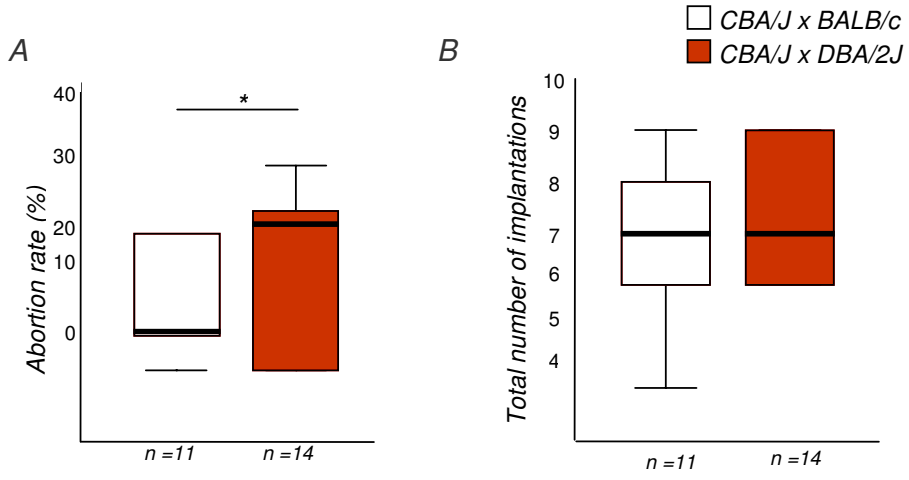

Fig. 20 *(A) Abortion rates. DBA/2J-mated CBA/J females presented increased abortion rate compared to BALB/c-mated CBA/J females on day 14 of pregnancy. (B) Implantation rates. The implantation rates were comparable between both groups. The data are shown as median ± 75% quartiles and are representative of two different experiments. Statistical significance was evaluated by the non-parametric Mann-Whitney-U test: $p<0.05$.*

5.1.2. Th1 and Th2 cytokines in abortion versus normal pregnancy

5.1.2.1. Decidual cells from abortion-prone mice produced significantly more TNF-α and less IL-10 when compared to normal pregnant mice

As already described by several studies an imbalance in the Th1/Th2 cytokine ratio could be associated with pregnancy failure (Clark *et al.*, 1999; Lin *et al.*, 1993; Wegmann *et al.,* 1993; Krishnan *et al.,* 1996; Raghupathy *et al.,* 1997, 1999). In this study, we analysed by flow cytometry the ability of decidual immune cells, isolated from abortion-prone or normal pregnant mice, to produce Th1 or Th2 cytokines after PMA/Ionomycin stimulation, which is known to stimulate mostly memory cells. This may serve as a mirror of the *in vivo* cytokine production. We observed a statistically significant augmentation in the TNFα/IL-10 ratio (Fig. 21) in decidual lymphocytes from abortion-prone mice when compared to normal pregnant mice on the 14th day of gestation, confirming increased ability of decidual cells to produce Th1 cytokines and decreased Th2 cytokine production in abortion-prone animals.

5.1.2.2. Placental and decidual TNF-α mRNA levels were augmented in abortion-prone mice compared to the controls

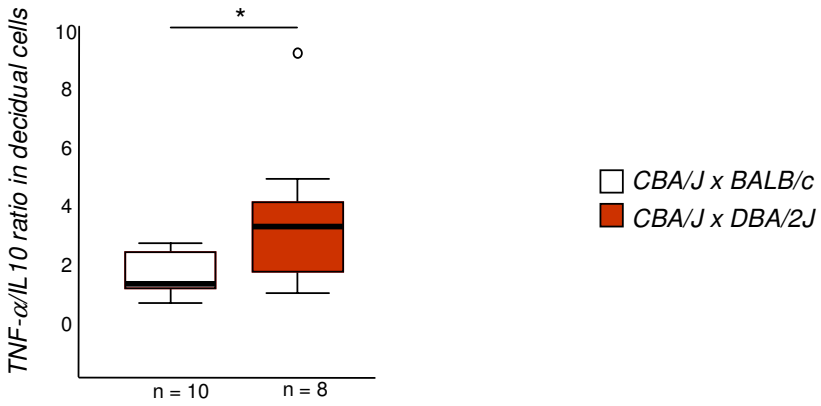

Fig. 21 *TNF-α/IL-10 ratio. The Th1/Th2 cytokine ratio of was significantly up-regulated in decidual immune cells from abortion mice compared to normal pregnant mice on the 14th day of pregnancy. Data are presented as median ±75% quartiles. Statistical significance was evaluated by the non-parametric Mann-Whitney-U test. *p<0.05. Circles represent outliers, which were included in the statistical analysis.*

Real time RT-PCR analysis confirmed a slight increase in TNF-α mRNA levels in decidua (Fig. 22 A) as well as in placenta (Fig. 22 B) from the abortion-prone group compared to the normal pregnancy. The increase was, however, non-significant. This may be due to the fact that the mRNA levels were measured in the whole tissue, comprising TNF-α from all cells (trophoblasts and immune cells in the case of placenta or decidual cells and immune cells in the case of deciduae), while the flow cytometry data represent solely the TNF-α produced by immune cells.

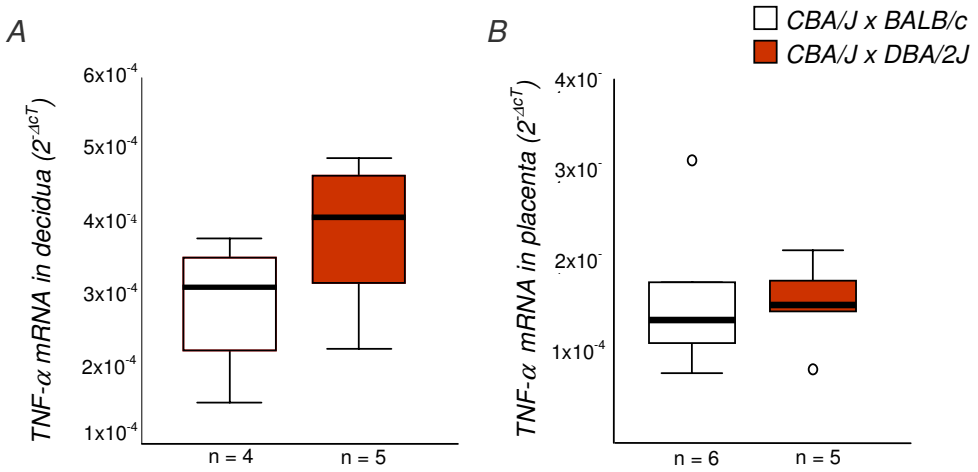

Fig. 22 *TNF-α mRNA levels. Slightly elevated TNF-α mRNA levels were observed in decidual (A) and placenta (B) tissues from the abortion group compared to the controls as analysed by real time RT-PCR and indicated by $2^{-\Delta cT}$. Data are presented as median ±75% quartiles. Statistical significance was evaluated by the non-parametric Mann-Whitney—U-test. Circles mean outliers, were included in the statistical analysis.*

5.1.3. Apoptosis in the murine combination CBA/J x DBA/2J

5.1.3.1. Spleen or decidual lymphocytes from abortion-prone and normal pregnant mice presented similar apoptotic rates

Considering the up-regulated levels of TNF-α protein we found in the abortion combination, and knowing that this pro-inflammatory cytokine is directly involved in inducing apoptosis via its receptors (TNF-RI and TNF-RII; Bowen *et al.*, 2000), we analysed by flow cytometry cells positive for annexin-V in spleen as well as decidual immune cells positive for annexin-V. Annexin-V is known as a marker for

early apoptosis, while cells positive for both annexin-V and propidium iodide are thought to be late apoptotic cells. Moreover, this assay allowed distinguishing apoptotic cells from viable (annexin-V^-/PI^-) or necrotic (PI^+) cells (Fig. 13). Our analysis revealed no significant differences in the apoptotic rate between local (decidual) or systemic (spleen) lymphocytes from DBA/2J-mated CBA/J females compared to BALB/c-mated CBA/J mice, suggesting that apoptosis is not involved in pregnancy failure as massive death of immune cells at this time point (Table 7).

Spleen	***Annexin-V$^+$cells***	***PI$^+$cells***	***Annexin-V/PI$^+$cells***	***Viable***
CBA/J x BALB/c n=8	8.04	6.90	21.57	55.80
CBA/J x DBA/2J n=9	4.65	11.47	38.58	31.39
Decidua	***Annexin-V$^+$cells***	***PI$^+$cells***	***Annexin-V/PI$^+$cells***	***Viable***
CBA/J x BALB/c n=6	26.11	1.40	8.23	59.69
CBA/J x DBA/2J n=5	20.90	1.57	5.65	65.67

Table 7 *Early and late apoptotic rates analysed by annexin-V-FITC and propidium iodide (PI) staining using flow cytometry. Data are shown as medians. No significant differences could be observed in any of the analysed parameters between the two groups when analysed by the non-parametric Mann-Whitney-U test.*

5.1.3.2. No signs of increased local apoptosis in the murine abortion combination compared to normal pregnant mice

The activity of caspase-3, a key enzyme in apoptotic process, was slightly higher in the abortion-prone group compared to normal pregnancy group (Fig. 23 A). However this difference was not statistically significant. Furthermore, the analysis of cellular DNA fragmentation by TUNEL assay confirmed similar apoptotic rates between both experimental groups on day 14 of pregnancy (Fig. 23 B). Representative pictures of TUNEL staining for both normal and abortion-prone mice are shown in Figs. 23 C and D. The negative control staining can be observed in Fig. 23 E

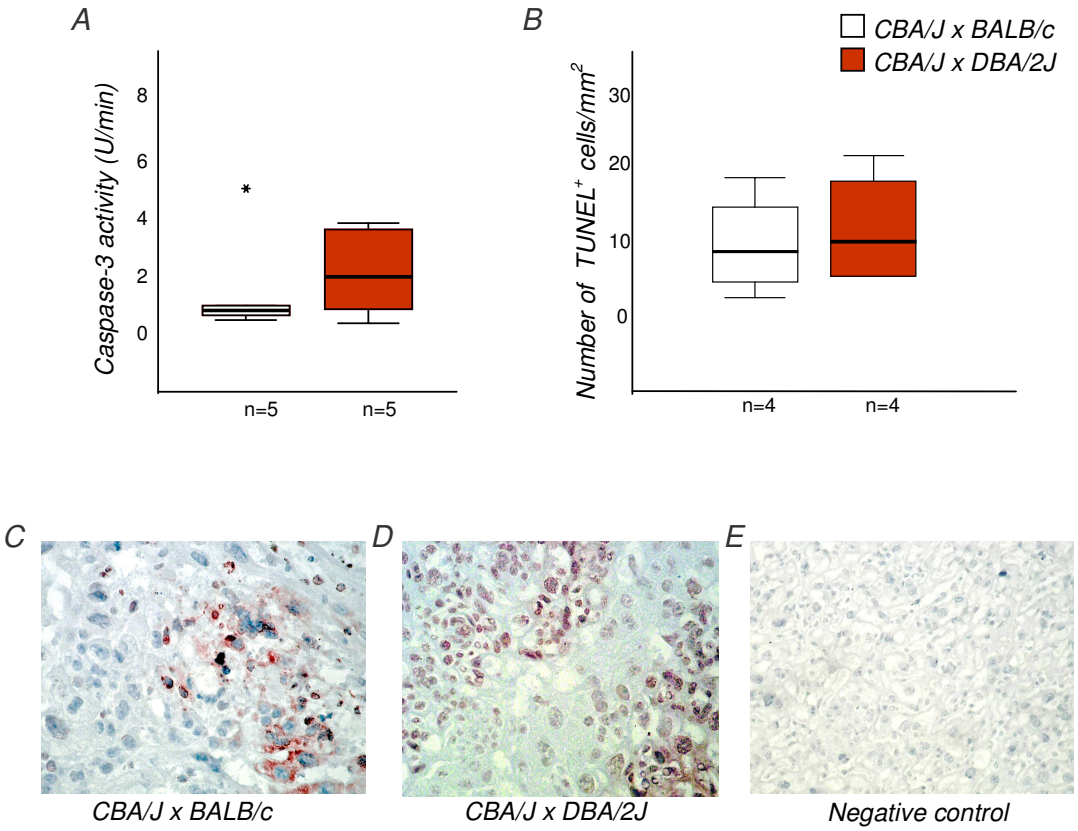

Fig. 23 *(A) Caspase-3 activity. Slightly, but not significantly augmented levels of caspase-3 activity were observed in placenta from abortion compared to normal pregnant mice. (B) TUNEL$^+$ cells. Analysis of DNA fragmentation by TUNEL in placental cells revealed a marginal and not statistically significant augmentation in the number of TUNEL$^+$ cells in abortion group compared to the controls. Data are presented as median ±75% quartiles. Statistical significance was evaluated by the non-parametric Mann-Whitney-U-test. Asterisks representing extreme values were included in the statistical analysis. (C, D, E) Representative pictures of TUNEL nuclear staining. The pictures show TUNEL$^+$ cells in normal (C) or abortion (D) samples developed with AEC Substrate Chromogen and counterstained with Hemalaun. The negative control (E) was performed by replacing the TdT enzyme with the label solution. Pictures were taken using a 20X magnification of the objective lens.*

5.1.3.3. Pro- and anti-apoptotic molecules at the feto-maternal interface

Having found comparable apoptotic activity at the feto-maternal interface from normal pregnant mice and mice undergoing immunological abortion, we aimed to analyse the expression of pro- and anti-apoptotic molecules.

5.1.3.3.1. Cellular localization investigated by immunohistochemistry (IHC): increased expression of Bcl-2 at the feto-maternal interface

The localization of pro- and anti-apoptotic molecules (caspase-3, Bcl-xS and Bag-1, Bcl-2 respectively) was investigated using IHC. Pro- and anti-apoptotic molecules were detected in all placental cell types (giant cells, spongiotrophoblasts, and labyrinthic cells) as well as in maternal decidual cells from both abortion-prone and control animals. When semi-quantifying the staining intensity, a significant increase in the expression of the anti-apoptotic Bcl-2 could be observed in decidual cells as well as in spongiotrophoblasts and labyrinthic cells of placental samples from the abortion-prone group, when compared to the controls (Fig. 24 A-C). No differences could be observed for any of the other molecules analysed between abortion and normal pregnancy groups (Table 8).

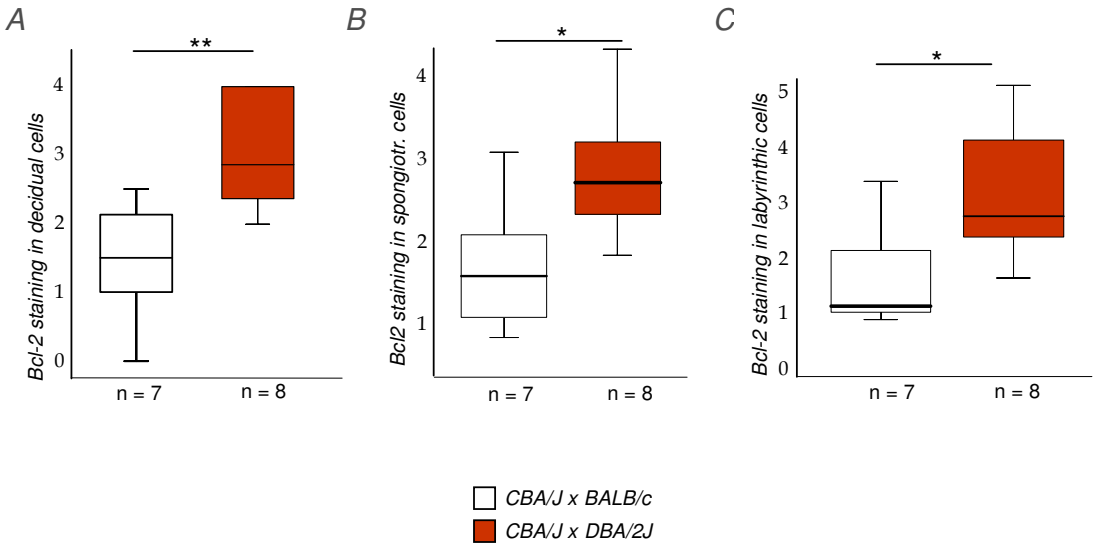

Fig. 24 *Expression of Bcl-2 in decidual cells (A) spongiotrophoblasts (B) and labyrinthic cells (C). Semi-quantitative analysis revealed significantly enhanced expression of Bcl-2 in decidual as well as placental cells. Data are presented as median ±75% quartiles. Statistical significance was evaluated using the non-parametric Mann-Whitney-U test. *: p<0.05 **p<0.01.*

Bag-1, an anti-apoptotic molecule as well, and the pro-apoptotic molecules, Bcl-xS and caspase-3 showed a comparable expression in decidual and placental cells between both experimental groups on day 14 of pregnancy (Table 8).

Bag-1	***Giant cells***	***Spongiotrophoblast cells***	***Labyrinthic cells***	***Decidual cells***
CBA/J x BALB/c n=7	0.50	0.20	0.50	0.50
CBA/J x DBA/2J n=8	0.50	0.50	0.75	0.50
Bcl-xS				
CBA/J x BALB/c n=7	3.00	2.00	1.50	3.00
CBA/J x DBA/2J n=8	2.50	2.00	1.75	3.00
Caspase-3				
CBA/J x BALB/c n=7	1.50	2.50	0.50	2.00
CBA/J x DBA/2J n=8	1.62	2.00	0.62	2.50

Table 8. *Expression of pro- and anti-apoptotic molecules as analysed by IHC. Data are shown as medians. No significant differences could be observed in any of the analysed parameters between the two groups as analysed by the non-parametric Mann-Whitney-U test.*

Representative staining patterns are shown in Figures 25 A-D together with the negative control (Fig. 25 E).

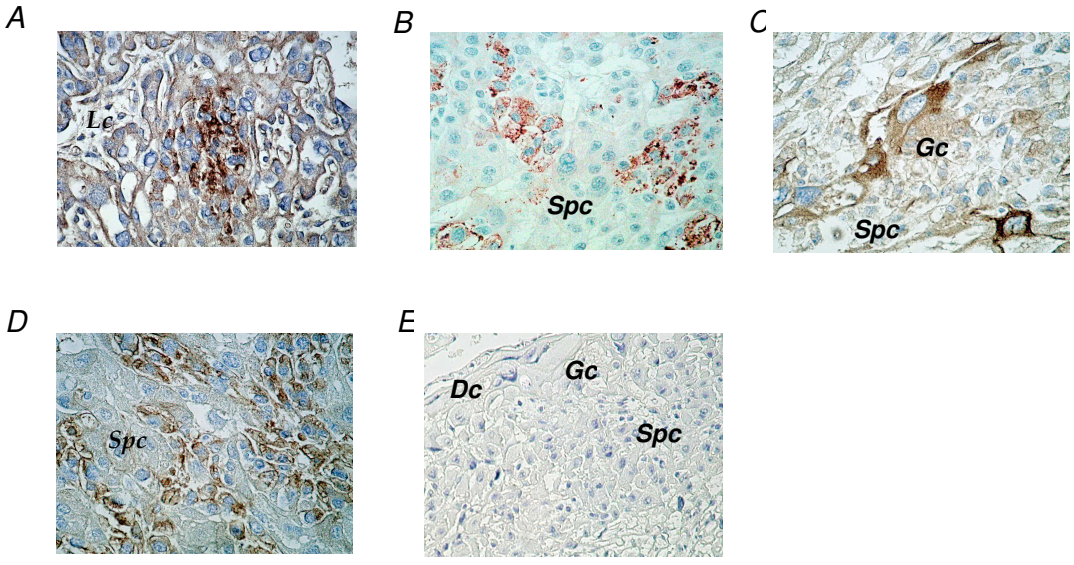

Fig. 25 *Picture representing IHC staining. The pictures show representative staining fields for Bcl-2 (A), Bag-1 (B), Bcl-xS (C) or caspase-3 (D) in different placental cell types (Dc: decidual cells; Gc: giant cells; Spc: spongiotrophoblast cells; Lc: labyrinthic cells) counterstained with haemalam.*

Pictures were taken using a 20X (B, E) or 40 X (A, C, D) magnification. (E) the negative control, performed without primary antibody or with the blocking peptide.

Considering that after 14 days of pregnancy we observed a significant up-regulated expression of Bcl-2 in decidual as well as in placental cells from abortion-prone mice compared to the controls, we decided to analyse this anti-apoptotic molecule in tissues coming from mice sacrificed on the 18th day of gestation. At this time point both, fetus and placenta are completely formed. With this complementary analysis we aimed to analyze whether the expression of Bcl-2 was still up-regulated during late pregnancy (day 18 of gestation). It is important to remark here that the tissue specimens included in the study were "healthy" placentas in close proximity to resorbed fetuses. Increased Bcl-2 levels at both time points could indicate a mechanism to avoid death. When semiquantified the staining intensity, a significant increase in the expression of Bcl-2 could be observed in decidual cells from abortion-prone mice, supporting the data obtained with samples from 14th day of pregnancy. This further suggests that the up-regulation of Bcl-2 in abortion could be involved in the activation of a protective mechanism tending to push the correct development of the placental organ and consequentially of the embryo, due to its closeness to rejected tissues (resorbed fetuses).

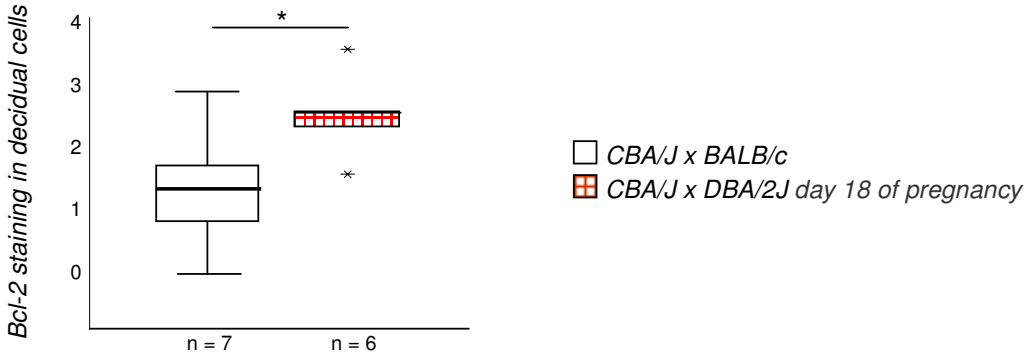

Fig. 26 *Expression of Bcl-2 in decidual cells of tissues coming from abortion and normal pregnancy mice sacrificed at day 18 of gestation. The semi-quantitative analysis revealed significant expression of Bcl-2 in decidual cells from abortion compared to the control. Data are presented as median ±75% quartiles Statistical significance was evaluated using the non-parametric Mann-*

*Whitney-U test. *: p<0.05. Asterisks representing extreme values were included in the statistical analysis.*

5.1 3.3.2. Pro- and anti-apoptotic protein expression: up-regulation of Bcl-2 in CBA/J x DBA/2J mice compared to the controls

We quantified the expression of pro- and anti-apoptotic proteins (Bcl-xS, Bcl-2 and Bag-1) in whole placental extracts from mice sacrificed after 14 days of pregnancy using Western Blot assay. Positive bands for all molecules were detected in both, normal pregnant and abortion-prone mice. Quantification of the band intensities by ImageQuant TL software from Amersham (*www.Amersham.com*) allowed us to confirm statistically increased Bcl-2 levels in placental tissues from abortion-prone mice when compared to normal pregnant mice.

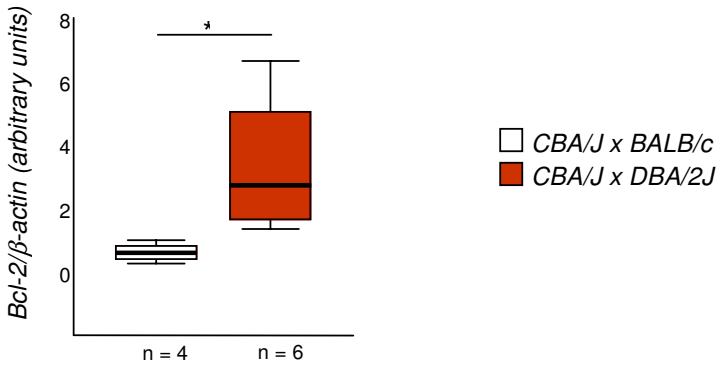

***Fig. 27** Quantification of Bcl-2 expression. The intensity of the bands for Bcl-2 in placental homogenates from normal pregnant and abortion-prone mice was analysed densitometrically referred to β-actin band intensity using the ImageQuant TL software (Amersham). We observed a significant up-regulation of Bcl-2 in the abortion-prone group compared to the control. Data are presented as median ± 75% quartiles. Statistical significance was evaluated by the non-parametric Mann-Whitney-U test. *: p<0.05.*

In accordance with the previously discussed IHC data, no changes could be observed for Bag-1 or Bcl-xS protein expression by Western Blot either (Table 9).

Anti-apoptotic protein	***CBA/J x BALB/c n=4***	***CBA/J x DBA/2J n=6***
Bag-1	2.15	1.81
Pro-apoptotic protein	***CBA/J x BALB/c n=4***	***CBA/J x DBA/2J n=6***
Bclx-S	1.43	1.6

Table 9 *Pro- and anti-apoptotic protein expression analysed by Western Blot and quantified densitometrically referred to β-actin. No significant difference could be observed in any of the analysed parameters between the two groups as analysed by the non-parametric test Mann-Whitney-U test. Data are presented as median.*

5.1. 3.3.3. mRNA levels of pro- and anti-apoptotic molecules by real time-RT-PCR

The mRNA levels of Bax (pro-apoptotic), Bcl-2, Bcl-xL and Bag-1 (anti-apoptotic) were analysed in placental and decidual samples from both experimental groups by using real time RT-PCR. Bcl-2 mRNA levels were slightly augmented in abortion-prone animals as compared to normal pregnant individuals. However, no statistically significant differences could be observed between normal pregnant and abortion-prone mice for Bcl-2 in placental samples (Fig. 28). Bcl-xL and Bax mRNA placental expression was also comparable in both groups (Table 10).

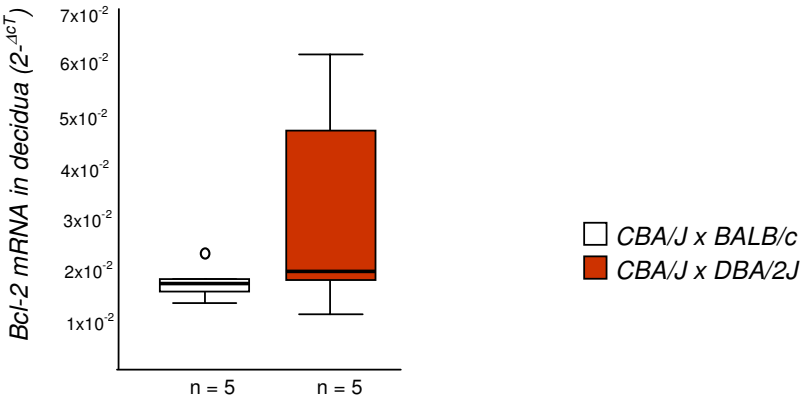

Fig. 28 *Bcl-2 m-RNA levels in decidual tissue. The m-RNA levels for Bcl-2 were analysed in placenta using real time RT-PCR and indicated by $2^{-\Delta cT}$. Bcl-2 was slightly but not statistically significantly up-regulated in decidua from abortion-prone group compared to the control. Data are presented as median ± 75% quartiles in box blot due to their non-Gaussian distribution. Circles*

representing outliers were included in the statistical analysis. Statistical significance was evaluated by the non-parametric Mann-Whitney-U test.

In decidual samples, the mRNA levels for Bcl-2 were again up-regulated in abortion-prone mice as compared to normal pregnant mice, supporting IHC and WB data. These augmented levels did not reach significance levels. No changes could be observed for Bag-1, Bcl-xL or Bax in decidual tissue (Table 10).

Anti-apoptotic protein		***Placenta***	***Decidua.***
Bag-1	*CBA/J x BALB/c, n=6*	*0.1127*	*0.4491*
	CBA/J x DBA/2J, n=9	*0.3301*	*0.5724*
Bcl-xL	*CBA/J x BALB/c, n=6*	*0.0219*	*0.0146*
	CBA/J x DBA/2J, n=8	*0.0396**	*0.0178*
Pro-apoptotic protein		***Placenta***	***Decidua.***
Bax	*CBA/J x BALB/c, n=5*	*0.0341*	*0.0206*
	CBA/J x DBA/2J, n=8	*0.0303*	*0.0183*

Table 10 m-RNA levels of pro- and anti-apoptotic molecules in placenta and decidua. *Data are expressed as $2^{-\Delta cT}$ and shown as medians. *: $p<0.05$ as analysed by the non-parametric test Mann-Whitney-U test.*

5.2. Heme oxygenase

5.2.1. Abortion rates after up- or down-regulation of HO-1 by means of Co-PP and Zn-PP

We analysed the abortion (Fig. 29 A) as well as the implantation rate (Fig. 29 B) of mice previously treated with Co-PP or Zn-PP during the implantation window (i.e. day 4th of pregnancy). This treatment allowed us to up- or down-regulate the protective enzyme HO-1, thus analysing the effect of augmented or diminished HO-1 levels on pregnancy outcome. As expected, the abortion rate of DBA/2J-mated CBA/J females treated with PBS was significantly higher than the PBS-treated normal pregnancy control mice (Fig. 29 A). Co-PP treatment was effective in diminishing the abortion rate, thus preventing fetal rejection, whereas Zn-PP treatment significantly boosted the abortion rate. These data strongly suggest that the absence or down-regulation of HO-1 negatively influences the pregnancy outcome, while its up-regulation has a positive effect. The experiments presented in the graphics below were done in collaboration with André Sollwedel from our laboratory.

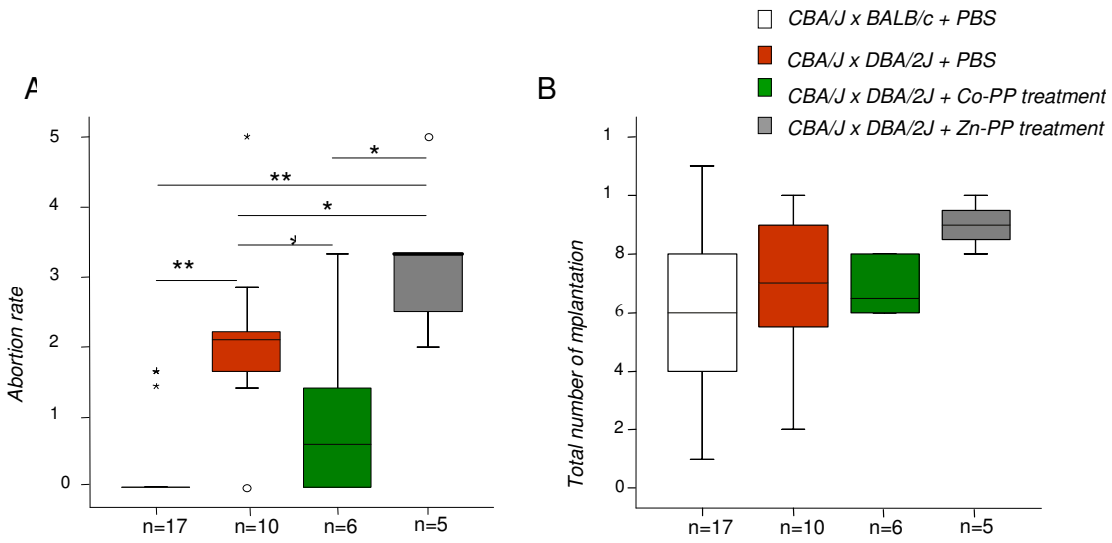

Fig. 29 *(A) Abortion rate. DBA/2J-mated CBA/J females presented increased abortion rate compared to BALB/c-mated CBA/J females on day 14 of pregnancy. Co-PP application significantly diminished the abortion rate, while Zn-PP treatment boosted fetal rejection. (B) Implantation rate. The implantation rate was comparable between the two groups. Data are presented as median ±75% quartiles in box blot due to their non-Gaussian distribution. Statistical significance was evaluated by the non-parametric Kruskall-Wallis test followed by the Mann-Whitney-U test between*

*two particular groups. *: p<0.05; **: p<0.01; ***: p<0.001. Circles and asterisks representing outliers and extreme values were included in the statistical analysis.*

5.2.2. Anti-apoptotic properties of heme oxygenase during pregnancy

5.2.2.1. Heme oxygenase down-regulates apoptosis in CBA/J females treated with Co-PP

Heme oxygenase-1 was shown to have anti-apoptotic and tissue-protective properties (Wagener *et al.*, 2003; Song *et al.*, 2003; Sollwedel *et al.*, 2005). Considering these facts, we up- and down-regulated HO-1 by Co-PP and Zn-PP applications, then we analysed the activity of caspase-3 enzyme and the DNA fragmentation by TUNEL in those samples from animals treated with Co-PP or Zn-PP. Interestingly, we observed a slight diminution of the caspase-3 activity in abortion-prone mice treated with Co-PP as compared to abortion-prone mice treated with PBS (Fig. 30 A). Moreover, the analysis of apoptotic cells by TUNEL (Fig. 30 B) further confirmed that apoptosis is tendentially down-regulated in Co-PP-treated animals as compared to the controls. These data point to a tissue protective effect of HO-1 by diminishing the incidence of apoptosis at the feto-maternal interface, similarly to the data reported for other tissues (Wagener *et al.*, 2003; Song *et al.*, 2003).

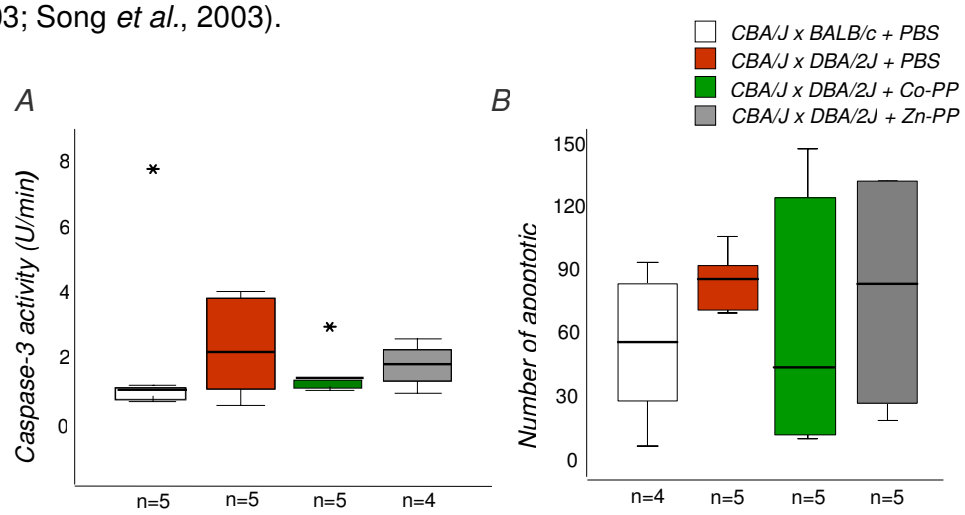

Fig. 30 *(A) Caspase-3 activity. Slightly, but not significantly diminished levels of caspase-3 activity were observed in placenta from Co-PP-treated mice compared to the controls. (B) TUNEL$^+$ cells. Analysis of DNA fragmentation by TUNEL in placental cells revealed a marginal and not statistically significant dimininution in the number of TUNEL$^+$ cells in Co-PP group compared to the controls. Data are presented as median ±75% quartiles. Statistical significance was evaluated by the non-*

parametric Mann-Whitney-U test after the Kruskall-Wallis test. Asterisks representing extreme values were included in the statistical analysis.

5.2.2.2. Bag-1 expression is up-regulated after HO-1 augmentation by Co-PP treatment

In order to investigate if HO-1 augmentation could prevent abortion by diminishing apoptosis, we further investigated the levels of apoptosis-related molecules such as Bag-1, Bcl-2 and Bcl-xL. Interestingly, Bag-1 protein expression (Fig. 31 A) and mRNA levels (Fig. 31 B) were significantly up-regulated in placental homogenates after treatment with Co-PP as compared to the PBS-treated control groups or Zn-PP treated abortion-prone animals. Our data suggest that Bag-1 induction after HO-1 up-regulation may play an important role in protecting the fetuses from rejection.

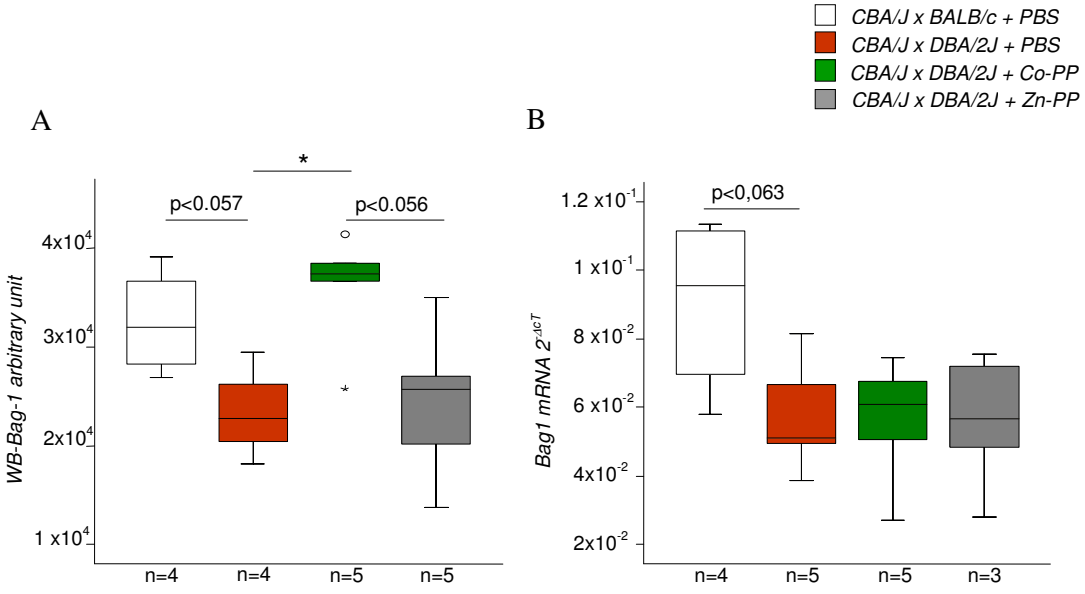

Fig. 31 *Quantification of protein expression (A) and mRNA levels (B) of Bag-1 in placental homogenates. In A, the intensity of the bands for Bag-1 was analysed densitometrically referred to β-actin using ImageQuant TL software (Amersham). We observed a significant up-regulation of Bag-1 protein expression in the group receiving Co-PP compared to the abortion-prone or Zn-PP group. In B, a similar tendency could be observed at mRNA levels for Bag-1 in placental tissue. Data are presented as median ± 75% quartiles. Statistical significance was evaluated by the non-parametric Mann-Whitney-U test. *: p<0.05. Circles and asterisks representing outliers and extreme values were included in the statistical analysis.*

Immunohistochemistry analysis supported up-regulation of anti-apoptotic molecules (i.e. Bcl-2 and Bag-1) in placental cells as well as in deciduas of treated samples compared to the controls. On the other hand, pro-apoptotic molecules have a quite similar expression in all the groups. The data are summarized in Table 11.

Bcl-2	***Giant cells***	***Spongiotrophoblast cells***	***Labyrinthic cells***	***Decidual cells***
CBA/J x BALB/c n=7	0.50	0.20	0.50	0.50
CBA/J x DBA/2J n=8	0.50	0.50	0.75	0.50
CBA x DBA/J + Co-PP n =7	3 **	4 **	3.7 **	3.5 *
CBA x DBA/J + Zn-PP n =7	3.2	3.6	3.5	3.7 **
Bag-1	***Giant cells***	***Spongiotrophoblast cells***	***Labyrinthic cells***	***Decidual cells***
CBA/J x BALB/c n=7	0.50	0.20	0.50	0.50
CBA/J x DBA/2J n=8	0.50	0.50	0.75	0.50
CBA x DBA/J + Co-PP n =7	0.6	1	2.2 *	0.75
CBA x DBA/J + Zn-PP n =7	0.5	0.9	1.1	1.5 *
Bcl-xS	***Giant cells***	***Spongiotrophoblast cells***	***Labyrinthic cells***	***Decidual cells***
CBA/J x BALB/c n=7	3.00	2.00	1.50	3.00
CBA/J x DBA/2J n=8	2.50	2.00	1.75	3.00
CBA x DBA/J + Co-PP n =7	3	4	3.7	3.5
CBA x DBA/J + Zn-PP n = 7	3.2	3.6	3.5	3.7
Caspase-3	***Giant cells***	***Spongiotrophoblast cells***	***Labyrinthic cells***	***Decidual cells***
CBA/J x BALB/c n=7	1.50	2.50	0.50	2.00
CBA/J x DBA/2J n=8	1.62	2.00	0.62	2.50
CBA x DBA/J + Co-PP n = 7	3	4	3.7	3.5
CBA x DBA/J + Zn-PP n =7	3.3	3.6	3.5	3.7

Table 11 Expression of pro- and anti-apoptotic molecules analysed by IHC. Data are shown as medians. No significant differences could be observed in any of the analysed parameters between the two groups as analysed by the non-parametric test Kruscal Wallis and Mann-Whitney–U tests.

6. Discussion

The mechanisms leading to successful pregnancy are still poorly understood. Novel pathways are being studied in pregnancy-related research.

In the past, several theories were proposed to explain why the fetus is not rejected from its mother, despite its paternal inheritance. The explanation provided by one of these hypotheses, the "Th1/Th2/Th3 paradigm", is that augmented Th2/3 cytokines synthesis accompanied by diminished Th1-cytokines production could be involved in avoiding immunological rejection of the fetus (Wegmann *et al.*, 1993; Clark *et al.*, 1999; Raghupathy *et al.*, 1997, 1999; Lin *et al.*, 2003). This hypothesis has triggered a heated debate, since several studies suggest this to be an over-simplification (Svensson *et al.*, 2001; Zenclussen *et al.*, 2002a; Chaouat *et al.*, 2003).

The murine combination CBA/J x DBA/2J represents a well-known experimental model of Th1-induced abortion, with increased local TNF-α levels at the feto-maternal interface (Tangri and Raghupathy, 1993; Clark *et al.*, 1999; Raghupathy, 2001). TNF-α is also known as potent inductor of apoptosis (Baud and Karin, 2001; Gupta, 2002). It has become clear that apoptosis plays a role in many aspects of reproduction like implantation (Hardy, 1999), placental growth and development (Joswig *et al.*, 2003). Pregnancy loss and the occurrence of inborn structural anomalies are often preceeded by excessive apoptosis in targeted embryonic and extra-embryonic tissues (Toder *et al.*, 2002). Considering these facts, we therefore aimed to investigate the incidence of cell death in this model in order to understand if miscarriage was preceded by augmented apoptotic rates. We analysed several key features of apoptosis such as the caspase-3 activity and the DNA fragmentation together with pro- and anti-apoptotic molecules, in order to establish their role at the feto-maternal interface, by employing immunohistochemistry, flow cytometry western blot and molecular biology methods.

Despite augmented levels of Th1 cytokines at the feto-maternal interface, apoptosis seems not to play a big role in the onset of abortion. Moreover, our data suggest the up-regulation of Bcl-2 as a possible mechanism to keep safe the

development of placentas closed to resorbed sites, in order to maintain the correct physiology and function of this organ to ensure the fetal growth until birth.

During gestation, heme oxygenase enzyme–1 (HO-1) seems to be protective. As a matter of fact, data from our laboratory suggest that HO-1 if up-regulated by Co-PP injection could prevent abortion, whereas when down-regulated (by employing Zn-PP), enhance miscarriage. In this work we investigated the possible mechanisms leading to successful pregnancy induced by up-regulation of HO-1. Considering the anti-apoptotic properties of HO-1 (at least *in vitro*), we checked if the protective effects of HO-1 during gestation could be due to changes in the apoptotic rate and in the expression of apoptotic-related molecules.
This study strongly supports a very important role for the enzyme heme oxygenase-1 in protecting the fetus from maternal rejection, suggesting up-regulation of Bag-1 by HO-1 as the probable tissue protective mechanism to ensure successful pregnancy.

6.1. Do the decidual lymphocytes coming from the CBA/J x DBA/2J murine combination produce more TNF-α (pro-inflammatory) and less IL-10 (anti-inflammatory) at the feto-maternal interface as compared to normal pregnant mice?

In recent years, it was hypothesized that cytokines play a determining role in pregnancy outcome. The "Th1/Th2/Th3 paradigm" proposes that the ratio of pro- (Th1) and anti- (Th2/Th3) inflammatory cytokines could be critical during gestation (Wegmann *et al.*, 1993; Chaouat *et al.*, 1995, 2003; Clark *et al.*, 1999). In this context, Th2 or Th3 cytokines such as IL-4, IL-10 or TGF-β, would favor the maintenance of pregnancy, whereas excessive pro-inflammatory cytokine (i.e. TNF-α or IFN-γ) production would be involved in determining fetal rejection (Raghupathy, 1997, 1999; Clark *et al.*, 1999; Chaouat *et al.*, 2003; Zenclussen *et al.*, 2003a; Lin *et al.*, 2003). Accordingly, administration of IL-10 prevents naturally occurring fetal loss in CBA/J x DBA/2J combination (Chaouat *et al.*, 1995); successful human pregnancy is totally correlated with the presence of anti-inflammatory cytokines (i.e. IL-4 and IL-10; Dealtry *et al.*, 2000) and the *in vivo* blocking of the trafficking of Th1 lymphocytes migration to the feto-maternal interface by administration of mAbs against selectins improved pregnancy outcome in CBA/J x DBA/2J combination compared to the controls (Zambon Bertoja *et al.*, 2005). However, recent works questioned the importance of Th1/Th2 theory as IL-4 and IL–10 KO mice have normal pregnancies (Svensson *et al.*, 2001), pro-inflammatory cytokines are essential to placenta immunity (Barber E.M. *et al.*, 2005), and immune cells from patients suffering from spontaneous abortion produce less IL-12 than cells from normal pregnant patients (Zenclussen *et al.*, 2002a).

In this work, we employed a murine combination representing a well-established murine model of abortion, as described by Clark (1980), where CBA/J (H-2^k) female mice spontaneously show high abortion rates (about 20-40%) when mated with DBA/2J (H-2^d) males and low abortion rates (usually 0 %) when mated with other $H2^d$-bearing males like BALB/c. Several studies carried out employing this combination showed augmented pro-inflammatory cytokine production by decidual lymphocytes or placental cells from the abortion-prone-CBA/J females, compared

to the normal pregnant controls (CBA/J x BALB/c; Chaouat *et al.,* 1995; Clark *et al*., 1999; Zenclussen *et al.* 2003a, 2005; Zenclussen M.L. *et al*., 2005). In our study, we analysed the ability of decidual lymphocytes to produce Th1 and Th2 cytokines, respectively TNF-α and IL-10, after PMA and Ionomycin stimulation, known to stimulate mostly memory cells as a mirror of the *in vivo* cytokine production. We confirmed increased ability of decidual cells to produce TNF-α along with decreased production of IL-10 in abortion-prone animals when compared to normal pregnant mice. Considering that trophoblast cells are also responsible for synthesis of cytokines, we further analysed total TNF-α mRNA levels in both decidua and placenta of abortion-prone animals as compared to normal pregnancy group. This augmentation was however only tendentially but not statistically significant. Our data confirm the Th1/Th2/Th3 paradigm at least for these two clue cytokines, TNF-α and IL-10, while showing that immune cells produce TNF-α when a rejection is taking or has taken place. Moreover, these results confirmed once more, that abortion events are accompanied by imbalances between Th1 and Th2 even when Th2 cytokines are not essential for pregnancy outcome or augmented Th1 cytokines can be seen in normal pregnancies. Considering the important role of pro-inflammatory cytokines (i.e. TNF-α) in the onset of miscarriage in this model, and the ability of TNF-α in triggering apoptosis in several other models (Sidoti-de-Fraisse *et al*., 1998), we aimed to investigate whether apoptosis is up-regulated in animals undergoing abortion after observing augmented Th1 levels at the feto-maternal interface.

6.2. Is the apoptosis of immune cells modified after occurrence of immunological abortion?

Programmed cell death is essential for the development and maintenance of cellular homeostasis of the immune system in mammals, because it avoids aberrations such as immunodeficiency, autoimmunity and cancer (Vaux and Korsmeyer, 1999). We were interested in studying the *ex-vivo* apoptotic rate (early and late) of spleen and decidual lymphocytes immune cells, in order to investigate whether apoptotic process could be altered after resorption time (day 8 of gestation), i.e. at the day 14^{th} of pregnancy. The data revealed a similar apoptotic rate in decidual or spleen lymphocytes from abortion-prone mice compared to normal pregnant mice, suggesting that apoptosis of immune cells is not being modified after occurrence of immunological abortion. For a long time, apoptotic cells were considered to be neutral in immune response. Interestingly, recent studies demonstrated that apoptotic cells actively regulate the immune response (Voll *et al*., 1997; Fadok *et al*., 1998; Byrne and Reen, 2002). Thus, the presence of apoptotic cells during monocyte activation increases the secretion of IL-10 and decreases the release of TNF-α and IL-12, clearly modifying the Th1/Th2 ratio. Moreover, apoptotic cells release TGF-β1, a potent immunosuppressor, which contributes to the local suppression of immune response (Chen *et al*., 2001), and after being ingested by macrophages, they can actively alter the biological behavior of macrophages, acquiring immunosuppressive properties (Zhang and Zheng, 2005). Interestingly, necrotic tumor cells help macrophages to kill tumor cells, but apoptotic cells decrease the competence of macrophages in clearance tumor cells, promoting their growth (Reiter and Krammer, 1999). Taking these facts into consideration, we can hypothesize a scenario where during resorption formation (namely on day 8 of pregnancy), increased apoptosis in immune cells may boost Th2 lymphocytes expansion over Th1, in order to contrast the occurring inflammation and to re-establish the immunological environment existing before resorption. But, once resorption is formed and placental remodeling processes are also complete (day 14, when we analysed), apoptosis does not affect immune cells.

6.3. *Are the increased TNF-α levels at the feto-maternal interface from mice undergoing abortion accompanied by a higher incidence of apoptosis?*

Studies suggest that de-regulation of the apoptotic process is strictly associated with abnormal and ectopic pregnancies, fetal growth restriction or pre-eclampsia (Allaire *et al.*, 2000; Levy *et al.,* 2002). We wondered if apoptotic activity might be modified in whole tissue (immune + non-immune cells) in the pathological situation of immunological abortion. Therefore, we analysed the activity of caspase-3 enzyme, which mediates nuclear events during apoptosis by cleaving approximately 40 of 70 caspase substrates (Nicholson, 1999), and which represents one of the most important signs of whether cells or tissue are undergoing apoptosis (Cohen, 1997). Knofler and colleagues (2000) demonstrated that pro-inflammatory cytokines such as TNF-α may induce apoptosis in primary and immortalized first trimester cytotrophoblasts by increasing their caspase-3 activity. Therefore, our hypothesis was that apoptosis would be augmented in these mice as TNF-alpha levels were up-regulated. Contrary to our expectations, the analysis of caspase-3 activity revealed a slight and non-significant up-regulation of this enzymatic activity in placental tissues from mice undergoing abortion compared to the controls. Accordingly to the caspase activity data, further investigations about apoptotic process such as the analysis of DNA fragmentation - through TUNEL assay – confirmed statistically non-significant differences in the number of apoptotic cells at the feto-maternal interface from both experimental groups. Taken together, these results suggest that apoptosis might not be up-regulated, thus also not involved in a Th1-induced murine abortion. In other words high Th1 levels at the fetal-maternal interface were not able to boost apoptosis in abortion-prone mice or if so, this is contrasted by other mechanisms. With these data, we then focused our attention on NF-kappa B (NFκB), a transcription factor playing important roles in inflammation, autoimmune response, cell proliferation and apoptosis by regulating the genes involved in these processes (Pahl, 1999). As matter of fact, NFκB is activated by various intra- and extra- cellular stimuli including cytokines such as TNF-α. This pro-inflammatory cytokine negatively regulates its own ability to induce apoptosis by concomitantly activating NF-kappa B (Baldwin, 1996). In the absence of TNF-alpha stimulation, NFκB is associated with its inhibitor I-kappa B in the cytoplasm, whereas with TNF-alpha, the inhibitor

is degraded through ubiquitination (Pimentel-Muinos and Seed, 1999). The ability of NFκB to suppress apoptosis is one of the main NFκB functions determining its pro-inflammatory activity and implicating it in the pathogenesis of inflammatory diseases, oncogenesis and cancer therapy resistance (Karin and Lin, 2002). Therefore, NFκB could be activated directly by TNF-alpha to antagonize its own ability to induce apoptosis, thus protecting fetal development. Another possibility of no apoptosis despite high TNF-α levels is that the time point of analysis we chose was too late. As already indicated, we included in these study samples obtained on day 14 of pregnancy, a period during which placental remodeling processes have already taken place and the placenta is completely formed. However, we analysed samples on day 8 of pregnancy and found similar results. In addition, the interpretation of the results obtained from an earlier time point of analysis may be hampered by the fact that apoptosis is actively taking place for the normal development of tissue. Recently, Savion and colleagues (2002) analysed the role of apoptosis in murine pregnancy loss induced by LPS. In this context, they observed a marked correlation between abortion and apoptosis. Their study described that during resorption formation apoptosis is up-regulated, while the anti-apoptotic molecules are not. On the contrary, they observed down-regulation of apoptosis when resorption is complete, thus underlying a complex mechanism reflecting a specific regulation of apoptotic process tending to protect and maintain safety the physiology of the neighbor placentas, which have not been resorbed. However, it is important to say that LPS injection is a much more potent stimulus when compared to our model, in which the abortion events occur spontaneously. In the light of this consideration, we decided to analyse pro- or anti-apoptotic molecules in the CBA/J x DBA/2J combination using as controls BALB/c-mated animals as controls.

6.4. Pro- or anti-apoptotic molecules and regulation of apoptosis in a Th1-induced mouse abortion model. Is Bcl-2 the main player?

Considering that several studies revealed that the different cytokine profile at the feto-maternal interface influences pregnancy outcome (Lin *et al*. 1993; Chaouat *et al*., 1995, 2003; Clark *et al*., 1999; Raghupathy *et al*., 1997, 1999, 2001.), we wondered whether a naturally occurring TNF-α augmentation in the abortion-probe combination could interfere with the apoptotic process. Surprisingly, our study revealed no increased apoptosis incidence at the feto-maternal interface from mice undergoing abortion when compared to controls, despite high levels TNF-α, a well-known apoptosis-inductor. This shows that apoptosis does not play the main role in perpetuating abortion at least on day 14 of gestation. We then decided to analyze the levels of pro- and anti-apoptotic molecules to investigate whether there is a dysregulation at this level.

The apoptotic process is regulated by the Bcl-2 family, a large family of related, conserved protein products, each associated with either the inhibition or augmentation of cell death (Vander Heiden *et al*., 1999). The caspase family (cysteine proteases) represents the major executioners of programmed cell death (Hengartner, 2000). Proteins of the Bcl-2 family regulate apoptosis in part by affecting the mitochondrial compartmentalization of cytochrome *c*. Expression of Bcl-2 or Bcl-xL prevents redistribution of cytochrome c, while bax promotes its release (Hengartner, 2000). Bcl-2 was the first proto-oncogene identified, which inhibits apoptosis in a variety of cells (Hockenberry *et al*., 1990; 1991). All the proteins belonging to the Bcl-2 family share structural homology in four peptide sequences called Bcl-2-homology (BH) domains. Specific features of the four BH domains determine the function of these proteins. Caspases are best known as executioners of apoptotic cell death and their activation is considered a “point of no return” commitment to cell death (Nicholson, 1999). Based on structure and function, caspases are divided into two groups such as initiator – activated through oligomerization - and effector caspases, which required cleavage by an initiator caspase to be activated (Thornberry and Lazebnick, 1998). Despite this role, studies suggested that caspases might have a function outside apoptosis. In addiction to the well-establish role of caspase-1 in the production of active IL-1β and IL-18 in inflammation (Dinarello, 1998), it has been observed that cells

survived despite activated caspases. As first reported by Miossec *et al.*, during T lymphocyte stimulation caspase-3 is activated without inducing apoptosis, (1997). This opens a debate on a possible non-apoptotic function of caspases in a complex scenario with several other players. In the present work, we analysed mRNA and protein levels pro- and anti-apoptotic molecules by employing real time RT-PCR and WB respectively. With IHC we aimed to analyse the cellular localization of the proteins to identify their cellular source. We did this in samples obtained from abortion-prone as well as from normal pregnant mice. We analysed Bcl-xS, Bax, caspase-3- as the cell death inductors (pro-apoptosis) and Bcl-xL, Bag-1, Bcl-2, which are apoptotic repressors (anti-apoptosis). Ratts and colleagues (2000) showed the cellular expression of three important members of Bcl-2 family, Bcl-2, Bax and Bak, in human placenta. It has been observed that the expression of both anti- and pro- apoptotic molecules during normal tissue turnover, such as in placental remodeling, counterbalances the proliferation of trophoblast cells (Danihel *et al.*, 2002). Other studies confirmed the important roles of the Bcl-2 family members in different tissues. For example, bcl-2 deletion in mice allows offspring to complete embryonic development, but the fetuses display growth retardation and the newborn die early (Veis *et al.*, 1993). On the other hand, deletion of bcl-xL gene in mice causes fetal death at day 13^{th} of pregnancy (Motoyama *et al.*, 1995), whereas bax deletion gene results in normal development but infertility (Knudson *et al.*, 1995).

In all molecules we investigated, we observed that mice undergoing abortion had elevated levels of Bcl-2 protein in placenta when compared to normal pregnant mice as analysed by immunohistochemistry and Western Blot. Bcl-2 represents a multiple-function molecule which lays the a main role during apoptosis. In fact, Bcl-2 expression may regulate the mitochondrial physiology by inhibiting the generation of reactive oxygen species and intracellular acidification (Vander-Heiden and Thompson, 1999), or stabilizing the mitochondrial membrane potential and its proton flux (Hengartner, 2000). Bcl-2 is able to attenuate apoptosis induced by TNF-alpha (Smyth *et al.*, 1997). In fact, TNF-alpha binding to its receptor activates sphingomyelin hydrolysis, generates the second messenger ceramide, which induces apoptosis (Smyth *et al.*, 1997). The anti-apoptotic molecule Bcl-2 can interfere with the ceramide signal. Danihel *et al.* (2002) delineated a protective role for Bcl-2 during human pregnancy. Using immunohistochemical studies, they

observed that Bcl-2 protein was exclusively localized in the cytoplasm of syncytiotrophoblast throughout gestation with a similar abundance in preterm as well as in term placentas. Taking these facts into account, it is tempting to speculate that this anti-apoptotic marker is indispensable to ensure the correct placental physiology together with development of the fetus. In our analysis, all molecules (pro- and anti-apoptotic) were expressed in all placental cell types (giant cells, spongiotrophoblast and labyrinthic cells) as well as in decidual cells in different patterns and intensities. Our study revealed that mice undergoing abortion had elevated levels of Bcl-2 protein in the placenta when compared to normal pregnant mice. Moreover, despite up-regulated levels of TNF-alpha, caspase-3 activity, thus apoptosis, is not up-regulated. Bcl-2 might protect from apoptosis by blocking caspase activity, which is necessary to end apoptosis. In accordance with this, Cory and Adams (2002) proposed a model where Bcl-2 might control several initiator caspases, which are upstream or independently of any mitochondrial breach. However, the nature of the immediate effectors of Bcl-2 function remains an open point. Interestingly, Bcl-2 mRNA was slightly up-regulated in decidual but not in placental tissue. This may indicate the decidua as a preferential site for regulating cell death. Decidual tissue posseses several functions that are vital for pregnancy maintenance, such as immunomodulation, nutrition, endocrine functions and regulation of invasion. Therefore, this tissue represents a key point during gestation by orchestrating cross talk between several molecules and processes. In a recent work, Wei *et al*. (2005) investigated for the first time the expression of Bcl-2 and p53 in relation to apoptosis at the feto-maternal interface of the rhesus monkey at very early stages of gestation, reporting that the localization of Bcl-2 protein follows that of apoptotic nuclei, and its expression level increases as the development of the placenta progresses. They proposed that the expression of Bcl-2 is not sufficient to completely inhibit apoptosis but becomes a protective mechanism tending to limit the degree of nuclear degradation boosting cell survival. Savion and colleagues (2002) analysed the role of apoptosis in murine pregnancy loss induced by LPS, focusing their attention on Bcl-2 and p53 expression. Interestingly, they described high apoptosis and lower Bcl-2 when the resorption is being formed, while this situation changes completely to higher Bcl-2 levels and low apoptosis when the resorption process was complete, probably to protect the fetuses which have survived. In agreement

with these observations, our analysis revealed up-regulated levels of Bcl-2 on day 14, thus 6 days after abortion took place. Previous data proposed a similar inhibitory effect of Bcl-2 on apoptosis, suggesting that bcl-2 might attenuate cell death either directly or through its dimerization with a related gene product, bax. Therefore, we hypothesized that Bcl-2 augmentation in our model may reflect a compensatory mechanism tending to protect and maintain the physiology of the placentas which have been not resorbed. This hypothetical scenario is summarized in Fig. 32.

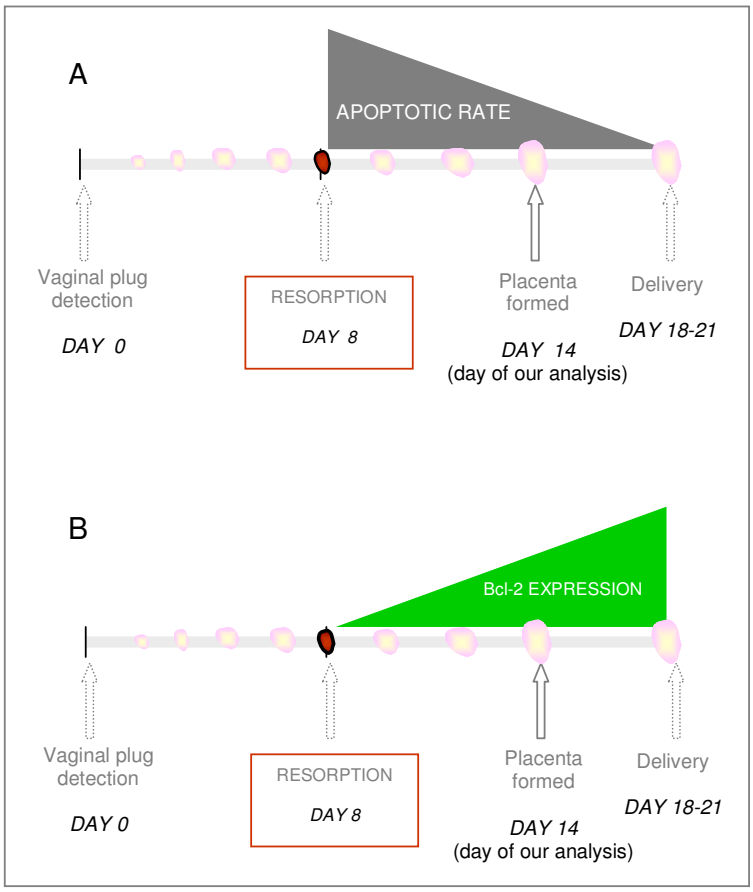

Fig. 32. *Hypothetical scenario proposed for the cross talk between Bcl-2 and other components of the feto-maternal interface. On day 8 of pregnancy, when resorption takes place, apoptosis may be up-regulated, whereas Bcl-2 expression diminished (A). Insufficient Bcl-2 levels could prevent apoptosis. After the resorption process is complete, the healthy placentas closed to the resorbed continue to grow in order to ensure a correct fetal development. At that point a higher expression of Bcl-2 may help the tissue to grow, while leading to down-regulation of programmed cell death. Bcl-2 up-regulation may represent a compensatory mechanism tending to protect the physiology of the unresorbed placentas in order to permit fetal growth until delivery (B).*

6.5. *Is the pregnancy protective effect of HO-1 related to diminished apoptosis at the feto-maternal interface?*

Recent data on heme oxygenase-1 suggest that this enzyme might be a potential target of novel therapies designed to protect the fetus from maternal immunological rejection (Zenclussen *et al.*, 2005; Sollwedel *et al.*, 2005; M.L. Zenclussen *et al.*, 2006). Interestingly, HO-1 down-regulation is directly related with graft rejection (Soares *et al.*, 1998) or murine abortion (Zenclussen *et al.*, 2005; Sollwedel *et al.*, 2005; M.L. Zenclussen *et al.*, 2006). Moreover, a significant reduction of HO-2 expression was observed in placenta samples from patients with pregnancy complications compared to normal pregnant women (Zenclussen *et al.*, 2003b). The protective effect of HO-1 on pregnancy outcome could be due to the avoidance of free heme accumulation or to protective effects of its metabolites (CO and ferritin). Up-regulation of HO-1 via gene therapy protects fetus from rejection and augments Th2 cytokine production locally (Zenclussen M.L. *et al.*, 2006). However, up-regulation of HO-1 by Co-PP did not augment Th2 or diminish Th1 and was nevertheless pregnancy-protective. Thus, other mechanisms may be involved: one possibility is that HO-1 protects the fetus from rejection due to its well characterized anti-apoptotic properties, Petrache et al. (2000) and Brouard et al. (2000) observed that anti-apoptotic effect of HO-1 may be mediated via carbon monoxide. Accordingly, over-expression of HO-1 in fibroblast or in endothelial cells (Soares *et al.*, 1998) protects these cells from TNF-α mediated apoptosis. Ferris *et al.* (1999) proposed that the anti-apoptotic action of HO-1 is correlated with its ability to regulate intracellular iron. Moreover, HO-1 up-regulation by gene therapy was associated with diminished apoptosis at the feto-maternal interface and augmented Bag-1 levels (Zenclussen M.L. et al., 2006). In the context of this work, HO-1 has been up- or down-regulated by means of Co-PP or Zn-PP applications, respectively, in DBA/2J-mated CBA/J female mice during implantation. When we analysed important hallmarks of apoptosis such as caspase-3 enzyme activity and the DNA fragmentation, we observed slightly diminished apoptotic activity in those mice previously treated with Co-PP as compared to PBS-treated abortion-prone mice. These results suggest an anti-apoptotic effect of HO-1 at the feto-maternal interface. After observing an anti apoptotic effect of HO-1, we further analysed and quantified anti- and pro-apoptotic-related molecules at the protein and mRNA levels. No changes could be

observed for caspase-3, Bcl-xS, Bcl-xL and Bcl-2. However, when analyzing the anti-apoptotic molecule Bag-1, both mRNA and protein analyses revealed its up-regulation in samples from animals previously treated with Co-PP as compared to the controls. These data confirm the data obtained on diminished apoptosis and strongly suggest a tissue protective effect of HO-1 up-regulation during implantation. Moreover, Zn-PP application, which diminished HO-1 and HO-2, also diminished Bag-1 mRNA and protein, confirming our hypothesis on HO-1 up-regulating Bag-1. Considering these facts, we postulate herein that HO-1 could protect the fetus from abortion by up-regulating the cytoprotective molecule Bag-1. Bag-1 belongs to the Bcl-2 family and its ability to suppress apoptosis has been demonstrated in various cells (Townsend *et al.*, 2003). The mechanisms involved are not fully characterized yet. A possible target of action could be Bcl-2 protein, a Bag-1 interacting partner (Hengartner, 2000). Interestingly, Townsend *et al.* (2003) have shown that Bag-1 protects cardiac myocytes from apoptosis through association with heat shock proteins such as HSC70/HSP70, thus forming a specific complex with them. In the light of our results, we can speculate that Bag-1 mediates fetal tissue protection, being the mechanisms involved in its cytoprotective effect still under investigation.

7. Summary

Several burning questions remain unanswered in pregnancy-related research. Pro- and anti- inflammatory cytokines orchestrate an intriguing interaction leading either to the development of a normal individual or to its rejection.

Augmented Th1 cytokine production is involved in immunological rejection of the fetus. Of these Th1 cytokines, particularly TNF-α is know to trigger apoptosis. The present work was aimed to investigate the incidence of apoptosis in a well-known experimental model of Th1-induced abortion, characterized by increased local TNF-α levels. Despite elevated Th1 levels at the feto-maternal interface, mice undergoing abortion presented apoptotic rates comparable to normal pregnant animals. Interestingly, we found a significant up-regulation of the anti-apoptotic Bcl-2 protein at the feto-maternal interface of abortion-prone mice, while no changes could be observed for pro-apoptotic molecules. We observed no evidence of increased apoptosis in mice undergoing immunological abortion in spite of elevated TNF-alpha levels. This is probably due to a selective up-regulation of anti-apoptotic pathways (i.e. Bcl-2) at the feto-maternal interface as compensatory and/or protective mechanisms.

Therefore, in this study we proposed that Bcl-2 could represent an important modulator to contrast apoptosis after resorption formation in order to keep the physiology of surviving placentas safe to enable them develop normally to nourish the fetuses to be born.

It has been proposed that HO-1has a protective role during allogeneic pregnancy. Our data show that the up-regulation of HO-1 by Co-PP injection prevents abortion, while it is able to enhance Bag-1 levels, a well-known suppressor of apoptosis. These data strongly suggest that apoptosis, despite having a secondary role in the normal situation is involved in pregnancy success as its blockage is associated with fetal protection. Moreover, it is tempting to define HO-1 as a novel target for successful pregnancy-related therapies, considering that its mechanism of action could involve anti-apoptotic molecules such as Bag-1 in order to ensure correct fetal development.

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Erklärung an Eides Statt

Hiermit erkläre ich, dass diese Dissertation von mir selbst und ohne die Hilfe Dritter verfasst wurde. Sie stellt auch in allen Teilen keine Kopie anderer Arbeiten dar. Die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.

Cordenons, den 5 Dezember 2006,

Annarosa Zambon Bertoja

Der Lebenslauf wird aus Datenschutzgründen in der elektronischen Fassung meiner Arbeit nicht veröffentlicht.