Rejection and Immunosuppression at Uterus Transplantation: an experimental study in rodents

Klaus Groth

Department of Obstetrics and Gynecology
Institute of Clinical Sciences
at
Sahlgrenska Academy
University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover picture: One of the earliest illustration of uterine anatomy (9th century). The drawing was based on the studies of Soranus of Ephesus

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"I'm not a magician, Spock, just an old country doctor." (TOS: "The Deadly Years")

...to Jenny, Gustav and Valter

Abstract

Uterus transplantation is developed as a possible treatment for patients with absolute uterine factor infertility. There has been one attempt to transplant a human uterus, which however failed and more basic research is needed before another attempt is performed. The aim of the thesis was to describe the rejection process after uterus transplantation in rodent models and to study the effects of the most widely used immunosuppressant, cyclosporine A (CsA) on this process. The effect of CsA on fertility was also studied in exposed mice and their offspring.

In a fully allogenic mouse model microscopic signs of rejection were found from day five. Blood flow was lower as compared to the native uterus. The gross morphological signs of rejections were initial swelling of the transplant and later the transplant became firmer in texture with a clear color change. There was an early infiltration of macrophages into the myometrium of the graft from day 2 and in the endometrium at day 5. Density of CD8+ cytotoxic T-cells increased in the graft from day 5 but there was only a transient increase in CD4+ T-helper cells. In a semi-allogenic mouse model different doses of CsA were tested. In the non-treated transplanted animals pronounced inflammation was seen. In the CsA treated groups inflammation was less pronounced. The tissue density of CD8+ cytotoxic T-cells was higher in treated group. Similar microscopic findings of rejection were also present in an allogenic model in the rat where CsA was used. It was found that mRNA levels of interleukin-1α were decreased and the levels of galectin-1 mRNA were increased in the CsA group. The study on CsA:s effect on reproduction, in two generations showed that high doses of CsA reduced implantation rates/fetal survival and did also reduce adolescent growth in offspring but not fertility. Reduced fetal weight was seen in offspring of female exposed to CsA in utero.

The collective result from these studies form a base for future studies of rejection of uterus transplants and of studies aiming to optimise immunosuppression to inhibit rejection and minimise the negative effects of immunosuppression on fertility, pregnancy and future health of offspring.

Key words: *cyclosporine A, fertility, mouse, pregnancy, rat, rejection, transplantation, uterus*

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Abbreviations

AFS	The American Fertility Society
AIH/AID	assisted insemination husband/donor
APC	antigen presenting cell
ART	assisted reproductive technologies
ASRM	American Society of Reproductive Medicine
ATG	antithymocytic globulin
CsA	cyclosporine A
CD	cluster of differentiation
CTA	composite tissue allo-transplantation
DC	dendritic cell
DNA	deoxyribonucleic acid
FDA	US Food and Drug Administration
FSH	follicle stimulating hormone
Gal	galectin
GREs	glucocorticoid response elements
HLA	human leukocyte antigen
HPV	human papilloma virus
Ig	immunoglobulin
IL	interleukin
INF	interferon
IUA	intra uterine adhesion
IUE	intra uterine exposure
IVF	in vitro fertilisation
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
ME	maternal exposure
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTOR	mammalian Target of Rapamycin
NaCl	natriumchloride
NFAT	nuclear factor of activated T cells
NK	natural killer
NTPR	National Transplantation Pregnancy Registry
SLE	systemic lupus erythematosus
STD	sexual transmitted diseases
T-cell	thymus cell
TCR	T-cell receptor
Th1/2	T helper cell type 1 or 2
TNF	tumor necrosis factor
Treg	regulatory T-cell
UW	University of Wisconsin
WHO	World Health Organisation

Introduction

Infertility

The total number of infertile adults in the world may be as many as 70 million (Boivin *et al.*, 2007; Fathalla *et al.*, 2006). The causes of infertility are both male and female factors as well as combinations of these. Furthermore, infertility is generally divided into primary (no previous pregnancy) or secondary (previous pregnancy). However, it should be stated that accurate estimations of infertility prevalence in different populations are rather difficult to perform, and there exist wide differences in methodologies used to define infertility and to investigate infertility rates in the studies in the field. Very few epidemiological studies in the field of infertility have examined the infertility rate in a complete population. Many studies have extrapolated data from prevalence rates in various selected populations, as exemplified by a French study where all infertile couples that consulted medical care for primary or secondary infertility were included in the calculations (Thonneau *et al.*, 1991). This methodology will most likely underestimate the prevalence of infertility. The estimated prevalence of infertility among women in countries of the developed world is approximately 9% (Boivin *et al.*, 2007). In countries of the developing world surely many women do not seek health care for infertility problems because lack of medical resources for primary care and for infertility treatment. A paradox is that many of these countries with a high prevalence of infertility also have high birth rates. It is reported that many countries in Northern-Africa, Southeast-Asia, and Latin-America that have fertility rates around and over 3, also have secondary infertility prevalence around 15-25% (Nachtigall, 2006).

The classification between male and female factor infertility is based on if the likely anatomical/pathophysiological cause of infertility within the couple is present within the woman or man of the couple. Male factor infertility can be due either poor sperm quality (pre/intra-testicular cause), low sperm numbers (pre/intra-testicular cause), or due to any type of obstruction of the male reproductive ducts (post-testicular cause). Female factor infertility could be divided into oligo/amenorrhoic disorders and others. The former disorders are classified according to WHO (Table 1). The other types of causes of female infertility are adhesions within the uterine cavity, various congenital Műllerian malformations, adhesions within the abdomen or within the oviduct that affects transport of oocyte, sperm or embryo secondary to inflammatory conditions or infections, endometriosis, disorder involving cervical mucus, and the group that still is classified as unexplained female infertility.

Today, modern medical care has the possibility to treat most couples with infertility to achieve parenthood within the couple. Infertile couples with tubal factor and/or male factor, due to low sperm count or sperm mobility, are helped with in vitro fertilisation (IVF) (Steptoe and Edwards, 1978) and intracytoplasmic sperm injection (Palermo *et al., 1992*). Women with oligo/amenorrhoic infertility of WHO classes I and II are generally successfully treated by clomiphene or gonadotropin stimulation, either by follicle stimulating hormone (FSH)

WHO I	Hypothalamic - pituitary failure: Amenorrhoeic women with no evidence of endogenous oestrogen production; non-elevated prolactin levels, low FSH levels (hypogonadotrophic hypogonadism), and no detectable space-occupying lesion in the hypothalamic-pituitary region.
WHO II	Hypothalamic - pituitary dysfunction: Women with a variety of menstrual cycle disturbances (e.g. lutealphase insufficiency, anovulatory cycles, anovulatory polycystic ovary syndrome, and amenorrhoea) with evidence of endogenous oestrogen production, and normal prolactin and FSH levels.
WHO III	Amenorrhoeic women with no evidence of ovarian production and with elevated FSH levels, but non-elevated prolactin levels.
WHO IV	Congenital or acquired genital tract disorder: Amenorrhoeic women who do not respond with withdrawal bleeding to repeated courses of oestrogen administration.
WHO V	Hyperprolactinaemic infertile women with a space-occupying lesions in the hypothalamic pituitary region: Women with a variety of menstrual cycle disturbances (e.g. luteal phase insufficiency, anovulatory cycles, or amenorrhoea) with elevated prolactin levels and evidence of a space-occupying lesion in the hypothalamic-pituitary region.
WHO VI	Hyperprolactinaemic infertile women with no detectable space occupying lesion in the hypothalamic - pituitary region: Same as group V women except that there is no evidence of a space-occupying lesion.
WHO VII	Amenorrhoeic women with non-elevated prolactin levels and evidence of a space-occupying lesion in the hypothalamic-pituitary region: Women with low endogenous oestrogen production, normal or low prolactin and FSH levels.

Table 1: The WHO classification based on the levels of endogenous gonadotropins (LH and FSH), prolactin and estrogens.

or human menopausal gonadotropin (hMG). Females with WHO V or WHO VI have high prolactin levels, that cause ovulatory dysfunction, and treatment with dopamine agonists or in rare cases surgery/radio-therapy will in most cases normalise the prolactin levels and re-establish cyclicity with ovulation. The cause of WHO VII is treatable. Females with WHO III can become gestational mothers by the use of donated oocytes. Treatment with IVF is today standard procedure to increase the fertility rates in females with endometrioses, where the cause may be either adhesions but where also more subtle defects relating to fertilization and implantation have been discussed (Dmowski *et al.*, 1986; Mulayim and Arici, 1999). The female with cervical factor as source for the infertility can be treated by assisted insemination of sperms from husband/ partner (AIH) or with sperms from spermdonors (AID) or IVF.

Despite the developments in assisted reproductive technologies (ART), as mentioned above, there are still some women that are unconditionally infertile. A group of these women are those that have a non-functional uterus or those who lack a uterus. They belong to the WHO IV group of oligo/amenorrhoic infertility with infertility cause that may be either congenital or acquired. For women with this type of infertility, the use of gestational surrogacy offers a chance to become genetic mothers, albeit they will never become gestational mothers (Goldfarb *et al.*, 2000). It is possible to divide the group of surrogacy treatments into traditional/straight surrogacy and gestational/IVF surrogacy. The term traditional/straight surrogacy is used when the surrogate mother uses her own oocyte that is fertilised with the intended father's sperm. Gestational/IVF surrogacy is when the surrogate mother carries the intended parents' genetic offspring after conception by IVF. The attitudes towards surrogacy, and especially gestational surrogacy, in different countries and societies of the world vary due to religious, ethical and/legal concerns. In some countries such as Argentina, Australia (a majority of states), Brazil, Ecuador, El Salvador, Greece, Israel, Korea, Netherlands, Peoples Republic of China, Romania, Russia, United Kingdom, Venezuela and many states in United States (Nakash and Herdiman, 2007) gestational surrogacy is legal, but with differences whether the surrogacy is allowed to be commercial or only compassionate. The term commercial surrogacy is used when the gestational surrogate mother achieves economic compensation for the surrogacy, which is far greater than the direct expenses, or loss of income, which are caused by the pregnancy and delivery. In compassionate surrogacy there is no economic incentive for the surrogate mother. Other countries are exploring the possibility for legalisation of gestational surrogacy, as exemplified by Singapore (Heng, 2007). The Catholic church is strongly against gestational surrogacy (McCormick, 1992; Ratzinger, 1987). According to the Jewish law there is a duty for families to have children (Schenker, 1997) and therefore there are no religious obstacles in the Jewish religion for gestational surrogacy. The Islamic religion state that only the one who gives birth to a child could be the child's mother and the gametes must be from the husband and wife, thereby ruling out the use of donor sperms or donor oocytes (Husain, 2000). However, there are some differences between the different orientations of Islam (Aramesh, 2009).

For women with uterine factor infertility, which of personal reasons or due to the regulations of the society cannot use gestational surrogacy to acquire genetic motherhood, uterus transplantation can in the future become a realistic alternative. Uterine factor infertility can, like most other types of infertility, be subdivided into primary and secondary infertility. Another distinction concerning uterine factor infertility is between congenital forms that are present from birth and those that are acquired during childhood or during fertile life.

Several types of uterine malformation can be regarded as partial, since the uterus is present but is not normal in its anatomy. The uterine malformations belong to the group of Müllerian anomalies that originate from defects in the development of the fusion of the Müllerian (paramesonephric) ducts during embryogenesis. The diagnosis and

classification of the various partial uterine malformations are difficult procedures since they often require investigations by several diagnostic methods. The American Fertility Society (AFS), now referred to as the American Society of Reproductive Medicine (ASRM) have put forward a classification system over Müllerian anomalies that is generally used today. The subdivisions are:
unicornate uterus - failure in development of one paramesonephric duct
didelphic uterus - failure of the lateral fusion of the paramesonephric ducts
bicornate uteri - failure of the lateral fusion of the paramesonephric ducts with duplication
septate uteri- failure of generation of the midline body

The exact prevalence of congenital uterine anomalies is unknown since this would require multiple investigations also by invasive methods of a population-based cohort or a random sample of women. A comprehensive survey of the field including five relevant studies with more than 300 patients with congenital uterine anomalies, all of fertile age and in contact with health care due to sterilisation, contraception consultation or abnormal bleeding, found that the prevalence of any uterine malformation in the general female population was about 4.3% (Grimbizis *et al.*, 2001). In this material of five studies the mean incidence of septate uterus was 34.9%. The rates of arcuate uterus, bicornaute uterus, unicornaute uterus and didelphys uterus were 18.3%, 26%, 9.6% and 8.4%, respectively. A more recent survey of the literature found that the total prevalence of congenital uterine anomalies, depending of the examination method, ranged between 0.05% and 9.7% in the fertile population (Saravelos *et al.*, 2008). In the latter study the most common malformations were septate and arcuate uterus. In another study the total prevalence of Műllerian malformations in women was estimated to be about 2-3% with an incidence of around 1:200 to 1:600 in childbearing women, with a quarter of these having fertility problems females (Lin *et al.*, 2002). The fertility problems mainly involved maintenance of pregnancy and not decreased ability to conceive, with high frequencies of spontaneous abortion and premature birth. The anatomical defects also rendered the proportion of abnormal fetal presentations being increased. When assessing only an infertile population the prevalence of congenital uterine anomalies was approximately 7.3% (Saravelos *et al.*, 2008). In this infertile population the prevalence of septate uterus ranged between 1.3% and 35.6% depending on the method of investigation and the prevalence of arcuate uterus ranged between 0.3-14.0%. In the same publication a thorough literature search was done and it was found that uterine malformations were reported in approximate 16.7% of the patient with recurrent miscarriage (Saravelos *et al.*, 2008).
It is agreed that the septate uterus is the most common malformation of the uterus (Taylor and Gomel, 2008) and that septate uterus is associated with a high incidence of spontaneous abortion in the first or second trimester (Raga *et al.*, 1997) as well as being strongly associated with infertility (Pabuccu and Gomel, 2004). On the other hand, hysteroscopic metroplasty will to a large extent increase the fertility chance among women with septate uterus. The rate of miscarriage decreased with 74% and live births were seen among 80% of the patients after metroplasty compared with 3% before

metroplasty (Homer *et al.*, 2000). Taken together, around 25% of women with uterine septate malformations have infertility problem (Ansbacher, 1983; Harger *et al.*, 1983).

The prevalence rates of the other uterine malformations are much lower than the prevalence of septate uterus. There is an estimated prevalence of unicornuate uterus of around 1-2% in the total female population and up to 10.5% among infertile patients (Saravelos *et al.*, 2008). Didelphys and bicornuate uterus are rarer. Most types of uterine malformations as described above are not associated with total infertility, but rather to subfertility which can be partially cured by surgery in many instances. It shall also be noted that in many patients with uterine malformations, and even other structural alterations of the uterus such as presence of leiomyoma (as discussed further below), pregnancy rate may be almost normal but there is an increased rate of spontaneous abortion (Ventolini *et al.*, 2004).

The rarest form of Müllerian malformation is Müllerian agenesis, which is commonly called the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome and was initially described in 1829 by the German anatomy professor Mayer. The MRKH syndrome is a complete agenesis of structures derived from the Műllerian ducts. Thus patients with this syndrome will not have a uterus, cervix, vagina or oviduct although thin fibrous tissues may be present at the anatomical sites of these structures. The incidence of the MRKH syndrome is estimated to be about 1 in 4000 to 5000 female births (Folch *et al.*, 2000; Griffin *et al.*, 1976; Guerrier *et al.*, 2006). In a study comparing the prevalence in women of fertile age that were seeking health care for various reason, including menstruation disturbances, a prevalence of 2.9% was found (Grimbizis *et al.*, 2001).

In the meta-analysis by Saravelos they also found that the prevalence based on class Ia (the investigations were capable of accurately identifying congenital uterine anomalies and classifying them into appropriate subtypes (accuracy >90%)) studies showed that among the infertile women 9.4% of the uterine anomalies consisted of hypoplastic uterus (Saravelos *et al.*, 2008). The syndrome is characteristically diagnosed when the patients are evaluated for primary amenorrhea (Carson *et al.*, 1983; Timmreck *et al.*, 2003).

There exist a large group of causes of uterine factor infertility that are acquired and these include lesions within the uterine cavity, lesions within the endometrium, a combination of these or that the uterus has been surgically removed. Intrauterine adhesions (IUAs) are caused by an insult to the endometrium that engenders adhesion of the uterine walls so that the uterine cavity gets partly or totally obliterated. The symptoms of IUA may be reduction or loss of menstrual bleeding, infertility or early pregnancy loss. The overall prevalence of IUA, as estimated by hysterosalpingogram (HSG), is calculated to be about 1.5% (Dmowski and Greenblatt, 1969; Al-Inany, 2001). The prevalence of the full IUA (synechia uteri in total) is steadily increasing world-wide and an increase is also seen in countries of the western world such as Denmark, Israel and Greece (Schenker, 1996). The most common reason for the endometrial insult that cause IUA in the western-world is surgical curettage (Schenker, 1996), and not genital infections,

such as genital tuberculosis, which most likely is the major cause in societies of the developing world. The reproductive outcome when IUA is present is poor. Pregnancy can often take place in cases of mild or moderate IUA, and one study reported pregnancies in 45% of women with IUA, but of these did 40% and 23% ended in miscarriage and preterm delivery, respectively (Schenker and Margalioth, 1982). The mode of treatment of IUA has varied over time and country. The adhesions were traditionally treated by blind lysis using a sharp curette but nowadays hysteroscopic lysis is the treatment of choice. The live birth rate after hysteroscopic treatment of IUA is about 33% and the cumulative miscarriage rate during the first and second trimester was about 25% (Fernandez *et al.*, 2006). In another study they reported a term pregnancy rate ranging between 32% and 81%, with the rate being related to the severity of IUA (Valle and Sciarra, 1988).

Uterine leiomyoma (myoma) is also a common cause of uterine factor infertility/subfertility. This disease is fairly common among women of reproductive age but with certain difference with age and race. Thus, uterine myoma is more common in Afro-American women in the US than in Caucasian women in the same country (Parker, 2007). Thus, a prevalence-study from the USA, including women aged 35 to 49 years, found a cumulative prevalence of over 80% in Afro-American women and just below 70% in Caucasian women (Day Baird *et al.*, 2003). In a Swedish study of a somewhat younger population (33 – 40 years of age) they found myomas in 7.8% of the women (Borgfeldt and Andolf, 2000). It is also estimated that 5-10% of women that seek medical attention for infertility have at least one myoma (Donnez and Jadoul, 2002). It is nowadays accepted that submucosal and intramural myomas that affect the endometrial cavity should be removed to improve the pregnancy rate (Lin, 2004).

Endometrial polyps are benign overgrowths of the endometrium and it is generally a treatable cause of uterine factor infertility. In one randomised study it was shown that fertility rates increased to 63.4% after hysteroscopic polypectomy compared to 28.2% in those who did not undergo this procedure versus (Perez-Medina *et al.*, 2005).

Another group of women with uterine factor infertility are those who have had their uterus removed because of large symptomatic myomas or other benign conditions, postpartum bleeding and malignancy, especially cervical cancer. Taken together, this group in total is most likely the numerically largest group of women with uterine factor infertility. According to an IVF surrogate gestational pregnancy program it was established that around 50% of the females enrolled because of uterine factor infertility were hysterectomized (Goldfarb *et al.*, 2000), thus indicating the relatively large size of this group. In a study it was found that 600,000 women each year in the USA undergo hysterectomy (Farquhar and Steiner, 2002). In Sweden, numbers from the Swedish National Board of Health and Welfare (Socialstyrelsen) showed that during the years 1998-2007 the number of hysterectomies in the population group aged 20-54 were around 4000/year and in the subgroup of women aged 20-39 around 450/year (Socialstyrelsen). Emergency peripartum

hysterectomy is seldom performed but may be a life-saving procedure in cases of severe post-partum haemorrhage. In the Swedish National Board of Health and Welfare's database it was shown that during 1998 - 2007 an average of 11.4/women per year went through a hysterectomy and caesarean section at the same time (Socialstyrelsen). However, the specific reasons for hysterectomy at the time for caesarean section were not reported and it should be noted that the rate is very low considering the average number of deliveries of about 93000/year during this period. Even though there has been a development of new effective uterus compression sutures (El-Hamamy and B-Lynch, 2005; Sziller *et al.*, 2007), the rate of emergency peripartum hysterectomy seems to increase. One can speculate that this is due to the increased rate of delivery through caesarean section.

Gynecological malignancies are fairly rare in women of the reproductive ages and as a total group the peak incidence is during the post menopausal period. In fertile women malignancy in the ovary and the cervix are the most common sites. Cervical cancer is the most common gynaecological malignancy world-wide (Quinn *et al.*, 2006) but in developed countries the incidence is much lower due introduction of effective screening programs (Andrae *et al.*, 2008; Gustafsson *et al.*, 1997; Smith *et al.*, 2007). It is estimated that 30% of the cervical cancers affect women under 40 years of age (Sonoda *et al.*, 2004; Quinn *et al.*, 2006). Since this cancer has a relative high prevalence in the fertile population and that the median age, especially in the western world for the first child is increasing there are some women who will be nulliparous when diagnosed. The treatment for the early stages (I-IIa) of cervical cancer is performed by surgery. Squamous cell carcinoma in the cervix stage Ia1 is treated with an extensive cervical cone and stage Ia2 and Ib, of smaller size, could be candidates for trachelectomy if the pelvic lymph nodes do not have any metastasis (Einstein *et al.*, 2009; Schlaerth *et al.*, 2003). Larger cervical cancers of stage Ib or IIa are treated with a radical hysterectomy and these patients will of course become uterine factor infertile after the surgery, in spite of that the ovaries are generally left in situ. Concerning a possible role for uterus transplantation in this patient group it should be noted that a uterus recipient will undergo immunosuppression therapy and there are some risks that it could reactivate a genital HPV infection (Seshadri *et al.*, 2001; Kane *et al.*, 2008), which could lead to vaginal dysplasia and a risk for cancer.

Collectively, all these groups of patients with uterine factor infertility, as mentioned above, could in the future become candidates for uterus transplantation if conventional therapy has not been able to reverse their infertility.

Transplantation of solid organs and tissues

Transplantation of organ and tissue can be divided into directly life-saving types of transplantation such as heart, lung, and liver, transplantations that prolong life-expectancy substantially such as transplantation of the kidney and to some extent intestinal transplantation, and those types which can be considered more of life-improvement procedure such as transplantations of cornea, hand, forearm, face and diaphragm.

Since the first transplantation, to replace skin damage on soldiers (Gibson and

Medawar, 1943; Medawar, 1948), that took place during the Second World War, there has been a tremendous development in this area and for many disorders transplantation of solid organ is a clinical reality as the treatment of choice. The main obstacle to overcome in the beginning of the era of transplantation surgery was the surgical techniques with blood vessel anastomosis, ischaemic injuries of the graft and rejection. Moreover, there were also organ-specific anastomosis techniques that had to be optimised such as the connection of the ureter to the bladder in renal transplantation, the bile ducts in liver transplantation and the bronchial ducts in lung transplantation.

The kidney was the first solid organ to be transplanted (Toledo-Pereyra and Toledo, 2005). Over the year there has been an increase in the magnitude of kidney transplantation and the indication for transplantation has widened. Today kidney transplantation is more or less a routine procedure and the surgical complications are low. The main problem is long-term graft survival. Overall there are about 200-300 (Socialstyrelsen) kidneys being transplanted every year in Sweden today.

In the late 1960`s (Barnard, 1968) the first heart transplantation was performed but the graft survived only for 18 days (Thomson, 1967). The first Swedish person who received a grafted heart underwent the heart transplantation procedure year 1982 in the UK (Cullhed and Nilsson, 1982) and some years later the first transplantation of a heart that took place in Sweden occurred (William-Olsson *et al.*, 1984). Today there are about 30-40 heart transplantations performed every year in Sweden (Socialstyrelsen).

Lung transplantation is sometimes performed as combined heart-lung transplantation and sometimes as single lung transplantation. The first lung transplantation was performed in the early 1960s (Hardy *et al.*, 1963) but it was first during the 1980s that the graft survival increased to an acceptable level and the one-year survival is today above 80% (Christie *et al.*, 2008). The incidence of lung transplantation in Sweden lies about 1/100000 inhabitants (Socialstyrelsen) and word wide about 150000 lung transplantations have been performed (Christie *et al.*, 2008).

Transplantation of the liver was first performed in the early 1960s (Starzl *et al.*, 1963). In Sweden there are approximately 100 liver transplantation performed every year and there is an increase over the last years (Scandiatransplant, 2008; Socialstyrelsen).

Transplantation of the small intestines was first attempted in the late 1960s (Lillehei *et al.*, 1967) but the intestinal transplantation, which was considered to be the first successful took place more than 20 years later (Deltz *et al.*, 1990). The surgical techniques in small bowel transplantation are similar to anastomosis after bowel resection and the vascular anastomosis with the large vessels are performed with good access. During the last decade the outcome of intestinal transplantation has advanced considerably but there are some major problems with the process of acute rejection (Farmer *et al.*, 2001). This may due to that the small intestine has an effective mucosal immune system. The heavily impact to the intestinal immune system of bacteria, viruses, and protozoa has necessitated development of such system with special

types of plasma cells secreting IgA antibodies, high density of antigen presenting cell (APC) cells, especially dendritic cells. There are also other leukocyte subtypes present outside and within the Peyer`s patches, which is a unique immunological site for maturation of T-cells. In Sweden up to 2008, about 25 intestinal transplantations have been performed (Socialstyrelsen).

Transplantation of the pancreas as a single organ or together with the intestine and/or kidney is still a fairly rare type of organ transplantation and the line of development in this area has been to transplant cell suspensions of beta-cells (Meloche, 2007; Niclauss *et al.*, 2009). The first pancreas transplantation case was reported in 1967 (Kelly *et al.*, 1967; Lillehei *et al.*, 1967). Since then about 30 pancreas transplantations have been performed in Sweden (Socialstyrelsen).

The type of transplantation which is numerically the largest world-wide today is cornea transplantation, which also has the highest success rate. The first attempts were done in the late 1950`s and early 1960`s (Payrau *et al.*, 1961). This type of tissue transplantation is nowadays performed very routinely with no need for immunosuppression postoperatively (Niederkorn, 2003) and with a success over 90% at the first attempt. This is one of the new types of quality-of-life enhancing transplantations and in Sweden around 350 of these transplantations are carried out each year (Socialstyrelsen). Approximately, 2300 corneal transplantations are performed each year in the UK and over 33000/year in the USA.

During recent years other types of quality-of-life enhancing types of transplantations have been introduced and in the year of 1999 the first successful human hand transplantation was presented (Dubernard *et al.*, 1999) by a group in France. More recently, in the year of 2006, the first face transplantation was performed by the same group that performed the first hand transplantation (Devauchelle *et al.*, 2006). These types of transplantations involve tissues of several types and are commonly referred to as composite tissue allotransplantation (CTA) (Tobin *et al.*, 2007; Swearingen *et al.*, 2008). The first attempts in CTA, carried out in the late 1960s, were that of transplantation joints such as the knee joint (Porter and Lance, 1974) and work along these lines are still continued (Siliski *et al.*, 1984). A handful of successful abdominal wall transplantation have been performed in patients who previously have been repeatedly operated through the abdominal wall and the technique was in some cases used in conjunction with multivisceral transplantation (Selvaggi *et al.*, 2004).

In animal models, other types of CTA and novel types of organ transplantations have been explored. Thus, the diaphragm was transplanted in the dog (Krupnick *et al.*, 2008). There have also been some trials with bladder transplantation in the rabbit (Yamataka *et al.*, 2001a) and the rat (Wang *et al.*, 2001). Experimental work to transplant the oesophagus was performed in rats (Yamataka *et al.*, 2001b). Penile allotransplantation has been performed in animal models during the last decade (Koga *et al.*, 2003; Sonmez *et al.*, 2009) to find techniques to transplant a penis after trauma where re-(auto)transplantation (Tuerk and

Weir, 1971) is unsuccessful or after resection due to diseases or trauma.

Uterus transplantation

The present thesis deals with experiments on uterus transplantation, which also should be classified as a quality-of-life enhancing type of transplantation. The first and up until today the only attempt of human uterus transplantation was performed year 2000 in Jeddah, Saudi Arabia. The uterus transplanttation was performed in a 26-year-old woman who had lost her uterus some years before at emergency peri-partum hysterectomy carried out due to haemorrhage at caesarean section (Fageeh *et al.*, 2002). This trial was unsuccessful since the uterus graft only survived for 3 months. However, it is likely that this effort to perform uterus transplantation in the human may have led to that the field of uterus transplantation, which had been dormant since the 1960s to 1970s, was reinitiated.

During the 1960s and 1970s attempts were made to transplant the uterus together with the adnexa as a mean to treat tubal infertility. Transplantation of the Fallopian tube by vascular anastomosis had been attempted in many animal species and there are also pregnancies reported (Winston and Browne, 1974). In the human, some attempts were conducted to allotransplant oviducts but no pregnancies were reported (Sillo-Seidl, 1975; Cohen *et al.*, 1976; Wood, 1978). The concept of utero-tubal transplantation had the potential to improve the results since the vessels to be anastomosed would be much larger in their diameter and since anastomosis of the oviduct would not be needed. When IVF was introduced during the 1980s, gradually a new and effective method to bypass tubal factor infertility was on the clinical scene and there was no need for further research in that field.

It is my opinion that the human uterus transplantation case (Fageeh *et al.*, 2002) was done to early considering the limited research in this field up to the time when it was performed. Uterus transplantation involves risks for several individuals (living donor, recipient, prospective child) and in such situation it is wise to use extensive animal research to optimise the procedure and to minimise the risks. The animal research on uterus transplantation performed before and in parallel to this thesis work is summarised below. For clarity, the research conducted in each species is summarised separately.

Mouse

In the year 2002, Randa El-Akouri with coworkers from my institution, presented the first mouse model for uterine transplantation (Racho El-Akouri *et al.*, 2002). In this model, pregnancy was demonstrated for the first time in a transplanted uterus, but the pregnancies reported in this initial study did not go to term. It was stated that the advantage with the mouse in comparison to other experimental animals was the low cost of the animal and that genetically modified strains and recombinant species-specific proteins would be available for further research. Since the uterine vessels of the mouse are very thin it was established that the vascular anastomosis had to be done at the site of the largest vessels, the aorta and the vena cava. The graft included one uterine horn, the common cavity, and the cervix with a vaginal rim. The harvesting of the uterus was prepared by excision of one uterine horn and the ovary. The uterus was flushed in situ with physiological saline,

which was supplemented with xylocaine for vasodilatation, and heparin as anticoagulant. The cold ischaemic time was about 35 min and the warm ischemia during vascular anastomosis about 50 min. In several of the animals a swollen uterus was seen some weeks after transplantation and the swelling was due to intraluminal accumulation of fluid/mucus. Thus, the techniques was modified so that the cervix with its vaginal rim, that previously was kept at an intra-abdominal position, was brought through the abdominal wall to a form a vaginal-cutaneous stoma (Racho El-Akouri *et al.*, 2003a). In this model the uterus did not get swollen and the pregnancy rate, after embryo-transfer, was similar to that of the native uteri and the uteri of sham-operated controls. Pregnancies went to term in this model and this was the first demonstration of live births after proper uterus transplantation, although in a syngenic setting. By the use of the same surgical methodology, the time limit for cold ischemia for a mouse uterus was investigated. The uterus was flushed as above and then stored in the commonly used preservation medium University of Wisconsin (UW) solution for 24h, 48h or 72h (Racho El-Akouri *et al.*, 2003b). The results showed that a mouse uterine graft that had been preserved for 24h in cold UW solution showed myometrial contractility, normal morphology and could harbour pregnancy to term after transplantation (Racho El-Akouri *et al.*, 2003b). Longer preservation times (48h, 72h) resulted in necrosis of the uterus after transplantation.

Rat

The first attempt to transplant a rat uterus with oviducts and ovaries en bloc was reported in 1995 (Lee *et al.*, 1995a) and later another group presented a slightly modified technique (Jiga *et al.*, 2003). In the latter report they describe a cold ischaemic time of around 30 min but no estimation is made of the second warm ischaemic time, during reanastomosis of the graft. The grafts were examined after 24h and 72h by laparotomy and they found thrombosis in most grafts 72h postoperatively. In our research group, Wranning with colleagues, (Wranning *et al.*, 2008a) used a modification of the donor operation developed for the mouse (Racho El-Akouri *et al.*, 2003a). The left uterine horn, both oviducts and the ovaries were excised from the transplant specimen during the retrieval procedure. Thus, the transplant included the right uterine horn, the common uterine part, the cervix and a vaginal rim. In the report (Wranning *et al.*, 2008a) the uterus was flushed with ice cold Ringer Acetate supplemented with heparin and xylocaine and the cold ischaemic time was about 60 minutes. The second ischaemic time, when the anastomosis in the recipient was performed, was around 90 minutes.

In the rat models, where the uterus transplantation was performed en bloc together with ovaries and oviducts (Jiga *et al.*, 2003; Lee *et al.*, 1995), the vessel anastomosis were either end-to-side to the aorta and vena cava (Lee *et al.*, 1995) or side-to-side to the right femoral vessels (Jiga *et al.*, 2003). The entire utero-tubal-ovarian specimen was placed in an orthotopic position and after the vessels were anastomosed the vaginal ends were anastomosed.

In the rat model of proper uterus transplantation (Wranning *et al.*, 2008a) the right common iliac artery and vein that were connected to the uterine graft were anastomosed end-to-side to the mid-abdominal part of the aorta and vena cava of

the recipient. The native uterus remained intact as an internal control and the graft was placed in a heterotopic position with the cervix and vaginal rim connected with a cutaneous stoma. No offspring has so far been reported after transplantation of the uterus in rats but experiment on fertility after syngenic rat uterus transplantation are presently conducted in our research facilities.

Rabbit

There have only been two studies that have examined the feasibility of uterus transplantation in rabbit. The first report from 1986 (Confino *et al.*, 1986) describes a procedure to surgically isolate the uterus for non-vascular transplantation to the surface of the broad ligament. The graft was washed outside the body with lactated Ringer solution at a temperature of 37˚C. The subtotal hysterectomy specimen was attached to the recipient cervix and then fixed to the incision site in the broad ligament. The viability rate of the auto-transplanted uteri one month after autotransplantation was around 75%. Another technique for uterus retrieval in the rabbit (Sieunarine *et al.*, 2005a) was developed in rabbit cadavers, and the procedure included attainment of large vessel patches of the aorta and vena cava, similar to that used at human multiorgan transplantation.

Dog

The dog was probably the initial species that was exposed to uterine transplantation research. One of the first trials was performed in the mid 1960s but they did not carry out actual uterus transplantation (Eraslan *et al.*, 1966) since the uterus was not removed from the body. They dissected the vessels of the uterus and divided them proximally at the level of the common iliac vessels, followed by vascular surgery involving end-to-end anastomosis. During this time the vagina was clamped but not divided. After vascular reanastomosis had been completed, the vagina was divided and then reanastomosed. During the time of vaginal clamping the uterus was flushed with physiological saline solution and the ischaemic time was estimated to around 30 minutes. When the blood flow was re-established to the uterus the vagina was divided and then reanastomosed. This means that the uterus was never disconnected from the body although the circulation was interrupted during the vascular reanasto-mosis. Some years later another group (Truta *et al.*, 1969) presented a similar method to carry out the dissection and anastomosis procedures but the vaginal connection was left intact throughout the whole procedure. The ischaemic time was estimated to around 45 minutes and the uterus was flushed with heparinised saline solution. It was not until the early 1970s that the first true autotransplantations of a uterus was performed (Barzilai *et al.*, 1973; Paldi *et al.*, 1975), although it has to be emphasized that the graft was not only the uterus but that the oviducts and the ovaries were also included in the graft. Parallel with the previously described experiments there were some groups trying to do utero-tubal-ovarian allo-transplantation with different vessel techniques (Wingate *et al.*, 1970; Yonemoto *et al.*, 1969; Mattingly *et al.*, 1970). The warm ischaemic times were estimated to be around 30 minutes and the specimens were flushed with saline solution with heparin *ex vivo*.

The anastomosis techniques in the early experiments often involved end-to-end

anastomosis of the common iliac artery and end-to side anastomosis to the common iliac veins (Eraslan *et al.*, 1966; Truta *et al.*, 1969; Mattingly *et al.*, 1970; Paldi *et al.*, 1975; Yonemoto *et al.*, 1969). In one study with allo-transplantation they used end-to-side anastomosis of the aorta and vena cava of the graft to the recipient's aorta and vena cava (Wingate *et al.*, 1970). Different and simplified techniques for uterus retrieval and transplantation were reported in studies of vascular uterus transplantation in dogs. (O'Leary *et al.*, 1969; Scott *et al.*, 1970). These methods had very short ischaemic times since they did not involve dissection of the vessels and retransplantation was through omental wrapping for revascularization. Both reports demonstrated viable uterine tissue several weeks after the transplantation.

In the dog models using auto-transplantation with vascular anastomoses the accumulated pregnancy rate was 11% (3 pregnancies/18 animals (Eraslan, 1966), 1 pregnancy/10 animals (Truta *et al.*, 1969), 2 pregnancies/7 animals (Mattingly *et al.*, 1970), 1 pregnancy/12 animals (Barzilai *et al.*, 1973) and 1 pregnancy/12 animals (Paldi *et al.*, 1975) and some live births (Eraslan *et al.*, 1966) reported.

Pig

The domestic pig is a large animal with many anatomical and physiological similarities to the human, which are reasons that it often has been used in practise and development of surgical procedures that will be used in the human. Thus, it was natural that two independently working groups selected this species as a large animal model to practice surgery for uterus transplantation (Sieunarine *et al.*, 2005b; Wranning *et al.*, 2006). The surgical procedures of pig uterus auto-transplantation were similar. After dividing the round ligaments, the oviducts were separated from the uterine horns and subtotal hysterectomy was performed. It should be noted that the uterine vessels were divided above the level where they cross the ureter. The group from our institution (Wranning *et al.*, 2006) flushed the uterus with ice cold Ringer Acetate for about 90 minutes and the second warm ischaemic time was also about 90 minutes. The other study (Sieunarine *et al.*, 2005b) used UW solution or Celsior solution with cold ischemia for around one hour and unreported length of warm ischaemic time. The uterine artery and veins were anastomosed end- to-end. The uterus was reattached to the round ligaments and to the cervix. The viability of the graft was evaluated differently in the two studies. In the study from our group (Wranning *et al.*, 2006) blood gases, lactate and thiobarbituric acid reactive species levels of the venous blood from the uterus were analysed ant there was a normalisation after 60 minutes. However, the study also noted some histological changes with an influx of neutrophils into the endometrium indicating some degree of ischemia-reperfusion damage. In the other study (Sieunarine *et al.*, 2005b) they used Doppler perfusion index together with oxygen saturation as a measurement of viability and it was stated that there was adequate uterine perfusion after transplantation. After longer post-operative durations investigations of histology of the uterine grafts revealed thrombosis and it was also noted in the latter study that the porcine uterus transplantation model is highly susceptible to postoperative infections. Moreover, the relatively large size of the uterine horns (around 1 meter in length) and the inaccessibility for vessel

dissection deep in the pelvis led to the conclusion of both research groups to search for a more suitable large animal model for uterus transplantation, at least when using a concept to train for uterus transplantation from living donor.

It may well be that the pig is a suitable large animal experimental species in development of techniques for uterus transplantation, using deceased donors. Thus, dissection of the uterine vessels up to aorta and vena cava can be achieved in pig cadavers (Sieunarine *et al.*, 2005a) and recently another group presented a model in miniature swine where they used a similar technique (Avison *et al.*, 2009). In the study the vessels of the uterine graft were dissected free to include the aorta and vena cava up to the levels of renal vessels and down to the levels of external iliac vessels. Flushing was accomplished in situ with chilled UW and the allogenic transplantation then involved side-to-side transplantation of the major vessels and the vaginal vault was exteriorized as a stoma. The uterus was also fixed to the abdominal wall. Ten transplantations were performed and six of them were major histocompatibility complex (MHC) matched. Five animals died during the evaluation period. The immunosuppressant protocol, previously used in this animal model for experiments involving kidney transplantation, consisted of steroids and for 12 days post-operative treatment with tacrolimus iv which was shifted to cyclosporine A (CsA). In the MHC-mismatched groups, rejection episodes occurred and these animals were treated with higher doses of steroids. This first allogenic uterus transplantation model in the pig may have been more utilizable than the previous since it used retrieval of large vessels for anastomosis and since minbreed pigs were used. However, problems with infections such pneumonia and endometritis were encountered.

Sheep

The sheep was suggested as a more appropriate large animal model for surgical training towards human uterus transplantation. Anatomically the ewe has wider pelvis than the pig and the body size is fairly similar to a young women. In the early 1970s there were some autotransplantation experiments performed with the uterus or the uterus together with the oviduct and ovary being placed at the heterotopic site of the neck of the ewe, with anastomosis to the carotid artery and vena jugularis (Baird *et al.*, 1976; McCracken *et al.*, 1971). During last year a number of studies on uterus transplantation in the sheep were presented including auto-transplantation (Dahm-Kähler *et al.*, 2008; Ramirez *et al.*, 2008; Wranning *et al.*, 2008b). In the technique by our group (Dahm-Kähler *et al.*, 2008; Wranning *et al.*, 2008b) the round ligaments were divided and one uterus horn was removed so vessel dissection was only needed on one side. The internal iliac artery was identified just below the aortic bifurcation and the artery was then dissected caudally with all branching vessels being ligated. The common uterine-ovarian vein was dissected free up to the internal iliac vein and after the ureters had been mobilised from the cervix the vagina was divided. The uterus was flushed with either Ringer-Acetate or Perfadex® in situ and then stored cold *ex vivo*. The cold ischaemic time was about 70 minutes and the second warm ischaemic time, at anastomosis surgery, was about 60 minutes. The artery was anastomosed end-to-side to the external iliac artery and the uterine-ovarian vein was also anastomosed end-to-side to the external

iliac vein (Dahm-Kähler *et al.*, 2008). The vaginal rim was afterwards anastomosed and the uterus body was fixed to round ligament to prevent torsion. During a reperfusion time of 3h measurements of the uterine venous blood concerning blood gasses and parameters that would indicate oxidative stress were performed (Wranning *et al.*, 2008b) and some minor differences were found in comparison to the levels of the parameters in uterine venous blood before perfusion. There was also an increase of neutrophilic density in the tissue and Perfadex® was in this regard more protective than Ringer-Acetate.

In a later study (Wranning CA *et al.*, 2009) it was shown that ewes auto-transplanted with a graft containing the uterus and the adnexae on one side could achieve spontaneous pregnancy. Accordingly, pregnancy occurred in 3 out of 5 auto-transplanted ewes.

A modified sheep uterus transplantation model was also presented last year (Ramirez *et al.*, 2008). The aim in this study was to anastomose the uterine arteries and veins end-to-end above the level of ureters. The vaginal arteries were ligated and the arteria and venae uterine were mobilised laterally and a total hysterectomy was performed. At transplantation the vagina was anastomosed followed by end-to-end anastomosis of the uterine vessels. Both auto- and allo- uterine transplantations were performed and the results 6 months after showed neovascularization and glandular endometrial tissue.

Non-human primate

In an early model to test the feasibility of utero-tubal transplantation to treat tubal infertility the uterine fundus with the tubes was harvest for auto- and allo-transplantation in rhesus monkeys (Scott *et al.*, 1971). Circulation was established by wrapping the graft in the omentum and subsequent neoangiogenesis.

For the purpose of uterus transplantation experimental training, baboons were used prior to the human uterus transplantation attempt (Fageeh *et al.*, 2002). Sixteen animals were used for autologous transplantation. The surgical technique for uterus retrieval is not detailed in the report but it is stated that the grafts were flushed with cold Euro-Collins preservation solution. The results were evaluated at laparotomy 6-12 weeks later by ocular examination and it was found that uterine vessel end-to-end anastomosis showed a low success rate and after conversion of the anastomosis technique to end-to-side to the external iliac vessels, results improved.

Human

Since the first human attempt (Fageeh *et al.*, 2002) there has not been any further human uterus transplantation attempts. However, research in the human is ongoing and uterus harvesting from heart-beating, brain-dead, multi-organ donors was descried (Del Priore *et al.*, 2007). The donors, who were between 30 and 45 years, had previously all given birth. The round ligaments were divided and the pararectal and paravesical spaces were developed followed by mobilisation of the ureters from the cervix and from the uterine vessels. The uterine artery was saved and the other branches from the internal iliac artery were ligated and cut. The veins were not specifically dissected and instead the parametrium surrounding the uterine artery was saved down to the internal iliac veins. The aim of this technique was to obtain vascular pedicles including the internal iliac vessels up to division on the common iliac vessels. It was achievable in two of seven

attempts and in the others the vascular pedicles were shorter or with a unilateral loss of the uterine artery.

Rejection

During evolution multi-cellular organisms and especially higher vertebrates have developed efficient systems to deal with the potential harmful intrusion of other organisms such as viruses, bacteria, fungi, protozoa and parasites. These so called immune systems are also involved in the destruction of harmful endogen tissues such as tumors and in the repair of injured tissue. It is therefore of extreme importance that these systems are correctly regulated and switched on and off at the right site and time.

The mammalian immune system can roughly be divided into two co-operative branches. There exists the fast and evolutionary old inborn innate immune system as well as the slow and evolutionary younger, acquired adaptive immune system. The innate immune system includes granulocytes, macrophages, dendritic cells (DC), natural killer (NK) cells and the complement system. The immune cells of the innate system survey the tissue and bloodstream to act as the first line of protection with immediate or very early response to infection or tissue damage. The innate system acts in a non-specific way and recognizes general pathogen surface molecules or mediators secreted during tissue stress. All cell types of the innate immune system have the ability to kill invading pathogens directly by secreting cytotoxic compounds or by phagocytosis. The innate immune system also has the ability, alone or together with parenchymal cells, to secrete a variety of mediators that directly or indirectly influences the adaptive immune system.

The adaptive immune system consists of two major cell types, namely T-cells and B-cells. These lymphocytes are highly specialized and will upon activation mount a tailored response to eliminate specific pathogens or pathogen-infected cells. After a primary infection, B- and T-cells also form long lived memory cells that, upon a second infection by the same pathogen, will mount a faster and more vigorous response.

Progenitor T-cells emerge from hematopoietic stems cells in the bone marrow and migrate to the thymus for maturity and selection. In the thymus the progenitor T-cells (thymocytes) expand and undergo somatic hypermutation of the variable V(D)J-region of the T-cell receptor (TCR) (Cobb *et al.*, 2006). This gene-rearrangement generates a wide diversity in the ability to recognize different antigen presented by the MHC receptor on antigen presenting cells (APCs). As the thymocytes mature they pass several “check-points” that eliminate cells with TCRs that are defective or with no binding affinity to a peptide-MHC complex (positive selection) (Starr *et al.*, 2003) and cells with TCRs with very high binding affinity to endogenous peptide-MHC complexes (negative selection) (Starr *et al.*, 2003). These processes prevent the release of non-functional T-cells and self-reactive T-cells that would induce autoimmunity. During the process of positive selection the thymocytes also differentiate their expression of the TCR binding accessory molecules CD4 and CD8 so that cells with high binding affinity to MHC class I will express CD8 and cells with high binding affinity to MHC class II will express only CD4 (Singer *et al.*, 2008). The now naïve T-cells migrate through the bloodstream to the spleen, lymph nodes and

other secondary lymphoid tissue. An activated CD8 cell will drive the target cell into apoptosis and therefore this cell type will be specialized to destruct nucleated cell that present foreign peptide antigen such as viruses or tumor antigens. The CD4 positive cell, that recognize peptide antigen on special cells, will start a cascade to stimulate/regulate the immune system.

The CD4+ T- cell is activated when its' TCR and CD4 molecules bind an MHC class II receptor carrying a cognate antigen while simultaneously receiving co-stimulatory signals. The nature of the co-stimulation will influence the divergence of CD4+ T-cells into different sub-types of activated effector cells. For example, the presence of interleukin-1 (IL), IL-12 and tumor necrosis factor-α (TNF-α) during activation will steer the CD4+ T-cells to development into so called Th1-cells that secrete IL-2 and interferon-γ (IFN-γ) and are potent triggers of cellular immunity. The presence of IL-6 and IL-10 during CD4+ T-cell activation will instead stimulate the development of Th2 cells that produce IL-4, IL-5 and IL-10 and function as helper cells at B-cell activation and isotype switch (Romagnani, 2006). MHC class I, which is present on all nucleated cells, presents antigen to the CD8+ T-cell which also requires at least two signals to be activated and in the absence of the co-stimulatory signal the CD8+ T-cell undergoes apoptosis.

The other cell type that includes in the adaptive immune system is the B-cells, which also emerge in the bone marrow and undergo a gene- rearrangement both in the h-chain genes and l-chain genes that lead to a tremendous variety of antigen recognition combinations. Some of these membrane antibodies recognize however self-antigen and are therefore removed from the repertoire (Hardy and Hayakawa, 2001). The naïve B-cells will then migrate to the circulation and then to lymph nodes. If they come in contact with any antigen and are co-stimulated from a CD4+ T-cell they proliferate and differentiate into plasma cells, which secrete specific antibodies. The life time of plasma cells is about 4 weeks but some of the B-cells differentiate to memory cells. These latter cells are already prepared with IgG antibodies on their cell membrane which leads to a very fast and effect full respond to a new threat with the same antigen.

In a transplantation situation there are several crucial events that could activate or enhance the immune system. Firstly, there is the surgical trauma, which leads to an activation of the innate system as a response to tissue damage. The signals from injured parenchymal cells and endothelial cells could also trigger the adaptive immune cells to be more alert although the transplant antigens are not exposed only due to trauma. Inevitably, ischemia of the organ occurs when circulation is closed during retrieval, transport and vascular anastomosis of the transplant and this will also lead to tissue damage and necrosis. In modern organ transplantation flushing of the donor organ is standard. The flushing is done with cold solutions to induce hypothermia and to flush away blood cells that could trigger a hyperacute rejection due preformed antibodies. One of the major obstacles to overcome is the passenger dendritic cells (DCs) of donor origin that are residing in the parenchyma of the transplant. These DCs are activated by the inflammatory cascade caused by surgical trauma and ischemia and migrate to the recipient lymph nodes where they present foreign MHC to T-cells, the so-

called direct pathway of allorecognition. After these donor-derived DCs have died, parenchymal cells from the transplant that dies during normal cell turnover are engulfed by recipient APCs and their MHC fragments are presented via the so called indirect pathway of allorecognition (Game and Lechler, 2002). Recent research concerning the role of these different pathways of allorecognition in rejection and tolerance development indicates that the direct pathway is mainly responsible for acute rejection events while the more persistent indirect pathway upholds the vascular inflammation leading to chronic rejection but is also required for the development of transplantation tolerance (Li *et al.*, 2008; Li *et al.*, 2001; Xia and Kao, 2005).

Organ rejection

As stated above, activation of the immune system is unavoidable during transplantation of a solid organ. However, key factors such as the extent of surgical trauma, ischaemic time and HLA (human leukocyte antigen) incompatibility between donor and recipient can be controlled and reduced. Also, different organs have different vulnerability. For example, it is more difficult to prevent rejection of transplanted small intestines and lungs which are organs involved in the mucosal immune system as compared to kidney, heart and liver transplants (Report, 2007). It was not until the mid 80`s and 90`s, when one year graft survival rates of patients with lung and intestinal transplants reached acceptable levels. The enhanced graft survival of these organs and the increase in survival of kidney, liver and heart transplants is considered to be due to the introduction of new, immunosuppressive drugs such as cyclosporine A and later tacrolimus (Ghoneim *et al.*, 1993; Webster *et al.*, 2005) as well as the stronger induction immunosuppression that are used just prior to and during the first days after transplantation .

Pregnancy – a natural semiallogenic model

A natural semiallogen situation occurs every time a female is pregnant but a pregnancy can also be fully allogenic, in situations with donor oocytes or gestation surrogacy. The uterus also shows variation in the immune cell population during the ovarian cycle (Robertson, 2000) and during pregnancy (Chaouat *et al.*, 2007) with the local tolerance of a semiallogenic or allogenic fetus and placenta during pregnancy. It is not exactly clear what mechanisms that exist behind this tolerance of the semiallogenic/ allogenic pregnancy tissue to protect it from assaulted by the mother's immune system. It is speculated that a subset of T-cells, T-regulatory (T-reg) (Trowsdale and Betz, 2006) is activated locally during pregnancy. This T-reg cell seems to be guided by the hormonal status (Aluvihare *et al.*, 2004) and suppresses the activity of the adaptive immune cells and the NK-cells in the uterus (Croy *et al.*, 2003). Female steroid hormones also regulate the immune system in other ways. It is shown that the subset of inflammatory cells in the uterus varies with the estrous/menstrual cycle (Robertson, 2000). Oestradiol and progesterone also influence the APC function of the DC (Beagley and Gockel, 2003). The foetal tissues in addition express a subtype of HLA called HLA-G that seems to suppress the immune system (Le Gal *et al.*, 1999; Ristich, 2005; Sheshgiri *et al.*, 2008). One can therefore speculate that a failure in this regulation could be a reason for female

patients that are diagnosed with recurrent miscarriages. It could also be speculated that the unique capacity of the uterus to induce localised tolerance during pregnancy may be beneficial for an allogenic transplanted uterus and that the transplanted uterus could be helped to suppress rejection during pregnancy by this mechanism.

Immunosuppression

Despite careful surgical techniques and the use of hypothermia and special preservation solutions during transplantation, rejection of the transplant will occur due to the HLA mismatch and dissimilarity between donor and recipient if the recipients' immune system is not suppressed. There is a diversity of drugs that suppress the immune system in some way and usually a combination of three or more of these drugs are used to prevent rejection of a transplant (Fig. 1)

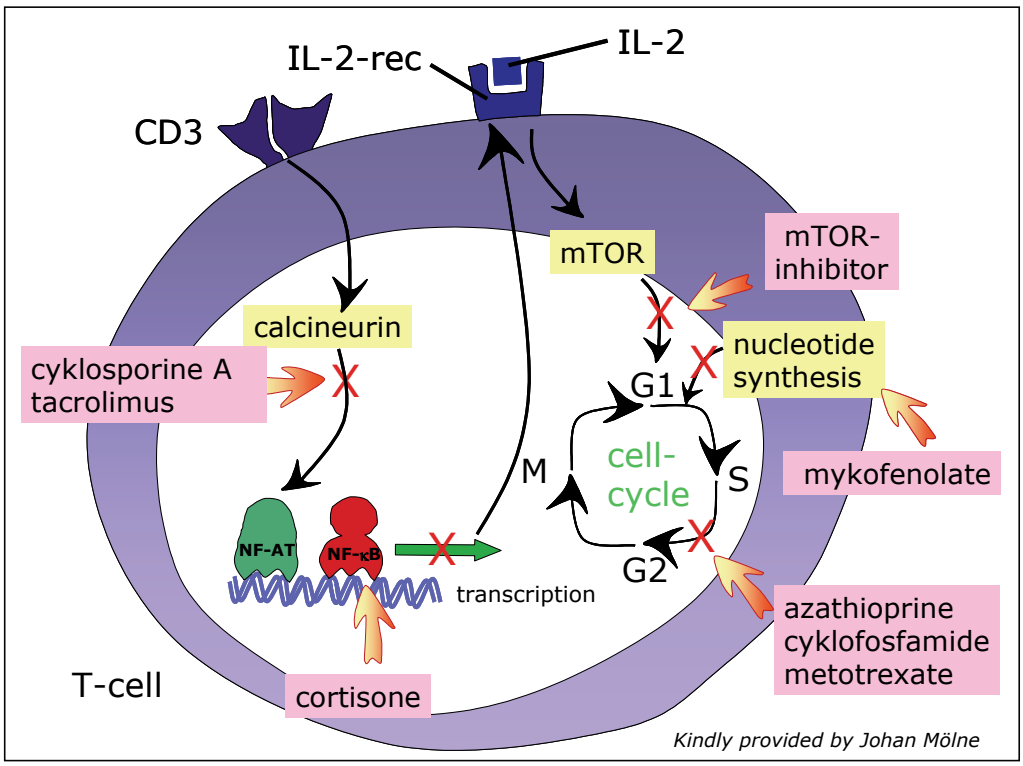

Figure 1. Main actions on the T-cell by immunosuppressive drugs.

Corticosteroids, including the endogenous corticosteroid cortisol, are known to be immunosuppressants and are used in a large extent to treat autoimmune diseases such as rheumatoid arthritis. In transplantation corticosteroids were first used in combination with 6-mercaptopurine (Calne, 1960) to suppress rejection. It is not exactly determined how corticosteroids suppress the immune system but it established that corticosteroids bind to its intra-cellular receptor with further effect in the nucleus on the so alled glucocorticoid response elements (GREs) that are specific DNA-binding sites. These GREs can be divided into two groups (Stahn and Buttgereit, 2008). The members of the first group are the positive GRE at transactivation, a process which results in induced synthesis of anti-inflammatory proteins such as IL-10, annexin 1 and inhibitors to NF-κB, an important intracellular pro- inflammatory mediator. The members of the second group are the negative GREs that suppress the expression of proteins such as pro-opiomelanocortin, α- fetoprotein and prolactin and thus explaining the widespread hormonal effects seen in cortisone treatment (Stahn and Buttgereit, 2008). Another process by which corticosteroids may affect gene-expression is transrepression.

Monomers of the cortisone-receptor complex bind to transcription factors such as NF-κB which prevents these proteins to bind to their DNA segments and ultimately inhibits the expression of several pro-inflammatory genes including IL-1, IL-2, TNF, IFN-λ and several prostaglandins (Clark, 2003).

Other ways to prevent rejection is to inhibit the adaptive immune system more specifically by the use of so called immunomodulating therapy. Azathioprine, that is rapidly hydrolyzed to the inidazol derivate 6-mercaptopurine, is incorporated into DNA and inhibits nucleotide synthesis by causing inhibition during the early stages of purine metabolism (Allison, 2000). This mechanism prevents mitosis of rapidly dividing cells, such as activated lympho-cytes. Azathioprine has accordingly little effect on established immune response and is for that reason most effective in the prevention of acute rejection and not treatment of rejection. Mycophenolic acid decreases the synthesis of the guanosine nucleotide. T- and B-cells are dependent on the primary synthesis of guanosine and can not use alternative ways to synthesis this nucleotide and for that reason, the immune response is suppressed by induced apoptosis (Allison, 2000). Sirolimus suppresses T-cell proliferation by blocking the calcium dependent and calcium non-dependent intracellular signaling. It has been proposed that sirolimus binds to FKPB-12 which suppress mammalian Target of Rapamycin (mTOR). This prevents the progression of T-cells from the G1 to the S cycle by blocking signaling downstream of the IL-2 receptor (Kirken, 2003). Sirolimus is therefore able to bloc delayed hyper-sensitivity reaction, cytotoxic leukocyte activation and humoral response.

Tacrolimus (FK506), a rather new drug that is derived from fungi, also binds to FKPB and the FKPB-FK506 complex binds and inactivates the calcium dependent serine/ threonine phosphatase calcineurin (Liu *et al.*, 1991). Calcineurin regulates the nuclear translocation and activation of nuclear factor of activated T-cells (NFAT) transcription factor one essential step for cytokine expression in activated T-cells. However, it

is also been shown that the FKPB-FK506 complex can inhibit the JNK and p38 activation pathways (Matsuda and Koyasu, 2003). There are some similarities between sirolimus and tacrolimus as seen above and there are also some similarities with the historically most widely used immuno-suppressive agent, cyclosporine A (CsA).

Cyclosporine A was first isolated from the fungus *Tolypocladium Inflatum Gam* in the late 1950s. In the 1970s a screening program was initiated to review the sample and it was established that CsA possesses three main properties: (i) immunosuppression activities, (ii) no non-specific cytostatic action and (iii) nephrotoxicity (Borel *et al.*, 1976). CsA binds to cyclophilin and the CsA-cyclophilin complex binds and inhibits the calcium- and calmodulin-dependent phosphatase calcineurin. Calcineurin is a phosphatase and the consequence of activation of calcineurin is the nuclear translocations of nuclear factor of activated T cells (NFAT) and NFAT together with other factors induce DNA transcription. The inhibition of calcineurin as a result leads to inhibition of the synthesis of proteins including IL-2 that leads to decreased IL-2 dependent proliferation and differentiation of T-cells.

Initially CsA was used experimentally to suppress rejection (Calne, 1979; Calne *et al.*, 1979; Zimmermann *et al.*, 1979) but eventually it was approved to be used as an immunosuppressant in transplantation. Today CsA is one of the most commonly used immunosuppressants in transplantation, often in combination with corticosteroids and sometimes with a third immuno-suppressant drug that acts at a different pathway to achieve synergy effects (Calne *et al.*, 1979). Since the introduction of CsA patient and graft survival has increased dramatically (Jamieson *et al.*, 1979).

One major side effect of CsA treatment is however nephrotoxicity. Acute renal failure can be a consequence of CsA treatment and the symptoms are similar to those indicating acute rejection of a kidney transplant with decreased glomerular filtration rate leading to impaired urine concentration and sodium retention. In histological analysis, CsA-induced nephrotoxicity can be differentiated from acute renal rejection by the absence of extensive infiltration of immune cells. It seems that CsA in high concentrations acts as a toxin to the proximal tubuli (Mihatsch *et al.*, 1988) and that it also induces renal vascular injury (Shulman *et al.*, 1981) with thrombosis and arteriopathy as results. The acute CsA-induced nephrotoxicity is considered to be dose-dependent and can be reversed if the treatment is terminated or the doses reduced. The chronic nephrotoxicity is characterized by the development of structural changes such as tubulointerstitial fibrosis, which is irreversible and may lead to end-stage renal failure (Kopp and Klotman, 1990). Another concern regarding CsA has been the possible effects on the endocrine system and the reproductive system.

Immunosuppression and pregnancy

In animal studies, a dose dependent reduction by CsA of the implantation rate and an increased abortion rate (Brown *et al.*, 1985; Fein *et al.*, 1989) have been shown. Moreover structural differences in sperm morphology (Masuda *et al.*, 2003) and reduction of male fertility (Srinivas *et al.*, 1998) have also been reported.

About 50 years ago the first post-transplantation pregnancy was reported (Murray *et al.*, 1963) and since then more than 14 000 births among transplanted women have been reported (McKay and Josephson, 2006). There are three major registries in the world that collect data on pregnancy outcome: the European Dialysis and Transplantation Association Registry, the UK Transplantation Pregnancy Registry and the National Transplantation Pregnancy Registry for the US. According to the US Food and Drug Administration (FDA) and the Swedish FASS the current pregnancy risk for corticosteroids are category B (no evidence of risk in the human) for corticosteroids; category C (risk cannot be ruled out) for cyclosporine, mycophenolic acid, tacrolimus, and rapamycin; and category D (positive evidence for risk) for azathioprine. Other risk categories are A (no risk) and X (contraindicated). These categories are mainly based on animal data and cannot directly be applied to human pregnancies. The placement of azathioprine in category D is mainly based on animal research (Githens *et al.*, 1965) and currently it is not recommended that azathioprine treatment should be withdrawn during pregnancy (Armenti *et al.*, 1998; McKay *et al.*, 2005) if there is no alternative drug for the transplanted pregnant woman. However, a recent study found a higher frequency of malformations in children exposed to azathioprine in utero (Cleary and Kallen, 2009). There are also some concerns about mycophenolic acid, which recently has been shown to have teratogenic effects and there are ongoing discussions whether to place mycophenolic acid in category to D (Armenti *et al.*, 2008).

According to Armenti (Armenti *et al.*, 2004) there is in total no increased rate of major malformation in the children born after intrauterine exposure to CsA. In this report (Armenti *et al.*, 2004) it was also found that the major risk for the fetus was preterm delivery (mean around 36 weeks) and low birth weight (<2500g) and for the pregnant woman a higher risk for hypertension and pre-eclampsia (Armenti *et al.*, 2004; McKay *et al.*, 2005). This has also been confirmed in a Swedish study (Kallen *et al.*, 2005). This study is a complete population-based study on the pregnancy outcome after transplantation based on the Swedish Medical Birth Registry. As a subgroup all women that had undergone transplantation were identified from the nation-wide hospital discharge registry. In this well performed study it was established that the miscarriage risk was increased for transplanted women both before and after the transplantation. In this population there also existed increased risks for preterm delivery, pre-eclampsia and SGA but the odd ratios were similar before and after transplantation, suggesting that the cause was the underlying disease/-s and not the transplantation or immunosuppressant drugs. Smaller studies have been performed to more specifically look at the health of the children of immunosuppressed mothers and no decrease in renal function has been detected in children exposed to immuno-suppressants in utero (Cochat *et al.*, 2004). Also, to date no study has identified detectable differences in the immunological composition in children exposed to immunosuppressants in utero as compared to children born to non-immunosuppressed mothers (Cimaz *et al.*, 2004). However,

there is some data pointing towards a disturbance in the mechanism that inhibit the development of auto antibodies (Classen and Shevach, 1991). Thus there are concerns about the long-term effect on the immune system after intra uterine exposure to immunosuppressants (Scott, 2002; Scott *et al.*, 2002) and especially that of cortisone and azathioprine.

In the future when it is time to attempt human uterus transplantation again it is necessary to have evaluated and studied all the aspect of uterine transplantation. The surgical procedure, with vessel anastomoses and fixation of the graft to avoid torsion has to be studies in detail and also developed in large animal models. The different aspects to control rejection to avoid side effects of immunosuppressive agents in the recipient have to be well-studied. The impact on fertility and implantation and miscarriage should also be looked upon in detail in appropriate models. The different side effect on the fetus and offspring also in the long term most be evaluated and critical examined. If the results of these and other studies are favorable it may be time for the second human uterus transplantation attempt.

Aims

The general aim of this thesis is to increase the knowledge of rejection of a transplanted uterus in two rodent models as well as to study the effects of cyclosporine A (CsA) treatment on uterus rejection and on reproductive performance in mothers and offspring.

The specific aims are:

- To describe the time-dependent changes in development of macroscopic and histological signs of rejection in a fully allogeneic mouse uterus transplantation model (*paper1*).

- To determine the time-specific influx of leukocyte subtypes into the endometrium and the myometrium during rejection of a transplanted uterus in a fully allogeneic mouse model (*paper III*).

- To establish whether treatment with CsA as a single immunosuppressive agent reduces the course of rejection or alters the composition of T-lymphocyte subsets in a transplanted uterus in a semi-allogeneic mouse model (*paper II*).

- To determine whether CsA modifies the course of rejection and the uterine expression of mRNA for some specific cytokines that have been stated to be involved in the mechanisms behind rejection as well as in uterine reproductive processes (*paper V*).

- To determine if CsA treatment of mice during pregnancy alter the reproductive outcome in animals that are exposed to this immunosuppressant either directly or in utero (*paper IV*).

Material and method

Below follows a brief summery of the materials and methods used in this thesis. For more detailed descriptions, please study the material and method sections in the original papers.

Animals

All animals were supplied by accredited breeders. Mice used in papers I, II and III were purchased from M&B A/S (Copenhagen, Denmark), mice for paper IV were supplied by Harlan Laboratories (Horst, Netherlands) and rats used in paper V came form Charles River Laboratories (Suzfeldt, Germany). Housing and care of the animals followed the rules and guidelines issued by the Animal Care Agency in Sweden and the experiments were approved by the Animal Ethics Committee in Göteborg, Sweden.

To study rejection patterns we used an allogeneic model and therefore mouse strains (BALB/c as donor and C67Bl/6 as recipient) that exhibit dissimilarities in all alleles except one were used to receive a pronounced rejection (paper I and III). In paper II, the aim of the experiments was to study the effect of CsA and its immunosuppressing effects and here a semi-allogeneic mouse model was used (B6CBAF1 (F1 hybrid of C57Bl/6 and CBA/ca) as donor and C57Bl/6 as recipient) (paper II) that is more relevant to a clinical situation. In paper V an allogenic rat model was used with Brown Norwegian rats as donors and Lewis strain as recipients. In the study of the reproductive performance under immunosuppression by CsA (paper IV) we used C57CBA-F1 hybrid mice (cross between C75BL/6 females and CBA/ca males).

Surgery

The surgical techniques were basically the same in all the papers involving transplantation (paper I, II, III and V) and this technique is described in more detail therein. Briefly, in the donor the right uterine horn and cervix was isolated with supplying and draining vessels including the uterine, internal and common iliac vessels up to the level of the bifurcation from the aorta and vena cava (paper V) or including the abdominal portion of the aorta and vena cava (papers I, II, III). The specimen was flushed and put on ice. In the recipient the aorta and the vena cava (papers I, II, III) or the right common iliac vessels (paper V) were mobilized and used for end-to-side anastomosis of the graft vessels. In papers I, II and III, the native uterus was left in place and the cervix of the grafted uterus exteriorized and sutured as a stoma to the cutaneous tissue of the abdominal wall and then the laparotomy was closed. In the modified version (paper V) the entire uterus in the recipient was removed before the graft was placed in an orthotopic position with a vagina-to-vagina anastomosis.

In the papers where the effect of CsA was examined (paper II, IV and V) a small mini osmotic pump (Azlet Osmotic Pumps, Cupertino, CA, USA) containing the substance was placed sc dorsally of the neck. The mini-osmotic pumps were changed according the instruction by the manufacturer.

Drugs

Cyclosporine A (Sandimmun®, Novartis Pharma AG, Basel, Switzerland) was diluted in 90% propylene-glycol (Fluka, Buchs, Switzerland) to the desired concentration resulting in daily doses of 0, 10 or 20 mg/kg (paper II), 0 and 10 mg/kg (paper V) and 0, 10, 20 and 30 mg/kg (paper IV) and then placed in the mini-osmotic pump according to the instructions by the manufacturer (paper II, IV and V).

Analyses

Macroscopic signs of rejection were analysed by grading the severity of swelling, darkening of color and fibrosis (paper I, II, III and V) and the uteri were grossly divided into groups.

Laser Doppler flowmetry was used to estimate the tissue blood flow in both the grafted and native uterus. A mini probe (Perimed, Järfälla, Sweden) was placed inside the uterin cavity and the blood flow was recorded (Periflux 5000 flowmeter; Perimed, Järfälla, Sweden) (paper I and II).

Microscopic signs of rejection were mainly analysed by grading morphology as well as the presence of thrombosis, apoptosis, necrosis and infiltrating cell density (paper I, II and V). In paper III the morphology was noted but not presented.

Immunohistochemistry was used to evaluate the density of leukocyte subtypes; CD3+ T-cells (rat anti- mouse CD-3 molecular complex; BD PharMingen, San Diego, CA, USA) (paper I), CD4+ T- cells and CD8+ T-cells (anti-CD8; BD Biosciences, Franklin Lakes, NJ, USA) (paper II and III), macrophages (anti Mac-3 BD Biosciences, Franklin Lakes, NJ, USA), neutrophils ((neutrophil allotypic marker (MCA 771G); Serotec, Oxford, UK) and B- cells (anti-CD19; BD Biosciences, Franklin Lakes, NJ, USA (paper III).

Tissue sections of kidneys taken from animals exposed to CsA directly (paper II and V) or in utero (paper IV) were examined for morphological changes related to CsA toxicity such as tubular degeneration, striped or diffuse interstitial fibrosis, nodular arteriolar hyalinosis and tubular calcification.

Analysis of CsA concentration in whole blood was performed by enzyme immunochemistry using a CsA assay (Emit®2000, Dade Behring, Milton Keynes, UK) according to manufacturer's instruction (paper II and IV). Serum creatinin was analyzed using a reagent kit (CREAplus R1, R2, Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's instructions (paper II and IV). These analyses were performed by the department of Clinical Chemistry, Sahlgrenska University Hospital, Göteborg, Sweden.

Quantification of mRNA was performed by real-time quantitative PCR (QT-PCR). Uterine tissue biopsies were immediately immersed in RNAlater® (Ambion, Huntingdon, UK) and frozen until analysis. Taqman MGB probes (Applied Biosystems, Applera Corp., Foster City, CA, USA) targeting IL-15 (Rn00689964_m1), IL-1α (Rn00566700_m1), CD200 (Rn01646320_m1), LIF (Rn00573491_g1) and Gal-1 (Rn00571505_m1) and control (β-actin) were used and analysis were run using ABI Prism 7000 Sequence Detector (Applied Biosystems, Applera Corp., Foster City, CA, USA) according to the manufacture's specification. Each amplification reaction consisted of 20 ng cDNA, 1 x probe-mix and 1 x TaqMan Universal PCR mastermix (Applied Biosystems, Applera Corp., Foster City, CA, USA) to a final volume of 25 µl. The relative expression of target genes were

presented with the comparative Ct method (ΔΔCt) (Livak and Schmittgen, 2001) and normalized to the amount of β-actin mRNA (paper V).

Reproductive performance in mice exposed to CsA was evaluated in two sets of experiment. In the first set of experiments three groups of female mice received CsA at doses of 10, 20 or 30mg/kg/day throughout the experiment and were mated. On day 18 after mating the animals were scarified and the numbers of viable fetuses and resorption-sites were counted. The placenta and fetuses were also weighed (paper IV). In the second experiment, mice that had been exposed to CsA in utero (maternal doses of 0, 10 and 20 mg/kg/day) were followed by weighing until sexual maturity (7 weeks) and mated with unexposed partners of proven fertility. Females exposed to CsA in utero were scarified on day 14 of pregnancy and fetuses and placentas were counted and weighed. Unexposed female mating partners to male mice exposed to CsA in utero went to term and the numbers of pups were counted and weighed (paper IV).

Statistics

For analysis of quantitative data computer assisted calculation (SPSS 15.0 (SPSS, Chicago, IL, U.S.A.)) was performed (Kruskal- Wallis analysis, Mann- Whitney U-test and Wilcoxon signed piared test). For detection of any correlation between event and given dose drug Spearman Rho correlation test was used. Differences in weight or numbers between exposed or not exposed to CsA were analyzed by the independent two-sample t-test. The mRNA data was calculated from the comparative Ct method (ΔΔCt). A P- value of less than 0.05 was considered significant.

Results and discussion

One of the major difficulties in transplantation is to overcome the rejection process that starts when the recipient's immune system recognizes a potential threat from the non-autologous tissue. This recognition initiates a cascade of complex signal pathways, which results in an activation of immune cells that eventually destroys the transplanted tissue. To prevent rejection, the transplanted patient is treated with drugs that inhibit or interfere with cellular signalling pathways that lead to activation and/or proliferation of immune cells. In the clinic, these drugs are effective in preventing rejection and negative side effects of the drugs can be controlled. However, these drugs pass over the placenta and can interfere with fetal development. The main aims of this thesis are to increase the knowledge of mechanisms behind rejection of a uterine allograft and to study the effects of CsA, the most widely used immunosuppressant, on uterine rejection and female and offspring fertility in general. These are only some of the many aspects of uterus transplantation that should be investigated thoroughly in animal models before human uterus transplantation attempts.

The following sections present and discuss the results from the studies included in this thesis and are organized around i) the progression of rejection (papers I and III), ii) the effects of the immunosuppressant drug CsA on rejection (papers II and V) and iii) the effects of CsA exposure, directly and in utero, on fertility and reproduction (paper IV).

Rejection

In the studies of the time-frame of uterine rejection (paper I and III) a fully allogenic mouse model that has dissimilarities in all alleles except one was used to induce a pronounced rejection response after transplantation. All data of these studies indicates that a severe rejection takes place in all the tissue compartments of the transplanted uterus. On the macroscopic level this could initially be seen at day 5 post-transplantation as a swelling and darkening color of the transplant, indicating edema and microcirculatory blood flow stasis, at least in the capillaries and venules on the serosal surface of the uterus. This can be assumed to be a response by the innate immune system to early inflammatory mediators released from the transplanted tissue that recruits inflammatory cells. The composition of this first wave of inflammatory cells was systematically investigated in paper III and is described in more detail in the following paragraph. When the rejection progressed (day 10) the transplant became darker in color as a sign of further blood stasis and necrosis of the tissue. Later on (day 15) the graft shrunk and became gray and hard as a sign of complete tissue death and fibrotic changes. Despite the early macroscopic indications of changed blood flow in the transplant, no change in this parameter, in relation to the day 2 value, could be detected by laser-Doppler flowmetry until day 10 after which a gradual cessation of blood flow was evident. However, it should be pointed out that there was a relatively wide variation in the blood

flow levels and it may well be that a minor blood flow decrease was present already at day 5. The laser-Doppler flow-metry method is rather sensitive for the exact placement of the detection probe and it was difficult to standardize the experimental method concerning intra-uterine positioning of the probe. Interestingly, the blood flow of the transplanted uterus was lower than that of the native uterus already at postoperative day 2. An explanation would be that the surgical trauma and/or early rejection mechanisms already at that time point influenced the blood flow or that the fact that the uterus was only supplied by one uterine artery influenced the blood flow. The single uterine artery would of course supply the whole common part of the uterus and cervix, which in the physiological situation are supplied bilaterally by the uterine arteries but also by the vaginal arteries. However, it is likely that such a negative influence would be corrected during a longer time period by compensatory vascularization and growth of the transplanted vessels. This is indicated by results in the initial study of mouse syngeneic uterus transplantation where blood flow was similar in grafted and native uterus at postoperative day 30 (Racho El-Akouri *et al.*, 2002). On a microscopic level, there were histological signs of rejection from day 2 and 5 with edema, inflammatory cell infiltration, apoptosis and thrombosis in the smaller blood vessels of the myometrium. Scattered lymphocytes were also seen in the endometrium at this time point. On day 10, the entire uterine wall was heavily inflamed, with a great density of lymphocytes in the endometrium and apoptosis were seen focally in the graft. At day 15 there were additional signs of heavy rejection with arterial thrombi and at day 28 necrosis were abundant and no viable myometrial or endometrial tissue was observed (paper I). There was a greater density of T-lymphocytes in the grafted uterus as compared to the native and also an increase over time in the graft during the initial observation time (paper I). At later times the tissue necrosis made it difficult to assess density of T-lymphocytes. In the rat model (paper V) macroscopic and histological signs of rejection, corresponding to those found on day 5 in the mouse, were also present on postoperative day 7. In paper V 5 out of 5 grafted non-CsA treated uteri showed macroscopic signs of rejection with swelling (4 of 5) and faint color (5 of 5) when compared to the native uterus.

The rationale behind paper III was to study the influx of different leukocyte subtypes into the uterine graft during rejection in detail, since such a description would be necessary in future studies aiming at assessing the effect of various immunosuppressive strategies to prevent uterine graft rejection. In paper III the leukocyte subtypes within the tissue were identified by immunohistochemistry and the densities were determined at different time points during rejection. Initially, neutrophilic granulocytes and macrophages invaded the transplant, followed at later time points by CD4 and CD8 positive T-cells. Very few CD19 positive cells (B-cells) were found in the tissue at all time points.

The density of neutrophils was low compared to the density of macrophages and T-cells at all time points. However, an increase in neutrophil density was found in the myometrium at day 5 and 10 and in the endometrium at day 10 after transplantation. Markedly higher numbers of macrophages were seen in the grafted uteri compared to the native from day 2 in the myometrium

and from day 5 in the endometrium. The density of CD8+ as well as CD4+ T-cells in the myometrium and endometrium increased from day 5. The ratio of CD8+/CD4+ T-cells at day 2 and 5 ranged between 0.38 and 1.9 with no difference between native and grafted tissue. At day 5 the ratio increased, in the myometrium to 3.41 and in the endometrium to 4.79 compared to the native uterus.

The large variation in numbers of infiltrating immune cells (especially macrophages) between the various specimens of the same groups in paper III is most probably a consequence of the fact that the animals (donors and recipients) were not hormonally synchronized before transplantation. Since the rodent estrus cycle is only 4 to 5 days long, all different cycle stages could in theory be represented in the material. The density of resident immune cell populations in the uterus vary with hormonal status during the cycle (Chegini *et al.*, 2002) and this is likely to influence the results herein. Also, progesterone has an anti-inflammatory effect (Gibson *et al.*, 2005) that possibly would reduce the magnitude of post transplantation inflammation. To avoid variations induced by hormonal cycle phase differences, future studies should use hormonally synchronized animals as donors and recipients. This can be achieved by the use of a GnRH analog (Vickery and McRae, 1980) or by induction of pseudopregnancy (Olofsson and Selstam, 1988) to minimize inter-variability between the native and grafted uterus and to minimize the intra-variability inside the experimental group. Further more, despite that a practiced person performed all surgeries in order to reduce the inter-personal differences of surgical skills (Wranning *et al.*, 2008), quality of an individual's surgical performance varies from day to day and this could also influence the impact of surgical trauma and ischemia time on outcome.

In paper II, a semi-allogenic model was used to study the effect of CsA treatment after uterus transplantation. In the rejecting control group (vehicle treated) the macroscopic and microscopic parameters indicative of rejection and inflammation were milder compared to what was seen in the fully allogenic model (papers I and III) but followed essentially the same pattern. Here also, immunohistochemistry was used and the influx of CD4+ T-cells and CD8+ T-cells was demonstrated to be increased compared to the syngenic negative control. The CD8+/CD4+ ratio in the vehicle group was 0.38 at day 10 compared to 1.39 to the syngeneic negative control.

The rejection of the untreated transplanted rodent uterus follows the same rejection pattern as has been found for some other transplanted solid organs. After uterus transplantation the first immune cell to invade the graft are the neutrophilic granulocytes. These cells lack receptors for direct allo-recognition but play important roles in rejection by initiating and mediating stimuli of early inflammatory reactions that bridge over the adaptive response (Land, 2007). Neutrophilic granulocytes have been described in biopsies of human kidney grafts during rejection (Wakabayashi *et al.*, 1986). Infiltration of neutrophilic granulocytes was also a major event seen in auto-transplanted uterus in the sheep (Wranning *et al.*, 2007) where no rejection takes place which suggests that the neutrophilic granulocytes respond to the direct trauma of surgery and ischemia at uterus transplantation as in other organs (Dragun *et al.*, 2000).

Macrophages are present at relatively constant densities in most organs. However,

in the ovary (Brännström *et al.*, 1993; Brännström *et al.*, 1994) and in the uterus (Keenihan and Robertson, 2004) macrophage density vary with the reproductive cycle. In paper III macrophages accounted for about 20% of the total number of leukocytes in rejecting uteri. Studies of cellular infiltration in transplanted hearts showed that the number of infiltrating macrophages correlated with the severity of rejection (Ahmed-Ansari *et al.*, 1988). Similar patterns of macrophage infiltration were found in rejecting liver allografts in non-human primates (Donato *et al.*, 1993) and in mice (Zhang *et al.*, 1996). This relative high density of macrophages during rejection is even more obvious in studies of organs involved in the mucosal defence, such as the small intestine (Clark *et al.*, 1990; Zhang *et al.*, 1996). It seems that during rejection macrophages have a specific role in enhancing the rejection process both as destructors of allogenic tissue and as APC's (Wyburn *et al.*, 2005).

A finding of paper I was that the density of CD3 positive cells (accessory molecule of the T-cell receptor and present on all peripheral T-cells) increased from day 2, starting in the myometrium. In a later paper (paper III) however, it was found that the increase of CD8+ T-cells and CD4+ T-cells started at a later time point. Since CD3 antigen also is present on other T-cells such as NK-like T-cells (Godfrey *et al.*, 2004; van de Wetering *et al.*, 2009) and T-regs (Trowsdale and Betz, 2006; Aluvihare *et al.*, 2004) this finding implicates that either one or both of these sub populations of T-cells are also present during the early phases of rejection of the transplanted uterus. The delayed infiltration of CD8+ T-cells and CD4+ T-cells is probably due to the time taken for activation of these antigen-specific effector cells. APCs within the grafted tissue will be activated by early inflammatory signals caused by surgical and ischemic trauma, migrate to secondary lymphoid tissue and present (directly or indirectly) antigen to cognate T-cells. These will be activated, undergo clonal expansion and migrate to the target tissue. This process of T-cell activation after solid organ transplantation has been shown to take about 3 days in rodents with elevated levels of IL-2 already at day 1 post-transplantation in the mouse (Liang *et al.*, 2006). The predominance of CD8+ T-cells in rejecting uterine tissue is indicated by the ratio between CD8+ to CD4+ cells and was correlated to the severity of rejection as judged by histology analysis. Interestingly, the ratio between CD8+ to CD4+ cells in peripheral blood was used to determine whether rejection of the uterine allograft took place during the human uterus transplantation attempt (Fageeh, 2002). On day 9 when the patient experienced abdominal pain, fatigue, malaise and body pain, that are clinical signs of rejection, the CD4/CD8 ratio in blood was 3.4. The patient was then treated with prednisolone, elevated doses CsA, azathioprine and antithymocytic globulin (ATG) and it was stated that the rejection episode was resolved, and the CD4/CD8 ratio decreased to 1.3. Many studies have been performed to investigate if the peripheral ratio of CD4 and CD8 could be used to early discover a rejection episode (Schuurman *et al.*, 1989; Sheikh *et al.*, 1995). However, the method to diagnose early rejection by this mean seems to be inadequate comparing to ordinary biopsies (Prohaska *et al.*, 1998; Yang *et al.*, 2003). Recently, research has been performed to find other less invasive methods to discover rejection mainly by blood samples. Soluble peptides (Grunewald

et al., 2000) or other antigens (Yang *et al.*, 2003) have been investigated. Similar relations between number of CD4+ and CD8+ cells in the tissue as those of the present thesis (paper III) were found in a study with DBA/2 mice as heart donors and C57Bl/6 mice as recipients (Bishop *et al.*, 1992). Also, treatment with anti-CD4 monoclonal antibody has been shown to inhibit infiltration of both types of T-cells while only CD4+ T-cells, although fewer in numbers than CD8+ T-cells, were demonstrated to be necessary for rejection (Bishop *et al.*, 1993). This dominant role of CD4+ T-cells in rejection may be coupled to its facilitating role in stimulation of proliferation of the CD4+ T-cell itself as well as of CD8+ T-cells.

In paper III of the current thesis, the absolute number of CD4+ T-cells respectiveely CD8+ T-cells were higher in the myometrium while the ratio between CD8+ and CD4+ T-cells was higher in the endometrium at day 5 after transplantation. This suggests that rejection starts in the myometrium while the endometrium is more affected by CD8+ T-cells, correlating to what was also found by histology analysis (paper I). This predomination of CD8+ T-cells has also been found in several early studies of human renal allografts (McWhinnie *et al.*, 1986; Hancock *et al.*, 1983; Platt *et al.*, 1982). The possible importance of ratio of CD8+ to CD4+ T-cells is supported by a study in renal transplantation with grafts from deceased donors (Stelzer *et al.*, 1984), where a high CD4+ to CD8+ T-cell ratio in tissue biopsies was a predictor of milder rejection and prolonged graft survival. In the semi-allogenic model (paper II) the vehicle-treated, rejecting control group also displayed a high CD8+/CD4+ T-cell ratio, however slightly reduced compared to the allogenic model (paper III) correlating to the milder rejection in this model.

We could not detect any elevation in the density of tissue bound B-cells in rejecting uterine tissue but this finding does not necessarily mean that B-cells are not participating in the cellular rejection of a transplanted uterus. In clinical observations of rejecting kidneys, B-cell infiltration is associated with worse clinical outcome (Hippen *et al.*, 2005) while the mechanisms behind these findings remains to be elucidated. However, studies involving transplanted hearts they could not find any correlation between B-cell numbers and severity of rejection (Ahmed-Ansari *et al.*, 1988). These findings might again reflect organ differences of leukocyte infiltration during acute rejection.

Conclusion

Rejection of the transplanted uterus starts with infiltration of neutrophils and macrophages. The uterine tissue shows these early signs of inflammation with edema and cell infiltration followed by a higher density of lymphocytes and tissue damage together with macroscopic alterations and decreased blood flow. In a semi-allogenic model, the rejection at day 10 was less pronounced as in the fully allogenic model. It also seems that histological signs of rejection in the myometrium correlate with rejection of the endometrium and therefore it is possible that biopsies obtained by curettage can be used as a diagnostic tool for monitoring rejection in a future clinical trial. This must however be further investigated in studies of uterus transplantation in large animal model such as the pig, sheep or a non-human primate model.

Cyclosporine A and rejection

To study the effects of CsA treatment on the rejection process, a semi allogenic mouse model (paper II) and a medium responder, fully allogenic rat model (paper V) was used. These models were chosen since they offer a more clinically relevant situation for studies of immunosuppression at uterus transplantation, where a mother or older sister would be suitable living donors.

In the mouse paper (paper II) transplants were evaluated on day 10 after surgery in groups treated with either vehicle or 10 or 20 mg/kg*day of CsA. CsA at 20 mg/kg*day decreased the macroscopic signs of rejection so that 3/5 showed normal color, 2/5 showed moderate darkening and 4 of 5 uteri displayed normal texture with no swelling as well as pulsations of the grafted vessels and myometrial bleeding when incised. Slightly more signs of rejection were seen in the 10 mg/kg*day group. Five of 5 showed moderates darkening, 2 of 5 were harder and enlarged and only 2of 5 bled when the uterine tissue was incised in situ. In the vehicle group, 2 of 5 uteri were markedly darker and the rest moderately darker than the native uteri. Three of 5 grafts were swollen and only 1 of 5 grafted uteri bleed from the myometrium when cut. These findings also correlated with the appearances of the cervical/vaginal tissue of the stoma. In the CsA treated transplanted rat uteri the signs of rejection were less obvious than in the untreated group (paper V) with only 2 of 5 grafted uteri showing macroscopic signs of rejection compared to 5 of 5 in the untreated group.

The histological analysis of transplanted uteri in mice treated with CsA or vehicle (paper II) showed findings correlating to the macroscopic evaluation. Four of 5 uteri in the vehicle group displayed edema, stasis and extravasation of erythrocytes and 1 of 5 was necrotic. There were also occasional infiltrating immune cells in the muscle layer and endometrium. When treated with CsA at 10 mg/kg*day there were less edema, stasis and bleeding and only small areas of necrosis were seen. In the cells of the endometrium numerous apoptotic bodies were seen. Even less signs of rejection were seen when 20 mg/kg*day of CsA was used. No edema, stasis or bleeding were shown in 4/5 of the specimens. There were only occasional apoptotic bodies seen. The numbers of infiltrating immune cells were lower compared to the vehicle and 10 mg/kg CsA groups. The histological overview showed also better preserved endometrial glands.

The number of infiltrating CD4+ T-cells was found to be increased in the semi-allogenic groups compared to the syngeneic group and in the two treatment groups a slight decrease was seen compared to the vehicle group but this decrease was not significant. Surprisingly, there was also a rise in the numbers of CD8+T-cells in the two groups receiving CsA. The CD8+/ CD4+ ratio in the syngeneic group was 1.39 and in the vehicle 0.38. The ratio in the treatments group were 0.56 respectively 0.9.

In rats transplanted with allogenic uteri and either treated with CsA at 10 mg/kg*day or not treated (paper V) the microscopic picture showed less signs of rejection in the CsA treated group compared to the untreated transplanted group. There were less edema and a marked decrease in density of infiltrating cells. Furthermore, the luminal and glandular area were better preserved in the CsA group and the endometrial lining was intact. In this CsA treated group there were no signs of

thrombosis and no apoptotic cells were seen. In both the mouse (paper II) and the rat (paper V) the signs of rejection of transplanted uteri on both macroscopic and microscopic levels were decreased when CsA was used. It has been shown that the doses of CsA needed to suppress rejection vary with different strains and organs (Wang *et al.*, 2003; Tanaka *et al.*, 2005; Vessie *et al.*, 2005). Furthermore, when comparing the stage of rejection in paper II to paper I, III and to the vehicle group on the same post operative day there are signs of a milder rejection in the uteri of paper II. This difference probably depends on different factors such as that the non-steroid anti-inflammatory drug carprofen, which inhibits cyclooxygenase-2, was used as analgesia (paper II), since it has been shown that cyclooxygenase-2 inhibition can enhance graft survival (Ma, 2002). Furthermore - and perhaps a more plausible explanation - is that a semi-allogenic model, where half the genome of the donor is identical to that of the recipient, was used and that this is an underlying cause of a milder rejection (Zhang, 1996, Xia and Kao, 2005) (paper II). The number of infiltrating CD4+ T-cells was on the other hand not significantly reduced when CsA was used in the concentration 10 mg/kg and 20 mg/kg per day. It can be assumed that these doses were suboptimal to inhibit CD4+ T-cell proliferation. Surprisingly the numbers of CD8+T-cells were elevated when CsA was used. It has been hypothesized that soluble major MHC complex-peptide complexes induce activation-induced cell death (AICD) in T-cells as shown in vitro and this mechanism is inhibited in the presence of CsA (Cebecauer *et al.*, 2005) and that a portion of the CD8+ T-cells identified in the present material (paper II) are in fact non-cytotoxic. However, this remains to be demonstrated.

Cyclosporine A also seems to directly or indirectly influence the expression of different cytokines/membrane proteins. Accordingly, in paper V the mRNA levels of IL-15, CD200, LIF, IL-1α and Gal-1 in normal and transplanted uteri from rats, with or without CsA treatment were measured. There were no detectable differences between non-transplanted, untreated transplanted uteri and CsA treated transplanted uteri in the expression of IL-15, CD 200 and LIF. However, there was a large variation in the levels of LIF within groups. The mRNA levels of IL-1α were higher in the transplanted control group compared non-transplanted controls and in the CsA treated transplanted uteri levels were lower than in the non-treated, transplanted group. The levels of Gal-1 mRNA was higher in uteri from CsA treated animals compared to the transplanted, non-treated animals and the levels of Gal-1 mRNA in the rejecting non-treated transplanted uteri was lower compared to the non-transplanted control group.

Interleukin-15 has been implicated in the regulation of early pregnancy via down regulation of uterine NK cell (uNK) activity (Verma *et al.*, 2000) and it has also been shown that impaired activation of uNK cells could lead to mild reduction of fetal weight (Barber and Pollard, 2003). CD200 is known to promote graft survival in mouse renal transplant (Gorczynski *et al.*, 1998) rat islet xenograft in mouse (Gorczynski *et al.*, 2002) and heart and skin transplant in mouse (Gorczynski *et al.*, 2009). The expression of CD200 may be related to the development of feto-maternal tolerance in the early stages of pregnancy as shown in

the mouse (Clark *et al.*, 2001). However, in our experiments in rats IL-15 and CD200 levels were not altered by rejection or CsA treatment.

Neither did LIF expression change significantly in our study. LIF is a cytokine produced and secreted by the endometrium and it is implicated in the receptivity of the endometrium at the time of implantation (Bhatt *et al.*, 1991). Accordingly, LIF expression varies with the estrous cycle in the mouse (Lee *et al.*, 2005) and the upregulation of its receptor is promoted by progesterone in sheep (Song *et al.*, 2009). The requirement for LIF at implantation has been shown by several studies. For example, hLIF inhibitor deposited in the uterine lumen of mice at the time of embryo implantation decreases implantation rate (Mohamet *et al.*, 2009) and while LIF knock-out female mice produce oocytes that can be fertilized, these embryos fail to implant in the knock-out but not in the wild type mouse (Stewart *et al.*, 1992). In human endometrium, LIF expression increase by approximately six times during the mid- to late secretory phase and early human embryos expresses the LIF receptor at the time for implantation (Charnock-Jones *et al.*, 1994). Additionally, LIF has been shown to be up-regulated in female transplant patients during rejection episodes (Blancho *et al.*, 1993) and results from a study on alloreactive human T lymphocyte clones from rejecting kidney transplant patients cultured with or without CsA, indicates that LIF expression is suppressed by CsA (Bentouimou *et al.*, 1993). Therefore, it was hypothesized that LIF expression would decrease in CsA exposed allogenic uteri compared to the non-treated, rejecting uteri. However, in our study no significant difference in LIF expression could be seen between groups. Since the animals used were not hormonally synchronized, it can be assumed that they were in different stages of the hormonal cycle and this would explain the large variation in LIF expression within groups. It might be that if this variation would be reduced if the animals were hormonally synchronized and analyzed at the same cycle phase and that a difference between groups then would be present. However, this remains to be shown.

The levels of the pro-inflammatory cytokine IL-1α mRNA was reduced by CsA treatment in our study. This was expected since CsA was predicted to reduce the general inflammatory reaction when IL-2 expression and CD4+ T-cell proliferation is reduced by the drug. IL-1α has also been shown to be regulated by ovarian steroids and varies with the estrous cycle. In mice IL-1α expression has been shown to peak at estrous (Lee *et al.*, 2005) and in humans a large increase is seen during the secretory phase of the menstrual cycle (Tabibzadeh and Sun, 1992). It seems that an upregulation of IL-1α by the decidua is implicated in the feto-maternal cross talk at implantation (Simon *et al.*, 1997; Segerer *et al.*, 2009) while it's been shown that systemic inflammation induced by lipopolysccharides (LPS) in mice and with a global IL-1α upregulation reduces implantation (Deb *et al.*, 2004). Thus, from our results it is difficult to predict how the observed CsA induced down-regulation of IL-1α in transplanted uteri would affect implantation.

Galectin-1 (Gal-1) belongs to a group of glycan-binding proteins that recognizes multiple galactose-β1, 4-N-acetylglucosamine units on cell surface glycoconjugates (Rabinovich *et al.*, 2007) and thus binds to

leukocyte surface antigens. In transplantation Gal-1 might have immunoregulatory effects since it has been shown to induce the expression of IL-10 (van der Leij *et al.*, 2004), induce apoptosis in T-cells (Perillo *et al.*, 1995) and might it self be induced by immunosuppressants (van der Leij *et al.*, 2004). Gal-1 is also expressed in the endometrium both in humans (von Wolff *et al.*, 2005) and rodents (Phillips *et al.*, 1996) and is implicated in the development of feto-maternal immunological tolerance since Gal-1 deficient mice showed a high rate of fetal loss in allogenic mating and that treatment with recombinant Gal-1 restored the implantation rate (Blois *et al.*, 2007). In paper V we show that the expression levels of Gal-1 is reduced in rejecting uterine tissue but normalized by CsA-treatment. This finding indicates that CsA, by its inhibition of the activity of CD4+ T-cells (He and Baum, 2004), contributes to restoration of the suppressed Gal-1 expression, most likely caused by the massive inflammation at rejection. The results in paper V needs to be confirmed in a larger study and related not only to the morphological grade of rejection but also to other parameters connected to rejection grade such as quantification of infiltrating leukocytes and pro-inflammatory cytokine levels. Again, the large variation in Gal-1 expression within groups is most probably due to hormonal cycle phase differences between individual rats that can be assumed since the animals were not hormonally synchronized.

Conclusion

Cyclosporine A treatment of mice or rats after allogeneic uterus transplantation reduces the macroscopic and histological signs of rejection and induces changes in the expression of several markers of rejection on the mRNA level. However, CsA does not alter the number of infiltrating T-cells and therefore it can be assumed that rejection was not completely abolished by the doses used. In clinic, combination therapies consisting of different immunosuppressants are routinely used to target several pathways leading to rejection and this will also allow reduction of each drug in order to minimize negative side effects. In rodents monotherapy with CsA can be successful in achieving long term graft survival in both rats (Siemionow *et al.*, 2005) and mice, depending on strain combination and organ (Wang *et al.*, 2003). However, in the models used in the studies presented in this thesis it might be necessary to use CsA in combination with glucocorticosteroids if long term graft survival is to be achieved. The expression of some key cytokines and membrane proteins involved in both rejection and implantation seems to be more normalized when CsA is used and this could indicate that CsA is a suitable candidate for treatment of rejection of transplanted uterus in the future.

Cyclosporine A and fertility

In paper IV we investigated the effects of direct or intra uterine CsA exposure on reproductive capacity in non-transplanted mice.

In non-pregnant, adult females, direct exposure to CsA at doses of 10, 20 and 30 mg/kg*day for 7 weeks induced mild anorexia in a dose dependent manner. However, the doses of CsA did not alter serum creatinine concentrations or kidney morphology, indicating that even if the doses were relatively high they did not lead to detectable changes in an organ that has been shown to be sensitive to CsA side

effects. At introduction of treated sexually mature females to untreated males of proven fertility, the highest CsA dose reduced the frequency of observed vaginal plugs (6 out of 9 mice). Also, the total number of visible implantation sites on day 18 after mating decreased with increasing CsA dose (correlation coefficient = -0.520, $p<0.01$). Moreover, the percentage of absorbed fetuses in relation to total numbers of implantation sites correlated positively to increased concentration of CsA (correlation coefficient = 0.367, $p <0.05$). The weight of live fetus was however not altered by treatment at any dose. The concentration of circulating CsA at a given dose was markedly reduced during pregnancy.

In offspring to CsA (20 mg/kg*day) or vehicle treated mothers no differences in birth weight were seen. However, at 4 weeks of age the female pups that were exposed to CsA in utero had a lower body weight than vehicle exposed females ($p<0.01$). This difference persisted throughout the observation period until week 7 (week 5, $p<0.01$; week 6 $p<0.01$; week 7: $p<0.01$). Male offsprings to CsA exposed mothers were also of lower weight at 4 and 5 weeks of age (week 4 $p<0.05$; week 5 $p<0.001$) but this was not seen at later tomes of the study period.

The reproductive performance in both male and female mice exposed to CsA in uteri was not different to controls. In the female offspring group (IUE) 100% frequency of vaginal plugs were seen when mated with males of proven fertility. Also, females of proven fertility were mated with CsA exposed males and showed 100% frequency of delivery. There was no difference in the number of fetuses/pups produced by animals exposed to CsA in uteri when compared to controls and no increase in the rate of resorbed pregnancies in exposed females. However, body weights of fetuses at gestational day 14 was significantly decreased in females exposed to CsA in uteri ($p<0.05$) while birth weight of pups from CsA exposed males were similar to controls. Serum creatinine concentrations and kidney morphology of in uteri exposed female mice on pregnancy day 14 was not changed when compared to controls.

In general, high doses of CsA given to mice before and during pregnancy reduced implantation rate and increased intrauterine death of fetuses. Animals exposed to CsA in utero were of normal weight at birth but females showed a reduced growth rate later and also carried smaller fetuses. However, no functional disturbance in mating behavior and implantation rate, which was likely to be caused by intrauterine exposure to CsA, could be detected. Further no harmful effects on kidney function and morphology could be detected in this mouse hybrid during this observation periods.

Since the introduction of CsA in the clinic as an immunosuppressive agent to be taken by patients with organ transplants the number of reported pregnancies and children born to CsA treated, transplanted mothers have accumulated. There are no evidence that the frequency of major congenital malformations is increased in children born to transplanted mothers treated with CsA but there is evidence of a slightly increased risk for miscarriage, preterm delivery and birth of babies that are small for gestational age (Armenti *et al.*, 2002a; Armenti *et al.*, 2002b; Kallen *et al.*, 2005).

In mice, reduced birth weight of pups born to CsA treated dams has not been shown (Fein *et al.*, 1989) which is also consistent with our findings in paper IV. According to

the NTPR there are significantly more premature births, up to 50% of the children born to transplanted mother are premature (Armenti *et al.*, 2003) and more children growth retarded (Armenti *et al.*, 2003). It is however shown in another study that pregnancy before and after kidney transplantation went with premature deliveries to the same extent before and after transplantation (Kallen *et al.*, 2005), indicating that the underlying disease leading to transplantation in these women may be a contributing factor to the observed prematurity and growth impairment. Similar observations were done in rats given 25 mg/kg*day of CsA gestations day 8-14 (Brown *et al.*, 1985). Here a reduction of the fetus weight but no differences in the litter size was found while the number of resorption was increased. When the rats received CsA on gestational day 1-7 there was no change in fetal weights but a small but significant reduction of litter size (Brown *et al.*, 1985). In the study presented in this thesis (paper IV) the mice received CsA before and during mating and throughout pregnancy and in accordance with previous findings (Brown *et al.*, 1985) we found a dose dependent increase in resorption rate and reduction in implantation rate.

In paper IV the mice given CsA showed a dose dependent decrease in blood CsA concentrations during pregnancy which is consistent with findings in pregnant transplanted women (Fischer *et al.*, 2005). This decrease in blood CsA can not solely be explained by the increase in plasma volume during pregnancy (Pirani *et al.*, 1973) but also by the accelerated expression of CYP3A isoform that increase the metabolism of CsA. This upregulation is known to be induced by both pregnancy (Zhang *et al.*, 2008) and CsA (Lemahieu *et al.*, 2004; Nakamura *et al.*, 1994). It is also shown that the influx of CsA in the placental circulation and in maternal-fetal direction was dependent of the maternal inflow of CsA and even higher if a P-glycoprotein inhibitor was (Pavek *et al.*, 2001) present. This phenomenon could partly explain some of the feto-toxicity seen in high doses of CsA and the decrease in blood of CsA seen in paper IV and by Fisher. Both the directly exposed group (maternal exposure = ME) and the indirectly exposed group (intra uterine exposure = IUE) of both sexes had the ability to mate. However, in the ME 30 mg/kg group there were two animals that did not show any vaginal plug. There is no similar experiment in the literature to be found. However, since calcineurin inhibitors such as CsA also affect calcineurin activity in other cells than T-cells, many groups have investigated behavioral and memorial alterations and it is shown that high doses in mice reduced motor activity, increased the anxiety and decreased the social behavior (Sato *et al.*, 2007). In paper IV behavior was not specifically studied but there were fewer vaginal semen plugs (and fewer pregnancies) found in the highest dose-group at direct exposure. Plausible explanations can be CsA induced neurological changes or steroid production alterations that impaired mating behavior but the mechanism behind the reduced mating frequency remains to be elucidated. It is clear though that this effect only concerns direct exposure since all male and female animals in the IUE groups succeeded to reproduce.

Since the important mechanism of CsA is to inhibit the phosphatase activity of calcineurin there are concerns for the direct effect of immunosuppression on the fetus with increased risk for induction of auto-

antibodies as shown in mice (Classen and Shevach, 1991) and this could in turn lead to autoimmune diseases in adulthood. It was also shown in rabbits that CsA exposure during fetal life induces nephron deficit (Tendron-Franzin *et al.*, 2004). There have also been some concerns of the sperm quality after CsA treatment. It has been shown that male rats given CsA during aldoscence show lower fertility (Srinivas *et al.*, 1998) and changes in sperm morphology (Masuda *et al.*, 2003).

When exposed directly to CsA (ME) there was a decrease in implantation rate and also a decrease in number of live fetuses, which both correlated with CsA dose. This decrease could not be found in the female exposed to CsA in uteri (IUE) and neither any differences in the litter size and weight of pups fathered by male IUE mice. Similar studies in the 2nd generation have not been performed previously. In a study with higher doses of CsA (50 mg/kg) given on gestation day 12 in two different mouse strains an increase in incidence of the intra uterine death of fetus (Gasser *et al.*, 1992). In a study utilizing the rat as the experimental animal CsA (25mg/kg) was given during different development windows during gestation and it was found that administration during the first week resulted as mentioned previously in a reduction in litter size and when the rats were exposed later in the pregnancy (day 8 to 14) an increased rate of fetal resorption was seen (Brown *et al.*, 1985). All these findings suggest that there are some direct embryotoxic effects of CsA and that the toxicity is concentration dependent since the implantation rate decreased from 9 with 10mg/kg*day CsA to 7 respectively 6 with higher concentration (20 and 30 mg/kg*day) (paper V). The fetal toxicity was even more obvious since the resorption rate increase 3 and 5 fold when exposed directly to CsA (ME) but not when exposed intrauterine (IUE) (paper V).

There are concerns raised about negative CsA effects on the immune system at intra uterine exposure (Cimaz *et al.*, 2004). In a case report it was described that a transplanted female treated with azathioprine and prednisolone gave birth to a daughter who as an adult developed systemic lupus erythematosus (SLE), went through several miscarriages and developed pre-eclampsia during pregnancy (Scott *et al.*, 2002). Others have on the other hand failed to find any immunological differences in children that had been exposed to immunosuppressants in utero when the children were studied up to an age 12 months (Motta *et al.*, 2007).

Findings of the present study (paper V) was the difference in weight trajectory and that there was a significant weight reduction of the fetus from female mice exposed to CsA in uteri (IUE). It is impossible to know if the low fetus weight depends on intra uterine growth reduction or due to small for gestational age. It most be remembered that these female animals was not exposed to CsA after birth so all difference must be at random or due to the intrauterine exposure. A plausible explanation for the weight reduction in fetuses could be that the pre-pregnancy weight of the mother and fetal weight is linked together since it is shown that pre-pregnant body weight in human correlate to birth weight (Frederick *et al.*, 2008). Calcineurin is a ubiquitous phosphatase involved in many processes in the organism including muscle growth and the reduction in adolescent growth of female mice exposed to CsA in utero can possibly be linked to CsA exposure. A study on calcineurin Aα-/-, and Aß-/- mice found that

although the relation between bone length and muscle weight was not changed, the overall body weight was reduced (Parsons *et al.*, 2003). However, the mechanism behind this effect by calcineurin suppression remains to be investigated.

The normal serum creatinine levels in the mouse differ between different strains of mouse so that normal levels between 6 and 67 micromol/l have been reported (Meneton *et al.*, 2000). During a normal pregnancy the level of creatinine decreases in humans (Wichman and Ryden, 1986) and this is also the case for transplanted women (Fischer *et al.*, 2005). Our present study did not show any differences in serum creatinine between the treatment groups or the vehicle group both in the ME experiment and the IUE experiment. However, the females in the IUE experiment carried fetuses that were slightly but significantly smaller than those of vehicle-exposed females. Considering the well-documented nephrotoxic effect of CsA, also in mice (Masri *et al.*, 1988) it could be suspected that physiological changes related to impairment of kidney function, such as hypertension, also could be the underlying cause of the low fetal weight. However, neither serum creatinine concentrations nor analysis of kidney morphology indicates CsA induced impairment of kidney function. Nevertheless, it cannot be ruled out that subtle functional renal changes influencing body growth were present.

Conclusion

In mice, direct exposure to high doses of CsA has negative effects on reproductive performance and pregnancy outcome. Intrauterine exposure to CsA reduces growth rate in young mice but it does not alter mating behavior, implantation rate and fetal survival. A decrease in fetal weight in female mice exposed to CsA in uteri is observed: This may be related to the smaller body weight of these animals. No alterations in the kidney morphology or the serum creatinine levels were found that indicated kidney damage by CsA.

Concluding remarks

A major difficulty and concern in relation to uterus transplantation have been the elaborate surgery which is involved when long vascular pedicles have to be obtained deep in the pelvis with its prominent and convoluted vascular system. This is especially true for the venous side of the uterine vascular system. During recent years models that have successfully auto- or syngenically transplanted the uterus in several animal species have been brought forward. When this surgical development also has reached the stage of non-human primates it seems that the issue concerning suitable immunosuppression at uterus allo-transplantation will be the main focus of this research field. The theme of this thesis is to study rejection of a uterine allograft and to do initial studies of immunosuppressants to suppress uterine rejection and to look at the issue of safety and fertility potential of these drugs. A major concern is thus to make sure that the immunosuppressants used after transplantation surgery is the most effective and as nontoxic for the recipient and the fetus/future child. Results from studies in this thesis (paper I, II, III and V) together with other studies in rat (Jiga *et al.*, 2003), sheep (Ramirez *et al.*, 2008), pig (Avison *et al.*, 2009) could be the basis for a classification system in grading rejection after uterus transplantation. There are developed grading systems for rejection of other organs such as the Bannf-system for the kidney (Solez *et al.*, 1993). In higher animal species and in the human it would by easier to receive samples for grading of rejection by endometrial sampling, cervical biopsies or also needle biopsies through the vaginal fornix to obtain myometrial biopsies. It is of course problematic to properly diagnose uterine rejection during pregnancy since endometrial samples cannot be obtained and given that there is increased risk of bleeding when a cervical biopsy is taken. In paper III it was clearly shown that there is a correlation between the composition of leukocyte subsets in the myometrium and the endometrium. This knowledge could be used when a pregnant female is monitored so that if there are indirect signs of rejection a myometrial biopsy could be taken by a transvaginal or transvesical approach. This would most likely not interfere with the pregnancy in uteri.

The issue concerning which immuno-suppressive drug would be recommended after uterus transplantation remains to be elucidated. This thesis has not specifically investigated that issue. The pharmacological agent CsA is a well-known and widely used immunosuppressor and so far it has not been demonstrated to exert any teratogenic effect. However, all the accumulated data in this regard is from data registries and case series, with the possibility of selection concerning what data are reported to the registries and what case series are presented. It can however be concluded that the risk is not considerably higher than in the normal population, with the normal influence of infections, systemic diseases, pollutants and pharmaceuticals which may be teratogenic. The well described side effects of CsA when used during a long time are the effects on the kidney and also that viral infections and certain malignancies can emerge. In the research field of transplantation immuno-

logy today there is much focus on induction of tolerance and the role of the T-reg cells in that process (Zelenika *et al.*, 2001). In any case of transplantation, and especially if the patient will carry the transplanted organ the rest of the life, it is important to minimize the intake of immunosuppressive drugs. Several studies are done in this field and it seems that the liver is the organ (Di Cocco *et al.*, 2009; Donckier *et al.*, 2009; Wallgren *et al.*, 2006), which after some time needs the lowest doses of immunosuppressants and in some cases a natural tolerance can be developed so that the liver recipient can be weaned off from immunosuppressive agents. The use of CsA and tacrolimus prevents this tolerance due to its mechanism of action since T-reg depend on IL-2 for their function (Miroux *et al.*, 2009) and that rapamycin do not affect the T-reg (Segundo *et al.*, 2006) since it disturb the cell cycle and altogether it seems that rapamycin is a better drug to use if tolerance is desirable (Wekerle, 2008; Donckier *et al.*, 2009). On the other hand the goal with uterus transplantation is to produce one or possibly to normal pregnancies in the recipient and after delivery, preferably by cesarean section the uterus will be removed. Thus the time span for the transplant is relatively short so the goal would not be to induce tolerance by rapamycin or other drugs after transplantation but rather to induce tolerance to the organ before transplantation. Research along these lines are carried out in experimental animal models but several still include irradiation of the bone marrow of the recipient (Sachs, 2000) which would of course be detrimental to the ovaries of a uterus recipient. It should also be mentioned that there exist very few reports on rapamycin-exposed pregnancies and the risks for the fetus can not yet be estimated (Armenti *et al.*, 2004). The uterus has the unique ability to harbor a semi-allogenic and sometimes an allo-allogenic implant during 40 weeks when a female is pregnant. It is possible that in a future uterus transplantation situation that it would be possible to expand this inherent mechanism of immune suppression during pregnancy so that the use of immunosuppressive drugs could be minimized during pregnancy. The natural pregnancy-related immunosuppression then has to include all layers of the uterus, the cervix and the uterine vessels. A difficulty with an approach that could allow lowering of the intake of immunosuppressing drugs during pregnancy is that it would be difficult to estimate the doses of for example CsA that are need to prevent rejection. In a recent study (Fischer *et al.*, 2005) there was a need for CsA to be elevated during pregnancy to maintain the therapeutic window and in paper IV the concentration of CsA decreased in blood during pregnancy.

In our studies in rodents of uterine rejection and immunosuppression it was shown that the rejection process is somewhat inhibited but not abolished by the concentrations of CsA (paper II and IV) used. Importantly, the fertility potential of the offspring was not altered (paper IV). However, it should be pointed out that the fertility potential is just one basic aspects of the physiology of the offspring and the normality of this does not rule out other influences by CsA. However it is important that the three large registries of pregnancies in transplant patients (the European Dialysis and Transplantation Association Registry, the UK Transplantation Pregnancy Registry and the National Transplantation Pregnancy Registry) keep and complete population-based studies such as the recent Swedish study (Kallen *et al.*, 2005) and also follow the children born,

concerning suggested risks such as development of renal failure, auto-immune diseases and future pregnancy complications. This is of course data that would not only apply to a situation of uterus transplantation but to all female patients of fertile age that has received an organ transplant and that would plan a pregnancy.

Swedish summary

Transplantation av livmodern utvecklas idag som en möjlig metod att behandla de kvinnor som har en infertilitet där orsaken beror på avsaknad av eller defekt livmoder (livmoderfaktor infertilitet). Kvinnor med Mayer-Rokitansky-Küster-Hausser (MRKH) syndrom, kvinnor som opererat bort sin livmoder p.g.a. godartad eller malign orsak eller kvinnor med sammanväxningar i livmoderhålan eller som har stora icke operabla myom är de stora patientgrupperna som kan främjas av livmodertransplantation. Det har gjorts ett försök tidigare att transplantera en livmoder på människa, vilket dessvärre misslyckades. De senaste åren har det bedrivits forskning inom livmodertransplantation och ett flertal djurmodeller har utvecklats för att studera olika aspekter inom livmodertransplantation och för att optimera metoden inför användande på människa.
Målsättningen med denna avhandling var att undersöka avstötningsprocessen efter allogen livmodertransplantation i djur-modeller, i mus samt råtta. Därtill har effekten av det mest använda immuno-suppressionsläkemedlet, cyklosporin A (CsA), studerats i en semiallogen, samt allogen modell. Vidare har CsA:s effekt på fortplantning i två generationer studerats.

I en helt allogen model, där livmoder transplanterades mellan två olika raser av mus (BALB/C givare och C57Bl/6 mottagare), studerades blodflöde, makro-skopiska och mikroskopiska förändringar för att erhålla en bakgrundskunskap om avstötning av transplanterad livmoder för att ha som bas inför framtida experiment. Mikroskopiska tecken på avstötning uppträdde efter fem dagar samtidigt med något nedsatt blodflöde och mindre inflammatoriska förändringar. Det nedsatta blodflödet var sämre vid samtliga under-sökningstillfällen under experimenttiden på 28 dagar. De makroskopiska tecknen på avstötning var initialt svullnad av transplantatet och senare genomgick livmodern förändring av konsistens till fastare och färgen ljusnade.

I en uppföljande studie studerades inflödet av några specifika vita blodkroppar i den transplanterade livmodern. Mängden vita blodkroppar i transplanterad livmoder jämfördes med den i icke transplanterad livmoder kvar i muskroppen 2,5 och 10 dagar efter transplantationen. Vi fann ett tidigt inflöde av makrofager redan dag 2 i livmodermuskeln och dag 5 i livmoder-slemhinnan. I den här tidiga ökningen av celler var även neutrofiler närvarande. Det skedde en ökning av CD8+ T-lymfocyterna från dag 5 i både muskel och slemhinna i transplanterad livmoder men det var en ringa ökning av CD4+ T-lymfocyterna., Det fanns ingen ökning av B-celler. Studien visar ett ökat inflöde av celler, framför allt neutrofiler, makrofager och T-celler, i en allogen musmodell dag 2-5 efter transplantation. Resultat från denna studie kan ha implikationer till vilket val av läkemedel man kan välja för att förhindra avstötning av transplanterad livmoder.

I en semiallogen muslivmodertransplan-tationsmodell användes två olika doser av CsA. I den icke behandlade musgruppen

skedde en utpräglad inflammation som följdes av nekros tydande på avstötning. I de CsA-behandlade grupperna var denna avstötning mycket mildare beroende på dos. Vi fann dock att de CD8+ T-lymfocyterna var högre i de grupper som erhöll CsA. Det tycks som om CsA dämpar avstötningen men ej hämmar den helt om den används i dessa doser som singelterapi och att det krävs kombinationsbehandling för att hämma avstötningen helt.

CsA användes även i en allogen livmodertransplanterad råttmodel (Brown Norway som givare och Lewis som mottagare). Livmodern transplanterades här till ortotop plats och råttorna erhöll 10 mg/kg CsA dagligen. En markant inflammation kunde ses hos de icke CsA behandlade råttorna men endast en mindre inflammation kunde ses hos de CsA-behandlade. Real-tids PCR användes för utvärdering av mRNA-nivåer av vissa proteiner som kan ha en roll både i avstötning och reproduktion. Nivåerna av mRNA hos interleukin-1α (IL-1α) var sänkt i den grupp som behandlats med CsA, medan mRNA-nivån av galectin-1 var förhöjd i samma grupp. Resultatet visar att CsA minskar graden av avstötning i denna råttmodell av transplanterad livmoder och att denna minskning också kunde ses i mängden mRNA för vissa nyckel-mediatorer.

CsA är det immunonedtryckande läkemedel som används mest på kvinnor i fertil ålder. Studien utformades så att man kunde undersöka om maternell eller i livmodern exponering av CsA påverkar den reproduktiva förmågan av moder eller avkomma hos mus. Honmöss erhöll 3 olika doser av CsA via en pump som applicerades strax före befruktning och denna behandling avslutades i och med förlossningen. Det visade sig att höga doser CsA sänker implantationsfrekvensen och embryo/foster-överlevnaden, samt sänker tillväxten under aldoscensen av avkomman. Avkommans fertilitet både kvinnlig och manlig var dock ej påverkad. Studien visade dock reducerad fostervikt hos de honor som exponerats för CsA i livmodern. Denna studie indikerar att höga doser CsA påverkar graviditet och avkomma. Uppföljande studier inom detta, inkluderat andra arter, krävs.

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Live long and prosper

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