I give permission for public access to my thesis and for copying to be done at the discretion of the archives' librarian and/or the College library.

_____ _____

Signature Date

Macrophage Phenotype Differentiation in the Rat Uterus during Normal and Diabetic Pregnancies

By

Eleni Stampouloglou

A Paper Presented to the

Faculty of Mount Holyoke College in

Partial Fulfillment of the Requirements for

The Degree of Bachelors of Arts with

Honor

Department of Biological Sciences

South Hadley, MA 01075

May, 2011

This paper was prepared

under the direction of

Professor Sarah J. Bacon

for eight credits.

ACKNOWLEDGEMENTS

I want to thank my thesis advisor, Professor Sarah Bacon, whose expertise and guidance helped me complete this project. Professor Bacon was as zealous as I was throughout the course of this past year; she was a great source of inspiration and motivation. She is the person who more than anyone helped me shape the ways I think about science and the scientific method today.

I also want to thank Professor Gary Gillis for personal support, thoughtful critiques and assistance throughout the process.

Many thanks to the third member of my committee, Professor Karen Hollis, who has shown interest and enthusiasm towards my work.

Special thanks to Sue Lancelle for answering critical questions for the development of a methodology for this study.

Part of the reading process for this thesis was made most enjoyable thanks to Cassidy Bommer and Ge Wang, with whom I had constructive conversations on relevant literature.

I would also like to express my gratitude to my advisor, Professor Jeff Knight, and the Biology Department.

Finally, my heartfelt appreciation goes out to my family and friends, for their emotional support and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Macrophages are the most common type of immune cell in the pregnant uterus. Depending on the tissue microenvironment, macrophages assume one of two broad phenotypes. In general, M1 macrophages destroy extracellular pathogens and present their antigens to cells of the innate immune system. When all invaders have been destroyed, M2 macrophages remove apoptotic cells, remodel tissue, and suppress further inflammation. But which phenotypes predominate at different stages of pregnancy? In this study, macrophage phenotype in the uterus in normal pregnancy was characterized using a rat model. The ratio of M1 to M2 macrophages was estimated at two crucial stages of pregnancy (the first day after mating, and the twelfth day of pregnancy, which is a critical period for embryonic organogenesis) using immunohistochemical labelling. Immunohistochemical staining suggested that M1 macrophages predominate in the uterus right after mating. M2 macrophages are in the minority in the postmating uterus, but predominate on day 12 at the close of the period of organogenesis. This may be because factors in semen induce a transient inflammatory response that is suppressed before embryos enter the uterine horn. So while mating is an M1 phenomenon, during trophoblast invasion, vascular remodeling, implantation and placentation, M2 macrophages prevail. Their role may be to suppress inflammation, to help in vascular remodeling and to clean up apoptotic cells. In pregnancies complicated by diabetes, embryonic abnormalities are common, possibly because the balance of the controls of macrophage phenotype is disturbed. Maternal metabolism was perturbed by injecting pregnant rats with streptozotocin (STZ, induces Type-I diabetes), and its effect on pro-inflammatory macrophages during the period of embryonic organogenesis was studied. Immunohistochemical labeling revealed an increase in M1 levels for the STZ treated dams. Products secreted by M1 macrophages during organogenesis in diabetic pregnancies have been shown to decrease teratogenesis, hence the role of M1 macrophages in diabetic pregnancies could be an ameliorating one.

INTRODUCTION

Introductory Note

Mammalian pregnancy is a physiological context in which cells display a wide range of functions that would be strange or even unhealthy in the mature tissues of the adult body. Epithelial cells peel themselves off of their basement membrane and migrate (Abrahamsohn and Zorn, 1993). Apoptosis of healthy cells is both widespread and normal (Straszewski-Chavez *et al*, 2005). Genetically foreign tissue is allowed to invade deep into the healthy uterus, even into maternal blood vessels (Goldman-Wohl and Yagel, 2002; Vercruysse *et al*, 2006). Unequal expression of maternally and paternally-inherited genetic material is widespread. And the mother's immune cells, upon leaving the blood stream and migrating within the pregnant uterus, also behave in an unexpected manner (Moffett and Loke, 2004). Immune cells associated with clearing pathogens can display a wide range of functions in the uterus: clearing apoptotic cells, angiogenesis, vascular and tissue remodeling, embryo attachment and implantation (Abrahams *et al*, 2006; Nagamatsu and Schust, 2010a). Those unexpected activities are linked to the success of pregnancy. Indeed when immune cells in the pregnant uterus display the more expected canonical functions of clearing pathogens, presenting antigens and contributing to inflammation, that is often when pregnancy is the most at risk (Nagamatsu and Schust, 2010b). In this thesis I developed a method to investigate the phenotype,

and phenotypic shifts, in one uterine immune cell type, the macrophage, during pregnancy. In addition, I ascertained whether macrophage phenotype is perturbed in a rat model of diabetic pregnancy. Understanding how macrophages work in pregnancy complicated by diabetes seems to be an important task, given the detrimental effect the disease has on embryos and the already established significance of macrophages for a successful pregnancy.

Macrophage Definition and Phenotypes: M1 and M2

Macrophages are immune effector, phagocytic cells that are generally involved in erythrocyte clearance, in the removal of cellular debris that is generated during tissue remodeling, and in clearance of apoptotic cells. They show remarkable phenotypic plasticity and they change their phenotype in response to signals from their microenvironment (Mosser and Edwards, 2008). There is a wide spectrum of possible forms of macrophage activation, the extremes of which are called M1 and M2 (Mantovani *et al*, 2004). M1 macrophages are pro-inflammatory. Macrophages can adopt the pro-inflammatory M1 phenotype in response to the presence of cytokines like INF-γ or TNF. These cytokines are produced in response to intracellular pathogens or elevated apoptosis and inefficient cleaning of apoptotic cells. The “educated”, polarized M1 macrophage will in turn produce superoxide anions, oxygen and nitrogen radicals and TNF, and pro-inflammatory cytokines such as IL-12, IL-1 and IL-6 to promote inflammation and killing of intracellular pathogens (Mills *et al*, 2000;

Mantovani *et al*, 2004; Mosser and Edwards, 2008; Classen *et al*, 2009). M2 macrophages, in contrast, have an immunomodulatory or tissue remodeling function. Extracellular pathogens, stress, presence of apoptotic cells or being at a later stage of an immune response and needing to limit inflammation will cause the production of cytokines like IL-4, IL-13 and IL-10 which will polarize tissue macrophages towards an M2 phenotype. The M2 macrophage will produce even more IL-10 and TGF-β for tissue repair/remodeling, wound healing, anti-inflammatory activity and suppression/down-regulation of inflammatory cytokine production, promotion of angiogenesis and scavenging of debris (Mills *et al*, 2000; Mantovani *et al*, 2004; Mosser and Edwards, 2008; Classen *et al*, 2009).

Macrophages appear to play a role in (a) the immunological tolerance of the semiallogeneic fetus, (b) promotion of implantation, (c) clearance of apoptotic cells during tissue remodeling, (d) promotion of angiogenesis and maintenance of pregnancy (Abrahams *et al*, 2006; Niederkorn, 2006; Nagamatsu and Schust, 2010a). In the next section, the roles of macrophages in these functions are discussed in greater detail.

Macrophages and Immune Tolerance of the Fetus

Mating and pregnancy introduce the mother to a semiallograft, since half of the fetal genes are derived from the father (Trowsdale and Betz, 2006). It is remarkable that the fetus is not rejected by the mother, since it presents paternal antigens that can elicit an immune response. The fetus is called semiallogeneic

since it confronts the maternal immune system with paternal alloantigens, antigens that are part of the paternal self recognition system and which should be recognized as non-self by the mother. Acceptance of the semiallograft is possible, not as a result of immune unawareness, but through tight control of the maternal immune system and by immune suppression (Robertson *et al*, 2002). Macrophages, which can be one of the first cells to encounter alloantigens, can be involved in the instigation of inflammatory responses as well as the induction of tolerance (Nagamatsu and Schust, 2010a, b).

After insemination, a post-mating inflammatory response is observed in the uterus of mice and rats (Robertson, 2005). Seminal transforming growth factor-β (TGF-β) interacts with epithelial cells in the female's cervix and uterus to induce production of pro-inflammatory cytokines like granulocyte-macrophage colony-stimulation factor (GM-CSF), IL-6 and other chemokines (Robertson, 2005). In turn, these cell-signaling molecules recruit cells of the immune system. Dendritic cells and macrophages are the main populations recruited into uterine tissue after mating (Robertson, 2005). They phagocytose, process and present seminal antigens on their surface causing the activation of immune responses against antigens in semen and other paternal antigens, thus eliciting what is termed the "post-mating inflammatory response" (Robertson, 2005). However, immune responses against seminal antigens are detrimental to sperm tolerance and pregnancy. Surprisingly, the immune activation triggered by semen does not cause the rejection of male antigens, because of the presence, in seminal plasma,

of TGF-β, which is a powerful immune deviating agent (Robertson *et al*, 2002; Robertson, 2005). TGF-β helps in inducing tolerance by initially triggering inflammation and resolving inflammation right afterwards (since inflammation can be detrimental to tissue) (Robertson *et al*, 2002). Resolution of inflammation could be achieved by a reduction in sensitivity of macrophages to TGF-β after activation (Robertson *et al*, 2002). A refractory state arises, where pro-inflammatory cytokine production is suppressed and deactivation occurs (Robertson *et al*, 2002).

In addition, macrophages in the placenta and decidua have been shown to express several members of the B7 family ligands (Nagamatsu and Schust, 2010a, b; Petroff and Perchellet, 2010). These ligands participate in immune responses by transmitting a costimulatory or coinhibitory signal that will either activate or inactivate T-cells (Collins *et al*, 2005). At the maternal-fetal interface, the B7-H1 and B7-DC inhibitory receptors are involved in tolerance, by binding to programmed death-1 (PD-1) receptor on T cells, causing T cell apoptosis or anergy (Nagamatsu and Schust, 2010a, b; Petroff and Perchellet, 2010).

The inflammatory response is transient and stops by the time of embryo implantation (Robertson, 1998). After the "immune privilege" of the fetus has been established, the immune system becomes involved in different functions: promotion of implantation, clearance of apoptotic cells during tissue remodeling, promotion of angiogenesis and maintenance of pregnancy (Abrahams *et al*, 2006; Niederkorn, 2006; Nagamatsu and Schust, 2010a). One could expect macrophage

phenotype to shift as the necessary functions of the immune system shift in pregnancy.

Pregnancies Complicated By Diabetes

Diabetes mellitus as a maternal illness results in major congenital malformations at a rate of 6-10% among infants of diabetic mothers, and accounts for 40% of all perinatal deaths among offspring of diabetic mothers (Eriksson et al, 1996). Maternal hyperglycemia (high blood sugar) is likely the major contributor to congenital malformations (Reece and Eriksson, 1996). Further, research indicates that there is a correlation between hyperglycemia, the teratogenicity of diabetes and distorted maternal immune responses (Savion *et al*, 2004). For instance, immune stimulation of diabetic mice has been shown to ameliorate some of the disease's teratogenic effects (Savion *et al*, 2004; Punareewattana and Holladay, 2004).

In pregnancies complicated by diabetes, as well as by other problems such as preeclampsia and pre-term delivery, disturbances in the tight control of macrophages are observed (Savion *et al*, 2004; Nagamatsu and Schust, 2010a, b). For example, TNF-α (a pro-inflammatory cytokine produced by M1 macrophages) production is enhanced during diabetic pregnancies (Pampfer *et al*, 1995; Mantovani *et al*, 2004). TNF-α production is enhanced and IL-10 production is suppressed in maternal peripheral blood in the placentas of women with preeclampsia. Preeclampsia is a common pregnancy complication that seems

to be initiated by poor trophoblast invasion, and results in high blood pressure that could result in maternal death and fetal demise (Redman and Sargent, 2005). It has been hypothesized that since both TNF-α and IL-10 can be produced by macrophages, macrophages may be involved in the pathogenic pathways of preeclampsia (Nagamatsu and Schust, 2010b). In this study, the focus is on the ways in which macrophages could be involved in pathogenesis in pregnancies complicated by diabetes.

Reactive Oxygen Species Might Influence Diabetic Embryopathy

Elevated blood glucose is the major contributor to congenital malformations in diabetic pregnancy (Reece and Eriksson, 1996). For example, it has been shown that in a mouse model of diabetic pregnancy, high glucose levels increase neural tube apoptosis in the embryo which leads to neural tube defects and malformations (Fine *et al*, 1999). Due to increased glucose utilization, increased oxidative metabolism, and immaturity of the embryo's enzymatic system for managing ROS (its antioxidants), free radicals are elevated (Reece and Eriksson, 1996; Fine *et al*, 1999; Myatt and Cui, 2004; Ornoy, 2007). Free radicals also damage cell membranes by causing lipid peroxidation (Ornoy, 2007). Since glucose is present in excessive amounts, excess molecules cross the mitochondrial membrane freely and overwhelm the still immature set of embryonic mitochondrial scavenging enzymes, which results in an excess of free oxygen radicals (Reece and Eriksson, 1996). This imbalance observed in embryos

of diabetic rats and in embryos cultured in high glucose is thought to contribute to diabetic embryopathy. Treatment with antioxidants and free radical scavenging enzymes reduce the effects of diabetic embryopathy (Reece and Eriksson, 1996; Fine *et al*, 1999; Ornoy, 2007). It has been proposed that the primary target of reactive oxygen species (ROS) resulting from disturbed glucose homeostasis is the yolk sac, the extra-embryonic membrane that sustains the developing embryo by providing nutrients though vessels (Reece *et al*, 1994). Due to free oxygen radicals, the yolk sac fails to form vessels and this vascular insufficiency causes embryo asphyxia and failure (Reece *et al*, 1994; Zhao and Reece, 2005). In addition to their initial effect on yolk sac, elevated free oxygen radicals could influence transcription factors and alter their abilities to regulate transcription, leading to further complications, such as elevated and uncontrollable apoptosis (Fine *et al*, 1999).

TNF-α: Role in Hyperglycemia induced Oxidative Stress and in Suppressing Diabetes-Induced Apoptosis

Tumor necrosis factor-alpha (TNFα) is one of the key molecules involved in the hyperglycemia-induced increase in the production of ROS: its expression is regulated by ROS and it regulates ROS production (Torchinsky and Toder, 2008). TNFα production is enhanced during diabetic pregnancies (Pampfer *et al*, 1995). Through an extensive review of literature and their own studies, Torchinsky and Toder (2008) postulate that TNFα can regulate the response of pre- and

postimplantation stage embryos to diabetes-induced catastrophic stimuli differently, depending on the stage of gestation. At the preimplanation and peri-implantation stages, TNFα has been linked to excessive apoptosis levels in the uterus, which lead to inhibition of implantation and pregnancy loss in diabetic pregnancies (Pampfer *et al*, 1995; Kawamura *et al*, 2007; Torchinsky and Toder, 2008). In the post-implantation stage of diabetic pregnancies, TNFα has been found to decrease the high apoptosis levels caused by the presence of ROS, leading to a lower degree of teratogenicity and thus having favorable effects (Torchinsky and Toder, 2008).

Excessive levels of apoptosis in diabetic pregnancies have been associated with increased ROS production, decreased activities of antioxidant enzymes and vitamins C and E, defects in the brains and yolk sacs of embryos as well as failure of the neural tube to close (Zhao and Reece, 2005; Ornoy, 2007; Torchinsky and Toder, 2008). In addition, TNFα (M1 macrophage product) synthesis is upregulated in the uterus of the pregnant diabetic rat and it has also been linked to excessive apoptosis levels prior to implantation (Pampfer *et al*, 1995; Pampfer *et al*, 1997; Kawamura *et al*, 2007; Torchinsky and Toder, 2008). However, after implantation it has been found that apoptosis levels in the brain of TNFα-/- embryos were higher than in the brain of their TNFα-positive counterparts (Torchinsky and Toder, 2008), suggesting that after implantation, TNFα (and therefore the M1 macrophages that produce it) might help in decreasing the severity of diabetes-induced malformations. It is thought that TNFα suppresses

apoptosis caused by diabetes by activating the NF-κB pathway, which inhibits apoptosis in the organogenesis stage (Torchinsky and Toder, 2008). Teratogens, like ROS, suppress the NF-κB pathway, which could be one mechanism by which teratogens increase apoptosis levels in embryonic structures (Fine *et al*, 1999; Torchinsky and Toder, 2008). TNFα could be corrective after implantation by rescuing the NF-κB pathway and thus suppressing teratogenic apoptosis (Torchinsky and Toder, 2008).

Diabetes teratogenicity has also been correlated with distorted maternal immune responses, such as alteration of the cytokine environment close to the embryo throughout pregnancy (Savion *et al*, 2004). Cells expressing macrophage and T cell subset markers in the spleen and lymph nodes tend to decrease in diabetic females (Savion *et al*, 2004). When the maternal immune system was strengthened by a splenocyte injection before mating or when the immune system was activated prior to hyperglycemia, embryonic resistance to the teratogenic effect of diabetes increased (Savion *et al*, 2004; Punareewattana and Holladay, 2004). Immunopotentiation normalized the level of immune effector cells present in the uterus and lymphoid organs of diabetic females, and made embryos resistant to hyperglycemia's teratogenic effects. These altered levels of immune effector cells could be the cause of the observed protective effect on embryos (Savion *et al*, 2004).

Purpose of Study

As explained above, there is evidence that macrophages are important in pregnancy in many ways, that their phenotype shifts during normal pregnancy and that those shifts are perturbed in abnormal pregnancies (pre-eclampsia, pre-term delivery). Diabetic pregnancies very commonly result in embryopathy, possibly through disordered cytokine signaling that could be related to altered macrophage numbers or macrophages polarizing away from their proper phenotype.

The first goal of this study was to develop a methodology that would allow me to assign macrophages to a specific phenotype and then to be able to quantify them. The second goal was to create a profile of macrophage activation and phenotype during two very different stages of normal pregnancy (post-mating and the period of organogenesis) and then test whether this profile of macrophage activation changed or was perturbed during diabetic pregnancies.

To achieve the second goal, I first determined macrophage phenotype in the uterus right after mating. The macrophage population present in the uterus post-mating has already been very well described. The descriptions match the M1 phenotype, but, to my knowledge, no study has used this term before. Hence, in this study, confirmation of the M1 phenotype after mating was attempted. Second, the degree of macrophage activation in the uteri of normal and diabetic female rats later in pregnancy, at the period of organogenesis, was determined. As explained above, TNF-α levels increase in diabetic pregnancies. Finally, I determined the phenotype of macrophages towards the end of organogenesis for

diabetic females. It was expected that in diabetic pregnancies, elevated M1 levels would be observed, as TNF-α is a product of M1 macrophages.

Model for Studying Diabetic Embryopathy

Maternal diabetes can be modeled in rats by injection of streptozotocin (STZ). STZ kills β cells of the pancreatic islets and impairs glucose oxidation by decreasing insulin biosynthesis (Szkidelski, 2001). In this model, the pregnant rats are given an intravenous or an intraperitoneal injection of STZ on day 6 of gestation and embryos are collected on day 12 for analysis (Reece *et al*, 1996).

The rat gestation period is 21-23 days long and organogenesis begins on day 9 and ends at around day 13 (Sharp and La Regina, 1998; Reece *et al*, 2006). Embryo implantation occurs on day 6 (Kaufman and Bard, 1999). Hence, day 6 is chosen as the day of STZ treatment, because injecting the rats prior to conception and implantation may cause infertility (Reece *et al*, 1996). In addition, STZ has a very short half life, so the effect we expect to observe later in the course of pregnancy will not likely be due to the pharmacological effect of STZ itself (Reece *et al*, 1996). By day 12, the basic structure of the circulatory, urogenital, skeletal and nervous systems is established. The components of four branchial arches are present, the otic vesicle has separated from the ectoderm, the olfactory and lens placodes have formed, the hindlimb and forelimb buds are present, the liver, kidneys and heart are under development and the caudal neuropore has closed (Kaufman and Bard, 1999). Thus, day 12 is chosen for embryo collection

since it is close to the end of organogenesis and STZ (and as a result, diabetes) has exerted its effects on organogenesis and development.

Anatomy of the Pregnant Rat Uterus and Focus for this Study

The two anatomical sites where uterine macrophages are found, and are thus areas of interest in this study, are the metrial gland and the decidua. A schematic representation of the pregnant rat uterus is shown in Figure 1 (figure modified from Soares *et al*, 1985). A layer of labyrinth tissue and trophoblast cells belonging to the embryo separate/connect the embryo from/to the decidua, which is the part of the placenta that belongs to the mother. The trophoblast sticks to the uterus and starts the placentation process (Goldman-Wohl and Yagel, 2002). The trophoblast migrates through and invades the uterine tissue and attaches the placenta to the uterus, specifically to the decidua (Goldman-Wohl and Yagel, 2002; Vercruysse *et al*, 2006). The metrial gland is a structure that is located in the mesometrial triangle (area between circular and longitudinal uterine muscle of the pregnant rat uterus) (Picut *et al*, 2009). It consists of a variety of cells, the majority of which are granulated metrial gland (GMG) cells (Picut *et al*, 2009). GMG cells are bone-marrow-derived leukocytes that proliferate in the pregnant uterus (Stewart, 1991). It is still unclear what the specific functions of GMG cells are (Allen and Nilsen-Hamilton, 1998). Some of the important functions they may serve are control of trophoblast invasion, lysis of virus-infected cells in the uterus and placenta, initiation of abortion, and cytokine production (Allen and Nilsen-

Hamilton, 1998). The point at which the trophoblast giant cells break (Figure 1) is the point where the trophoblast invades the decidua, it is the implantation site and the metrial gland is located in the mesometrium above the implantation site (Peel, 1989).

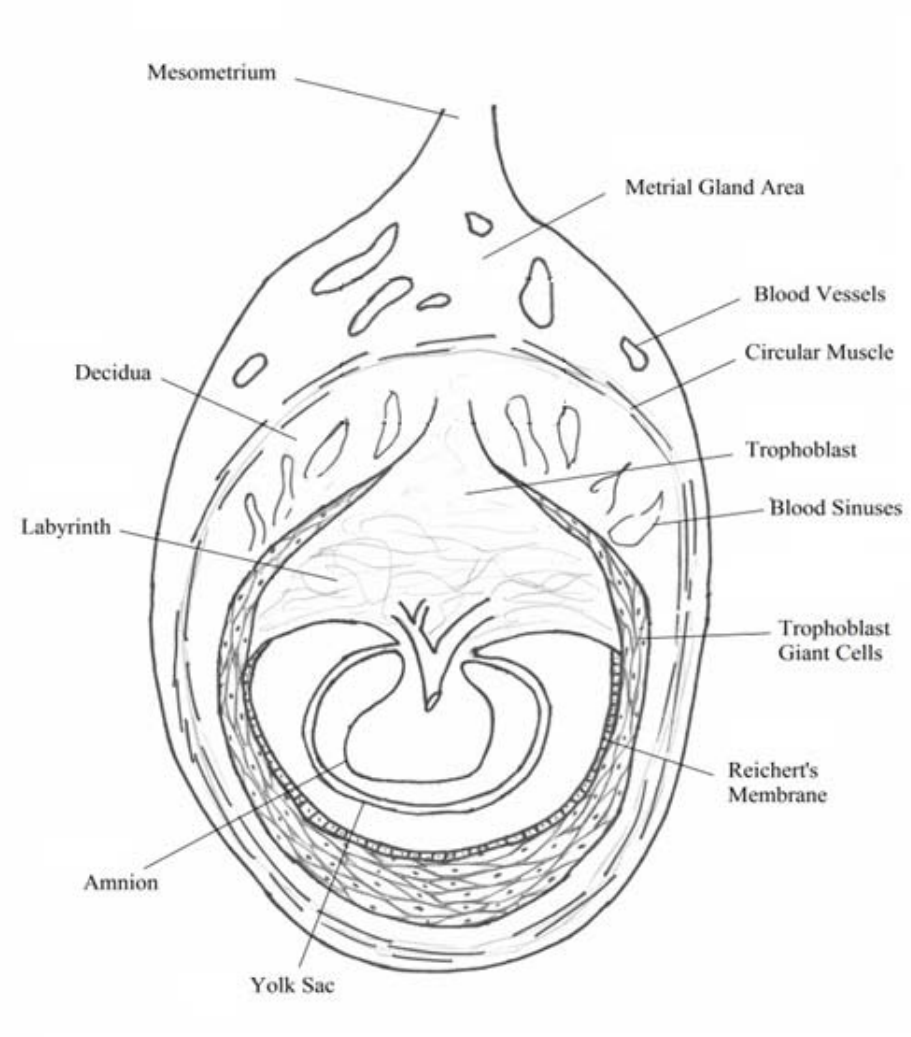

Figure 1: Schematic representation of the pregnant rat conceptus on day 12 (modified from Soares *et al*, 1985).

In this study, a methodology will be developed to allow for macrophage phenotype characterization and quantification. A profile of macrophage activation and phenotype at two different stages of normal pregnancy (post-mating and at the end of organogenesis) will be created. Macrophage phenotype and differentiation will also be characterized in the diabetic pregnant rat uterus, more specifically in the decidua and metrial gland areas towards the end of organogenesis, using a rat STZ model. It is hypothesized that macrophage phenotype in the uterus right after mating will be of the M1 phenotype. The macrophage phenotype during the period of organogenesis in metrial glands of normal pregnancies is hypothesized to be M2. In diabetic pregnancies, a shift from M2 to M1 phenotype is expected to be observed.

MATERIALS AND METHODS

Overview

Macrophage density and phenotype were quantified in female Norway rats (*Rattus norvegicus*) under three different experimental conditions: post-mating inflammatory response on gestation day 0 in the uterus, normal uterus on day 12 and diabetic uterus on day 12, late in the period of embryonic organogenesis. Two female rats were selected from each condition for analysis. Specimens for the day 0 and day 12 normal conditions were readily available in the laboratory from previous studies. Specimens from day 12 diabetic rats were obtained from an ongoing study in the lab. Male and female rats (Charles River, Wilmington MA) were paired to mate on the female's day of proestrus (ovulation) and mating was confirmed the following day by a vaginal saline lavage. If spermatozoa were present, that was day 0 of pregnancy. For the two rats sacrificed on day 0 of pregnancy, uterine horns were isolated and embedded in OCT (Sakura Tissue Tek) and stored at -80ºC. The other four rats were sacrificed on gestation day 12. Two of the dams had been injected with STZ on day 6 (Sigma-Aldrich, 60mg/kg body weight) and the other two were untreated but for a saline injection. In the diabetic subjects, on day 12, serum glucose levels were above 250mg/dL (normal glucose level in the rat is 90-110mg/dL).

Slide preparation

Rat uteri previously obtained from day 0 and day 12 of pregnancy and embedded in OCT, were sliced in 7µm sections using a cryostat, fixed in acetone, mounted on slides and then stored at -20ºC until staining. For day 12 specimens (both normal and diabetic), three sections were mounted on each slide. For day 0 specimens, three pairs of uterine cross-sections were mounted on a slide, the top row of sections cut from early in the tissue block, the bottom row cut late in the tissue block. Each of the three early-late pairs of sections could be stained using the same droplet of reagents during the staining procedure. In this way, any differences in staining between the sections could not be attributed to variability in the staining procedure, but rather, it would mean that there are differences in the amount of macrophages present at different regions of the day 0 uterus.

Immunohistochemistry

Macrophage markers were visualized with an indirect immunophosphatase staining method. Slides were removed from the -20ºC freezer and brought to room temperature in a humidity chamber. They were washed in Tris buffered saline (TBS) to remove the embedding medium. Non-specific binding of the primary antibody was blocked with 5% normal goat serum (Jackson Immunoresearch) and the slides were subsequently washed in TBS. Incubation with the primary antibody for one hour followed. The primary antibodies used were ED1 (CD68, clone IC7, BD Biosciences) and ED2 (CD163, clone HIS36, Santa Cruz

Biotechnology). ED1 is a monocyte and macrophage marker (both M1 and M2) and ED2 is a marker of M2 macrophages (Figure 2) (Holness and Simmons, 1993; Che *et al*, 2010). After incubation, the slides were washed, then incubated with secondary antibody (goat anti-mouse IgG linked to biotin, Jackson Immunoresearch) for 30 minutes, then washed again in TBS. The slides were incubated with streptavidin conjugated with alkaline phosphatase (Jackson Immunoresearch) for 30 minutes and washed again in TBS. The color reaction was developed with BCIP/NBT (Moss) with 1mM levamisole for ten minutes and washed in water to stop the color reaction. The slides were then mounted with glycergel (DAKO Cytomation). Immunostained cells were a dark purple-blue color. Because the sections were not counterstained with an additional histological stain such as hematoxylin, immunostaining was the only positive staining on the slide. This fact becomes important to the way in which the amount of positive staining was quantified. A summary of the samples used is given in Table 1.

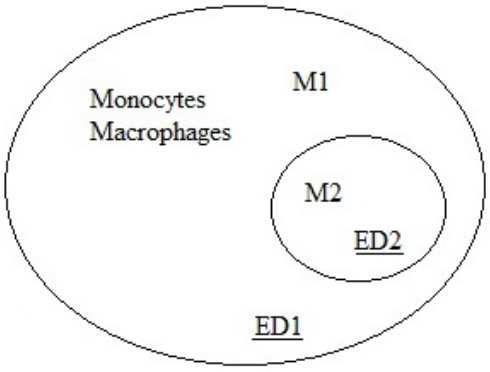

Figure 2: Markers for different kinds of immune cells. ED1 (CD68) is a marker for both M1 and M2 macrophages as well as monocytes and ED2 (CD163) marks M2 cells (Holness and Simmons, 1993; Ma *et al*, 2010)

One very important assumption made is that ED1 positive staining represents only M1 and M2 macrophages present in the uterus. ED1 is an antibody that binds to antigens of both macrophages and monocytes (Holness and Simmons, 1993). However, monocytes are not expected to be found in the uterus, because once they leave the blood stream, they differentiate to macrophages or dendritic cells (Swirski *et al*, 2009). ED2 is a marker for M2 macrophages, so it is reasonable to hypothesize that if degrees of ED1 and ED2 positive staining are equal, all the macrophages will be M2. In the same way, if one subtracts ED2 positive staining from ED1 positive staining, the result would be positive staining of M1 macrophages.

Sample Summary			
Day 0 Normal	♀ 1	Right uterine horn	8 sections/antibody, 4 early, 4 late
		Left uterine horn	8 sections/antibody, 4 early, 4 late
	♀ 2	Right uterine horn	4 sections/antibody, 2 early, 2 late
		Left uterine horn	4 sections/antibody, 2 early, 2 late
Day 12 Normal	♀ 3	6 embryos	3 sections/antibody/embryo
	♀ 4	4 embryos	3 sections/antibody/embryo
Day 12 Diabetic	♀ 5	4 embryos	3 sections/antibody/embryo
	♀ 6	4 embryos	3 sections/antibody/embryo

Table 1: Number of sections analyzed for each female in each condition.

Quantifying Macrophage Density and Phenotype

The immunostained slides were imaged using a Nikon E400 microscope with an Insight QE Color Digital camera. Quantitative analysis was performed using ImageJ (National Institute of Health). Specific binding of the antibody to the surface marker of the macrophage type of interest appeared darker than did the rest of the anatomical features in the pictures taken. Using the ImageJ "threshold" option, it was possible to selectively mark the positive immunostaining (the stained anatomical features were ignored) and compare it to the overall area (in pixels) of the anatomical area of interest. In this way, instead of counting the number of cells that exhibited positive staining, ImageJ would count the number of dark pixels on a white background in a given photograph. The number obtained was an indication of the degree of presence of a specific macrophage phenotype in the rat uterine compartment. The process is summarized below for a single day 12 normal metrial gland picture.

First, the anatomical area of interest was isolated by cropping the image (Figures 3, 4). Second, the area of the metrial gland was measured in pixels using ImageJ.

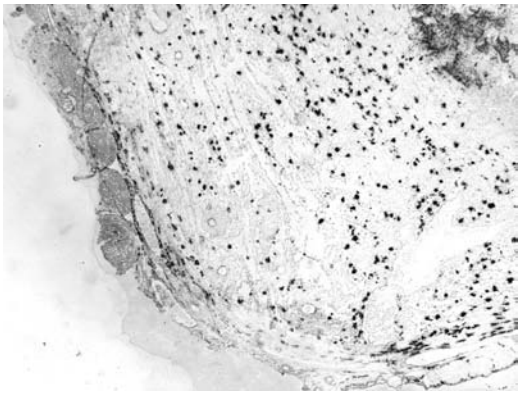

Figure 3: Uncropped image.

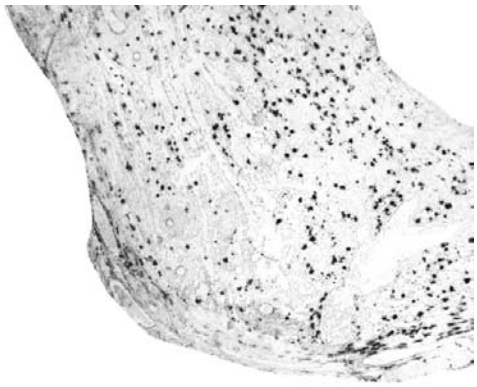

Figure 4: Cropped Image.

Third, the cropped image was thresholded to select positive staining. One could adjust the threshold, so that the whole area of positive staining in the cropped image was selected. However, several anatomical areas were stained faintly, apart from the specific binding of the stain to the macrophages of interest, which could also be selected during thresholding thus creating noise. Hence, deciding the appropriate threshold level was quite ambiguous, since areas that were not covered by macrophages could have been thresholded as well. It was decided that the appropriate threshold level was one where all the specific staining was covered, before more faint regions of the picture turned red. Examples of inappropriate and appropriate thresholding are shown in Figures 5 and 6.

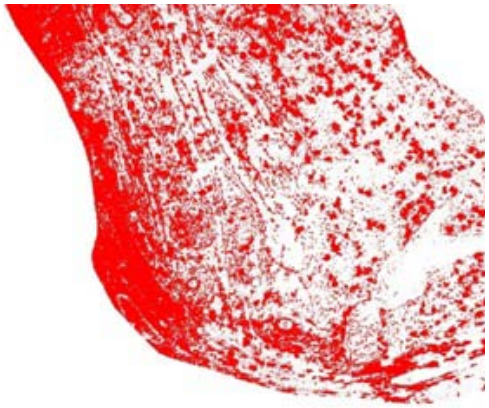

Figure 5: Inappropriate thresholding.

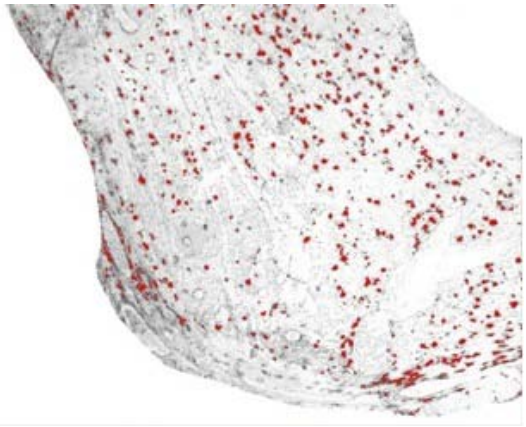

Figure 6: Appropriate thresholding.

Each piece of thesholded area was treated as a “particle” and its surface area could be measured. Particles smaller than 25 pixel units^2 were excluded, since they were small enough that in a manual count they would not have been identified as cells (Figures 7, 8). The 25 pixel units^2 limit was chosen by measuring the area of the biggest particle that was inappropriately thresholded, since it was not stained due to specific binding to the antibodies.

Figure 7: Particles thresholded as positive staining (stained macrophages plus noise).

Figure 8: Particles counted as macrophages (outlines). Any background noise is eliminated.

The values for each particle area were summed up to give the total area (in pixels) of the percentage of the uterine compartment that was covered by positive staining. The values for all three pictures that were used to cover the area of the metrial gland were summed up. By knowing the whole area of the anatomical area of interest, in this case the metrial gland, I could calculate the percentage of the whole area that was covered by positive staining.

RESULTS

Macrophage Phenotype after Mating and During Organogenesis

Macrophage phenotype at two very different stages of pregnancy (postmating inflammatory response on gestation day 0 vs. organogenesis on day 12) was measured. During the postmating inflammatory response on day 0, there was intense ED1 positive staining just under the epithelial cells surrounding the lumen of the uterus, as well as in the decidua (Figure 9A). There were fewer ED2 positive cells in the decidua, and almost none just under the uterine epithelium (Figure 9B). On day 12, ED1 and ED2 positive staining levels were very comparable in the metrial gland, suggesting that most of the macrophages present were M2 (Figure 9C and 9D). It should be noted that different anatomical areas are being compared for the two different stages of pregnancy.

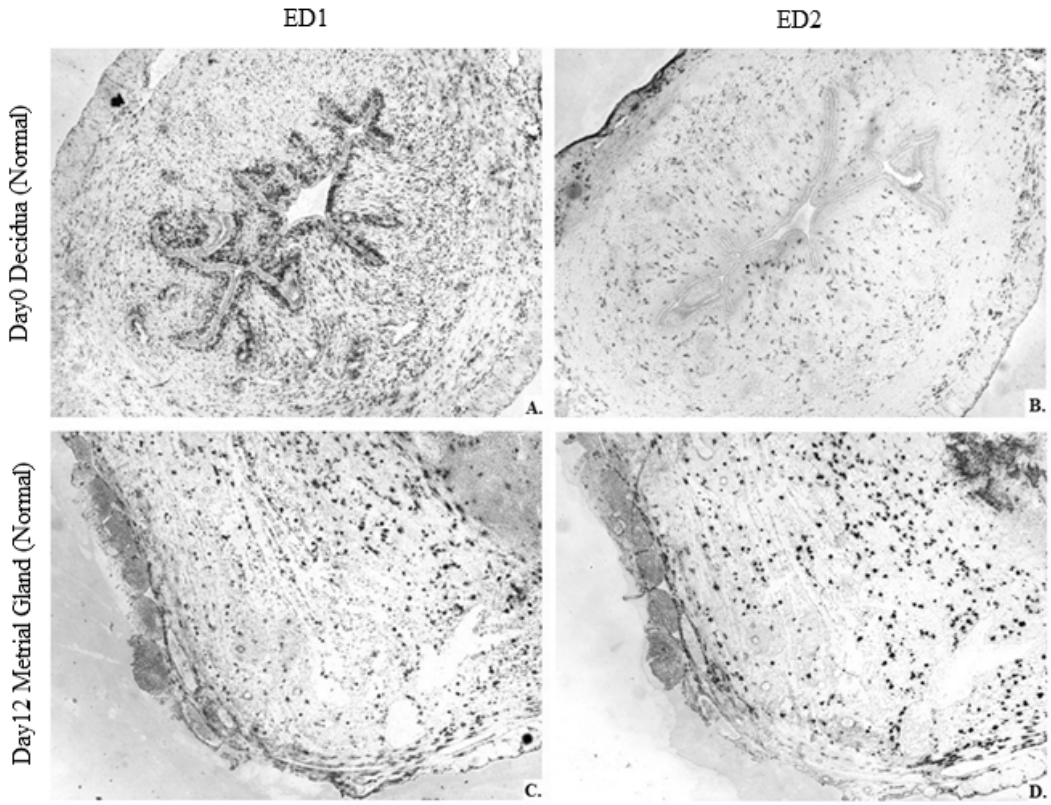

Figure 9. Representative ED1 and ED2 immunostaining in normal pregnant rat uterine decidua and metrial gland. A. ED1 in day 0 decidua. B. ED2 in day 0 decidua. C. ED1 in day 12 metrial gland. D. ED2 in day 12 metrial gland.

Positive staining for each antibody was quantified and the results appear on Figure 10. For day 0, on average 6.66% of the decidual area was covered by ED1 positive staining, and 0.95% was covered by ED2 positive staining. Hence, ED1 positive staining was approximately seven times more common than ED2 positive staining, suggesting that M1 macrophages predominate in the decidua of the postmating inflammatory response. On day 12 however, the proportion of the

metrial gland covered by ED1 and ED2 staining is almost equal (2.77% and 2.69% respectively), suggesting that almost all immunopositive cells were M2.

ED1 positive staining was very variable in day 0 decidua. Some sections exhibited very intense ED1 positive staining under the uterine epithelium (as in Figure 9A), whereas some others showed less staining in this area. This variability in the intensity of ED1 positive staining on day 0 resulted in a high standard deviation value (6.66±3.97).

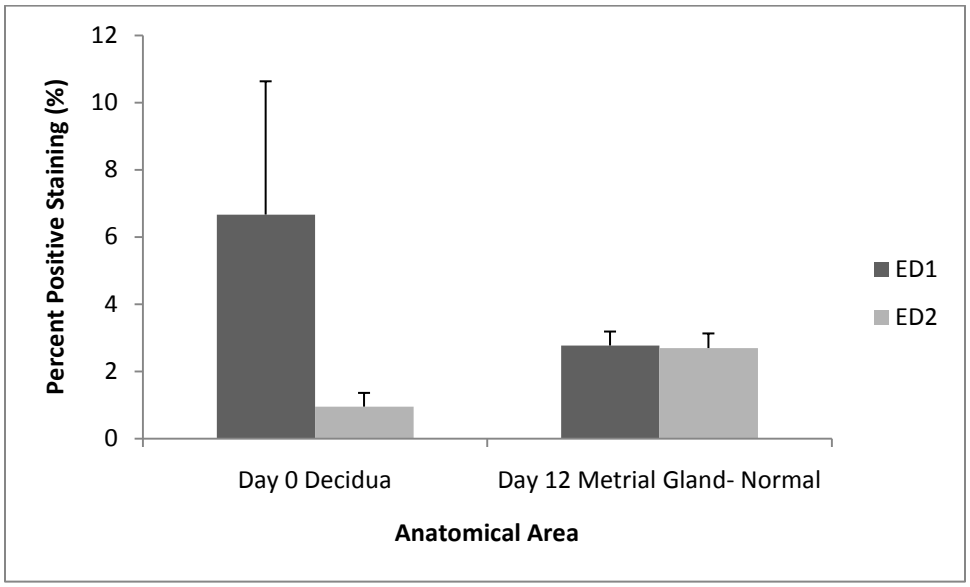

Figure 10. ED1 and ED2 positive staining in normal pregnant rat post-mating decidua and day 12 metrial gland.

Macrophage Phenotype in Diabetic Pregnancy

In order to ascertain macrophage activation and differentiation during normal and diabetic pregnancies during organogenesis (day 12), metrial glands and decidua of non-treated pregnant rats obtained at day 12 of gestation were compared to metrial glands and decidua of diabetic STZ-treated female rats obtained on day 12 of pregnancy.

In both the normal and diabetic day 12 metrial gland, nearly all ED1 positive staining is also ED2 positive staining (Figures 11 and 13) but in the diabetic metrial gland, the overall proportion of the metrial gland covered by immunopositive staining is lower (1.51% of the metrial gland area immunopositive in diabetes vs. 2.77% in normal pregnancy). This suggests that M2 macrophages predominate in the metrial glands of normal and diabetic pregnancies, but that diabetes may reduce the total number of macrophages in metrial gland on day 12.

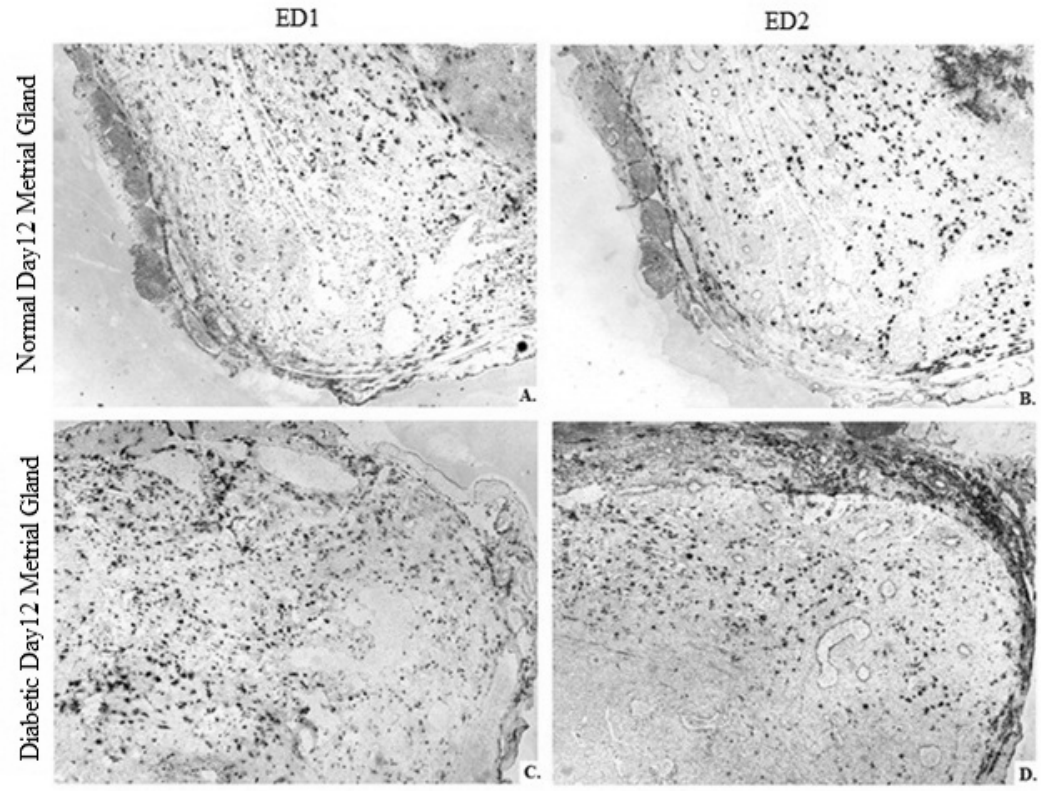

Figure 11. Representative images of Day12 normal and diabetic metrial glands. A. Normal pregnancy, ED1 staining. B. Normal pregnancy ED2 staining. C. Diabetic pregnancy, ED1 staining. D. Diabetic pregnancy, ED2 staining.

In day 12 decidua, diabetic rats had a higher level of ED1 positive staining than normal rats (Figure 12, panels A, C). ED2 staining was negligible (Figure 12, panels B, D), suggesting that M1 macrophages infiltrate near the developing embryo in diabetic pregnancies. In normal pregnancies, during organogenesis, there is almost no ED1 and ED2 positive staining in the decidua (Figure 12, panels A, B). In contrast, mean ED1 positive staining is very high for STZ treated

pregnant rats (1.17%) and ED2 positive staining is very low (0.07%). Results are plotted in Figure 13.

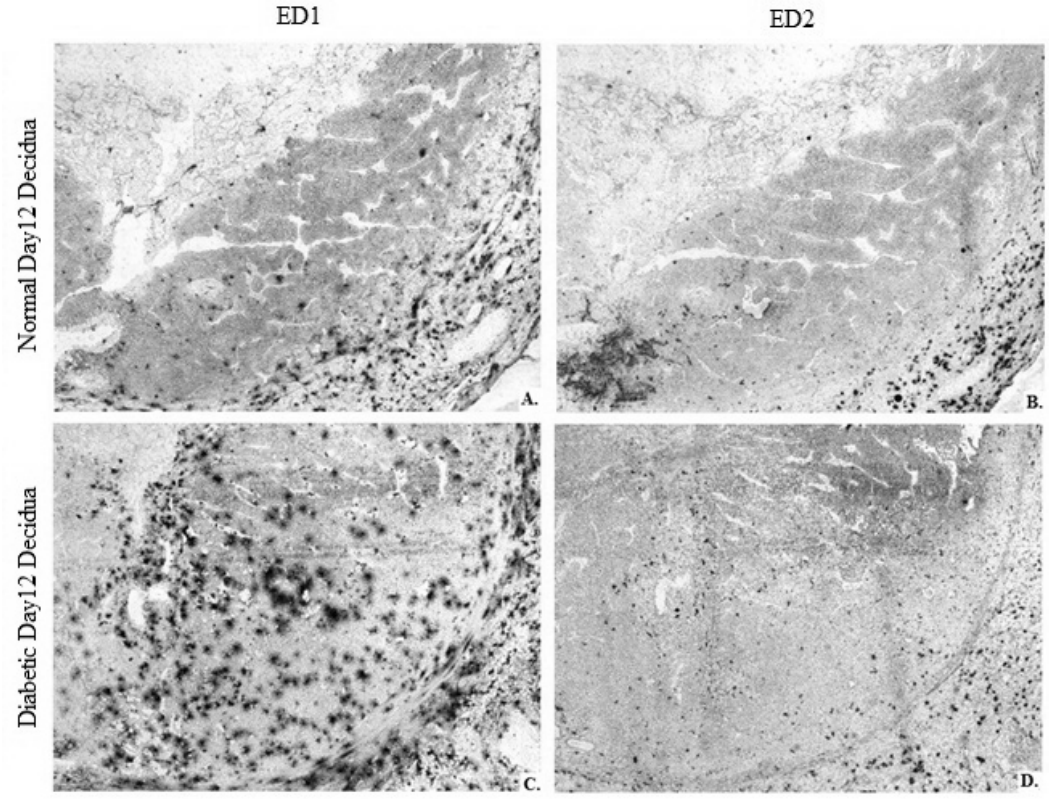

Figure 12. Representative images of Day12 normal and diabetic decidua. A. Normal pregnancy, ED1 staining. B. Normal pregnancy ED2 staining. C. Diabetic pregnancy, ED1 staining. D. Diabetic pregnancy, ED2 staining.

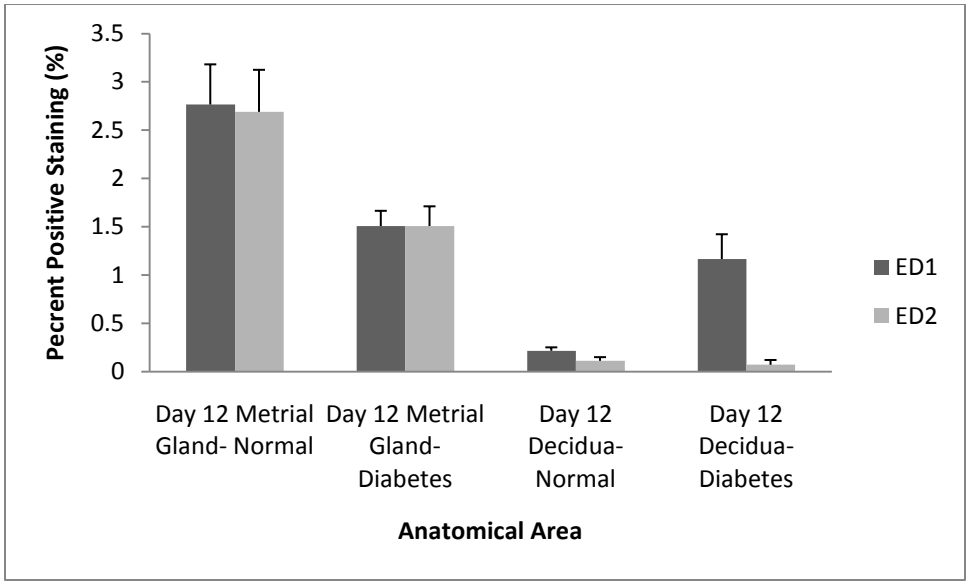

Figure 13. ED1 and ED2 positive staining in the metrial gland and decidua of diabetic and non-diabetic rats on gestation day 12, near the close of the period of embryo organogenesis. Data obtained from ten embryos of two untreated rats and eight embryos of two STZ-treated rats.

M1 and M2 Macrophages during Normal and Diabetic Pregnancies

As stated above, it is reasonable for one to assume that ED2 positive staining could be representative of just M2 macrophages and ED1 positive staining could be representative of both M1 and M2 macrophages present in the area under investigation. After having quantified the degree of ED1 and ED2 positive staining, one could then estimate the relative amounts of M1 and M2 macrophages present in the anatomical area of interest (Table 2, Figure 14).

Uterine Compartment	Percent Immunopositive		Percent M1 and M2	
	ED1	ED2	M1	M2
Day 0 Decidua	6.66 ± 3.97	0.95 ± 0.41	86.94	13.06
Day 12 Metrial Gland- Normal	2.77 ± 0.42	2.69 ± 0.44	4.24	95.76
Day 12 Decidua- Normal	0.22 ± 0.04	0.11 ± 0.04	N/A	N/A
Day 12 Metrial Gland- Diabetes	1.51 ± 0.16	1.51 ± 0.20	6.15	93.85
Day 12 Decidua- Diabetes	1.17 ± 0.26	0.07 ± 0.05	94.19	5.81

Table 2. Summary of ED1 and ED2 positive staining in rat decidua and metrial gland on day 0 and day 12 of pregnancy. Percent M2 present was calculated by dividing percent ED2 positive staining by ED1 positive staining and multiplying by a hundred. Percent M1 present was calculated by subtracting percent M2 present from a hundred.

For day 0 decidua, where ED1 positive staining is much more common than ED2 staining, macrophages are mainly of the M1 phenotype (87% M1 and 13% M2). ED1 positive staining was very variable, suggesting that M1 macrophages are not evenly distributed throughout the day 0 uterus. For both day 12 normal and diabetic pregnancies, the vast majority of macrophages could be characterized as M2 (95.8% and 93.9% respectively). In day 12 normal decidua, ED1 and ED2 staining were negligible (0.22% ED1 staining, 0.11% ED2 staining). In contrast, intense ED1 staining was observed in diabetic decidua. The vast majority of the macrophages present in day 12 diabetic decidua are probably M1 (94% of the total macrophage population).

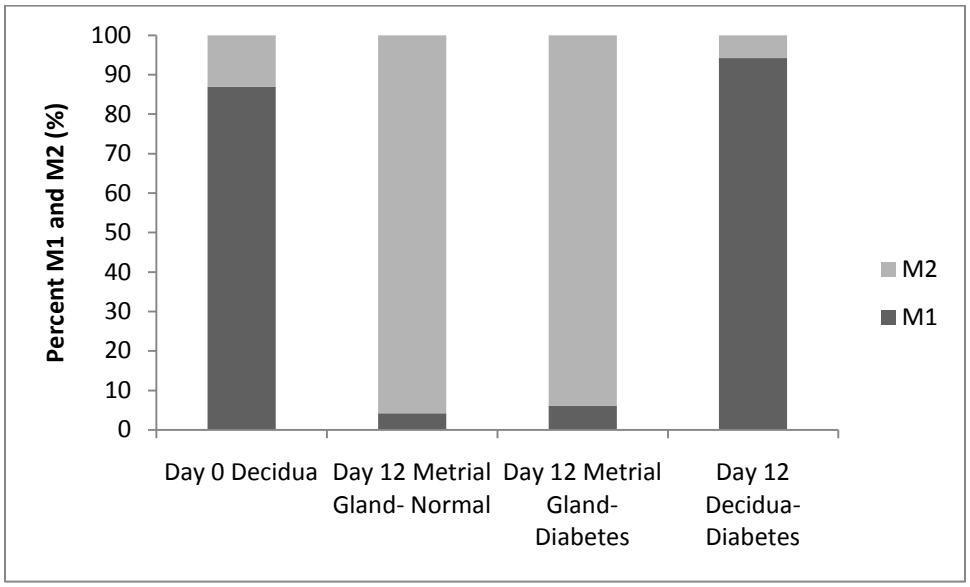

Figure 14: Degree of expression of each macrophage phenotype in each anatomical area and condition.

In summary, the post-mating decidua are characterized by an abundance of M1 macrophages, whereas in the metrial gland of day 12, M2 macrophages are predominating. There are almost no macrophages in the decidua of normal pregnancies on day 12. When pregnancies are complicated by diabetes, the predominant phenotype in the metrial gland is still M2, however the numbers of macrophages present in that anatomical area are lower. In contrast to day 12 normal decidua, diabetic decidua are infiltrated by M1 macrophages.

DISCUSSION

Summary of Results and Possible Interpretations

The role of macrophages in normal and pathological pregnancies has been studied extensively, and it has been shown to be an important one. However, research on macrophages during diabetic pregnancies still has gaps. The purpose of this study was to develop a methodology that would allow me to assign uterine macrophages in a rat to a specific phenotype and then quantify them, to create a profile of macrophage activation and phenotype after mating and during organogenesis and to test whether this profile of macrophage activation is perturbed during diabetic pregnancies.

I found that during the postmating inflammatory response on day 0, ED1 staining was more widespread than ED2 staining, suggesting that the majority of decidual macrophages are of the proinflammatory M1 phenotype. This finding is consistent with previous work that describes the uterus as being in an inflammatory state right after mating (Robertson, 2005). In contrast, the degree of ED1 and ED2 positive staining during the period of organogenesis (after the middle of pregnancy) is almost equal, suggesting that the dominant macrophage phenotype during that stage is M2. Macrophages play an important role in tolerating the semiallogeneic fetus and in maintaining pregnancy and these phenotypic shifts could provide insight on how macrophages contribute to these actions.

For instance, macrophages could induce immune privilege of the semiallogeneic fetus as follows: TGF-β in seminal plasma will cause uterine epithelial cells to secrete pro-inflammatory cytokines, like GM-CSF and IL-6. Macrophages present in the tissue will be educated by the cytokines and will assume an M1 phenotype causing inflammation. At the same time, the M1 macrophages will phagocytose, process and present the maternal antigens present in sperm, since they recognize them as non-self. During this time, epithelial cells become less sensitive to TGF-β, and a refractory period occurs, where no pro-inflammatory cytokines are produced. In the absence of inflammatory signals, inflammation is resolved and the new monocytes entering the tissue become macrophages of the M2 phenotype. These M2 macrophages will present the male antigens, but at the same time they display tolerogenic signals to T-cells (such as costimulatory ligands present in the deciduas and placenta).

Hence, immune deviation is achieved and the only part of the immune system that participates is the innate immune system, with the adaptive immune system being suppressed to avoid anti-sperm immunity (Robertson *et al*, 2002; Luppi, 2003). The now M2 macrophages can be involved in other activities for the rest of pregnancy such as clearing apoptotic cells, angiogenesis, vascular and tissue remodeling, embryo attachment and implantation. A uterine infection by a pathogen will stimulate Toll-like receptors on macrophages and the macrophage population will tend to shift from an M2 to an M1 phenotype (Nagamatsu and Schust, 2010b). M1 macrophages will then produce pro-inflammatory cytokines

as well as signals for recruitment of even more macrophages in the area (Nagamatsu and Schust, 2010b). This inflammatory response causes pregnancy complications and may result in preterm delivery (Nagamatsu and Schust, 2010b).

In pregnancies complicated by diabetes, ED1 positive staining was comparable to ED2 positive staining in the metrial gland, whereas in the decidua, ED1 staining was much more intense. Hence, macrophage phenotype in the metrial gland of diabetic pregnancies is probably M2, whereas in the decidua, it is probably M1. This is in great contrast to what happens in the decidua of normal pregnancies, where almost no macrophages are observed, let alone of the pro-inflammatory type. Also, the degree of macrophage recruitment in the area of the metrial gland was found to be lower in diabetic pregnancies than in normal pregnancies.

In trying to interpret the fact that pro-inflammatory macrophages are found in the decidua of pregnant diabetic rats, studies investigating the effect of TNFα were reviewed, since TNFα is a product of M1 macrophages. A number of studies report that TNFα has a deleterious effect on embryo development and implantation. TNFα synthesis is upregulated in the uterus of the pregnant diabetic rat (Pampfer *et al*, 1997). One of the most important and well-known activities of TNFα is inducing cell death (Pampfer *et al*, 1997). Rat trophoblasts are susceptible to the cytotoxic action of TNFα and TNFα has been found to induce a decrease in the total number of cells per rat blastocyst (Pampfer *et al*, 1997). Pampfer (2001) found that TNFα has a deleterious effect for the peri-implantation

embryo and the epithelial cells lining the uterine lumen. Blocking the expression of TNFα receptors protected rat blastocysts from these detrimental effects of TNFα (Pampfer *et al*, 1995).

Other studies indicate a more complicated role for TNFα. Torchinsky *et al* (2004) found that diabetic TNFα knockout mice had a higher pregnancy rate compared to diabetic mice that were $TNF\alpha^{+/+}$. At the same time, the knockout mice had a higher rate of fetal malformation than did the diabetic $TNF\alpha^{+/+}$ mice (Torchinsky *et al*, 2004). Diabetic TNFα knockout mice also showed higher rates of embryonic apoptosis, and lower levels of NF-κB , an inhibitor of apoptosis (Torchinsky *et al*, 2004). The authors interpreted these results in conjunction with other papers by saying that TNFα could contribute to death of peri-implantation embryos, but it could *protect* postimplantation embryos exposed to diabetes via activation of NF-κB-mediated anti-apoptotic signaling (Torchinsky *et al*, 2004; Torchinsky and Toder, 2008).

Hence, M1 macrophages present in the decidua of diabetic female rats after implantation and during organogenesis could indirectly control apoptosis and reduce malformation. High levels of ROS caused by hyperglycemia cause increased levels of cell death and malformations (Reece and Eriksson, 1996). However, TNFα secreted by M1 macrophages could activate NF-κB protein complexes to stop cell death and reverse the negative effects of ROS. Due to the fact that diabetes induces a decrease in macrophages and T cells during pregnancy, it is probable that the action of available M1 macrophages is not

enough to completely stop apoptosis and embryo malformations. In fact, when mice are immunized with splenocytes before the onset of diabetes, an increase in the number of T-cells and macrophages was observed (Torchinsky *et al*, 1997, 2004). Immunostimulated diabetic females also had fewer malformed fetuses than non-immunostimulated diabetic mice and their pregnancy rate was increased (Torchinsky *et al*, 1997, 2004). It is therefore reasonable to hypothesize that M1 macrophages present in the decidua of pregnant diabetic rats acts in favor of the developing fetus, but experiments need to be conducted to confirm this hypothesis.

But perhaps M1 macrophages are not helpful at all. M1 macrophages could be present in the decidua of diabetic pregnant female rats as part of the cascade of embryopathic factors acting to cause abnormality and death. Being a pro-inflammatory cytokine secreted by M1 macrophages, TNFα could be one factor that is involved in causing inflammation in the pregnant diabetic uterus, which is a harsh environment for the developing fetus. The steps that lead to the involvement of M1 macrophages in diabetic embryopathy could be as follows: ROS produced in high amounts due to diabetes could cause excessive apoptosis (Reece and Eriksson, 1996). Apoptotic cells are cleaned up by M2 macrophages (Mantovani *et al*, 2004). The clearance of apoptotic cells is very important, since if apoptotic cells are not efficiently engulfed, then secondary necrosis occurs, which causes the release of pro-inflammatory cytokines (Wu *et al*, 2001; Abrahams *et al*, 2006). This can be the case if the levels of apoptotic bodies are

elevated and M2 macrophages (present in reduced numbers in diabetic pregnancy) are overwhelmed (Abrahams *et al*, 2006). The pro-inflammatory microenvironment will cause macrophages to assume an M1 phenotype. M1 macrophages will in turn secrete even more pro-inflammatory cytokines like TNFα, which is upregulated in the uterus of diabetic rats, and reactive oxygen species, which are linked to embryopathy. M2 macrophages in normal pregnancy do not just function to engulf dead cells, but they also secrete tolerogenic factors that are immunosuppressive and anti-inflammatory (Abrahams *et al*, 2006; Nagamatsu and Schust, 2010b). If the M2 phenotype shifts to an M1 phenotype, then the protective role of the macrophages during pregnancy could be lost and M1 macrophages could be contributors to diabetic embryopathy.

Limitations of the present study

One of the goals of this study was to develop a methodology for assigning macrophages to a specific phenotype and quantifying them. ImageJ was used for this purpose. However, the method has certain limitations. Our method used the "thresholding" function to convert shades of grey in an 8-bit image of the tissue to binary black-and-white, with black areas counting as "positive staining." The darkest areas were selected first and as one lowered the threshold, lighter and lighter stained areas were counted as "positive." With low thresholds, it was sometimes hard to select the whole area of positive staining and so one had to increase the threshold, but as a result, staining that was not due to specific binding

was also selected. In order to correct for this ambiguity, multiple sections per embryo were analyzed. In addition, particles having an area smaller or equal to 25 pixel units^2 were excluded. This is the area of particles that are unlikely to be macrophages (since individual macrophages occupy a larger area on the image), but are most likely to be selected due to ambiguity in the thresholding process. The method still needs to be better standardized, but overall, it is highly sensitive to changes in positive staining and it gives you an idea of the trends of positive staining under different conditions, to say the very least.

Another limitation of this study is the fact that it was assumed that the amount of M1 macrophages present could be determined by subtracting ED2 positive staining, which only binds to M2 macrophages, from ED1 positive staining, which binds to M1 and M2 macrophages as well as monocytes. Although this assumption seems to be safe, it would be more accurate to use an antibody that would only recognize and bind to features of M1 macrophages. One such marker of pro-inflammatory macrophages is inducible nitric oxide synthase, or iNOS, an enzyme involved in the respiratory burst that produces ROS in M1 macrophages (Martinez *et al*, 2008). Hence, a better way to determine macrophage phenotype and degree of activation of each phenotype would be to use all three antibodies.

Third, when comparing macrophage phenotype and activation on day 0 and day 12 of pregnancy, different anatomical areas were used. For day 0, the anatomical area analyzed was the decidua and the epithelial cells surrounding the

uterine lumen. For day 12, the metrial gland was the anatomical area subjected to analysis. The reason is that on day 0, the inflammatory response is expected to occur in the epithelial cells and the decidua, and little positive staining was observed in the mesometrial triangle of the females. Metrial glands form in the mesometrial triangle of the pregnant rat uterus on day 8 through the end of pregnancy (Picut *et al*, 2009). On day 12, there was practically no positive staining observed in the decidua; rather positive staining was all located within the metrial gland area. Hence, a comparison of different anatomical areas for the different stages of pregnancy seems reasonable, but macrophage phenotype and activation could also be assessed for the mesometrial triangle after mating (from a few observations, it seemed that ED1 staining was more intense than ED2 staining, similar to what happened in the decidua of day 0 rats).

Fourth, the number of females tested in each condition (day 0, normal day 12, diabetic day 12) was low at two females per condition. Variability in positive staining between sections of one embryo, variability between embryos of the same female and variability between the few females that were tested for each condition seemed to be low, as indicated by the standard deviation values and by observation under the microscope. All embryos descend from the same female and share genetic material. Offspring of a single rat dam are more like each other than they are like offspring of a different dam, leading to a well-known “litter effect” in studies of rat and mouse embryonic development (Haseman and Hogan, 1975; Nelson *et al*, 1985). Individual embryos are not independent samples, and

the mother (or whole litter) is the appropriate experimental unit in a statistical analysis. In the present study, we did not perform statistical tests on our data because while the number of observations (ie, immunostained sections) was high (588 pictures in total), the number of independent experimental units (litters) was low. To evaluate statistically whether or not there is a significant difference in macrophage phenotype between day 0 and day 12, or on day 12 between normal and diabetic pregnancies, more pregnant dams must be (and will be) analyzed in future. The present study has established a method (and suggested some efficiencies) for that future work.

Implications and Future Directions

To our knowledge, this is the first time that the macrophage phenotype in the feto-maternal interface during diabetic pregnancies has been assessed. The finding that M1 macrophages are present in the decidua of diabetic pregnant females could help elucidate the role of macrophages in diabetic pregnancies. For instance, one could assess macrophage phenotype in the pregnant diabetic rats on day 12 of pregnancy following immunostimulation. If the number of M1 macrophages present in the uterus is increased, then this could mean that the role of M1 macrophages during diabetic pregnancies is a favorable one, given that immunostimulation decreases the degree of malformations.

In another study that that would help differentiate between the hypotheses that M1 macrophages are helpers or that M1 macrophages are part of the

problems caused by diabetes, one could inject diabetic pregnant rats with TNFα after implantation. Since TNFα is a product of M1 macrophages, then deterioration or improvement of malformations that would be attributed to TNFα could also be attributed to M1 macrophages.

In order to check whether the problem is excess apoptotic cells which cannot be efficiently engulfed by M2 macrophages and thus M1 macrophages are produced that contribute to embryopathy, one could inject pregnant diabetic rats with activated M2 macrophages at the site of inflammation (i.e. decidua) and then test for macrophage phenotype and degree of embryo malformations. If M1 macrophages are contributing to malformation and if the reason for their activation is excess apoptosis, then injecting with activated M2 macrophages will cause M2 levels in the decidua to rise, and hence clearance of apoptotic cells to be more efficient. Pro-inflammatory cytokine production will be reduced, and so will the number of M1 macrophages present in tissue.

Overall, this study shows that uterine macrophage phenotype varies with the stage of pregnancy, and further suggests that dysregulation of macrophage phenotype may be related to the embryo malformations so prevalent in maternal diabetes. Future studies will further clarify the functional consequences of increased pro-inflammatory macrophages present in the diabetic uterus.

REFERENCES

Abrahamsohn, P. A. and Zorn, T. M. 1993. Implantation and decidualization in rodents. *Journal of Experimental Zoology*. 266(6): 603–628.

Allen, M. P. and Nilsen-Hamilton, M. 1998. Granzymes D, E, F, and G are regulated through pregnancy and by IL-2 and IL-15 in granulated metrial gland cells. *Journal of Immunology.* 161: 2772-2779.

Classen, A., Lloberas, J. and Celada, A. 2009. Macrophage activation: classical vs. alternative. *Macrophages and Dendritic Cells, Methods in Molecular Biology*. 531: 29-43.

Collins, M., Ling, V. and Carreno, B. M. 2005. The B7 family of immune regulatory ligands. *Genome Biology*. 6(6): 223.1-223.7.

Fine, E. L, Horal, M., Chang, T. I., Fortin, G. and Loeken, M. R. 1999. Evidence that elevated glucose causes altered gene expression, apoptosis, and neural tube defects in a mouse model of diabetic pregnancy. *Diabetes*. 48: 2454-2462.

Goldman-Wohl, D. and Yagel, S. 2002. Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. *Molecular and Cellular Endocrinology*. 187(1): 233-238

Haseman, J.K. and Hogan, M.D. 1975. Selection of the experimental unit in teratology studies. *Teratology* 12:165-172.

Holness, C. L. and Simmons, D. L. 1993. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood*. 81: 1607-1613.

Kaufman, M. H. and Bard, J. B. L. 1999. The anatomical basis of mouse development. Academic Press, San Diego.

Kawamura, K., Kawamura, N., Kumagai, J., Fukuda, J. and Tanaka, T. 2007. Tumor necrosis factor regulation of apoptosis in mouse preimplantation embryos and its antagonism by transforming growth factor alpha/phosphatidylionsitol 3-kinase signaling system. *Biology of Reproduction*. 76:677-618.

Luppi, P. 2003. How immune mechanisms are affected by pregnancy. *Vaccine*. 21(24): 3352-3357.

Ma, J., Liu, L., Che, G., Yu, N., Dai, F. and You, Z. 2010. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. *Cancer*. 10(112): 1-9.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*. 25(12): 677-686.

Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. 2008. Macrophage activation and polarization. *Frontiers in Bioscience* 13: 453-461.

Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J. and Hill, A.M. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. *Journal of Immunology.* 164: 6166-6173.

Moffett, A. and Loke, Y. W. 2004. The immunological paradox of pregnancy: A reappraisal. *Placenta*. 25(1): 1-8.

Mor, G., Romero, R. and Abrahams, V. M. 2006. Immunology of pregnancy. Springer Science and Business Media, New York.

Mosser, D.M. and Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*. 8(12): 958–969.

Myatt, L. and Cui, X. 2004. Oxidative stress in the placenta. *Histochemistry and Cell Biology*. 122:369-382.

Nagamatsu, T. and Schust, D. J. 2010. The contribution of macrophages to normal and pathological pregnancies. *American Journal of Reproductive Immunology*. 63: 460–471.

Nagamatsu, T. and Schust, D. J. 2010. The immunomodulaotry roles of macrophages at the maternal-fetal interface. *Reproductive Sciences*. 17(3): 209-218.

Nelson, C. J., Felton, R. P., Kimmel, C. A., Buelke-Sam, J. and Adams, J. 1985. Collaborative behavioral teratology study: statistical approach. *Neurobehavioral Toxicology and Teratology* 7:587-590.

Niederkorn, J. Y. 2006. See no evil, hear no evil, do no evil: the lessons of immune privilege. *Nature Immunology*. 7(4): 354-359.

Ornoy, A. 2007. Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. *Reproductive Toxicology*. 24: 31-41.

Pampfer, S., Vanderheyden, I., McCracken, J. E., Vesela, J. and De Hertogh, R. 1997. Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-a in vitro. *Development*. 124: 4827-4836.

Pampfer, S., Vanderheyden, I., Vesela, J. and De Hertogh, R. 1995. Neutralization of tumor necrosis factor alpha (TNF alpha) action on cell proliferation in rat blastocysts by antisense oligodeoxyribonucleotides directed against TNF alpha p60 receptor. *Biology of Reproduction*. 52(6):1316-1326.

Pampfer, S., Vanderheyden, I., Wuu, W. D., Baufays, L., Maillet, O. and De Hertogh, R. 1995. Possible role for TNF-alpha in early embryopathy associated with maternal diabetes in the rat. *Diabetes*. 44(5):531-6.

Pampfer, S. 2001. Dysregulation of the cytokine network in the uterus of the diabetic rat. *American Journal of Reproductive Immunology*. 45(6): 375-381.

Peel, S. 1989. Granulated metrial gland cells. *Advances in Anatomy, Embryology and Cell Biology.* 115: 1-112.

Petroff, M. G. and Perchellet, A. 2010. B7 family molecules are regulators of the maternal immune system in pregnancy. *Americal Journal of Reproductive Immunology*. 63: 506-519.

Picut, C. A., Swanson, C.L ., Parker, R. F., Scully, K. L. and Parker, G. A. 2009. The metrial gland in the rat and its similarities to granular cell tumors. *Toxicologic Pathology.* 37(4): 474-480.

Punareewattana, K. and Holladay, S.D. 2004. Immunostimulation by complete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, or interferon-gamma reduces severity of diabetic embryopathy in ICR mice. *Birth Defects Research: Clinical and Molecular Teratology*. 70(1):20-7.

Redman, C. W. and Sargent, I. L. 2005. Latest Advances in Understanding Preeclampsia**.** *Science.* 308(5728): 1592-1594**.**

Reece, E. A., Pinter, E., Homko, C., Wu, Y. K. and Naftolin, F. 1994. The yolk sac theory: closing the circle on why diabetes-associated malformations occur. *Journal of the Society of Gynecologic Investigation*. 1(1): 3-13.

Reece, E. A., Wu, Y. K., Wiznitzer, A., Homko, C., Yao, J., Borenstein, M. and Sloskey, G. 1996. Dietary polyunsaturated fatty acid prevents malformations in offspring of diabetic rats. *American Journal of Obstetrics and Gynecology*. 175(4): 818-823.

Reece, E. A., Wu, Y. K., Zhao, Z. and Dhanasekaran, D. 2006. Dietary vitamin and lipid therapy rescues aberrant signaling and apoptosis and prevents hyperglycemia-induced diabetic embryopathy in rats. *American Journal of Obstetrics and Gynecology*. 194: 580-585.

Reece, E.A. and Eriksson, U. J. 1996. The pathogenesis of diabetes-associated congenital malformations. *Obstetrics and Gynecology Clinics of North America*. 23(1): 29-45.

Robertson, S. A., Ingman, W. V., O'Leary, S., Sharkey, D. J. and Tremellen, K. P. 2002. Transforming growth factor β—a mediator of immune deviation in seminal plasma. *Journal of Reproductive Immunology*. 57: 109–128.

Robertson, S. A. 2005. Seminal plasma and male factor signaling in the female reproductive tract. *Cell and Tissue Research*. 322: 43-53.

Robertson, S. A. 2007. GM-CSF regulation of embryo development and pregnancy. *Cytokine and Growth Factor Reviews*. 18: 287-298.

Savion, S., Gindon-Tabush, S., Fein, A., Toder, V. and Torchinsky, A. 2004. Diabetes teratogenicity is accompanied by alterations in macrophages and T cell subpopulations in the uterus and lymphoid organs. *International Immunopharmacology*. 4: 1319-1327.

Sharp, P. E. and La Regina, M. C. 1998. The Laboratory Rat. CRC Press LLC, Boca Raton.

Soares, M. J., Julian, J. A. and Glasser, S. R.1985. Trophoblast giant cell release of placental lactogens: Temporal and regional characteristics. *Developmental Biology*. 107(2): 520-526.

Stewart, I. J. 1991. Granulated metrial gland cells: pregnancy specific leukocytes? *Journal of Leukocyte Biology*. 50(2): 198-207.

Straszewski-Chavez, S. L., Abrahams, V. M. and Mor, G. 2005. The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. *Endocrine Reviews*. 26:877-897.

Swirski, F. P., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J. L., Kohler, R. H., Chudnovskiy, A., Waterman, P., Aikawa, E., Mempel, T. R., Libby, P., Weissleder, R. and Pittet, M. J. 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* 325(5940): 612-616.

Szkudelski, T. 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiology Research.* 50: 536-546.

Torchinsky, A., Gongadze, M., Orenstein, H., Savion, S., Fein, A. and Toder, V. 2004. TNF-alpha acts to prevent occurrence of malformed fetuses in diabetic mice. *Diabetologia.* 47:132–139.

Torchinsky, A., Toder, V., Savion, S., Shepshelovich, J., Orenstein, H. and Fein, A. 1997. Immunostimulation increases the resistance of mouse embryos to the teratogenic effect of diabetes mellitus. *Diabetologia*. 40(6):635-40.

Torchinsky, A. and Toder, V. 2008. TNFalpha in the pathogenesis of diabetes induced embryopathies: functions and targets. *The Review of Diabetic Studies*. 4(4):200-2

Tremellen, K. P., Seamark, R. F. and Robertson, S. A. 1998. Seminal transforming growth factor β_1, stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biology of Reproduction*. 58: 1217-1225.

Trowsdale, J. and Betz, A.G. 2006. Mother's little helpers: mechanisms of maternal-fetal tolerance. *Nature Immunology*. 7: 241 – 246.

Vercruysse, L., Caluwaerts, S., Luyten, C. and Pijnenborg R. 2006. Interstitial trophoblast invasion in the decidua and mesometrial triangle during the last third of pregnancy in the rat. *Placenta*. 27(1): 22-33

Wu, X., Molinaro, C., Johnson, N. and Casiano, C.A. 2001. Secondary necrosis is a source of proteolytically modified forms of specific intracellular autoantigens: implications for systemic autoimmunity. *Arthritis and Rheumatism*. 44(11): 2642-2652.

Zhao, Z. and Reece, A. 2005. Experimental mechanisms of diabetic embryopathy and strategies for developing therapeutic interventions. *Journal of the Society of Gynecologic Investigation*. 12(8): 549-557.