THE AUTOIMMUNE REGULATOR (AIRE) PRESERVES FERTILITY IN MALE AND FEMALE BALB/C MICE

BY

Bryce D. Warren

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Chair: Margaret G. Petroff, Ph.D.

Dale R. Abrahamson, Ph.D.

Lane K. Christenson, Ph.D.

Douglas E. Wright, Ph.D.

Thomas M. Yankee, Ph.D.

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The Dissertation Committee for Bryce Warren certifies that this is the approved version

of the following dissertation:

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Chair: Margaret G. Petroff, Ph.D.

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ABSTRACT

The Autoimmune Regulator (AIRE) contributes to central immune tolerance through the induction of self-antigen expression within the thymus. In humans, a defect in AIRE results in a multi-organ autoimmune disorder known as Autoimmune Polyglandular Syndrome Type-1. Both humans and mice with a deficiency in this gene develop autoreactive $CD4^+$ T cells, serum autoantibodies and elevated rates of infertility. Interestingly, the pathology of autoimmune infertility in *Aire*-deficient (*Aire*-KO) mice differs between males and females. Ovarian autoimmunity causing a depletion of follicular reserves is prominent in female *Aire*-KO mice over 10 weeks of age. However, 50% of mated six-week old female *Aire*-KO mice demonstrate embryo loss by embryonic day (ED) 7.5 despite having normal follicular reserves. We determined that ovulation (90% KO vs 100% WT; n=11), mating frequency (99% KO vs 100% WT; n>58), progesterone production (p=0.232; n=4) and decidualization are unaffected. However, 60% (9 of 15) of 6-8 week old *Aire*-KO female mice generated serum autoantibodies against the ovary and more frequently ovulated degenerated oocytes (31% KO vs 2% WT; n>71) When cultured, one-cell embryos from *Aire*-KO females failed to become 2-cell and blastocyst stage embryos at significantly reduced rate compared to WT controls (47% KO vs 76% WT and 19% KO vs 60% WT; n>67). Finally, embryos from *Aire*-KO dams are developmentally delayed at ED3.5 with a reduced trophectoderm outgrowth potential after 48 and 96 hours of culture ($0mm^2$ KO vs 7.1 mm^2 WT, and 16.5 mm^2 KO vs 30.9 mm^2 WT; p<0.001, n=4). Alternatively, male *Aire*-KO mice have greater variability in the autoimmune targets associated with reduced fertility (18% KO vs 80% WT; n=10). Signs of disease included reduced

testosterone (p=0.03; n=18), loss of vomeronasal organ-associated glands (86% KO vs 0% WT; n=7), prostatitis (90% KO vs 0% WT; n=20), epididymitis (75% KO vs 0% WT; n=20) and reduced *in vitro* fertilization rates (9% KO vs 54% WT; n>83 oocytes). Additionally a subset of males (23%; n=22) exhibited oligozoospermia with disruption of the blood-testis barrier. Collectively these results demonstrate the importance of central immune tolerance on fertility preservation by preventing autoimmune disease against the male and female reproductive systems.

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GENERAL INTRODUCTION

Preface

The adaptive benefits allowing for embryonic development within the female womb (*viviparity*), which include continuous nourishment and environmental protection, simultaneously presents a unique immunologic challenge. Ideal reproductive outcomes that avoid maternal infection as well as fetal loss and disease require a modulation to the maternal immune system such that exposure to reproductive neo-antigens does not elicit a harmful immune response. These antigens, which derive from a range of sources including male gametes, seminal fluid, the developing embryo and placenta are intimately exposed to the maternal immune system through a variety of mechanisms and should be recognized as “foreign”. In analogous tissue transplantation models (Auchincloss & Sultan, 1996; Gould & Auchincloss, 1999) a similar level of genetic discrepancy without immunosuppression would lead to host rejection of the graft within a few weeks of the procedure. However, the reproductive and immune systems of placental mammals have co-evolved such that the developing fetus experiences an “immune-privileged” environment that ensures species survival. Understanding how this balance is maintained and then extending those insights into broader areas of transplantation medicine, autoimmune disease and reproductive biology has been a long-term goal of our lab.

The following introductory chapter will include a broad overview of male and female infertility separated by anatomic location and presented from a clinical perspective. Next I will introduce the immune system, its two main branches and the mechanisms responsible for stemming autoimmune disease including the role of the AutoImmune REgulator (AIRE) gene. I will then include a detailed description of the interface between the immune and reproductive systems as well as the known causes of autoimmune infertility related to the ovary, testis and epididymis. Finally, this chapter will conclude with a clinical presentation of the Autoimmune Polyglandular Syndrome Type-I (APS-1), which is a disease resulting from a deficiency in the AIRE gene. Importantly, APS-1 patients present with elevated rates of autoimmune gonadal insufficiency. The work documented within this thesis using an *Aire*-deficient mouse model is an attempt to address a gap in our knowledge regarding the role of central immune tolerance on fertility perseveration.

INFERTILITY

Infertility is an unfortunately common and complex condition that involves couples instead of individuals and has profound demographic, social, physiologic and medical implications. Studies dating back to the 1940's (Guttmacher, 1956) as well several more recent ones (Gnoth, Godehardt, Godehardt, Frank-Herrmann, & Freundl, 2003; Slama et al., 2012; Zinaman, Clegg, Brown, O'Connor, & Selevan, 1996) have demonstrated two key parameters that characterize normal fertility. First, roughly 80-90% of couples who participate in regular intercourse will conceive over a 12 month duration and second, the reproductive potential (*fecundity)* of a female decreases with increased age.

While the precise numbers can vary by ethnicity and geographic location (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012), these estimates of normal fertility have in turn shaped the definition of infertility, which is the inability of a couple to conceive without the use of contraception over a period of 12 month in women under the age of 35, and six months in women over 35. Global estimates (Greenhall & Vessey, 1990) indicate that between 6-25% of couples meet the criteria for infertility or sub-fertility (reduced fertility with an extended period of non-conception). The reasoning for such variance is likely due to numerous factors including, but not limited to, survey methodology, geographic location, age of participants, primary vs. secondary infertility, inclusion of male infertility and the influence cultural stigma reporting accuracy. A more recent survey of 25 fertility population studies encompassing over 170,000 women (Boivin, Bunting, Collins, & Nygren, 2007), argued that the median prevalence of infertility worldwide was 9% in both developed and less-developed nations, of which 56% and 22% were seeking, and had obtained, medical treatment respectively. A pair of 2013 prospective studies regarding fertility status of women in the United States between the ages of 15 and 44 concluded a slightly higher value (12-18%), but also suggest that percentage of females meeting infertility criteria have declined since 1982 (Chandra A, 2013; Thoma et al., 2013). Regardless of a concrete value, the burden on the individual as well as the medical system is immense and likely to grow as factors including increased awareness and availability of assisted reproductive techniques, a desire to delay starting a family for personal or economic reasons and increased prevalence of sexually transmitted disease continue to broaden the percentage of individuals that could benefit from infertility treatment.

Causes of Infertility

Compromised fertility was originally attributed to female pathologies. However, it is now appreciated that pregnancy complications can be multifactorial and originate from the female, the male, or both. Beginning in 1992 and updated in 2000, the World Health Organization (WHO) published the *WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple (*Mahmoud, 2000*)*, which, among many other things, categorized the various causes of infertility. While the precise numbers vary between publications, generalized estimates are as follows: 35% female factor only, 30% male factor only, 20% contributions from both partners and 15% unexplained (Forti & Krausz, 1998; Hull et al., 1985).

Female Infertility

The following section will provide brief examples of pathologies leading to female infertility separated by anatomical location as their biology; medical evaluation and potential treatment options have been extensively reviewed elsewhere (Fiszbajn et al., 2000; Forti & Krausz, 1998; Hamilton-Fairley & Taylor, 2003).

Ovary

The primary responsibility of the ovary is the storage, development and release of a mature ovum and conditions that reduce (*oligoovulation*) or prevent (*anovulation*) ovulation can impair fertility. There are numerous potential factors that can impinge on these processes, include genetic abnormalities, autoimmune disease, aging, premature ovarian failure, secondary effects of medications like chemotherapeutics and

antidepressants or antipsychotics (Haddad & Wieck, 2004). The classification system established by the WHO for female anovulatory infertility includes three unique categories as well as hyperprolactinaemia. *1) Hypogondaotropic hypogonadism:* women with low levels of both pituitary and ovarian hormones. This category predominately reflects a pituitary disruption resulting in reduced levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol. Potential causes including very low body weight and extended periods of strenuous physical activity are more common than genetic causes including Kallman's syndrome (a genetic disruption of Gonadotropin Releasing Hormone (GnRH) production) and Sheehan's Syndrome (necrosis of the pituitary gland). *2) Normogonadotropic normoestrogenic:* women with normal production of both pituitary and ovarian hormones. The majority of women within this category present with Polycystic Ovarian Syndrome (PCOS), a complex genetic and endocrine disorder associated with hirsutism, obesity and elevated levels of androgens and LH (Balen et al., 1995). PCOS is thought to result in excessive recruitment of pre-ovulatory follicles that do not respond to physiologic levels of FSH, ultimately blocking the complete development and release of an oocyte by the Graafian follicle. Some women with PCOS will sporadically ovulate, however the rarity of this event is such that the chance of becoming pregnant is significantly reduced. *3) Hypergonadotropic hypoestrogenic:* women with elevated pituitary hormones in response to a lack of gonadal estrogen. This classification can be referred to "ovarian failure." The resulting LH and FSH elevation can be either a component of normal menopause, which occurs at ~age 50, or "premature", when it occurs alongside infertility in women < 40 years old. Genetic abnormalities may also cause hypergonadotropic hypoestrogenism. Early

detection in suspected cases of premature ovarian failure is important. The corresponding rise in FSH tends to precede complete ovarian exhaustion thus allowing for the possibility of fertility preservation through assisted reproductive intervention if diagnosed early.

Importantly, ovarian senescence leading to hypergonadotropic hypogonadism is a natural part of the aging process. The full endowment of oocytes peaks during midgestation and steadily declines over the lifetime of women until follicular reserves are depleted at menopause (Richardson, Senikas, & Nelson, 1987). However, fertility success diminishes prior to a complete absence of the female gamete as shown in a 2008 study from the Center for Disease Control which compared live birth rates following Assisted Reproductive Technologies (ART) using a patient's own eggs vs. donated ones as a function of age (Sunderam et al., 2013). The report showed that live birth rates from women using their own eggs between the ages of 35 and 45 continuously declined from 45% to 5%. Comparatively, the percentage of live births from women using donated eggs remained constant at >50% regardless of age. This age related decrease in fecundity is likely the result of lower egg quality, as well as quantity, leading to increased rates of meiotic disjunction failure producing chromosomal imbalances that block embryonic development (Broekmans, Soules, & Fauser, 2009).

Tubal-peritoneal

The Fallopian tubes are the location of several essential reproductive events including oocyte uptake, fertilization within the ampulla and early embryonic development prior to

passage into the uterus. Consequently, tubal disorders or local pelvic adhesions have the potential to impair fertility by preventing the normal transport of gametes. It is currently estimated that 25-30% of female infertility can be attributed to a tubal factor with the majority of cases involving an infectious pathogen leading to Pelvic Inflammatory Disease (PID) (Akande et al., 2003) as opposed to a congenital defect (Forti & Krausz, 1998).

Uterus

The hormonally primed and receptive uterus ultimately provides nutrients and protection for the growing fetus by facilitating implantation as well as placental and embryonic development. Anatomic or congenital uterine defects that impinge on implantation have the potential to reduce fecundity. Anatomic defects include intrauterine polypus, adhesions and neoplasias. Müllerian duct abnormalities represent a collection of congenital defects stemming from incomplete development, formation and/or fusion of the mesonephric ducts. It is currently estimated that these deformities impact approximately 3.8% and 6.2% of fertile and infertile females respectively, and are associated with recurrent pregnancy loss and preterm birth (Rackow & Arici, 2007; Raga et al., 1997).

Endometriosis

Endometriosis (Sampson, 1927) is a common and chronic disorder whose pathogenesis, incidence rate and ideal medical management are still being investigated. A complete diagnosis relies on the discovery of extra-uterine endometrial implants on

the ovaries or within the pelvic peritoneal cavity. Clinically, the condition is described as an estrogen-dependent inflammatory disease that often presents with pelvic pain, painful intercourse and elevated infertility rates (Falcone & Lebovic, 2011). However, the disease can be either asymptomatic or associated with a diverse range of symptoms (Rawson, 1991), therefore making the establishment of an incidence rate difficult. Current estimates suggest that 5-10% of the general female population has some degree of endometriosis and that the incidence rate is significantly elevated to a range of 25-40% in women with infertility (Strathy, Molgaard, Coulam, & Melton, 1982).

Cervix

The cervix and its mucosal lining are important for the transport of sperm to the site of fertilization. Ascending infections of the cervix and vagina have the potential to block mucus production or alter its pH range, both of which can inhibit sperm motility (Lai et al., 1997; Paavonen & Eggert-Kruse, 1999). Additional causes of infertility related to the cervix include trauma, congenital defects and the presence of anti-sperm antibodies within the mucus (Moghissi, Sacco, & Borin, 1980).

Genetic

The incidence of a genetic based cause of infertility is higher in men than women (Mau-Holzmann, 2005). In addition to the two predominate genetic causes of female infertility, Turner's Syndrome and PCOS, there are other single gene mutations associated with impaired fecundity. The majority of these genes are associated with the hypothalamic-pituitary (HPG) axis or steroidogenesis and consequently impair *both* female as well as

male infertility. Examples include mutation to the Kisspeptin receptor (GPR54) (Colledge, 2009), Gonadotropin-releasing hormone receptor (GnRH-R) (Cattanach, Iddon, Charlton, Chiappa, & Fink, 1977), Luteinizing hormone receptor (LH-R) (Arnhold, Latronico, Batista, & Mendonca, 1999) Follicle stimulating hormone receptor (FSH-R) (Lussiana et al., 2008), Steroidogenic factor 1 (SF-1) (Bashamboo et al., 2010) and fragile X mental retardation 1 (FMR1) (Wittenberger et al., 2007) (fragile X-syndrome).

Male Infertility

A complete evaluation of the infertile couple must include the male partner as it is currently estimated that 50% of infertility cases have a male specific component (Mahmoud, 2000). A male infertility assessment often begins with a semen analysis to address both quantity and quality. Many infertile and sub-fertile males will present some combination of oligo/azoospermia (decreased/no sperm in the ejaculate) with either asthenozoospermia (decreased motility) or teratozoospermia (abnormal morphology) or both. Unfortunately the assessment of male infertility is confounded by three factors. First, the primary clinical evaluation relies heavily on semen analysis using a light microscope, which can be vulnerable to both observer subjectivity and variability (H. W. Baker et al., 1981). Second, some infertile males display no abnormalities in quantity, morphology or motility. Finally, while an estimated 50-60% of infertilities can be categorized into disorders related to the hypothalamus/pituitary, testis or post-testicular sperm transport, this still leaves nearly half of male infertility cases with the diagnosis of idiopathic (de Kretser, 1997).

Pre-Testicular disorders

Male fertility involves a series of events beginning with testicular sperm development (spermatogenesis), epididymal sperm maturation/storage, contribution from accessory gland secretions and culminating with intercourse. Essential to many of these processes are downstream effects of gonadotropin signaling. Disruptions of hypothalamic GnRH or pituitary LH and FSH can produce secondary hypogonadism and infertility. Secondary hypogonadism, which is clinically distinguished by the combination of oligozoospermia, low serum testosterone and low or low-normal levels of LH and FSH, can originate from congenital, acquired or systemic illnesses.

Congenital secondary hypogonadism is typically due to Kallman's syndrome (MacColl, Quinton, & Bouloux, 2002) and afflicted patients present with eunuchoid habitus (small external genital, little or no body hair and low testicular volume). Other potential genetic alterations that can impair pituitary gonadotropins include mutations to GPR54 (de Roux et al., 2003), GNRH1 (Bouligand et al., 2009), leptin/leptin-receptor (Clement et al., 1998) and the β-subunits of LH/FSH (Phillip, Arbelle, Segev, & Parvari, 1998; Weiss et al., 1992).

Acquired disorders that impinge on the hypothalamic-gonadal (HPG) axis typically result in physical symptoms that are less dramatic than congenital disorders, but include asthenia as well as decreased libido, facial hair and ejaculate volume. The origin of acquired diseases are vast but the outcomes are typically separated into three categories; first, the destruction of GnRH neutrons or pituitary gonadotrophs, second, the functional suppression of GnRH or gonadotropin release, or third, the infringement of the hypothalamic-pituitary portal circulatory system. Examples resulting in functional

destruction include hypothalamic/pituitary tumors or cysts (Citron et al., 1996) as well as infiltrative disease like Sarcoidosis (Porter, Beynon, & Randeva, 2003). Alternatively, suppression of the hypothalamic-pituitary axis can result from hyperprolactinemia, prolonged opioid therapy (Daniell, 2002), chronic glucocorticoid use (MacAdams, White, & Chipps, 1986) and artificially elevated gonadal steroids (Turek, Williams, Gilbaugh, & Lipshultz, 1995). Finally, a disruption of the pituitary portal circulatory system, which prevents GnRH from reaching the pituitary or gonadotropins from entering systemic circulation, is usually caused by physical trauma, surgery or vascular infarction.

In addition to local effects on the hypothalamus or pituitary, there are systemic illnesses and nutritional conditions that can cause secondary hypogonadism. Examples of chronic illness include cirrhosis (Green, 1977), diabetes mellitus type 2 (Dhindsa et al., 2004) and renal failure (Albaaj et al., 2006). Additionally, severely altered nutritional statuses like anorexia nervosa and obesity (BMI 30 ≥kg/m^2) correlate with reduced fertility and low serum testosterone levels (Strain et al., 1982).

Primary gonadal

Primary hypogonadism results from diseases that directly affect the testis and have the potential to reduce serum testosterone and sperm quantity. Clinically, primary hypogonadism is distinguished from secondary by the corresponding elevation of both LH and FSH. However, presenting symptoms vary based on the cause of the disease and more importantly, if and when the loss of testosterone occurs. Additionally, male patients have a higher prevalence of gynecomastia due to the sustained increase in gonadotropins, which allows aromatase activity and consequently estrogen levels to rise

(R. E. Johnson & Murad, 2009). Finally, testicular disease, which can have genetic, environmental, infectious, physical and systemic origins, is estimated to provide the largest contribution to known causes of male-infertility at 30-40% (Mahmoud, 2000). The estimated range of genetic abnormalities in males with primary hypogonadal infertility is between two and eight percent. However, the association can increase to as much as 24% in azoospermic males (Dohle et al., 2002), with Klinefelter's Syndrome (KS) being the single largest contributor (Kamischke, Baumgardt, Horst, & Nieschlag, 2003). Other chromosomal abnormalities associated with male infertility include 46 XY/XO (Jackson, Hoffman, & Makda, 1966), various sex-chromosome translocations (Frydman et al., 2001; Guichaoua et al., 1990) and microdeletions within the Y-chromosome. The Yq11 region of the long arm of the Y-chromosome contains genes essential for spermatogenesis. The region, also known as the azoospermic factor (AZF), is subdivided into three parts (AZFa, AZFb and AZFc) (Tiepolo & Zuffardi, 1976; Vogt et al., 1996). Deletions impacting multiple genes within the AZFb and AZFc regions are more common than those within AZFa, due to large repetitive sequences (Krausz et al., 2006; Kuroda-Kawaguchi et al., 2001). While the exact role of AZF genes in spermatogenesis remains unclear, it is estimated these microdeletions contribute to approximately 10-15% of male infertility that present with severe oligozoospermia, azoospermia or Sertoli cell only syndrome (Martinez et al., 2000).

In addition to large-scale chromosomal abnormalities there are several single gene mutations that can impair male fertility. Some examples include mutations or polymorphisms to the genes responsible for the androgen receptor (Hiort et al., 2000), LH receptor (Latronico et al., 1996), FSH receptor (Tapanainen, Aittomaki, Min,

Vaskivuo, & Huhtaniemi, 1997), cystic fibrosis transmembrane conductance regulator (CFTR) (Cuppens & Cassiman, 2004), deleted in azoospermia-like (DAZL) (Teng et al., 2002), synaptonemal complex protein 3 (SYCP3) (Miyamoto et al., 2003) and insultin like 3 (INSL3) (Foresta & Ferlin, 2004). Finally, congenital disorders whose pathogenesis cannot be attributed to a single gene include cryptorchidism (undescended testes), testicular cancer and varicoceles (dilations of the pampiniform plexus).

Acquired testicular conditions can alter male fertility; however, the severity and associated symptoms vary depending on the causative agent, the timing of onset and whether sperm production, testosterone secretion or both are impaired. Several infectious conditions can result in infertility secondary to testicular inflammation (orchitis). Bacterial orchitis, often the result of a sexually transmitted infection (STI), typically begins as an epididymal infection that spreads to the testis (epididymo-orchitis) (Gonzales et al., 2004). Alternatively, viral orchitis is commonly derived from a mumps infection in post-pubertal males. Approximately one third of adult males that contract mumps will experience testicular involvement. In general, orchitis impairs male fertility through germ-cell disruption (Beard, Benson, Kelalis, Elveback, & Kurland, 1977).

A second significant source of acquired conditions that are harmful to male fertility includes exposure to various environmental and chemical agents. These products can impair spermatogenesis or androgen production with the extent of the damage being related to both dosage and duration. The sensitivity of the male reproductive system to environmental agents has been well documented (Spira & Multigner, 1998) and originates with case studies from the late 1970's involving the pesticide nematocide

(1,2-dibromo-3-chloropropane) (Whorton, Krauss, Marshall, & Milby, 1977). Other non-pesticide toxicants that can impair male fertility include ionization radiation (Ash, 1980) and alkylating/anti-neoplastic drugs like cyclophosphamide and cisplatin (Dohle, 2010). A third source of acquired testis-specific male infertility results from physical conditions. Examples include direct testicular trauma, bilateral orchiectomy, hyperthermia and testicular torsion.

Finally, many of the systemic illnesses, like cirrhosis (Green, 1977) and chronic renal failure (Albaaj et al., 2006), which cause secondary hypogonadism can also directly affect the testis. Other examples include men with HIV and sickle cell anemia. HIV positive patients, though fertile, tend to have lower testosterone levels (without the corresponding elevation in gonadotropins) compared to non-infected males (Dobs, Dempsey, Ladenson, & Polk, 1988; Sellmeyer & Grunfeld, 1996), whereas those with sickle cell anemia are often infertile, presumably due to testicular ischemic injury (Abbasi, Prasad, Ortega, Congco, & Oberleas, 1976).

The various biologic and molecular pathways involved in male sex determination, puberty onset and spermatogenetic development are extremely complex, and in many cases, still being investigated (McElreavey, Barbaux, Ion, & Fellous, 1995; Phillips, Gassei, & Orwig, 2010). Often these gaps in knowledge result in an ability to identify the target of the disorder but prevent a full understanding of disease etiology and pathogenesis. The consequence is that many cases of testicular-based infertility are diagnosed as idiopathic.

Post-testicular sperm transport

Spermatozoa entering the epididymis are both non-motile and incapable of fertilizing a female oocyte. During transit through the epididymal tubules, male sperm cells complete their maturation and are eventually stored in the caudal portion of the epididymis. During an ejaculatory event, caudal sperm pass through the vas deferens, are diluted by secreted products from the prostate, seminal vesicle and bulbourethral gland and eventually exit through the urethra.

Disorders that alter the ability of the epididymis to facilitate the later stages of sperm maturation, concentration and storage have the potential to impair fertility. Examples include bacterial sexually transmitted infections as well as other non-infectious abnormalities like trauma, vasectomy and neoplasia. Unfortunately, the current understanding of the conditions that impact the epididymis and their consequences is limited. The reasoning for this is two-fold; first, epididymitis, which is the most common intra-scrotal infection, only impacts approximately 0.4% of US males annually (Trojian, Lishnak, & Heiman, 2009) and second, disorders of the epididymis rarely impair spermatogenesis and therefore the ART of Intracytoplasmic sperm injection (ICSI) is still a viable option to maintain male fertility.

Lifestyle

The cumulative behavioral choices one makes appears to alter fecundity in both males and females. While the topic is still under investigation, there is variety of “lifestyle” factors that should be considered when assessing fertility complications. First, tobacco use in men can decrease sperm quality parameters including concentration and motility

(Vine, Margolin, Morrison, & Hulka, 1994) whereas in women, smoking more than 10 cigarettes per day is associated with an increased time required to become pregnant (D. D. Baird & Wilcox, 1985). Second, a Body Mass Index (BMI) above or below the normal range (18.5-25 kg/m^2) is statistically associated with anovulatory infertility in women and altered reproductive hormone levels in men (Clark et al., 1995). Third, prolonged strenuous exercise, particularly in females, is associated with infertility that is likely secondary to altered HPG axis hormone levels (Cannavo, Curto, & Trimarchi, 2001; Roupas & Georgopoulos, 2011). Forth, excessive alcohol consumption (≥ 14 drinks per week) is associated with increased times to become pregnant (Jensen et al., 1998), altered spermatogenesis (Pajarinen & Karhunen, 1994) and lower *In Vitro* Fertilization (IVF) success rates (Klonoff-Cohen, Lam-Kruglick, & Gonzalez, 2003). Finally, other "lifestyle" factors like caffeine intake, sexual behavior, environmental toxicant exposure, diet and stress are frequently mentioned in discussions regarding infertility but no clear consensus as to their mechanism of action has been established.

Unexplained

Unfortunately 15% of infertility cases fail to produce a definitive diagnosis. In these situations a single or a combination of slightly altered biologic properties is the most commonly offered rationale. Possibilities include mild decreases in sperm or egg quality (Lass et al., 1997), reduced semen quantity (Aitken, Best, Warner, & Templeton, 1984), impaired sperm/egg interaction (Tesarik & Testart, 1989) and incomplete ovarian luteinization (Li, Lenton, Dockery, & Cooke, 1990). While this diagnosis can be understandably frustrating, unexplained infertility is often not absolute as nearly 30% of

infertile women will become pregnant within a three-year window following diagnosis even without medical intervention (J. A. Collins, Burrows, & Wilan, 1995; Snick, Snick, Evers, & Collins, 1997). Additionally, assisted fertility counseling and treatments can improve prognosis. Options ranges from lifestyle adjustments (maintaining a normal body mass index (BMI = 20-27 kg/m^2), limiting caffeine, alcohol and smoking) to clinical intervention (clomiphene-induced ovulation with IVF) (Pandian, Gibreel, & Bhattacharya, 2012).

The reproductive system, and by extension, fertility success, is dependent upon cooperative interactions with other systems within the body including the immune system. The immune system is not only responsible for the efficient removal of infectious agents, but is also integral to many normal physiologic functions. Inappropriate activation of the immune system can produce many human diseases. The following sections will provide a background on the general principles of the immune system, its involvement with normal reproductive physiology and conclude with examples of immune disorders that impair human fertility.

THE IMMUNE SYSTEM

The immune system is a network of cells and organs broadly divided into two categories (innate and adaptive) that work together to protect a host against invasion from the multitude of potentially pathogenic microorganisms that share our environment. In the event of an infection, the immune system recognizes, responds and remembers the responsible foreign antigen such that the initial as well as any subsequent infection by

that pathogen can be eradicated.

Innate Immunity

Pattern Recognition Receptors and Pathogen Associated Molecular Patterns

The innate branch of the immune system, which is evolutionarily the oldest, is essentially the first line of host defense. It relies on a limited repertoire of germline-encoded receptors known as Pattern Recognition Receptors (PRR) that recognize and respond to highly conserved molecular motifs known as Pathogen Associated Molecular Patterns (PAMPs) found on various pathogens but absent within the host. This is a simple method allowing for a rapid distinction of harmless self from infectious non-self during the initial phase of an infection. The PRRs are broadly separated into two categories; secreted and cell associated. The circulating forms of pattern recognition receptors allow for direct microbial toxicity, increased phagocytosis (opsonization) and work synergistically with other transmembrane PRRs. While the list of secreted PRRs is extensive, they can be broadly grouped into antimicrobial peptides (Zasloff, 2002), pentraxins (Garlanda, Bottazzi, Bastone, & Mantovani, 2005), lectins (Holmskov, Thiel, & Jensenius, 2003) and collectins (Holmskov, Malhotra, Sim, & Jensenius, 1994; Holmskov et al., 2003). Alternatively, membrane bound pattern recognition receptors are constitutively expressed at the plasma membrane and on intracellular vesicles of many of the innate immune cells as well as by professional antigen presenting cells (B cells, monocytes/macrophages and dendritic cells). Engagement of these cell-associated receptors triggers an immune response by activating pathways that increase PRR expression, facilitate phagocytosis and stimulate the release pro-inflammatory

cytokines, chemokines and type I interferons. The classic transmembrane PRRs are the family of Toll-like receptors (TLRs) (Akira & Takeda, 2004; Takeda, Kaisho, & Akira, 2003) which are broadly expressed across several cell types, including dendritic cells, macrophages, epithelial cells and neutrophils, can recognize a variety of PAMPs including nucleic acids, proteins and portions of microbial cell walls and have domains that are highly conserved across species (O'Neill & Bowie, 2007; Yamagata, Merlie, & Sanes, 1994). Additional cell-associated PRRs include Rig-1 like receptors (Yoneyama & Fujita, 2007) and C-type lectins.

Cells of the Innate Immune System

Cells of the innate immune system are defined by their expression of PRRs and serve many different immune functions. Epithelial and mast cells reside in regions of high antigen exposure and provide a sentinel role. Stimulation of their PRRs results in the production of proinflammatory cytokines like TNF-α and IL-8 and other immune mediators including histamines, leukotrienes and proteases. Neutrophils, monocytes/macrophages and natural killer cells provide the effector functions of the innate immune system. Neutrophils (Mantovani, Cassatella, Costantini, & Jaillon, 2011), which are the most abundant circulating phagocyte, are the initial immune component recruited to a site of inflammation. Activated neutrophils phagocytize opsonized microbes and attempt to destroy them within the phagosome via reactive oxygen species (ROS), proteases and other anti-microbial products. Bone marrow derived monocytes complete differentiation into macrophages upon entering into a tissue. Similar to neutrophils, they phagocytize and destroy microbes, but also provide

additional immune functions including antigen presentation (Martinez-Pomares & Gordon, 2007) and the secretion of a much larger repertoire of inflammatory products (Takemura & Werb, 1984). Natural killer cells (NK cells), although derived from the lymphoid lineage, are still a component of the innate immune system. Besides the standard set of PRRs, NK cells have an additional battery of activating and inhibitory receptors (Lanier, 1998) that regulate and preferentially direct their response towards cells that are virally infected or malignant.

Communication between the Innate and Adaptive Immune System

The two branches of the immune system are distinguished by their developmental lineage and the origin of their antigen recognition receptors. But they do not function independently of one another. PAMP recognition by the innate immune system can both activate antigen presentation pathways and function as an adjuvant, thereby alerting the adaptive immune system to a pathogen and generating a sustained vigorous response. The dendritic cell (DC) is arguably the most important cellular mediator of communication between the innate and adaptive immune systems. DCs become activated through direct recognition of PAMPs and exposure to proinflammatory cytokines including type I interferons and IL-12 (Langenkamp, Messi, Lanzavecchia, & Sallusto, 2000). This maturational process facilitates key events including phagocytosis and destruction of microbes; the upregulation of cytokine receptors, major histocompatibility complex (MHC) II molecules, and costimulatory molecules (CD80 and CD86); and the subsequent presentation of antigens derived from phagocytosed microbes. Activation culminates in the migration of the mature DC from the initial site of

inflammation to a draining lymph node where it can initiate an adaptive immune response by presenting antigen to naïve T cells (Stoll, Delon, Brotz, & Germain, 2002). Importantly, this communication pathway is not unidirectional, as effector products released from activated lymphocytes can influence the cellular response of DC through feedback signaling pathways. This concept is illustrated by the following examples. First, opsonization of microbes by specific B cell generated antibodies aids in efficient phagocytosis by innate immune cells. Second, activated T cells secrete IFN-γ that feeds back on macrophages to enhance their ability to eliminate microbes by stimulating enzyme pathways that reduce ROS into superoxide radicals.

Adaptive Immunity

The adaptive immune system evolved as a mechanism of host defense in response to various pathogens that developed the ability to evade innate immunity. Key features of the adaptive immune system that are distinct from innate immunity include near limitless antigen specificity and immunologic memory.

Humoral Immunity

B Cell Development

Humoral (non-cellular component of blood) immunity refers to immune defense mediated by antibodies, which are the secreted effector product of mature and activated B cells. B cell development first occurs in the fetal liver and is gradually transitioned to the bone marrow at around 12 weeks of gestation (Hardy & Hayakawa, 2001). The creation of a B cell from a common lymphoid progenitor involves a series of highly

regulated steps controlled by lineage-specific growth factors, transcription factors and enzymes. Further, the transition between stages requires the successful rearrangement and expression of the Ig (immunoglobulin) heavy and light chains that constitute the B-cell Receptor (BCR), which is in essence a membrane-bound antibody. In brief, the first stage of B cell development (pro-B cell) is initiated by extrinsic factors including IL-7, stem cell factor and IGF-1 and results in the intracellular expression of recombinase activating genes (RAG) 1 & 2 and the membrane expression of CD19. Transition to the pre-B cell stage occurs when the RAG genes complete a successful V-D-J recombination (Brack, Hirama, Lenhard-Schuller, & Tonegawa, 1978; Schatz, 2004) of the Ig heavy chain, which is then expressed (along with a surrogate light chain) at the cell surface. Other key markers of this stage include expression of CD40 and TdT (terminal deoxytransferase). Finally, the pre-B cell becomes mature when the Ig light chain is rearranged and combined with the heavy chain to generate an IgM molecule that is expressed at the cell surface. Mature B cells, still within the bone marrow, that have a high affinity for self antigens can attempt to edit their antigen receptor (Tiegs, Russell, & Nemazee, 1993) through a reactivation of the V(D)J recombination process. It is estimated that nearly half of all peripheral B cells have activated this mechanism of receptor editing in order to assemble an Ig that is nonresponsive to autoantigens (Retter & Nemazee, 1998). B cells with Ig receptors that are ignorant of self-antigens leave the bone marrow and migrating to secondary lymphoid organs like the spleen, tonsils and lymph nodes, whereas those with Ig receptors that are still specific for self-antigens are removed via apoptosis (negative selection).

B cell Activation

Activation of a mature/resting naïve B cell requires two initial stimulatory signals. First is the simultaneous engagement of multiple B cell receptors. The second is a cooperative signaling event from either circulating cytokines or cell-cell contact. The consequence of B cell activation depends on the type of antigen driving the response and whether or not it is the initial exposure to that antigen. Type I and II Thymus-Independent (T-Independent) antigens are typical polysaccharides that activate B cells through intrinsic repeated structural motifs and produce a response that is generally low affinity, IgM-based and creates little to no immunologic memory. Thymus-Dependent (T-dependent) antigens are protein in origin and rely on cooperation between B and T cells to create a polyclonal, high affinity response that produces long-lived immunologic memory (Stein, 1992).

The steps involved in the T-dependent activation and maturation of a naïve B cell during a primary immune response are quite extensive and well-reviewed (Baumgarth, 2000; Parker, 1993). Briefly, an antigen-activated T cell within a lymph node will recognize an activated B cell presenting the same antigen on its surface in the context of an MHC molecule. The T cell will provide the secondary activating signal through ligation of the cell surface molecules CD40-CD40L. These signaling events induce B cell hyperproliferation within a germinal center and led to Ig class switching and hypermutation through the activation of the DNA altering enzyme activation-induced cytosine deaminase (AID). The end result is expanded populations of B cells that make different subclasses of Ig molecules with high affinity for the stimulating antigen. Mature B cells have three basic fates. Most mature B cells are lost in the germinal

center to unsuccessful affinity maturation of the Ig molecule wherein they no longer recognize the stimulating antigen. Second, a percentage of B cells with a productively rearranged Ig molecule will differentiate into plasma cells, migrate to the bone marrow and secrete large amounts of circulating antibodies. During the primary response, the time from initial exposure to production of high affinity, class-switched antibodies can take nearly two weeks. Finally, a small percentage of the stimulated B cells will differentiate into $CD27^+$ memory B cells (Klein, Rajewsky, & Kuppers, 1998) that can survive for decades. Upon subsequent exposures, memory B cells commence a secondary immune response by rapidly differentiating into plasma cells that release high affinity antibodies. Compared to a primary response, the secondary immune response is much faster and more robust.

Cellular Immunity

T Cell Development

The majority of circulating T cells complete their development within the thymus and express a T cell receptor (TCR), which is composed of a heterodimer of an α and a β chain (Garcia et al., 1996). Early T cell progenitors are first detected in the fetal liver around the sixth week of gestation (McCune et al., 1988; Touraine, Roncarolo, Royo, & Touraine, 1987); by the end of the second trimester, hematopoietic development transitions to the bone marrow (Haynes, Martin, Kay, & Kurtzberg, 1988; Spits, Lanier, & Phillips, 1995). These bone marrow-derived progenitors migrate hematogenously to the thymus, where they enter through high endothelial venules at the corticomedullary junction and gradually migrate to the outer cortex (Kwan & Killeen, 2004). Within the

thymus, specialized epithelial cells control the recruitment, development, expansion and differentiation of early T cell progenitors through cell-to-cell contact, which activates the notch-signaling pathway, and a variety of secreted products including IL-7, thymopoietin and thymulin (Takahama, 2006). Briefly, immature T cells within the cortex undergo a massive IL-7-dependent expansion, and expression of the cluster of differentiation (CD) markers CD7, CD1 and CD2 is induced. At this stage, cortical T cells are 'double negative' (DN), i.e., they lack expression of both of the CD4 and CD8 co-receptors. These DN thymocytes progress through four stages that are identified by the differential expression of CD44 (E-selectin ligand) and CD25 (IL-2 receptor alpha chain) (Starr, Jameson, & Hogquist, 2003). A key event during these DN stages is RAG-dependent recombination of the TCR beta chain, followed by surface expression of the pre-TCR together with the lineage-specific CD3 co-receptor complex. Signaling through the pre-TCR promotes several important outcomes including cell survival, further differentiation, allelic exclusion of the TCR beta chain (Borgulya, Kishi, Uematsu, & von Boehmer, 1992), recombination of the TCR alpha chain and the co-induction of CD4 and CD8. Finally, "double positive" (DP) thymocytes experience positive selection (see below) in the cortex before migrating to the medulla where T cells complete maturation by undergoing negative selection (see below) and acquiring the singular expression of either CD4 or CD8. Ultimately, only 2-5% of T cells complete this process and provide peripheral immune surveillance.

T Cell Activation

The specific activation of T cells to eradicate an infection requires a controlled series of

events. Professional antigen presenting cells (APC), become activated by ligation of their inflammatory cytokine receptors and PRRs. Activated APCs migrate from the site of infection to a draining lymphoid organ where they present acquired antigens in the context of MHC I and II molecules to naïve T cells. MHC restriction ensures the activation of an appropriate T cell subset (see below) for a given infection. Class I MHC is critical for identifying virally infected cells by presenting peptides derived from intracellular proteins to $CD8^+$ T cells. Class II MHC molecules, which are selectively expressed on professional APCs, present peptides from phagocytized microbes to $CD4^+$ T cells to fight extracellular infections. The power of the adaptive immune system resides in its specificity. The variable recombination of the V, D and J gene segments (Schatz, 2004) of the TCR by RAG-1 and -2 allow for a theoretical limit of $2.5x10^7$ different antigen receptors (Casrouge et al., 2000). Specific antigen/MHC recognition by a unique complementary TCR induces intracellular signaling pathways leading to proliferative clonal expansion and the expression of co-stimulatory molecules and subset specific effector genes (see below). Similar to B cells, T cells require two linked stimulatory signals for a successful activation. The CD3 complex, which is non-covalently associated with the TCR, provides the initial signal in response to antigen recognition. The second co-stimulatory signal for T cell activation is provided through the ligation of CD28 on T cells with CD80 and CD86 molecule on APCs (Fujii, Liu, Smith, Bonito, & Steinman, 2004). These activation signals result in the upregulation of CD154 on T cells which binds to CD40 on macrophages, endothelial cells and B cells resulting in cellular activation and, in the case of B cells, affinity maturation and Ig class switching (Grewal & Flavell, 1998).

T Cell Subsets

Mature T cells are broadly separated into two categories: cytotoxic T cells, which mediate an immune response via direct cellular contact and modulatory T cells, which either activate or repress lymphocytes by secreting various cytokines. Cytotoxic T cells (Tc) are MHC class I restricted due to expression of the co-receptor CD8. Upon activation, Tc cells reenter the circulatory system in search of infected cells to synapse with and induce apoptosis through the Fas-FasL (Ju et al., 1995) pathway or, alternatively, through the release of granules loaded with perforin and granzymes. CD4+ T cells, which are MHC class II-restricted, are separated into four main effector groups (Th1, Th2, Th17 and Treg) based on their cytokine secretion profiles. T helper (Th) 1 cells, which release IL-2, IL-3, IFN-γ and TNF, are responsible for fighting intracellular infections by activating macrophages and cytotoxic T cells and by promoting B cell differentiation into antibody-secreting plasma cells. Th2 cells help fight parasitic infections by releasing cytokines and chemokines that recruit eosinophils and by inducing plasma cell production of IgE. Th17 cells (Harrington et al., 2005) are typically associated with chronic autoimmune inflammatory syndromes and develop after prolonged exposure to IL-6, TGF-beta and IL-1beta. These cells predominately secrete the proinflammatory cytokines IL-17, IL-22 and IFN-γ. Finally, T regulatory cells (Sakaguchi, 2004; Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 2011) (Tregs), are defined by expression of CD25 and the transcription factor Foxp3 (Hori, Nomura, & Sakaguchi, 2003), can develop *de novo* in the thymus or can be induced in the periphery, and serve as powerful suppressors of the immune system through release of IL-10 and TGF-beta (see below).

Mechanisms of Immune Tolerance

Regulation of mature lymphocytes is a constant and dynamic process that permits an immune response against harmful non-self while stemming immune recognition of harmless self. Immune tolerance mechanisms occur within the central lymphoid organs (thymus and bone marrow) during lymphocyte development, and continue in peripheral lymphoid tissues (spleen, lymph nodes and tonsils) where the immune response is initiated.

Central Tolerance

The critical ability of lymphocytes to differentiate between benign self-antigens and potentially pathogenic foreign antigens begins during their development within the thymus (T cells) and bone marrow (B cells). The basic premise of central immune tolerance, identification of lymphocytes that are capable of initiating an immune response (positive selection) and removal of those that vigorously respond to self-antigens (negative selection), applies to both B cells and T cells. However, details of this process are better understood for T cell development (Takahama, 2006). As discussed above, bone marrow-derived T cell precursors begin development in the thymic cortex where they rapidly proliferate, progress through the four DN stages, rearrange the TCR genes, and finally co-express CD4 and CD8. Continued survival of these DP T cells relies on interactions between the recombined TCR and peptide/MHC complexes found on APCs within the thymus. Low affinity recognition of self-peptide/MHC *positively* selects a developing T cell for survival, whereas lymphocytes with antigen receptors that fail to bind or demonstrate strong avidity for self-

peptide/MHC are “neglected” or actively removed via apoptosis, respectively (Surh & Sprent, 1994). It is estimated that nearly 95% of thymocytes fail to progress beyond the cortical DP stage of development (Egerton, Scollay, & Shortman, 1990). Low-avidity interaction between the TCR of surviving T cells and peptide-MHC complexes promotes further differentiation, which includes up regulation of CCR7 ligands (Kwan & Killeen, 2004), singular expression of either CD4 or CD8 (Campbell, Pan, & Butcher, 1999; Sohn, Forbush, Pan, & Perlmutter, 2001) and migration to the thymic medulla (Witt, Raychaudhuri, Schaefer, Chakraborty, & Robey, 2005). Within the medullary compartment, single positive (SP) thymocytes with a high affinity antigen receptor for self-peptide/MHC complexes are intentionally destroyed (*negative selection)* to prevent the release of mature self-reactive lymphocytes that could potentially initiate autoimmune disease.

Autoimmune Regulator (AIRE)

The breadth of self-antigen representation within the thymus for the purpose of negative selection and ultimately the removal of autoreactive T cells were considered to be limited to only ubiquitous antigens as recently as the late 1980’s. In addition, the identification of autoreative lymphocytes within the periphery (Guilbert, Dighiero, & Avrameas, 1982; Miller, Njenga, Parisi, & Rodriguez, 1996) resulted in the suggestion that peripheral, not central, immune tolerance had a larger role in preventing disease (Cohen, 1986). However, it is now recognized that the self-antigenic repertoire the thymus utilizes for central immune tolerance is extensive, representing nearly every peripheral organ and required to prevent the development of multiple autoimmune

disorders. This paradigm shift was initiated by three key observations. First was the detection of ectopic expression of transgenes driven by the insulin promoter within the thymus (Jolicoeur, Hanahan, & Smith, 1994). This observation resulted in the discovery of low levels of endogenous insulin expression within the thymus. Further investigation revealed that thymic insulin expression was important for preventing type-1 diabetes by eliminating insulin-specific T cells (Pugliese et al., 1997). Second was the development of multiple independent organ-specific autoimmune diseases following neonatal mouse thymectomy (Tong & Nelson, 1999). The removal of the thymus on the third day after birth results in disease by depleting the regulatory T cell population, which permits the unsuppressed activation of self antigen-specific T cells. Finally, the discovery of over 30 peripheral organ specific antigens in purified pools of thymic stromal cells by qRT-PCR suggested that the repertoire of tissue-specific antigens used for deletional tolerance might be larger than previously imagined. The antigenic expression profile of the thymus has now been fully characterized, and includes several thousand genes, some of which are otherwise restricted to only a few peripheral tissues, including those that are differentially expressed in relation to age, sex and pubertal development (Anderson et al., 2002; Derbinski, Schulte, Kyewski, & Klein, 2001). These thymic proteins that are selectively expressed throughout an organism are known as tissue restricted antigens (TRAs).

Thymic self-antigens are presented by resident APCs, cells that emigrate from circulation (B. A. Kyewski, Fathman, & Rouse, 1986), and the epithelial cells of the thymic cortex and medulla. However, an intrinsic property exclusive to the medullary thymic epithelial cells (mTECs), and to a lesser extent the cortical thymic epithelial cells

(cTECs), is the promiscuous gene expression (pGE) of tissue-restricted antigens that facilitates a comprehensive representation of antigenic self (Anderson et al., 2002; Derbinski et al., 2001). The mechanisms that result in mTEC pGE are still under investigation, however the study of this form of gene regulation has revealed five unique characteristics. First, promiscuous gene expression in the thymus occurs independent of the developmental parameters that controls their normal expression within a peripheral tissue. Second, expressed genes are commonly clustered within the genome. Third, different mTEC subsets preferentially express certain TRAs more frequently than others. Fourth, promiscuous gene expression mechanisms result in biallelic expression, even for those genes where one allele is normally silenced. Finally, single cell gene expression analysis has shown that each individual mTEC only express a few TRAs at one time and further that any overlap in expression of a given promiscuously expressed gene is limited to only 1-3% of mTECs (Derbinski, Pinto, Rosch, Hexel, & Kyewski, 2008).

The autoimmune regulator (AIRE) protein is integral to the pGE of TRAs within the thymus. AIRE is a 545 amino acid transcriptional co-activator that is localized to chromosome 21q22 (Nagamine et al., 1997) and is mainly expressed within the nuclei of mTECs (Gray, Abramson, Benoist, & Mathis, 2007). Unlike classic DNA binding transcription factors, AIRE has the unique ability to influence the expression of several thousand genes (Johnnidis et al., 2005). Currently, its specific molecular mechanism of action remains unclear. However, its known binding partners (CBP, PIAS1, DNA-PK, and P-TEFb) (Abramson, Giraud, Benoist, & Mathis, 2010) as well as its unique combination of peptide domains (Anderson et al., 2005; Peterson, Org, & Rebane,

2008) that are found in other chromatin binding proteins and transcriptional regulators, has yielded some clues. Co-immunoprecipitation with molecules like CBP and DAXX suggests AIRE influences broad control over gene expression through association with higher order transcriptional complexes (Meloni et al., 2010; Pitkanen et al., 2005). However, AIRE has also been shown to selectively access genes within the casein locus (Derbinski et al., 2008), suggesting individual gene regulation in addition to influencing large genomic regions. Finally, AIRE implements epigenetic regulation of gene expression by increasing methylation at H3K4 as well as decreasing methylation at H3K27 (Koh et al., 2008; Org et al., 2008; Org et al., 2009). Precise molecular mechanisms aside, AIRE-induced gene expression provides a considerable proportion of the thymic self-antigen repertoire for the purpose of deletional tolerance.

The full catalogue of AIRE-induced promiscuously expressed genes has been estimated at 3000, or roughly 5-10% of the genome (Gotter, Brors, Hergenhahn, & Kyewski, 2004). These genes provide extensive antigenic coverage and therefore significantly contribute to the tolerance of the various cell types and organ systems within the body. The combined discoveries of pGE and the AIRE protein have dramatically broadened the importance of central tolerance in stemming autoimmunity against not only common antigens but also to those that are rare or restricted in distribution. This shift in thinking has particular relevance to reproductive fitness. Many of the reproductive antigens expressed within the thymus are present simultaneously within their endogenous tissue distribution. However, some genes, including those for the casein family of proteins, HLA-G and AFP are only expressed following pubertal development and pregnancy (Derbinski et al., 2008; Lane et al., 2014; Mallet et al.,

1999). The unique ability of AIRE to influence gene expression regardless of developmental status allows the immune system to become tolerized to antigens such as these prior to their normal expression in the periphery. The complete immunologic tolerance necessary for reproduction requires not only peripheral tolerance mechanisms (see below) including the partial sequestration of male germ cell antigens behind the blood-testis barrier (Yule, Montoya, Russell, Williams, & Tung, 1988) and lack of class I MHC expression on oocytes (Roberts, Taylor, Melling, Kingsland, & Johnson, 1992) but also likely dependent on centrally induced tolerance to a host of reproductive antigens.

Peripheral Tolerance

The thymic removal of autoreactive T cells is efficient, but not absolute. Peripheral immune tolerance is required for controlling the few high avidity and the more common low avidity self-reactive mature lymphocytes that escape negative selection (Liu et al., 1995). Additionally, mechanisms of peripheral immune tolerance are responsible for specifically directing the activation of mature lymphocytes against foreign pathogens and containing the response once the infection has been cleared. Peripheral control begins with the requirement of multiple cooperative signaling events for complete B and T cell activation. The first signal, cross linking of multiple Ig receptors (B cells) or recognition of a complementary peptide/MHC complex (T cells), will only result in activation if it is accompanied by additional co-stimulatory signals. Engagement of CD40 on B cells with CD154 on an antigen activated T cell is the principle co-stimulatory pathway leading to B cell activation, Ig affinity maturation and Ig class switching. Similarly, complete T cell activation requires binding of CD28 on T cells with the co-

stimulatory molecules CD80/86 found on mature APCs (Fujii et al., 2004). Stimulation of the antigen receptor alone will render the lymphocyte unresponsive (Sloan-Lancaster, Evavold, & Allen, 1993) (anergic) and potentially initiate apoptotic pathways.

The immune response is very powerful and if left unchecked could produce large collateral tissue damage or malignant cellular proliferation. Activated lymphocytes have intrinsic inhibitory mechanisms designed to protect the host from these detrimental outcomes once an infection has been cleared. Importantly, these mechanisms are initiated by signaling through the antigen receptor, but there is a delay in fulfillment of their inhibitory potential allowing time for the lymphocyte to perform its effector role. Key examples of inhibitory pathways include activation induced cell death and signaling through the programmed cell death-1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) receptors (Jones, Chin, Longo, & Kruisbeek, 1990; Nishimura, Nose, Hiai, Minato, & Honjo, 1999; Siegel & Fleisher, 1999; Waterhouse et al., 1995). Following activation, T cells will reduce expression of CD28 and induce the expression of CD95, CD120b, PD-1 and CTL-4. Signaling through CD95 and CD120b by Fas-ligand and TNF-α, respectively, induces apoptosis, whereas PD-1 and CTLA-4 are receptors that prevent signaling from the T cell receptor.

Finally, Tregs provide a vital role in peripheral immune suppression by locally inhibiting several aspects of an immune response. Tregs are defined by an expression profile that includes CD4, CD25, CD127, CTLA-4 and the lineage-specific marker, Foxp3. They are activated in an antigen-specific manner, but inhibit nearby immune cells nonspecifically through the release of two potent anti-inflammatory cytokines, IL-10 and TGF-β. IL-10 signaling results in the down-regulation of MHC class II and costimulatory molecules on

APCs, and blocks the secretion of proinflammatory cytokines, including IL-2, TNF-α and IFN-γ. TGF-β signaling through the SMAD pathway (Conery et al., 2004) induces apoptosis thereby directly preventing peripheral lymphocyte activation.

IMMUNOLOGY OF REPRODUCTION

Immune System and Normal Reproductive Physiology

Female

Ovary

The ovaries are a pair of pelvic organs that produce gonadal steroids (estrogens and progesterone) as well as develop, store and release the female gamete. Briefly, primordial germ cells migrate to the gonadal ridge between the fourth and eight week of gestation. The absence of the SRY gene and Anti-Müllerian Hormone (AMH) allows for the establishment of the female gonad. The pool of oogonia expands mitotically through the first five months of gestation until a maximum allotment of approximately 5 to 7 million germ cells is reached (T. G. Baker, 1963). At this point the oogonia initiate meiosis and arrest at prophase I, thereby becoming an oocyte. Next, cells from the neighboring mesothelium and mesonephros colonize the ovarian cortex. These infiltrating cells provide the supportive granulosa cells that encapsulate each oocyte, forming the primordial follicles, which remain quiescent until before puberty. The initial growth and expansion of follicles (folliculogenesis) from the primordial to the pre-antral stage occurs independently of gonadotropin signaling. At the onset of puberty, pulsatile secretions of FSH and LH promote the resumption of the meiotic cycle, follicular growth and the initiation of menstrual cyclicity (Messinis, 2006). Ultimately a single dominant

follicle will be selected for ovulation from a small cohort of antral (2 – 5mm) follicles (D. T. Baird, 1987; Gougeon & Lefevre, 1983). The remaining subordinate follicles undergo a regressive process known as atresia that is likely apoptotic in nature (Tilly, Kowalski, Johnson, & Hsueh, 1991). Estrogen secreted from the dominant follicle prior to ovulation provides inhibitory feedback on GnRH secretion (Yamaji, Dierschke, Bhattacharya, & Knobil, 1972) and helps prepare the uterus for embryo implantation by stimulating uterine hyperplasia and inducing the upregulation of the progesterone receptor (van Dessel et al., 1996; Westergaard, Christensen, & McNatty, 1986). Following ovulation, the remaining theca and granulosa cells of the ovulatory follicle continue to grow, become vascularized and differentiate to form a corpus luteum(CL). The CL releases progesterone and to a lesser extent estrogen, which together ensure the receptivity of the uterus for the implantation of an embryo and maintains uterine quiescence during gestation. Although it should be mentioned that the role of luteal estrogen is not well understood and may not be necessary for pregnancy maintenance (Zegers-Hochschild & Altieri, 1995).

The immune system has an important role in many ovarian functions, including ovulation, luteinization and luteal regression. Various leukocyte populations are recruited to the ovary throughout the menstrual cycle. Both $CD4^+$ and $CD8^+$ T cells localize to the thecal layer of growing follicles and increase in density within atretic follicles and regressing CL (Suzuki et al., 1998). T cells are thought to play an important role in CL regression by activating macrophages and inhibiting steroidogenic pathways through the secretion of TNF-α and IFN-γ (Bagavandoss, Kunkel, Wiggins, & Keyes, 1988; Komatsu, Manabe, Kiso, Shimabe, & Miyamoto, 2003). Mast cells are also

present within in the ovary and their densities vary through the estrous cycle (Gaytan, Aceitero, Bellido, Sanchez-Criado, & Aguilar, 1991). Histamine released by mast cells is thought to promote ovulation through the induction of progesterone production and the vasodilation of the ovarian vasculature (Kobayashi, Wright, Santulli, Kitai, & Wallach, 1983). Macrophages and neutrophils are recruited to the ovary by the chemokines MCP-1 and IL-8, which are induced downstream of gonadotropin signaling (Arici et al., 1997). Both of these cell types localize to the apex or stigma (region of ovarian surface through which the mature ovum will be released) of the dominant follicle (Takaya et al., 1997) where they release matrix metalloproteinases. The breakdown of the extracellular matrix (ECM) by these secreted proteinases in combination with increase blood and intra-follicular pressure help to induce an ovulatory event. In addition, macrophages and neutrophils help promote neoangiogenesis within the developing CL after ovulation and are likely involved in the clearance of apoptotic luteal cells following a prostaglandin-induced regression.

Male

Testis

The testis is the primary site of male androgen production and germ cell development. Structurally, the testis is an encapsulated series of highly coiled tubes (seminiferous tubules), inside of which sperm develop. Specialized epithelial cells called Sertoli cells, which are themselves surrounded by peritubular myoid cells, line the seminiferous tubules. The inter-tubular space is a loose connective tissue, which houses the testosterone-producing Leydig cells. Safeguarding this organ is essential for both male-

specific development and fertility. There are numerous afflictions that can result in primary gonadal insufficiency (see above), including the careful regulation of the immune system within the testis to prevent autoimmune damage. The testis has long been considered an immune privilege site because of its ability to be successfully xenografted as well as accepting of xenografts (Honaramooz, Li, Penedo, Meyers, & Dobrinski, 2004; Selawry & Whittington, 1984). In general, immune regulation within the testis is considered suppressive, but not completely passive. This is important because immune components within the testis must still recognize and clear various infections (particularly bacterial) to prevent a permanent depletion of germ cells.

The Sertoli cell, which establishes the germ-cell specific microenvironment necessary for proper mitotic and meiotic development of sperm, is also integral to immune regulation within the testis. A defining function of Sertoli cells to establish the blood-testis-barrier (BTB), which is a unique series of basal tight junctions between the cells that isolates developing postmitotic spermatocytes from exposure to the immune system. In addition to the sequestration of sperm antigens, Steroli cells express TLRs 2-7 and the apoptosis-inducing Fas Ligand as well as release various immune modulating factors such as the tryptophan metabolizing enzyme indoleamine 2,3-dioxygenase (IDO) and complement inhibitors (Fallarino et al., 2009; Lee, Richburg, Younkin, & Boekelheide, 1997; Wu et al., 2008). Leydig cells release TGF-β which, in addition to promoting Sertoli and germ cell proliferation induces the differentiation of immunosuppressive Tregs (Avallet, Vigier, Leduque, Dubois, & Saez, 1994; Boitani, Stefanini, Fragale, & Morena, 1995; Huber et al., 2009; Mather, Attie, Woodruff, Rice, & Phillips, 1990).

Finally, the leukocytes within the testis includes dendritic cells, macrophages and T cells (Itoh, De Rooij, Jansen, & Drexhage, 1995). Under normal physiologic conditions, these immune cells reside in the intertubular space and appear phenotypically shifted to promote immune tolerance. Tolerogenic dendritic cells are a special subset of DCs that are capable of enhancing the suppressive function of Tregs and rendering antigen-specific T cells anergic (Aichele et al., 1994; Maldonado & von Andrian, 2010). Tregs and M2 macrophages both promote tolerance through the release of the immune suppressing cytokines TGF-β and IL-10 (Andersson et al., 2008; Bamboat et al., 2010).

Epididymis

The elongated spermotzoa that move from the testis to the epididymis are immobile and unable to fertilize an oocyte. The luminal environment of the epididymis provides the necessary signaling molecules that allow sperm to become progressively more motile and competent for capacitation, prior to being stored within the cauda. Structurally, the epididymis is, like the testis, a series of convoluted tubes; additionally, the epithelial lining is pseudostratified and the surrounding loose connective tissue contains abundant fibroblasts and smooth muscle.

While the testis is considered immunologically privileged, the epididymis is not, and thus is also susceptible to infection. Important immune-related differences between the two sites include TLR expression, permeability of the epithelial tight junctions, frequency of leukocyte infiltration and the distribution of immune cells. Similar to Sertoli cells, the epithelial cells of the epididymis express TLR 2 – 7, but they also expression TLR 9 and 11 (Palladino, Savarese, Chapman, Dughi, & Plaska, 2008). These cells also secrete

TGF-β and IL-10 (Bomgardner, Wehrenberg, & Rune, 1999). Compared to the BTB, the epithelial tight junctions of the epididymis are more permeable and have a classic apical location. Resident neutrophils are rare under normal conditions in the testis, but occasionally seen in the epididymis, and their density increases with age (Itoh, Miyamoto, Ohga, & Takeuchi, 1999). Finally, whereas the testis lacks leukocytes inside the BTB, the epididymal epithelium is replete with resident intraepithelial CD8+ T cells, macrophages, and dendritic cells (Da Silva et al., 2011; Serre & Robaire, 1999). Importantly, both of the antigen presenting macrophages and dendritic cells have been shown to extend dendrites towards the tight junctions of the blood-epididymal barrier (Da Silva et al., 2011). While the precise function of these intraepithelial APCs is not known, it has been suggested that they are either involved in maintenance of the mucosa (Geissmann, Gordon, Hume, Mowat, & Randolph, 2010) or are active participants in establishing immune tolerance to neoantigens present on post-testicular sperm (Da Silva et al., 2011).

Autoimmune Disease and Infertility

Susceptibility to autoimmune diseases involves a complex interplay between one's genetics and their environment. The overall prevalence of autoimmunity in the general population is estimated at 3-5% (Jacobson, Gange, Rose, & Graham, 1997) and classically, is attributed to an aberrant response to a single self-antigen (Marrack, Kappler, & Kotzin, 2001). Many of those afflicted with autoimmune disorders are of reproductive age, and clinical studies over the past four decades have implicated an intrinsic association between fertility and autoimmunity. This relationship, even during

episodes of remission, can impact the gametes of both sexes, as well as all stages of gestation from fertilization through parturition. Further, maternal autoimmune disease can extend its influence into early neonatal and infant development. Based on these concerns, as little as 25 years ago women with a diagnosed autoimmunity were encouraged to refrain from having children. While recent advances in disease management have improved prognosis, pregnancies in women with a known autoimmune disorder are maintained as “high risk” due to the potential for complications (Gordon, 2004).

Autoimmune disease can incite severe reproductive pathologies including premature ovarian insufficiency, infertility and recurrent pregnancy loss. In the general population there is a strong female gender bias in autoimmune disease presentation (Whitacre, 2001). Diseases, including autoimmune thyroid disease, systemic lupus erythematous (SLE), Sjogren's and scleroderma maintain a patient population that is over 80% female (Beeson, 1994; Borchers, Naguwa, Keen, & Gershwin, 2010). While the cause of the sexual dimorphism is still being studied it is likely due impart to gender differences in the immune system and sex steroids (estrogen, progesterone and testosterone). Sex related immune differences include higher CD4 T cell populations, increased Th1 cytokine produciton following vaccination and, in mice, incrase produciton of antibodies following immunization in females compared to males (Amadori et al., 1995; Eidinger & Garrett, 1972; Huygen & Palfliet, 1984). Evidence for hormonal influence on autoimmune disease includes estrogen-inhanced cytokine secretion *in vitro*, significantly reduced serum testosterone concentration in men with Rheumatoid arthritis (RA) and reduced disease severity for multiple sclerosis (MS) and RA during the third trimester of

pregnancy when estrogen and progesterone concentrations are at their peak (Birk et al., 1990; Gilmore, Weiner, & Correale, 1997; J. L. Nelson & Ostensen, 1997; Ostensen & Villiger, 2007; Spector et al., 1989)

Etiology and pathogenesis of autoantibodies

Autoimmune disease is caused by an aberrant recognition and subsequent response of the adaptive immune system against self-tissue. A hallmark of these conditions is the presence of self-antigen specific antibodies. Although not always pathogenic, these autoantibodies can appear prior to the onset of disease symptoms (Heinlen et al., 2007; Nielen et al., 2004) and are representative of the antigen driving the immune response, making them both predictive and diagnostic (ter Borg, Horst, Hummel, Limburg, & Kallenberg, 1990; Vencovsky et al., 2003). Determining both the originating antigenic stimuli and if the disease is mediated through a cellular or humoral mechanism has important research and clinical value. However, as disease etiologies have remained elusive for several autoimmune disorders, a complete understanding of the progression may facilitate effective management strategies without a definitive knowledge of the origin.

Direct evidence for humoral pathogenicity requires serum transfer followed by the development of disease symptoms in the recipient. While there are a few examples of passive disease transfer using human autoantibody-containing serum into immune-deficient mice (Anhalt, Labib, Voorhees, Beals, & Diaz, 1982; Robinson et al., 1998; Wooley et al., 1984), perhaps the best evidence for human autoantibody pathogenicity comes from transplacental transfer of autoantibodies during pregnancy leading to

neonatal disease. Maternal-fetal transfer of pathogenic antibodies has been demonstrated for range of autoimmune conditions including Graves' disease, myasthenia gravis, neonatal lupus and the antiphospholipid syndrome (G. M. Fenichel, 1978; Mahoney, Pyne, Stamm, & Bakke, 1964; Motta et al., 2012; Vonderheid, Koblenzer, Ming, & Burgoon, 1976). Excluding a few serious examples like congenital heart block and extensive thrombosis, the disease symptoms generally wane quickly after birth as the exogenous antibodies are cleared (Appan, Boey, & Lim, 1987; Buyon et al., 1998).

Indirect evidence for pathogenic antibody involvement in autoimmune disease derives from serum transfer experiments in a variety of animal model systems that reflect human diseases. Examples include collagen-induced autoimmune arthritis (Stuart & Dixon, 1983), anti-myosin antibodies in autoimmune myocarditis (Liao et al., 1995) and anti-cardiac troponin I antibodies from Program Death-1 (PD-1) KO mice resulting in cardiomyopathy (Okazaki et al., 2003). Other prominent autoimmune animal model systems include the multiple sclerosis-like demyelination in the Experimental Autoimmune Encephalomyelitis (EAE) mice (Bernard & Carnegie, 1975), multi-organ autoimmune disease in AIRE-deficient mice (Anderson et al., 2002) and the Type-I Diabetes-like syndrome in non-obese diabetic (NOD) mice (Makino, Muraoka, Kishimoto, & Hayashi, 1985). However in each of these models the disease symptoms are passaged through cellular transfer, not serum, indicating that the autoantibodies, despite the presence of high titers, are not pathogenic (Bernard, Leydon, & Mackay, 1976; Gavanescu, Benoist, & Mathis, 2008; Wicker, Miller, & Mullen, 1986).

The precise mechanisms by which autoantibodies produce tissue damage and

inflammation vary by disease. The production self-reactive, high affinity, non-IgM antibodies typically requires assistance from the Th2 subset of $CD4^+$ T cells (Allen, Okada, & Cyster, 2007; Shih, Meffre, Roederer, & Nussenzweig, 2002). Once in circulation autoantibodies can facilitate tissue damage through numerous pathways. Autoantibody ligation of a receptor can either neutralize or inappropriately active the signaling pathway. Similar to the normal function of antibodies, autoantibodies can opsonize their target resulting in the detection and destruction of normal tissues by innate immune cells. Finally, deposition of immune complexes (antibody plus soluble antigen) within various tissues can activate many inflammatory pathways resulting in tissue damage (D. Sylvestre et al., 1996; D. L. Sylvestre & Ravetch, 1994).

Ovarian Autoimmune Disease

Primary Ovarian Insufficiency (POI), previously termed premature ovarian failure or premature menopause, is defined by an elevation in pituitary gonadotropins, hypogonadism and amenorrhea in women with a normal karyotype before the age of 40. The overall incidence of POI is 1 in 100 and of this, approximately 4% is caused by an autoimmune oophoritis (Bakalov et al., 2005; Bannatyne, Russell, & Shearman, 1990; Sedmak, Hart, & Tubbs, 1987). The existence of isolated autoimmune POI is under debate as the disease is virtually always associated with other autoimmune disorders, most predominantly autoimmune adrenal disease (Bannatyne et al., 1990; Betterle et al., 1997; Sedmak et al., 1987). Association with other autoimmune diseases can include thyroid disease, myasthenia gravis and APS Type I and II (Betterle, Dal Pra, Mantero, & Zanchetta, 2002; Forges, Monnier-Barbarino, Faure, & Bene, 2004; Kim et

al., 1997; Ryan & Jones, 2004).

Autoimmune POI is uniquely distinguished by the presence of enlarged cystic ovaries in approximately half of all cases, thecal cell destruction in the absence of granulosa cell involvement, normal to elevated levels of inhibin B and the detection of anti-steroid cell antibodies (S. Chen et al., 1996; Falorni et al., 2002; Lonsdale, Roberts, & Trowell, 1991; Welt, Falorni, Taylor, Martin, & Hall, 2005). Indirect immunoflorescence detection of anti-21-hydroxylase antibodies (Bakalov et al., 2005) is the standard diagnostic marker; however, additional anti-steroid cell antibodies are also found. In the rare cases of autoimmune POI without adrenal disease, patients lack steroid-cell autoantibodies, but instead may possess anti-thyroid or anti- acetylcholine receptor autoantibodies (Bakalov et al., 2005; Dal Pra et al., 2003; Falsetti, Scalchi, Villani, & Bugari, 1999; Luo, Garza, Hunt, & Tung, 1993).

Pathogenesis of autoimmune POI remains under investigation (L. M. Nelson, 2009). The two predominant theories include an initial viral infection leading to molecular mimicry (Garza & Tung, 1995) and/or failed immune tolerance. Histologic evaluations of ovarian biopsies from women with POI have shown lymphocyte infiltration into the theca layer of growing follicles, as well as luteal tissue (L. M. Nelson et al., 1994). Consistent with an immune etiology of autoimmune POI, the absence of primordial cell destruction combined with case reports of menstrual cycle resumption with administration of glucocorticoids (Rabinowe, Berger, Welch, & Dluhy, 1986) suggests a possible window for fertility preservation if autoimmune POI is diagnosed early. Importantly, IVF protocols have been developed that use a combination of hormonal therapy, oocyte donation and intra-cytoplasmic sperm injection (ICSI) to facilitate a successful pregnancy in women

with POI (Lutjen et al., 1984).

Autoimmune models of ovarian disease

The principle model of autoimmune oophoritis is derived from the removal of the thymus three days after birth (D3TX). The consequence is a depletion of regulatory T cells which allows the development of spontaneous ovarian autoimmunity in over 95% of female mice (Tung, Smith, Teuscher, Cook, & Anderson, 1987). The disease, which develops 4 to 6 weeks following surgery, results from activation of naïve ovarian antigen-specific effector T cells and can be prevented by the adoptive transfer of polyclonal Tregs shortly following thymectomy (Asano, Toda, Sakaguchi, & Sakaguchi, 1996). Additionally, simultaneous thymectomy and oophorectomy deprives effector T cells of antigenic stimuli and as a result prevents development of disease (Alard et al., 2001). Several key conclusions about ovarian autoimmune disease have been derived from the use of this model. First, the disease is cellular in nature and passaged by CD4+ T cells, not CD8 T cell or antibodies. Second, disease prevention is facilitated by antigen-specific Tregs that require prior exposure to endogenous ovarian antigens. Third, the immune suppressive function of these specific Tregs occurs specifically in the lymph nodes that drain the ovary (Samy, Parker, Sharp, & Tung, 2005). Finally, the Maternal Antigen That Embryos Requires (MATER) has been identified as the ovarian specific protein responsible for oophoritis in the D3TX model (Taguchi, Nishizuka, Sakakura, & Kojima, 1980). Development of anti-MATER antibodies corresponds with initial ovarian lymphocyte infiltration suggesting that in this model, MATER is the primary immune target when immune regulation has been disrupted.

Orchitis

Systemic infections are known to impinge on reproductive success. In the male, the most common outcome is a suppression of the hypothalamic-pituitary-gonadal (HPG) axis, which results in reduced androgen production due to a failure in LH stimulation of Leydig cells (Kalyani, Gavini, & Dobs, 2007). Under normal physiologic conditions, intratesticular testosterone is purposely elevated through an association with androgen binding protein and its signaling is required for Sertoli cell function and spermatogenic support. In addition, Steroli cells express a battery of TLRs (see above) and cytokine receptors that can respond to inflammatory signals. Unfortunately, the exact mechanism of inflammation related male infertility is unknown. One possibility is that reduced androgen levels coupled with inflammatory signaling pathways is impairing spermatogenesis through a disrupt BTB integrity (Koksal et al., 2007). A second possibility is that inflammatory signaling through the seminiferous epithelium is disrupting normal Sertoli cell-germ cell interactions. Bacterial lipopolysaccharide induced changes within the seminiferous tubules include apoptosis of spermatocytes, loss of germ cell – Sertoli cell contact and vacuolization of spermatid nuclei (O'Bryan, Schlatt, Phillips, de Kretser, & Hedger, 2000).

Direct testicular inflammation generally results from a disseminating epididymal infection and has the same pathogenic origin. Isolated orchitis is less common than orchido-epididymitis (Kaver, Matzkin, & Braf, 1990) and is usually viral in origin (Masarani, Wazait, & Dinneen, 2006). Non-infectious orchitis can result from physical trauma, cancer, vasectomy or autoimmune disease.

Epididymitis

Inflammation of the epididymis is the most common intrascrotal infection (Luzzi & O'Brien, 2001) affecting approximant 600,000 males between the ages of 18 and 35 each year (Krieger, 1984). Epididymitis is classified as either acute or chronic and typically results from an ascending bacterial infection of either *N. gonorrhoease or C. Trachomatis* (Redfern, English, Baumber, & McGhie, 1984*)*, although several other infectious agents can cause epididymal inflammation. Similar to non-infectious orchitis, epididymitis can also result from physical trauma, cancer, vasectomy or autoimmune disease. While epididymitis can occur in isolation, it usually spreads to the testis, where the infection can impair spermatogenesis and steroidogenesis. Consequently the epididymis is affected directly by local inflammation and indirectly by a loss of androgen signaling.

Autoimmune models of testicular and epididymal disease

The experimental evidence for an autoimmune component to orchiepididymitis is derived from studies with *Aire*-KO mice, day 3 neonatal thymectomy (D3TX) and experimental models of autoimmune orchitis (EAO). A deficiency in AIRE in both humans and mice can result in male infertility. A study of the autoimmune components leading to male infertility is a principle focus of this dissertation and will be discussion in detail below.

The removal of the thymus at neonatal day 3 causes a multi-organ autoimmunity due to a disruption of peripheral T-reg-mediated tolerance (see above). Two of the specific targets of this model are the testis and the epididymis. Within the testis autoantibodies

are deposited on the surface of sperm as well as the nuclei of Sertoli cells (Tung et al., 1987). In the epididymis, male mice generate autoantibodies against the nuclei and luminal surface of the tubule epithelium as well as those against the surface of the sperm and the acrosome (Taguchi, Kojima, & Nishizuka, 1985; Tung et al., 1987). Approximately 30% of male D3TX mice will spontaneously develop orchitis. This is in contrast to the prevalence of oophoritis in female D3TX and is likely due to the local immune-modulatory environment of the testis compared to the ovary (Tung & Teuscher, 1995). Two key points have been derived from the study of male reproductive tract autoimmune disease using the D3TX model. First, the percentage of mice with orchitis increases to over 90% following vasectomy. Vasectomy is thought to result in the release of immune stimulatory sperm antigens. A similar response in witnessed in humans, where following vasectomy the prevalence of anti-sperm antibodies increases (Ansbacher, 1973). However, the pathogenicity of anti-sperm antibodies following vasectomy appears to be dependent on the presence or absence of T regulatory cells (Wheeler et al., 2011). Second, the presence of epididymitis always precedes orchitis in the D3TX model system (Taguchi & Nishizuka, 1981). This observation suggests that the inciting antigen in the D3TX model is present on mature sperm within the epididymis and the later testicular involvement is either through epitope spreading or a non-specific dissemination of the original inflammation (Vanderlugt et al., 1998).

A third model of autoimmune recognition of the male reproductive tract, known as Experimental Autoimmune Orchitis (EAO), is the result of artificial immunization with male reproductive tissue extracts. EAO is induced by injecting male reproductive tract antigens, with or without adjuvant, into the footpad. The majority of the initial studies

were preformed using guinea pigs as a model (Freund, Lipton, & Thompson, 1953). Induction of EAO in the mouse proved to be more difficult, requiring addition of particular extracts, repeated antigen injection or very strong adjuvants (Kohno et al., 1983; Sakamoto, Himeno, Sanui, Yoshida, & Nomoto, 1985; Sato, Hirokawa, & Hatakeyama, 1981). A possible explanation for the differences in these model systems is relative permeability of the blood-testis-barrier. The BTB in the guinea is more permeable to macromolecule and as a result exposure of sperm antigens to the immune system as well as passage of antisperm antibodies into the seminiferous tubules is likely increased (M. H. Johnson, 1970; Moroi et al., 1998). Similarly, increase susceptibility to orchitis in the mouse with the addition of Bordetella pertussis extract is likely due to a disruption of the BTB (Adekunle, Hickey, Smith, Tung, & Teuscher, 1987; Amiel, 1976; Bruckener, el Baya, Galla, & Schmidt, 2003). Additionally, the ability of repeated inoculation with male reproductive tract antigens to drive a testicular immune response, even without adjuvant, suggests the requirement of a memory response to induce EAO (Itoh, Hiramine, & Hojo, 1991). Finally, one important contradiction between the EAO and the D3TX model of autoimmune orchitiis is that the EAO protocols can be adjusted to allow for isolated epididymitis, orchid-epididymitis or isolated orchitis. For example, injection of testicular sperm extract without an adjuvant results in orchitis without a corresponding response against the epididymis (Itoh et al., 1991) and immunization with whole epididymal lysate or testiscular spermatids both with an adjuvant causes induction of epididymis (Itoh et al., 1992; Tung et al., 1987). These findings suggest that there are multiple male reproductive tract antigens capable of provoking autoimmunity and that the likelihood of their development can be influenced

by other ongoing immune activity.

Autoimmune Polyglandular Syndrome Type I (APS-I)

Epidemiology

The AIRE gene is a component of central immune tolerance whose function is to prevent autoimmune disease by removing developing lymphocytes with a high-affinity antigen receptor for self-peptides. Functionally disruptive mutations of this gene, of which over sixty have been documented (Heino et al., 2001; Mathis & Benoist, 2007), results in a multi-organ autoimmune disease known as Autoimmune Polyendocrine Syndrome Type-I (APS-1). APS-1 is a rare autosomal recessive (Nagamine et al., 1997) disorder that was first described in 1946 (Leonard, 1946). However, in some isolated populations the frequency of APS-1 can increase to 1:9000 for Iranian Jews, 1:14,000 for Sardinians and 1:25,000 for Finns (Bjorses et al., 1996).

Pathophysiology

APS-1 patients suffer from a multi-organ autoimmune disorder characterized by a high degree of target variability, CD4+ T cell infiltrates and the generation of autoantibodies against afflicted organs (Anderson et al., 2002; Ramsey et al., 2002; Vogel, Strassburg, Obermayer-Straub, Brabant, & Manns, 2002). The pathogenesis is clearly genetic, however there are environmental inputs that contribute to the variability of autoimmune targets. The best example of this comes from identical siblings with matching genetic disruptions in the AIRE gene that have a different constellation of symptoms (De Luca et al., 2008). Serum autoantibodies can be suggestive of autoimmune targets and may

exacerbate the severity, but are likely not the inciting factor. B cell deletion studies in *Aire*-deficient mice show reduced disease severity, particularly for chronic mucocutaneous candidiasis (Gavanescu et al., 2008). Alleviation of chronic mucocutaneous candidiasis in this model is likely due to an elimination of autoantibodies specific for two important cytokines in musical immunity, IL-17 and IL-22 (Kisand et al., 2010; Puel et al., 2010). However, serum transfer alone of autoantibodies from *Aire*-deficient mice alone was not sufficient for disease transfer (Gavanescu et al., 2008).

Clinical Presentation

The high degree of disease variability and timing of onset can make APS-1 difficult to identify and treat. A definitive diagnosis requires two of the three following symptoms; 1) Addison's disease, 2) hypoparathyroidism and 3) chronic mucocutaneous candidiasis (Betterle, Greggio, & Volpato, 1998). In humans, candida (yeast) infections are the most common symptom at presentation, affecting over 60% of patients and eventually present in all by the age of 40. Hypoparathyroidism is the most common endocrine dysfunction. It has a higher frequency in women compared to men and impacts more than 30% at presentation, eventually manifesting in over 80% of all APS-1 patients. Finally Addison's disease (adrenal insufficiency) is the third most common symptom appearing in approximately 60% of patients by the age of 15. In addition, a variable percentage of afflicted patients present with secondary autoimmune ailments, including thyroiditis (5%), ovarian insufficiency (60%), hepatitis (12%) and type 1 diabetes (T1D) (14%) (Kogawa et al., 2002; Podkrajsek et al., 2008). Two final complicating factors

include age of onset, which can range from two month to 18 years, and atypical symptoms. Unusual presentation is more common in young patients and involves are variety of symptoms including dry eye, hyperkalemia, nephritis and rash (Perheentupa, 2006).

Murine Models of Aire Deficiency

Similar to APS-1 patients, ablation of the *Aire* gene in mice produces a multi-organ autoimmune syndrome complete with autoantibodies and $CD4^+$ infiltrates (Jiang, Anderson, Bronson, Mathis, & Benoist, 2005; Kuroda et al., 2005). Analysis of several murine models on different genetic backgrounds has revealed that the pathogenesis of APS-1 is due to a loss in the expression of the majority of *Aire*-regulated antigens within mTECs, culminating in the escape of autoreactive T cells (Anderson et al., 2002). Consistent with this view, several authors have established a correlation between the thymic loss of TRAs and the autoimmunity that ensues, including MUC6 for gastritis, MATER for ovarian insufficiency, INS2 for T1D and IRBP for uveitis (Alimohammadi et al., 2008; Anderson et al., 2002; J. DeVoss et al., 2006; Gavanescu, Kessler, Ploegh, Benoist, & Mathis, 2007; Sabater et al., 2005). Disease severity and commonality of autoimmune targets are significantly influenced by strain differences. *Aire*-KO mice developed on the B6 background (Hubert et al., 2009) have a mild, almost autoimmune protected phenotype, whereas those on the non-obese diabetic (NOD) background have a very severe disease presentation including exocrine pancreatitis, gastritis and early morbidity (Niki et al., 2006). *Aire*-deficient mice on the Balb/c genetic background (Jiang et al., 2005) have an intermediate phenotype that includes dry eye, gastritis,

gonadal insufficiency (Jasti et al., 2012) and longevity of at least a year. Finally, some groups have utilized a B6/NOD hybrid background to precipitate and study certain autoimmune diseases, including chronic prostatitis (Hou et al., 2009), without elevated mortality rates.

Our understanding of pregnancy complications secondary to autoimmune disease is incomplete. Additionally, even less is known about the contributions of central immune tolerance on fertility. The discovery of the AIRE as the gene responsible for the disease known as the Autoimmune Polyendocrine Syndrome-I has provided new insights into the mechanisms of central immune tolerance. AIRE-induced gene expression within the thymus provides a pool of self-antigens used for the removal of developing autoreactive T cells. A disruption of this gene causes incomplete central immune tolerance and results in a multi-organ autoimmune disorder. Many of the proteins regulated by AIRE are co-expressed within the male and female reproductive tracts. In the following dissertation, I will use an *Aire*-deficient mouse model to explore the *hypothesis* that AIRE-mediated central tolerance to various reproductive tract antigens is required for male and female fertility preservation. Further, I will attempt to identify the specific targets leading to autoimmune-induced infertility.

CHAPTER 2:

Ovarian autoimmune disease in Balb/c Autoimmune Regulator (AIRE) deficient mice impacts fertility prior to follicular senescence.

Warren BD[1], Lynda K. McGinnis[3], William H. Kinsey[3], Lane K. Christenson[3], Brian K. Petroff[2] and Margaret G. Petroff[1].

Departments of 1) Anatomy and Cell Biology 2) Internal Medicine and 3) Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas.

ABSTRACT

The Autoimmune Regulator (AIRE) is responsible for establishing central immune tolerance through the induction of self-antigen expression within the thymus. A defect in the AIRE gene results in a multi-organ autoimmune disorder known as Autoimmune Polyglandular Syndrome Type-1. Humans, as well as mice, with a deficiency in this gene produce autoreactive $CD4^+$ T cells, increased concentrations of serum autoantibodies and present with elevated rates of infertility. Ovarian autoimmunity causing a depletion of follicular reserves is prominent in female *Aire*-deficient (KO) mice over 10 weeks of age. However, 50% of mated six-week old female *Aire*-KO mice demonstrate embryonic loss by embryonic day (ED) 7.5 despite having normal follicular reserves. We determined that ovulation (90% KO vs 100% WT; n=11), ability to mate (99% KO vs 100% WT; n= 71 and 58 respectively), serum progesterone production at ED5.5 (P=0.232; n=4) and the decidual response are unaffected by *Aire* deficiency. Additionally, *Aire*-KO females did not generate unique serum autoantibodies against the uterus, placenta or embryo. However, 60% (9 of 15) of 6-8 week old *Aire*-KO female mice generated autoantibodies against the ovary, and ovulated an increased percentage of degenerated oocytes (31% KO vs 2% WT; n=74 and 71 respectively). When cultured, one-cell stage embryos from *Aire*-KO females progressed to the 2-cell and blastocyst stage of development at significantly reduced rates compared to WT controls (47% KO vs 76% WT and 19% KO vs 60% WT; n=74 KO, n=67 WT). Finally, embryos from *Aire*-KO dams are developmentally delayed at ED3.5 and displayed a reduced trophectoderm outgrowth potential after 48 and 96 hours of culture (median

values: 0mm^2 KO vs 6.8 mm^2 WT, and 7.6 mm^2 KO vs 30.2 mm^2 WT; $P<0.001$, $n=4$). Collectively these results suggest that ovarian autoimmune disease due to a dysfunction in central immune tolerance can impact the health and quality of female oocytes leading to peri-implantation infertility.

INTRODUCTION

Infertility or subfertility are unfortunately common conditions with profound social, economic, medical and psychological implications, and are estimated to impact between 6 and 25% of couples world-wide (Boivin et al., 2007; Chandra A, 2013; Greenhall & Vessey, 1990). While the cause of compromised fertility can originate with either partner or as a combination of both, it is suggested that a female factors cause approximately 55% of infertility cases (Forti & Krausz, 1998; Hull et al., 1985; Mahmoud, 2000). Among the potential causes of female-factor infertility are a wide range of autoimmune disorders including autoimmune-primary ovarian failure, autoimmune-thyroiditis, autoimmune-Addison's disease. These conditions are associated with generation of autoantibodies against sperm, the ovary, phospholipids, steroidogenesis enzymes and nuclear antigens (Abalovich et al., 2007; Blumenfeld et al., 1993; S. Chen et al., 1996; Empson, Lassere, Craig, & Scott, 2002; Falorni et al., 2002; Hoek, Schoemaker, & Drexhage, 1997; Mathur et al., 1981; Motta et al., 2012; Oshiro, Silver, Scott, Yu, & Branch, 1996; Shatavi, Llanes, & Luborsky, 2006; Winqvist et al., 1995; Xu et al., 1990). Importantly, the prevalence of autoimmunity is higher in females and with a predilection to manifest during the reproductive years.

The regulation of the immune system, which is responsible for the identification and removal foreign pathogens while remaining tolerant to self-antigens, is essential for reproductive viability. The integration of female fertility and the immune system is very complex and requires a high degree of pliability. The female immune system must simultaneously maintain tolerance to the reproductive tract, avoid systemic inflammation

and remain ignorant or tolerant to a host of neo-antigens present within sperm, the placenta and the semi-allogeneic fetus (Moffett & Loke, 2006; Stimson, Strachan, & Shepherd, 1979; Suri, 2004; Taglauer, Adams Waldorf, & Petroff, 2010). However, the immune system cannot simply shut down since susceptibility to infection would increase and several key reproductive events, including ovulation, implantation and remodeling of the uterine spiral arteries require immunological involvement (Aluvihare, Kallikourdis, & Betz, 2004; M. K. Collins, Tay, & Erlebacher, 2009; Loke & King, 2000; Moffett & Loke, 2006).

Autoimmune disease is caused by the loss of immunologic self-tolerance resulting in an inflammatory response against self-antigens. These conditions and an individual's susceptibility have proven difficult to study as they often involve both host genetics and environmental factors (Marrack et al., 2001). Rare cases of monogeneic disorders leading to autoimmunity, as exemplified by polymorphisms in the gene encoding the autoimmune regulator (*AIRE*), can provide novel insight into the mechanisms behind these diseases (Akirav, Ruddle, & Herold, 2011; B. Kyewski & Derbinski, 2004). Autoimmune polyglandular syndrome type 1 (APS-1) (Ahonen, 1985), the disease resulting from a deficiency in *AIRE,* is a multi-organ autoimmune disorder that produces autoreactive T cells and high serum titers of autoantibodies (Soderbergh et al., 2004). Clinically, patients present with two of the following three pathologies: adrenal insufficiency, chronic mucocutaneous candidiasis and hypoparathyroidism (Ahonen, Myllarniemi, Sipila, & Perheentupa, 1990; Bjorses et al., 1996). Additionally, patients

often develop a broad range of secondary autoimmune-mediated complications, including elevated rate gonadal insufficiency (Ahonen et al., 1990).

The *AIRE* gene encodes a 58-kDa protein that is predominately expressed by thymic medullary epithelial cells (Bjorses et al., 1996; Heino et al., 2001) and possesses structural and functional attributes suggestive of a transcription factor (Anderson et al., 2002; Nagamine et al., 1997). While the exact mechanism remains unclear, AIRE, through its molecular motifs (Nagamine et al., 1997) and various binding partners (Abramson et al., 2010), has a vital role in establishing central immune tolerance by regulating the “promiscuous” expression and presentation (Kuroda et al., 2005) of numerous tissue-specific self antigens (Anderson et al., 2002; Derbinski et al., 2005; Derbinski et al., 2001). Importantly, developing autoreactive T cells are removed following exposure to these self-antigens (Anderson et al., 2005; Liston, Lesage, Wilson, Peltonen, & Goodnow, 2003), some of which are also found within, and even restricted in their non-thymic tissue distribution to the ovary, placenta and embryo (Derbinski et al., 2005).

Disruption of the murine *Aire* gene, which is 71% homologous with the human correlate (Halonen et al., 2001), effectively models many of the key attributes of APS-1. *Aire*-deficient mice fail to express many tissue restricted antigens within the thymus, (Anderson et al., 2002; Derbinski et al., 2005), which causes inappropriate escape of self-reactive T cells and culminates in development of autoreactive CD4+ T cell and antibodies (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et

al., 2002). In addition, disease onset and severity is highly variable both within and between genetic backgrounds (Jiang et al., 2005). Interestingly, several reports speculate that decreased fertility in homozygous *Aire*-deficient mice breeding pairs is due to a male specific factor (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002). However, we have previously shown 50% infertility in female *Aire*-deficient mice due to premature ovarian follicular senescence (Jasti et al., 2012). In the present study we explore the consequences of a disruption of AIRE-mediated gene expression on fertility in six-week old female Balb/c *Aire*-deficient mice prior to a complete deletion of their ovarian reserves.

METHODS AND MATERIALS

Animals

Aire-deficient (KO) mice on the Balb/cJ genetic background (>8 generations) (Anderson et al., 2002; Jiang et al., 2005) were a generous donation to our laboratory by C. Benoist and D. Mathis (Harvard Medical School, Boston, MA). Mice were housed under pathogen-free condition at the University of Kansas Medical Center (KUMC); all experiments both complied with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at KUMC. The *Aire*-KO female mice used in these experiments were generated using heterozygous breeders. Wild type (WT) Balb/cJ males and females were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Genotypes were confirmed by a single PCR reaction using the following primer set: FWD 5' GTCCCTGAGGACAAGTTCCA 3'; REV 5' GGGACTGGTTTAGGTCCACA 3'. WT mice generate a 758 bp product, *Aire*-KO mice yield a 297 bp product and heterozygous animals produce both.

Blood and Tissue collection

Wild type and *Aire*-deficient females were sacrificed for the collection of blood, ovaries, implantation sites and the recovery of transplanted ovaries. Mice were anesthetized with a sterile solution of 2,2,2-Tribromoethanol (250mg/kg IP, Sigma-Aldrich, St. Louis, MO) dissolved in 2-methyl-2-butanol (Sigma-Aldrich, St. Louis, MO). Serum was collected under anesthesia via cardiac puncture and stored at -20°C. Mice were then euthanized via cervical dislocation and opening of the cardiac cavity, after which the tissues were

removed, fixed in 4% paraformaldehyde (PFA) overnight, dehydrated in 70% ethanol, embedded in paraffin and stored at 4°C.

Fertility assessment and weight monitoring

Fertility was evaluated in 6-week-old WT controls and *Aire*-KO females at embryonic day 5.5, 7.5 and 10.5 ($n \geq 13$ for each genotype at each collection point). Females were cohabited with a single Balb/c WT male and checked daily for the presence of a copulatory plug. The duration to initial mating was recorded, and following mating their body weight was measured daily between 9 and 10 am until sacrifice at embryonic day 5.5, 7.5 or 10.5. Fertility was assessed by the presence of implantation sites at the time of sacrifice.

Embryonic day 0.5 embryo collection and in vitro culture

To assess the *in vitro* development of 0.5-day embryos, six-week old WT (n=11) and *Aire*-KO (n=10) females were cohabitated with a single Balb/c male mouse and evaluated daily for presence of a copulatory plug. The afternoon following a mating event, mice were anesthetized as above, and the oviducts were isolated by dissection. Oocytes were flushed from the oviducts using FHM-Hepes (EMD Millipore, Billerica, MA) and incubated in 0.3mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 10 minutes to remove cumulus cells. Oocytes were counted and evaluated for health (Nagy, Gertsenstein, Vintersten, Behringer, & Gersenstein, 2002) before being washed through and stored in microdrops of KSOM with amino acids and D-Glucose (EMD

Millipore, Billerica, MA). Embryos were visualized on an inverted light microscope 24 and 96 hours post fertilization and the numbers and stages of embryos recorded.

Determination of serum progesterone levels

To determine progesterone levels in mated wild type and *Aire*-KO females sacrificed at embryonic day 5.5, 7.5 and 10.5, serum progesterone was measured by radioimmunoassay at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA). The sensitivity of the assay is 0.1 ng/mL with a range of 0.1-40 ng/mL.

Immunoblotting

Presence of serum autoantibodies in WT and *Aire*-KO mice against components of the reproductive tract and fetal-placental unit was determined by immunoblot analysis using lysates from *Rag2*-deficient mice (C.129S6(B6)-Rag2 ^{tm1Fwa}, Taconic). The tissue was homogenized on ice in RIPA buffer and then boiled for five minutes in a buffer containing 10% glycerol, 40mM Tris (pH 6.8), 1% SDS, 10% 2-mercaptoethanol and 0.01% bromophenol blue. 400µg of lysate was run on a 4-20% TGX stain-free curtain gel (BioRad, cat# 567-8091, Hercules, CA) and transferred to a Whatman Protran nitrocellulose membrane (GE Healthcare Life Sciences) at 100 V for 20 minutes. The membrane was blocked for 3 hours at room temperature with a 3.5% solution of BSA (Sigma-Aldrich) in PBS (pH 7.4) before being placed inside a Mini-Protean II Multiscreen apparatus (BioRad). Sera (1:750 dilution) from WT (n=5) and *Aire*-KO (n=15) mice were loaded into the slots and incubated at 4°C for 18 hours. The

membrane was washed several times with PBS containing 0.1% Tween 20 and then incubated with an HRP conjugated anti-mouse secondary antibody (Sigma-Aldrich). Immunoreactivity was visualized with an ECL detection kit (GE Healthcare Life Sciences) per the manufacturer's instructions.

Immunoflorescence and immunohistochemistry

Cellular reactivity of serum autoantibodies was determined by immunofluorescence and immunohistochemistry using sera from *Aire*-KO mice to probe testis, epididymis and ovary. Briefly, tissues from *Rag2*-deficient mice (C.129S6(B6)-Rag2 ^{tm1Fwa}, Taconic) were flash frozen in dry ice-cooled 2-methylbutane (Sigma-Aldrich) for five minutes prior to embedding in OCT (Sakura, Torrance, CA). 10 µm thick sections where cryosectioned and fixed in 100% cold acetone for five minutes. Sections were washed three times in PBS (pH 7.4), blocked for one hour at room temperature with 2% bovine serum albumin (Sigma-Aldrich) supplemented PBS treated for endogenous peroxidase activity (0.5% H_2O_2 in methanol), and incubated overnight at 4°C with serum from WT (n=3) and *Aire*-KO (n=6) mice (1:40 dilution). The following morning the samples were washed in PBS and incubated with a goat anti-mouse IgG fluorescent secondary antibody (Jackson ImmunoResearch) in the dark at room temperature for 45 minutes before being mounted with Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA) and viewed on a Nikon 80i fluorescent microscope (KUMC KIDDRC image core). Alternatively, samples for immunohistochemistry were washed in PBS and incubated at room temperature for 45 minutes with a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). An HRP-Streptavidin conjugate (Invitrogen,

Carlsbad, CA) was applied prior to color development with aminoethyl carbazole (AEC substrate kit, Invitrogen, Carlsbad, CA). Finally, sections were washed in tap water, counterstained with hematoxylin and viewed by light microscopy on a Nikon 80i (KUMC KIDDRC image core).

RNA Isolation and qRT-PCR

To test whether the decidua reaction was impacted by the absence of *Aire* in mice, expression of key genes involved in decidualization were tested by RT-PCR. RNA was extracted from decidual tissue isolated from WT (n=3) and *Aire*-KO (n=4) embryonic day 5.5 implantation sites using TRI Reagent (Applied Biosystems, Foster City, CA.). RNA concentrations were determined using a BioPhotometer (Eppendorf, Hauppauge, NY) and 1µg of RNA was reverse transcribed to cDNA using Superscript III Reverse Transcriptase and random primers (Invitrogen, Carlsbad, CA). As controls, simultaneous reactions containing water in place of RNA, and reactions without reverse transcriptase were included to verify the absence of contamination and genomic DNA, respectively. For each sample TaqMan Gene Expression Assay kits (BMP2, Mm01340178_m1; CX43, Mm00439105_m1; Dprp, Mm01135453_m1; GAPDH, Mm99999915_g1) for real-time RT-PCR were purchased from Applied Biosystems (Grand Island, NY) and combined TaqMan Universal PCR Master Mix and 20ng of cDNA per the manufacturer's instructions in an optical reaction plate before being amplified using an Applied Biosystems 7300 Real Time PCR machine. All qRT-PCR samples were run in triplicate and the mean was created from the three C_T values for each sample. The mean value was then subtracted from the mean for GAPDH to

provide a normalized C_T value. These means were then combined to generate an average C_T value for each decidual marker.

ED5.5 implantation site evaluation

To determine morphology difference in ED5.5 impanation sites, six-week old WT (n=12) and *Aire*-KO (n=13) females were paired with WT males as described above and sacrificed on embryonic day 5.5. Whole implantation sites were removed, fixed in 4% PFA overnight, dehydrated in 70% ethanol, embedded in paraffin and stored at 4°C. 5μm thick serial sections were taken from WT (n=10) and *Aire*-KO (n=19) implantation sites, stained with hematoxylin and eosin (Dako, Carpinteria, CA) and viewed by light microscopy on a Nikon 80i (KUMC KIDDRC image core). The section with the widest embryo cross sectional area was selected for 2-D area measurements of the whole implantation site, region with decidualized cells and the embryo. Area measurements were recorded using ImageJ software (NIH, Bethesda, MD).

Blastocyst culture and trophoblast outgrowth

To determine the embryonic stage prior to implantation and address the ability for trophoblast cell to expand *in vitro*, six-week old WT (n=4) and *Aire*-KO (n=4) females were paired for mating with a WT male and sacrificed on embryonic day 3.5 as described above. The collection, staging and culture of ED3.5 blastocysts was performed as described by Nagy et al. Briefly, after removing the reproductive tract, each uterine horn was isolated by cutting the distal and proximal ends of each uterine horn was just below the fallopian tube and just above the cervix, respectively.

Blastocysts were flushed from each uterine horn using a 26G needle and 400µl of embryonic stem (ES) cell medium (DMEM containing 15% heat-inactivated fetal bovine serum, 0.2% glutamine, 0.2% non-essential amino acids, 0.1mM 2-mercaptoethanol, 0.2% sodium pyruvate and 50u/ml penicillin-streptomycin) washed through fresh microdrops of medium. Each blastocyst was transferred to a single well of a 24-well collagen Type I coated plate (EMD Millipore, Billerica, MA) and covered with fresh medium. The number and developmental stage of the ED3.5 embryos were determined at collection (Nagy et al., 2002). Additionally, images of trophoblast outgrowth were recorded on an inverted light microscope 48 and 96 hours post-collection. Total outgrowth area was determined for embryos derived from WT by WT (n=30 embryos from 4 mice) and *Aire*-KO by WT (n=30 embryos from 4 mice) breeding pairs using ImageJ software (NIH, Bethesda, MD).

Sub-renal transplant surgeries

To determine whether ovarian phenotype was due to effects of AIRE intrinsic or extrinsic to the ovary, wild type ovaries were transplanted into the kidney capsule of either *Aire*-deficient or wild type (control) female mice. Eight-10 week old wild type (n=3) or *Aire*-deficient recipients mice (n=6) were anesthetized as described above. Following exteriorization of the kidney, the renal capsule was perforated and an ovary from an age-matched WT mouse was maneuvered to the opposite pole of the kidney, after which the kidney was returned to the body cavity. The transplant recipient was sacrificed after 10 days, and the transplanted ovary together with the kidney and

overlying fat, as well as the recipient's endogenous ovary, were collected for his histopathology.

Statistical Analysis

Duration to initial mating, progesterone levels, weight change, gene expression, implantation site area, trophoblast outgrowth area and number of ovulation between wild type controls and *Aire*-deficient mice were analyzed by two-tailed Student's T test. Percentages of mice that mated, ovulated, had implantation sites at sacrifice were compared by Chi-square analysis. Additionally, the percentage of healthy oocytes and the percent of ovulated oocytes to reach 2-cell stage and blastocyst stage were compared by Chi-square analysis. Results were considered significantly different with a $P \leq 0.05$.

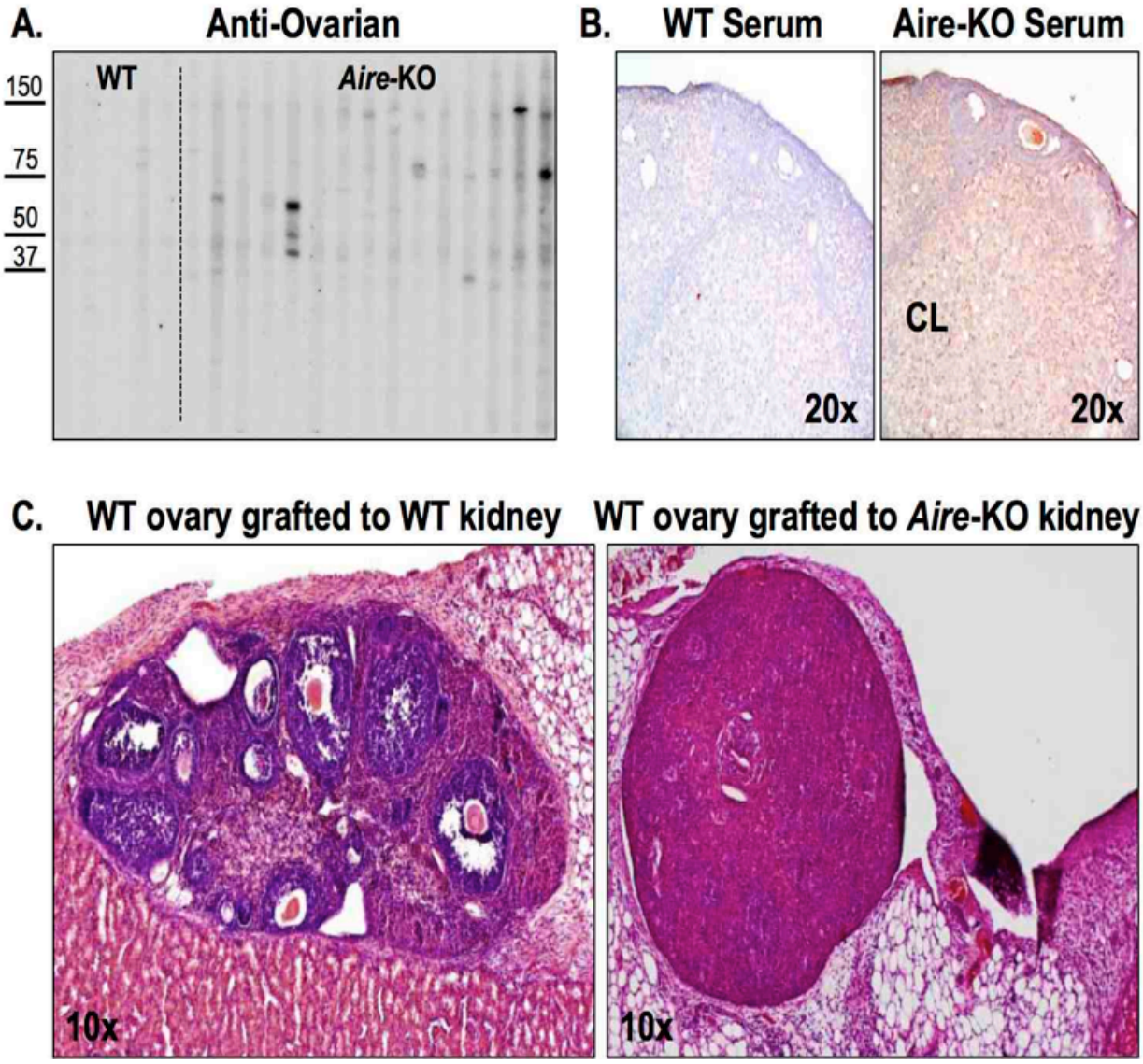
RESULTS

Ovarian autoimmunity.

Our initial study of the infertility in female *Aire*-deficient mice (Jasti et al., 2012) revealed ovarian immune targeting resulting in premature depletion of follicular reserves impacting approximately 60% of animals by 16-weeks of age. To determine the targets of an anti-ovarian immune response resulting in follicular depletion we probed an immunoblot of ovarian protein lysates from a Rag-deficient female mouse with serum collected from 6-8 week old mated WT and *Aire*-KO female mice with histologically normal ovaries. Nine of the 15 serum samples from *Aire*-KO females revealed unique reactivity not detected in serum from WT mice (**Figure 2.1A**). Next, we determined the cellular localization of antigenic targets by indirect immunostaining of frozen ovary sections from *Rag*-deficient mice. Compared to WT controls, serum autoantibodies from *Aire*-KO mice bound to multiple targets including stromal cells, luteal cells and the oocyte (**Figure 2.1B**).

Because expression of Aire mRNA has been reported in the ovary (Anderson et al., 2002; Halonen et al., 2001), we next sought to determine whether the altered ovarian environment in *Aire*-KO female mice is due to direct autoimmune targeting or an ovary intrinsic role of AIRE. To this end, we grafted WT ovaries under the kidney capsule of either WT controls (n=2) or *Aire*-deficient females (n=6). The health of the transplanted tissue was examined 10 days after the procedure. Ovaries transplanted to WT

Figure 2.1: Anti-ovary immune response. (A) *Rag*-KO ovarian lysate was probed with serum from WT and *Aire*-KO female mice. All serum samples are from mice approximately 7-8 weeks of age. **(B)** Ovaries from *Rag*-KO mice were stained by immunohistochemistry using serum from WT or *Aire*-KO mice. Images are 200x. **(C)** WT (left) or *Aire*-KO (right) female mice were engrafted with a donor WT ovary under the kidney capsule and sacrificed after 10 days. Images are 100x. WT, wild type; KO, AIRE-deficient mice; *Rag*-KO, Recombinase Activation gene-1 deficient mice.


A.
Anti-Ovarian
WT
Aire-KO
150
75
50
37
B.
WT Serum
Aire-KO Serum
CL
20x
20x
C.
WT ovary grafted to WT kidney
WT ovary grafted to Aire-KO kidney
10x
10x

recipients appeared healthy and revealed no signs of follicular loss. However, ovaries engrafted to *Aire*-KO females, whose own endogenous ovaries lacked follicles (4 of 6), displayed complete follicular depletion together with leukocytic infiltration (**Figure 2.1C**).

Fertility in female Aire-deficient mice.

Ovarian lymphocyte inflammation in *Aire*-KO mice precedes complete follicular loss (Jasti et al., 2012). To determine the impact of this autoimmunity on pregnancy we assess a variety of fertility parameters in six-week old congenic (>8 generations) *Aire*-deficient female mice on the Balb/c genetic background with histologically normal ovaries (determined post-mortem). *Aire*-KO females demonstrated no difference in the length of time to initial mating or in the percentage of mice that mated and successfully ovulated oocytes (**Figure 2.2A, B, C**).

We found that 50% of six-week old *Aire*-KO female mice were unable to produce a first litter despite over 90% (n=78) of animals having a normal complement of ovarian follicles. To assess the timing of embryonic loss in six-week old *Aire*-KO female mice we sacrificed animals on embryonic day (ED) 5.5, 7.5 and 10.5 following the detection of a copulatory plug and looked for the presence of healthy implantation sites. There was no difference in the percentage of pregnant females at ED5.5. However, there was a significant reduction in fertility at both ED7.5 and 10.5 (**Figure 2.3A**). The timing of pregnancy loss corresponded with growth curves recorded over the first half of pregnancy (**Figure 2.3B**). To address a possible cause of embryonic loss infertility, we

Figure 2.2: Timing of follicular loss and fertility parameters in AIRE deficient mice. **(A)** Mean duration between pairing and initial mating for six-week old WT and *Aire*-KO females. **(B)** Mean percentage of six-week old WT controls and *Aire*-KO females that produced a copulatory plug. **(C)** Mean percentage of six-week old WT and *Aire*-KO females to ovulate oocytes at ED0.5. WT, wild type; KO, AIRE-deficient mice; ED, embryonic day.

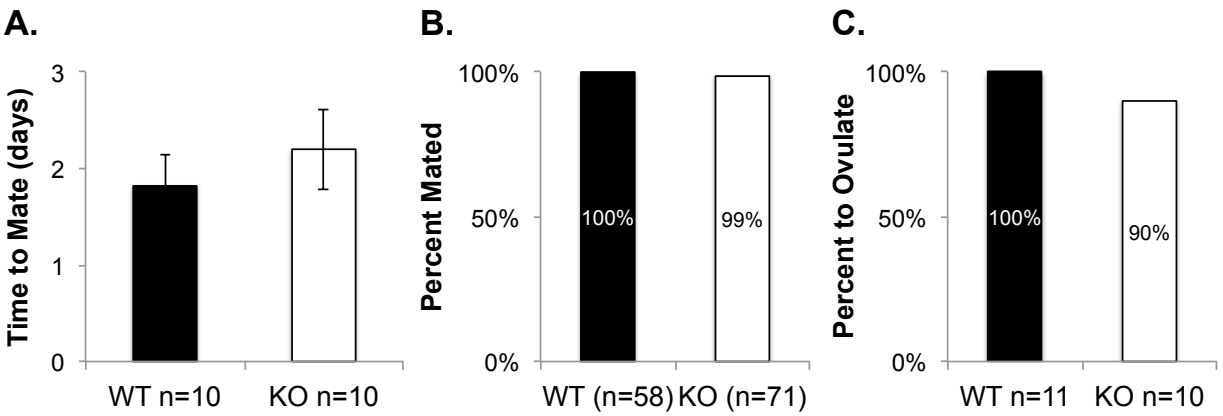
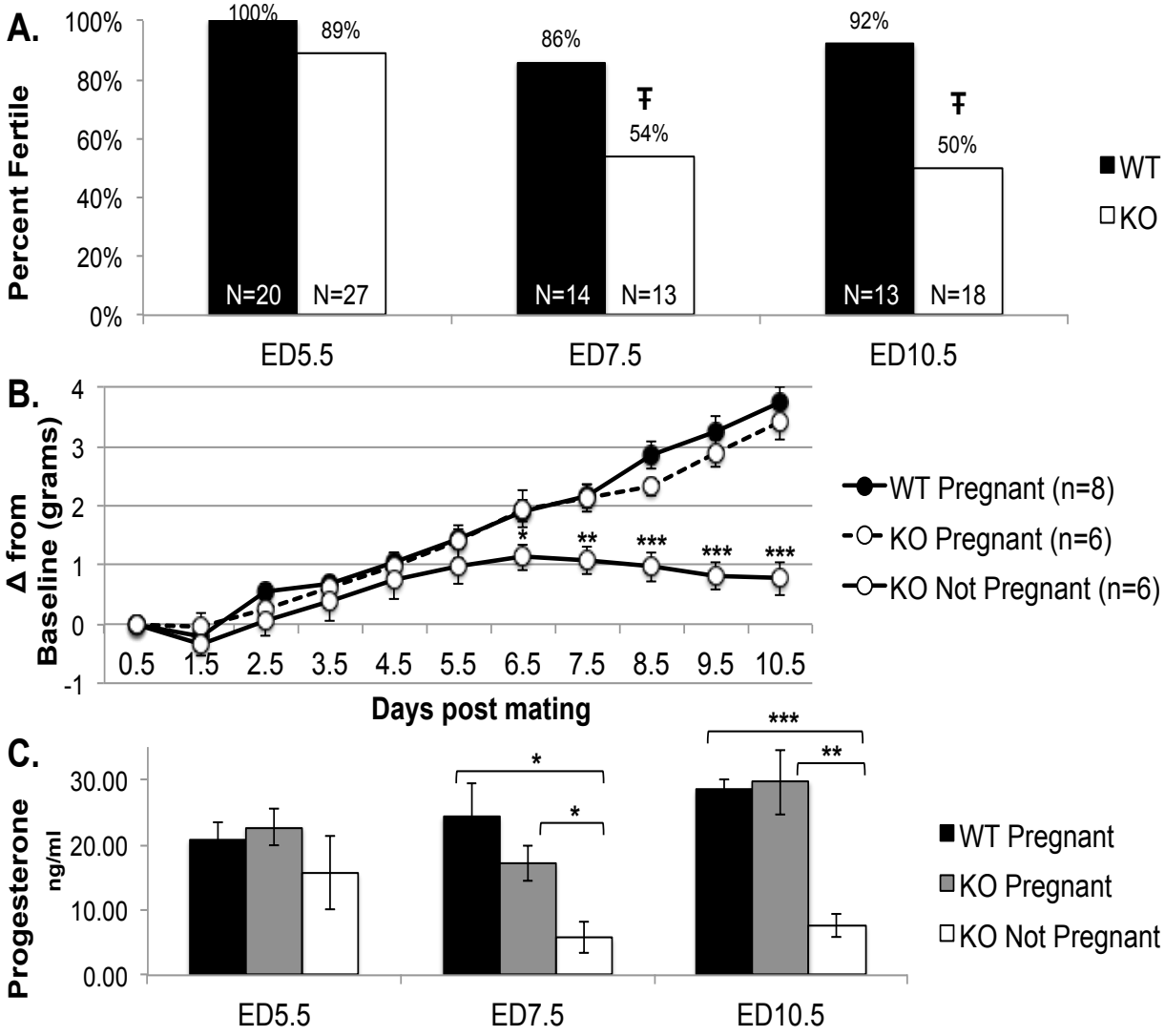

A.
Time to Mate (days)
3
2
1
0
WT n=10
KO n=10
B.
Percent Mated
100%
50%
0%
100%
99%
WT (n=58)
KO (n=71)
C.
Percent to Ovulate
100%
50%
0%
100%
90%
WT n=11
KO n=10

Figure 2.3: Timing of embryonic loss, pregnancy-associated weight gain and progesterone production. (A) Mean percentage of six-week old WT and *Aire*-KO female mice with implantation sites at ED5.5, 7.5 and 10.5. **(B)** Weight deviation from baseline at ED0.5 through ED10.5 in mated six-week old WT and *Aire*-KO females. **(C)** Mean values for serum progesterone. WT, wild type; KO, AIRE-deficient mice; ED, embryonic day. ∓, $P < 0.05$ by chi-square analysis; *, $P < 0.05$ by students t-test; **, $P < 0.01$ by students t-test; ***, $P < 0.001$ by students t-test.


A.
Percent Fertile
100%
80%
60%
40%
20%
0%
100%
89%
86%
54%
92%
50%
N=20
N=27
N=14
N=13
N=13
N=18
ED5.5
ED7.5
ED10.5
WT
KO
B.
Δ from Baseline (grams)
4
3
2
1
0
-1
0.5
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
Days post mating
WT Pregnant (n=8)
KO Pregnant (n=6)
KO Not Pregnant (n=6)
C.
Progesterone ng/ml
30.00
20.00
10.00
0.00
ED5.5
ED7.5
ED10.5
WT Pregnant
KO Pregnant
KO Not Pregnant

measured serum concentration of progesterone in mated WT controls as well as in and *Aire*-KO females, both with and without the presence of implantation sites at collection. All mice, regardless of genotype or pregnancy status had comparable progesterone levels at ED5.5. However, at ED7.5 and 10.5, serum progesterone was significantly reduced in non-pregnant females compared to pregnant animals of both genotypes (**Figure 2.3C**).

Female reproductive tract autoantibodies.

We next assessed the possibility that autoantibodies are generated in *Aire*-kO females against the uterus, placenta, embryo and testis protein lysates. To this end, tissue lysates were probed by immunoblot analysis using serum collected from WT and *Aire*-KO female mice. While serum from *Aire*-deficient mice displayed stronger reactivity towards female reproductive tract tissue antigens in general, no clear specific reactivity was detected when compared to WT controls (**Figure 2.4A, B, C**). Interestingly, both mated WT and *Aire*-KO females demonstrated increased reactivity against male testicular lysate compared to virgin animals. However, mated female *Aire*-deficient mice produced stronger reactivity and unique autoantibodies not seen in mated WT samples or samples from virgin mice of either genotype (**Figure 2.5A**). We confirmed specificity to sperm by probing frozen tissue sections of testis and epididymis from a *Rag*-deficient mouse with a selected serum sample (arrow head) (**Figure 2.5A**). Autoantibodies within serum samples from mated *Aire*-KO mice bound to testicular and epididymal sperm (**Figure 2.5B**).

Figure 2.4: Serum autoantibodies against uterus, placenta and embryo. *Rag*-KO protein lysate from **(A)** uterus **(B)** placenta and **(C)** whole embryo were probed with serum from WT and *Aire*-KO female mice. In each blot, columns 1-5 contain WT serum and columns 6-20 contain *Aire*-KO serum. All serum samples are from mated mice approximately 7-8 weeks of age. WT, wild type; KO, AIRE-deficient mice; *Rag*-KO, Recombinase Activation gene-1 deficient mice.

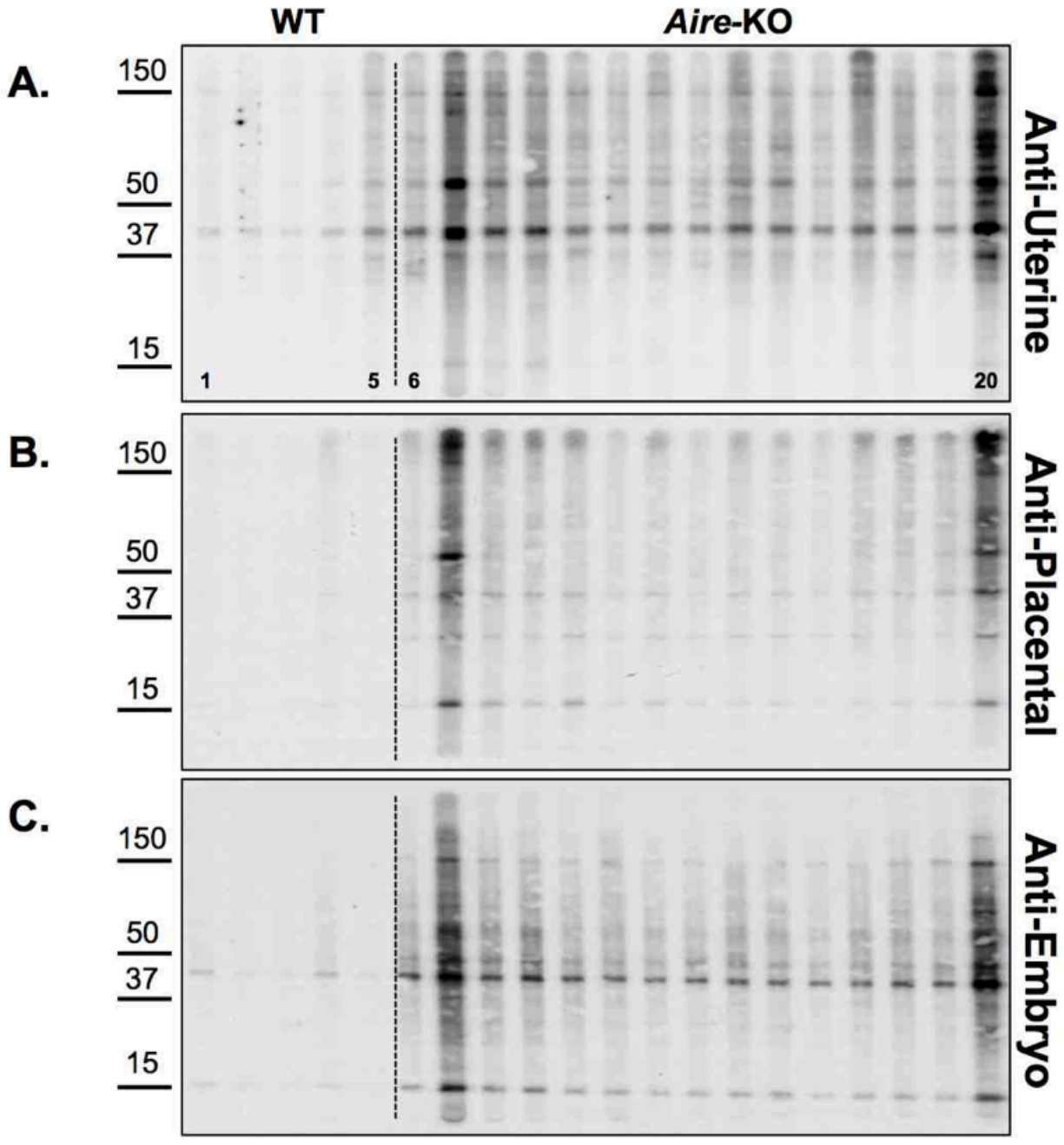
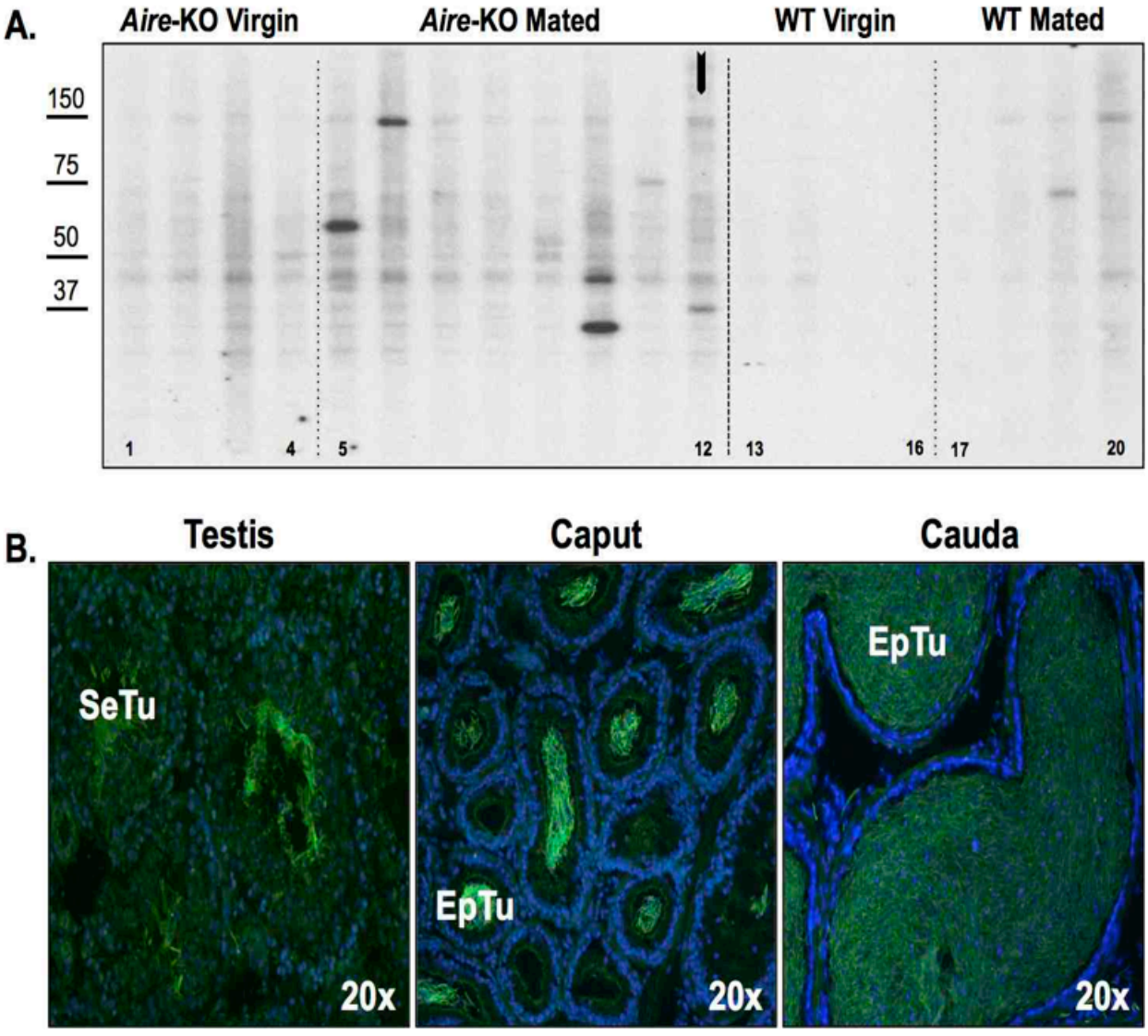

WT
Aire-KO
A.
150
50
37
15
1
5
6
20
Anti-Uterine
B.
150
50
37
15
Anti-Placental
C.
150
50
37
15
Anti-Embryo

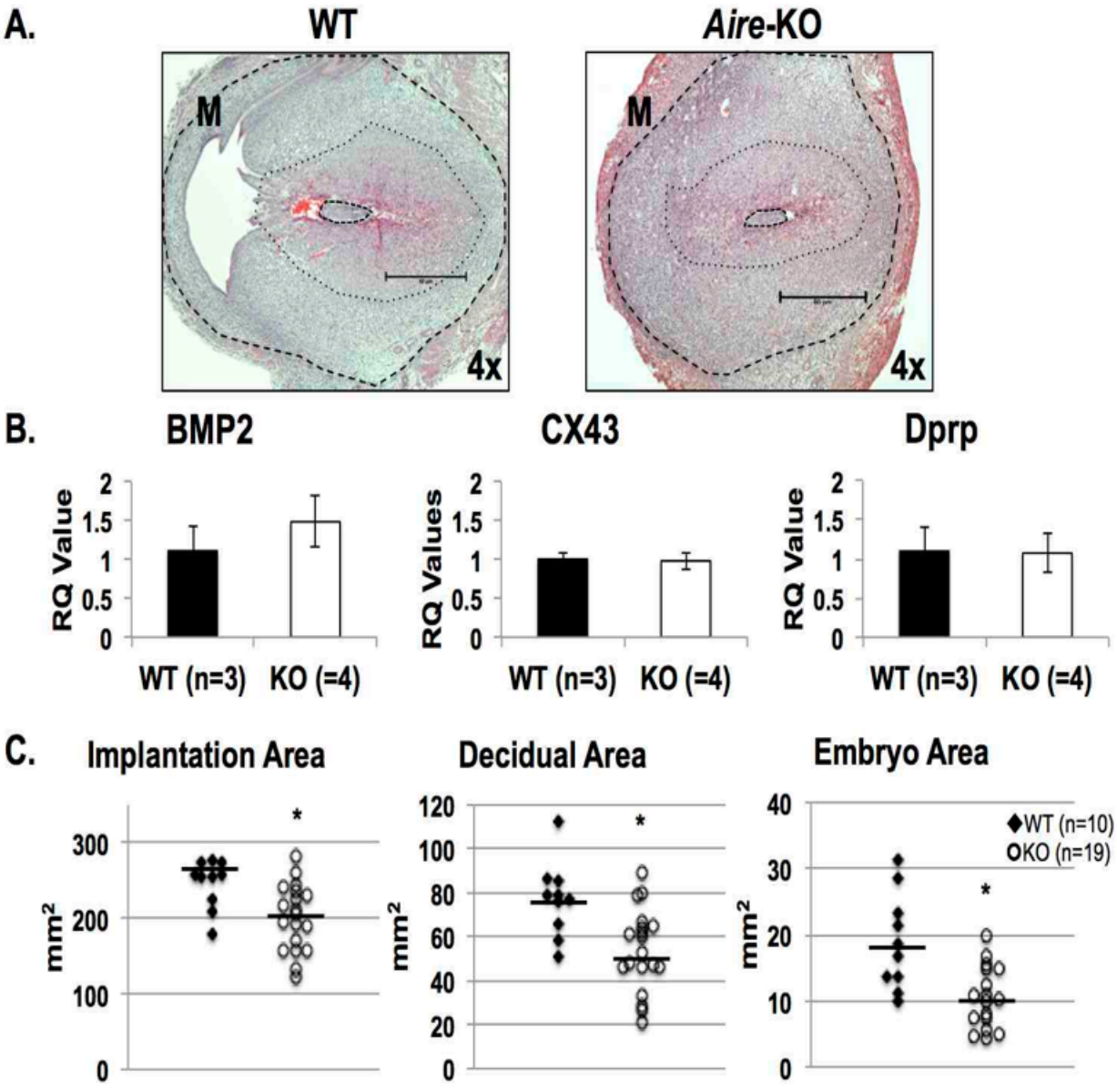
Figure 2.5: Mating induced generation of anti-sperm antibodies. (A) *Rag*-KO sperm lysate was probed with serum from virgin and mated WT and *Aire*-KO female mice. All serum samples are from mice approximately 7-8 weeks of age. **(B)** *Aire*-KO serum on frozen *Rag*-KO tissue sections. All images are 200x. Arrow indicates selected serum sample used for Fig. 4B. WT, wild type; KO, AIRE-deficient mice; *Rag*-KO, Recombinase Activation gene-1 deficient mice; EpTu, Epididymal tubules; SeTu, Seminiferous tubules.


A.
Aire-KO Virgin
Aire-KO Mated
WT Virgin
WT Mated
150
75
50
37
1
4
5
12
13
16
17
20
B.
Testis
Caput
Cauda
SeTu
EpTu
EpTu
20x
20x
20x

Histopathology of embryonic day 5.5 implantation sites and decidual marker gene expression.

We next performed histopathological evaluation of implantation sites from WT by WT and WT by *Aire*-KO mating pairs collected at ED5.5. No clear immune infiltration was noted. However, implantation sites from *Aire*-KO females were smaller and extensive gastrulation of the embryo was only seen in embryos from WT females (**Figure 2.6A**). Aire mRNA has been detected in the decidual stroma at gestational day 10.5 (Zhang et al., 2012). Therefore, Aire has a possible function in the decidua and to examine whether impaired decidualization of the uterine stroma could be responsible for smaller implantation sites or pregnancy loss observed at later time points, we analyzed gene expression of BMP2, CX43 and Dprp from isolated decidual tissue (**Figure 2.6B**). There was no difference in gene expression of these decidual markers between genotypes. To confirm the apparent size reduction we took area measurements of the total implantation site under the uterine musculature, the region containing the secondary decidual zone and that of the embryo (**Figure 2.6C**). The area measurements for all three were significantly smaller in implantation sites from *Aire*-KO females compared to WT controls.

Figure 2.6: Evaluation of implantation sites and gene expression decidualization markers at embryonic day 5.5. (A) Histopathology of ED5.5 implantation sites from pregnant WT and *Aire*-KO female mice. **(B)** Relative gene expression values for BMP2, CX43 and Dprp from isolated decidua from ED5.5 implantation sites. **(C)** 2-D area measurements of total implantation site, decidualized region and embryo taken from the serial section with the largest embryonic cross sectional area. All images are 40x. Bars represent median. Dashed lines in Fig. 5A are examples of regions used for measurements in Fig. 5C. WT, wild type; KO, AIRE-deficient mice; BMP2, Bone morphogenetic protein 2; CX43, Connexin 43; Dprp, Decidual prolactin related protein; ED, embryonic day; M, Mesometrial side. *, $P < 0.05$ by students t-test.


A.
WT
Aire-KO
M
4x
M
4x
B.
BMP2
CX43
Dprp
RQ Value
RQ Values
RQ Value
2
1.5
1
0.5
0
WT (n=3)
KO (=4)
C.
Implantation Area
Decidual Area
Embryo Area
mm²
300
200
100
0
120
100
80
60
40
20
0
40
30
20
10
0
*
WT (n=10)
KO (n=19)

In vitro culture of embryonic day 0.5 embryos.

To address the possibility of delayed embryo development, we cultured ED0.5 embryos from six-week old WT controls and *Aire*-KO females mated with WT males. Although the total number of ovulations was comparable between genotypes (**Figure 2.7A**), the number of degenerated oocytes from *Aire*-KO females was increased (**Figure 2.7B**). Embryos from WT by WT breeding pairs developed to 2-cell and blastocyst stage with a success rate of 76% and 60%, respectively. Comparatively, embryos from *Aire*-KO females were significantly reduced in their ability to reach the 2-cell and blastocyst stage of development with a success rate of 47% and 19%, respectively (**Figure 2.7C, D**). Compared to WT controls, of which 78% of 2-cell stage

In vitro blastocyst culture and trophoblast outgrowth.

Next we confirmed the delayed development by collecting ED3.5 blastocysts from six-week old WT and *Aire*-KO female mice that had been bred to WT males. 80% (24 of 30) blastocysts from WT females had a blastocyst cavity at collection on ED3.5, compared to 20% (6 of 30) of embryos from *Aire*-KO females. Of the remained 24 embryos, nine (30%) were morulla and 15 (50%) were between the 2 and 8-cell stage (**Figure 2.8A, B**). Collected blastocysts were then cultured to assess trophoblast outgrowth potential. By 48 hours post-collection, 87% of blastocysts from WT females had evidence of outgrowth compared to only 17% of blastocysts from *Aire*-KO females (**Figure 2.8A, C**). By 96 hours post-collection, all

Figure 2.7: *In vitro* embryo culture. (A) Mean number of ovulations from mated six-week old WT and *Aire*-KO female mice. **(B)** Mean percentage of visually healthy oocytes collected at ED0.5 from mated six-week old WT and *Aire*-KO female mice. **(C)** Mean percentage of ED0.5 oocytes to reach 2-cell stage 24 hours post-collection. **(D)** Mean percentage of ED0.5 oocytes to reach blastocyst stage 72-96 hours post-collection. WT, wild type; KO, AIRE-deficient mice; ED, embryonic day. ∓, $P < 0.05$ by chi-square analysis.

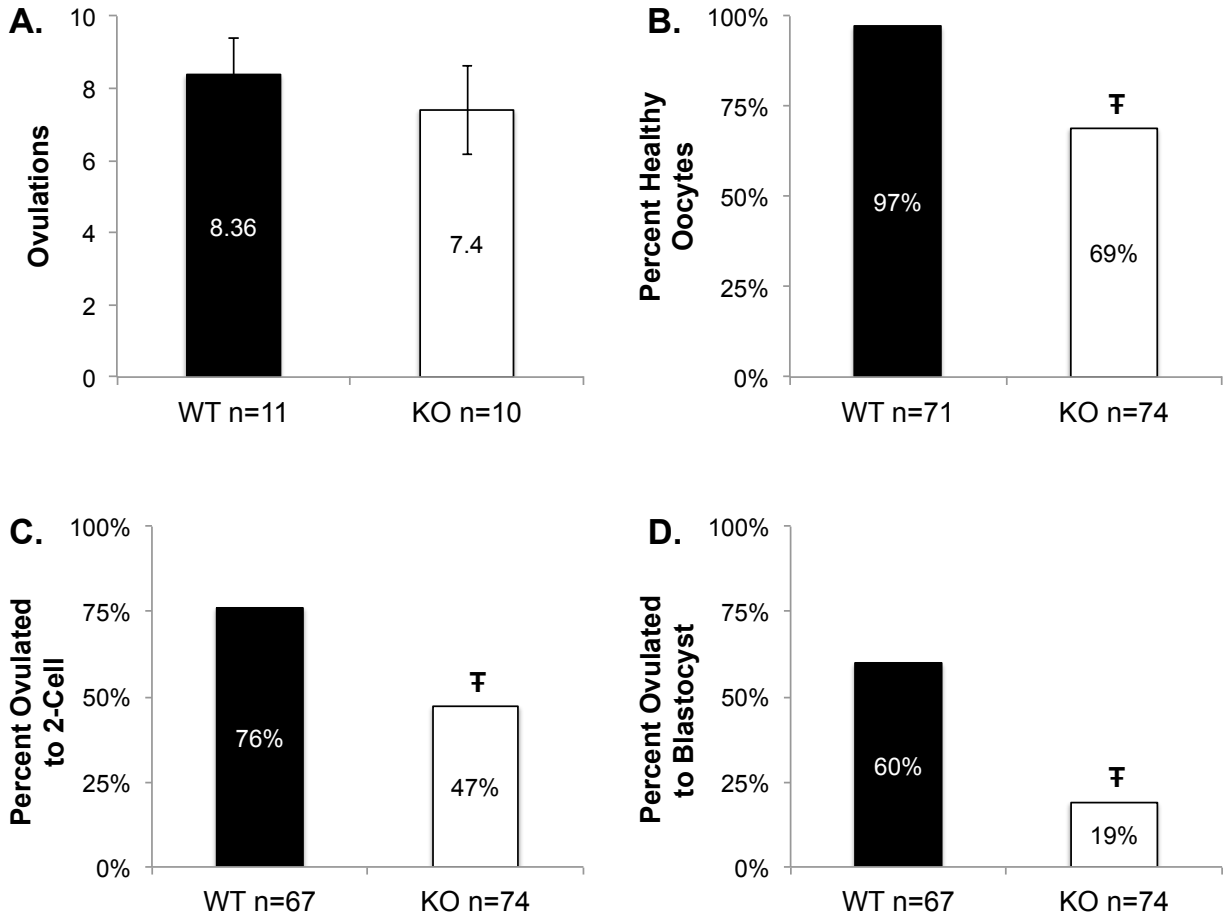
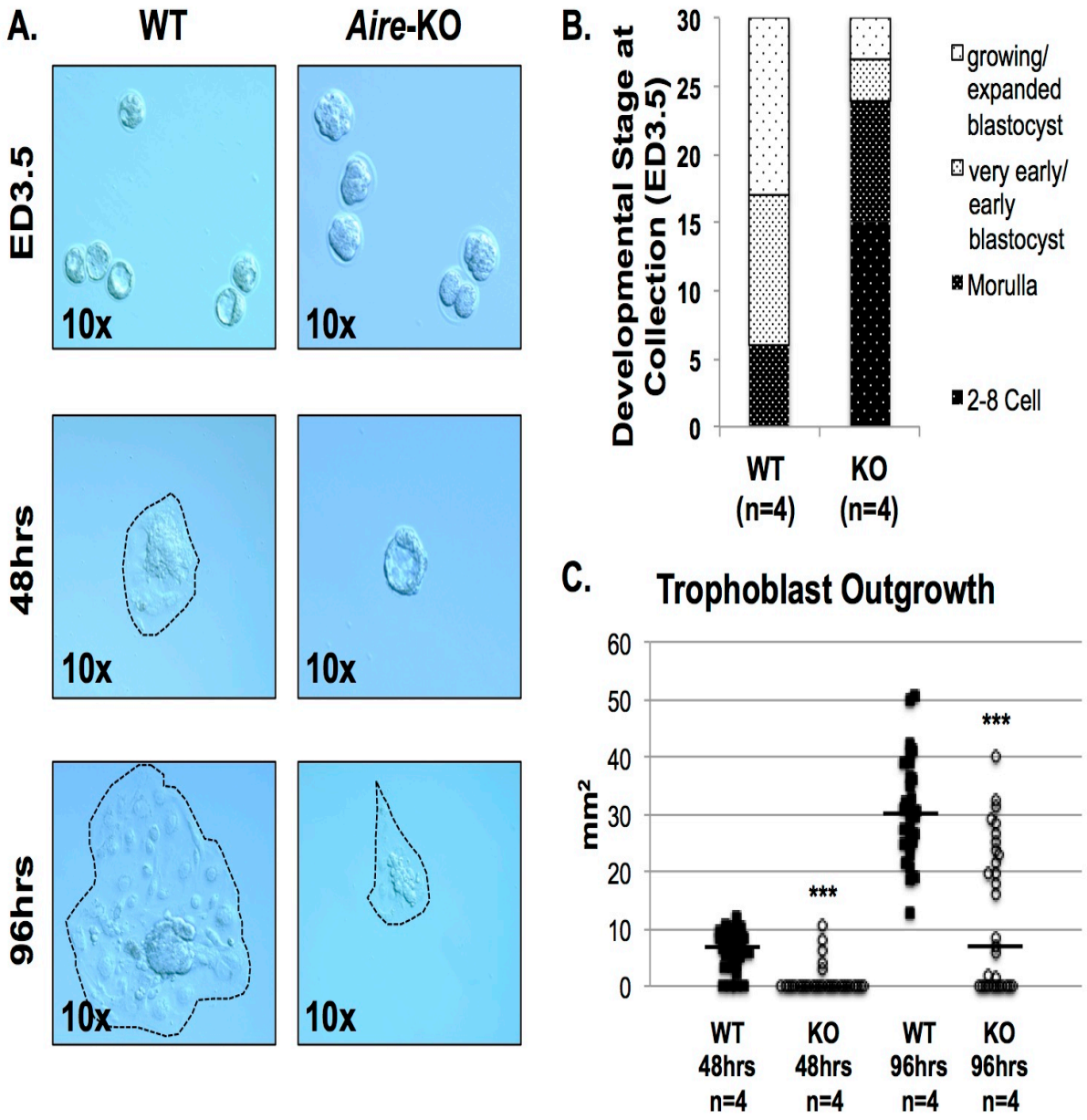

A.
Ovulations
10
8
6
4
2
0
8.36
7.4
WT n=11
KO n=10
B.
Percent Healthy Oocytes
100%
75%
50%
25%
0%
97%
69%
Ŧ
WT n=71
KO n=74
C.
Percent Ovulated to 2-Cell
100%
75%
50%
25%
0%
76%
47%
Ŧ
WT n=67
KO n=74
D.
Percent Ovulated to Blastocyst
100%
75%
50%
25%
0%
60%
19%
Ŧ
WT n=67
KO n=74

Figure 2.8: *In vitro* blastocyst culture. (A) Representative images of embryos from mated WT and *Aire*-KO female mice collected at ED3.5 and then cultured on collagen coated plates for 48 and 96hrs. **(B)** Assessment of embryonic stage at collection. **(C)** Individual area measure of trophoblast outgrowth from WT and *Aire*-KO embryos 48 and 96hrs post-collection. All images are 100x. Bars represent median. WT, wild type; KO, AIRE-deficient mice; ED, embryonic day. ***, $P < 0.001$ by students t-test.


A.
WT
Aire-KO
ED3.5
48hrs
96hrs
10x
B.
Developmental Stage at Collection (ED3.5)
30
25
20
15
10
5
0
WT (n=4)
KO (n=4)
growing/ expanded blastocyst
very early/ early blastocyst
Morulla
2-8 Cell
C.
Trophoblast Outgrowth
mm²
60
50
40
30
20
10
0
***
***
WT 48hrs n=4
KO 48hrs n=4
WT 96hrs n=4
KO 96hrs n=4

blastocysts from WT females had outgrowth compared to 63% of blastocysts from *Aire*-KO females (**Figure 2.8A, C**). At both time points the total outgrowth area was significantly larger for blastocysts from WT females compared to those from *Aire*-KO females (**Figure 2.8C**). Embryos progressed to blastocyst, only 40% of embryos from *Aire*-KO females transitioned from 2-cell to blastocyst stage.

DISCUSSION

The discovery of the Autoimmune Regulator (AIRE) and an appreciation of its role in central immune tolerance have changed our understanding of how the immune system becomes tolerized towards tissue specific antigens. AIRE-regulated gene transcription within the thymus leads to generation of self-peptide antigens necessary for the elimination of autoreactive T cells during their development (Anderson et al., 2002; Derbinski et al., 2005). Many of the genes controlled by AIRE, some of which are highly restricted in their non-thymic tissue distribution, are endogenous to the female reproductive tract as well as the developing placenta and embryo (Derbinski et al., 2001). A disruption of this gene in humans results in a condition known as the Autoimmune Polyendocrine Syndrome Type-1 (APS-1), which is a multi-organ autoimmune disease characterized by autoreactive CD4+ T cells and antibodies (Ahonen, 1985; Ramsey et al., 2002; Vogel et al., 2002). An engineered ablation of this gene in mice mimics many of the autoimmune endpoints seen in humans, including elevated rates of gonadal insufficiency (Anderson et al., 2002; Hubert et al., 2009; Jasti et al., 2012). In this study, we investigated the impact of ovarian autoimmune disease on fertility and oocyte quality in six-week old *Aire*-deficient prior to the premature depletion of their ovarian follicles, which is a hallmark of these mice on the Balb/c genetic background (Jasti et al., 2012).

Excluding the diagnostic triad for APS-1 of adrenal failure, hypoparathyroidism and candidiasis, gonadal insufficiency is the most common aliment in females, impacting 55% of patients by 20 years of age (Ahonen et al., 1990; Neufeld, Maclaren, & Blizzard,

1981; Vaidya et al., 2000). We have previously shown that this condition is mirrored in *Aire*-deficient mice, of which 60% demonstrate premature follicular senescence by 16 weeks of age. However, we have found that in 2-3 month old mice, which still have histologically normal ovaries, there is still a 50% rate of infertility. Initial investigation into the timing of embryo loss in *Aire*-deficient mice revealed comparable fertility rates just after implantation with complete embryonic loss being evident by embryonic day 7.5 (**Figure 2.3A**). This led us to assess possible post-ovulation causes of infertility including defects in progesterone production, decidualization of uterine stromal cells and the presence of autoantibodies specific for the placenta, uterus and embryo. Ultimately, we excluded these possibilities as the cause of pregnancy loss. Considering that APS-1 patients are known to generate a wide range of autoantibodies, of which large percentages are steroid cell specific (Krohn, Uibo, Aavik, Peterson, & Savilahti, 1992; Uibo, Perheentupa, Ovod, & Krohn, 1994; Winqvist et al., 1995), we were surprised that the non-pregnant *Aire*-deficient mice scarified at ED5.5 had comparable levels of serum progesterone (**Figure 2.3C**). The lack of progesterone at later time points likely reflects an absence of a positive feedback signal from placental lactogen (Soares & Talamantes, 1983; Talamantes, Ogren, Markoff, Woodard, & Madrid, 1980), since 50% of nonpregnant animals at ED7.5 and 10.5 lacked any detectable implantation sites, either healthy or resorbing. Next we considered a defect in decidualization as a mechanism for the lack the implantation sites in the nonpregnant females. However, isolated decidual tissue from implantation from *Aire*-KO females collected at ED5.5 had normal expression of three decidual specific genes (**Figure 2.6B**). Although we cannot formally rule out defective decidualization without directly

testing the decidua response in hormone-primed, unmated animals, the normal level of gene expression argues strongly that this is not the case. Finally, although we found an increase in the strength of autoantibody reactivity against placental, uterine and embryonic tissue compared to controls (**Figure 2.4**), the patterns of autoantibody reactivity were similar between WT and KO animals, without evidence of unique antibodies within *Aire*-KO serum. While we cannot complete discard the possibility that an over production of antibodies may be pathogenic we considered this unlikely as previous reports have failed to reproduce disease symptoms with serum transfer (Gavanescu et al., 2008).

Sperm specific antibodies are considered clinically significant when assessing infertility if more than 50% of the spermatozoa are coated in antibodies, fertilization is impaired and/or if there is a defect in the ability of sperm to penetrate the preovulatory cervical mucosa (N. J. Alexander, 1984; Heidenreich, Bonfig, Wilbert, Strohmaier, & Engelmann, 1994; Witkin & Toth, 1983). We were able to detect the generation of antisperm antibodies in the serum of previously bred *Aire*-deficient females (**Figure 2.5**). However, fertilization rates, as determined by the presence of the male pronuclei within the ovum (Jasti et al., 2012), were comparable between genotypes, suggesting that a sperm specific immune response is not responsible for the primigravida infertility.

The peri-implantation embryonic demise witnessed in six-week-old *Aire*-KO mice could have been the consequence of an anti-ovarian immune response that reduced oocyte quality prior to ovulation. Indeed ovarian antigens can drive an autoimmune response (P. Fenichel et al., 1997; Hoek et al., 1997; Tong & Nelson, 1999; Tung et al., 1987; Wheatcroft, Toogood, Li, Cooke, & Weetman, 1994) and previous studies have noted

the presence of antibodies specific for the oocyte and granulosa cells (Anderson et al., 2002). While we have not identified the responsible antigens for ovarian autoimmune disease in *Aire*-deficient mice, we do have multiple lines of evidence supporting that it is targeted. First, we have previously identified abnormal T cell accumulation within the ovaries of *Aire*-deficient mice as early as three-weeks of age. Second, serum from *Aire*-deficient mice contains autoreactive antibodies that are largely specific for proteins in the 60 and 75 kDa range and localize to multiple ovarian cell types including stomal cells, luteal cells and the oocyte (**Figure 2.1A, B**). Third, WT ovaries transplanted under the kidney capsule of *Aire*-KO experienced near complete follicular depletion in as short as ten days (**Figure 2.1C**). This result was only witnessed if the endogenous ovary of the transplant recipient was similarly devoid of oocytes, suggesting a rapid ovary-specific memory response. Ultimately, the outcome of this response was realized in the post-ovarian phenotype of the ovulated oocytes. Ovulated oocytes were degenerated at a significantly higher percentage (**Figure 2.7B**) and impaired in their ability to progress to the 2-cell and blastocyst stages of development (**Figure 2.7C, D**). Importantly, this observation was not just a consequence of culture conditions as embryos isolated from mated *Aire*-KO females just prior to implantation where often significantly delayed, with only 20% of embryos progressing beyond morulla (**Figure 2.8A, B**).

Diminished oocyte quality and reduced fertility are natural components of the age-related process of follicular senescence (Armstrong, 2001; Fitzgerald, Zimon, & Jones, 1998). Multiple *in vitro* fertilization and ovum donation studies have clearly linked declining oocyte quality due to aging with decreased pregnancy rates and impaired implantation (Cetin, Kumtepe, Kiran, & Seydaoglu, 2010; Chuang et al., 2003; Navot et

al., 1991). One possible explanation is that the process of follicular senescence results in low quality oocytes that, if fertilized, progress through the developmental stages at a rate that is not compatible with fertility. The steps between ovulation and implantation are complex and involve precise coordination between the embryo and the hormone responsive uterine environment (Paria, Reese, Das, & Dey, 2002). Successful implantation requires synchronization between the embryonic stage and the window uterine receptivity (Ma, Song, Das, Paria, & Dey, 2003). Changes in hormone levels, developmental stage and gene expression can derail this alignment and result in infertility (Dickman & Noyes, 1960; Palomino et al., 2005; Reese et al., 2001). It is possible that the infertility in 50% of *Aire*-deficient female mice is a result of an imbalance between these two components, resulting in embryonic demise shortly after implantation. A clear limitation of this study is that we did not completely isolate a uterine effect from an embryonic one. It may be necessary in the future to preform reciprocal blastocyst transfers into hormonally primed recipients female mice to confirm our assertion of infertility secondary to low oocyte quality resulting in embryonic developmental delay.

The discovery of the *AIRE* gene and its contribution to central tolerance has exposed new possibility for investigation into the mechanisms responsible for organ specific immune tolerance. *AIRE* induces the ectopic thymic expression of many genes that are also found in the ovary, placenta and embryo which are, especially with regards to the ovary, essential for fertility preservation. Here, we show that a deficiency in *Aire* in female mice results in autoimmune recognition of the ovary that culminates in low quality oocytes. Subsequently, these oocytes are more frequently degenerated at

ovulation, are impaired in their ability to become 2-cell and blastocyst stage embryos *in vitro*, are developmentally delayed at implantation and ultimately fail to maintain a viable pregnancy. This demonstration of the impact of ovarian autoimmune disease on oocyte health has implications not only for APS-1 patients but also extends our understanding of premature ovarian failure as a consequence of autoimmune oophoritis.

CHAPTER 3:

The Autoimmune Regulator (AIRE) protects against reproductive tract inflammation, germ cell loss and infertility in male Balb/c mice.

Warren BD[1], Leslie L Heckert[3], Timothy A Fields[4], Brian K Petroff[2], and Margaret G Petroff[1]

Departments of 1) Anatomy and Cell Biology 2) Internal Medicine 3) Molecular and Integrative Physiology and 4) Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas

ABSTRACT

Male-specific factors, including those with noninfectious autoimmune origins, contribute to approximately 50% of all infertility cases. However, an understanding of the etiologies and mechanisms behind autoimmune male infertility remain poorly understood, despite recognition that infertile males often develop antisperm antibodies. A deficiency of the autoimmune regulator (AIRE*)* gene impairs central immune tolerance by inhibiting the expression of thymic self-antigens. As a consequence, humans, as well as mice with an engineered ablation of *Aire*, develop a multi-organ autoimmune disorder that presents with elevated rates of infertility. The consequences of incomplete central immune tolerance on male fertility are still being determined. As such we sought to document the exact autoimmune targets contributing to infertility in male *Aire*-deficient (KO) Balb/c mice. In this study, 6-week old *Aire*-KO males exhibited reduced frequency of mating (46% KO vs. 100% WT; n=11 and 10 respectively $P<0.05$) and fertility (18% KO vs. 80% WT; n=11 and 10 respectively $P<0.05$) as well as potentially low litter size (2.00 vs. 5.08 pups/litter; n=2 and 10 respectively). Further investigation revealed primary hypogonadism with reduced serum testosterone (p=0.03; n=18), but a normal sperm count and pituitary gonadotropins. In addition, mature spermatozoa were consistently defective in their ability to fertilize wild-type oocytes *in vitro* (9%; vs.54%; n=102 and 83 oocytes respectively $P<0.05$). A subset of males exhibited severe oligozoospermia, fewer mitotically active germ cells and disruption of the blood-testis barrier. Morphologic alterations to the stroma and luminal epithelium of the epididymis, as well as the glands associated with the vomeronasal organ, occurred in 32% and 86%

of animals, respectively. Correspondingly, CD3+ T cell infiltration into the testis, epididymis, seminal vesicle, and prostate was evident in 5%, 75%, 84% and 90% of animals respectively. Finally, *Aire*-KO male mice generate autoreactive antibodies in an age-dependent manner against various components of the male reproductive tract, including sperm, testis, epididymis, prostate gland and seminal vesicle. Collectively, these findings reveal two important aspects of autoimmune infertility in *Aire*-KO male mice. First, the disorder has a high degree of heterogeneity between individual animals and is multifactorial within animals. Second, *Aire*-dependent central immune tolerance plays an integral role in maintaining fertility by stemming autoimmunity against multiple tissues within the male reproductive system.

INTRODUCTION

Infertility is estimated to affect 9-15% of reproductive-aged couples worldwide (Boivin et al., 2007; Rutstein & Shah, 2004). In the past, compromised fertility often was attributed by default to female pathologies, and our relatively broad knowledge base and range of treatment options reflect such a basis. However, it is now understood that roughly one-half of all infertility cases are caused by male-specific factors (Jarow et al., 2002). While many cases of male infertility are idiopathic, some of the known causes of male infertility include genetic and hormonal lesions at the pre-testicular level, oxidative stress, sperm arrest, and germ cell DNA damage within the testis. Post-testicular causes for infertility in men include infection, inflammation and congenital abnormalities associated with obstruction of sperm transport (Esteves, Hamada, Kondray, Pitchika, & Agarwal, 2012; Sullivan, 2004).

The immune system is tasked with identifying and removing foreign pathogens while remaining tolerant to self-antigens, including those associated with the reproductive system in both males and females. Thus, fertility preservation in males requires simultaneous tolerance to the immunogenic antigens on developing germ cells while also controlling the potentially damaging influence of reproductive tract infections (Baker, 1998; Suri, 2004). In addition, autoimmune and inflammatory diseases and associated orchitis, epididymitis, and autoantibodies are estimated to be associated with as many as 10% of all male-factor derived infertility cases (Jungwirth et al., 2012). Autoimmunity is the loss of immunologic self-tolerance and results in continual recognition and response against self-antigens by the adaptive immune system. These conditions and an individual's susceptibility have proven difficult to study as they often

involve both host genetics and environmental factors (Marrack et al., 2001). Rare cases of monogeneic disorders leading to autoimmunity, as exemplified by polymorphisms in the gene encoding the autoimmune regulator (AIRE), can provide novel insight into the mechanisms behind these diseases (Akirav et al., 2011; Kyewski & Derbinski, 2004). Autoimmune polyglandular syndrome type I (APS-1)(Ahonen, 1985), a disease resulting from a deficiency in AIRE, is characterized by autoimmune destruction of multiple organs and high serum titers of autoantibodies (Soderbergh et al., 2004). Clinically, the diagnostic criterion for APS-I requires patients to present with two of the following three pathologies: adrenal insufficiency, chronic mucocutaneous candidiasis and hypoparathyroidism (Ahonen et al., 1990; Bjorses et al., 1996). Additionally, patients often develop a broad range of secondary autoimmune-mediated complications, including elevated rate male infertility (Ahonen et al., 1990).

The *AIRE* gene encodes a 58-kDa protein that is predominately expressed by medullary thymic epithelial cells (mTEC) (Bjorses et al., 1996; Heino et al., 2001) and that possesses structural and functional attributes suggestive of a transcription factor (Anderson et al., 2002; Nagamine et al., 1997). While the exact mechanism remains unclear, AIRE, through its molecular motifs and various binding partners, has a vital role in establishing central immune tolerance by regulating the “promiscuous” expression and presentation of numerous tissue-specific self antigens in mTEC (Abramson et al., 2010; Anderson et al., 2002; Derbinski et al., 2005; Derbinski et al., 2001; Kuroda et al., 2005; Nagamine et al., 1997). Importantly, developing autoreactive T cells are removed following exposure to these self-antigens (Anderson et al., 2005; Liston et al., 2003).

Included within the battery of AIRE-regulated antigens are those found within, and even exclusive to, the male reproductive system (Derbinski et al., 2005).

Disruption of the murine *Aire* gene, which shares 71% homology with its human counterpart (Halonen et al., 2001), effectively models many of the key attributes of APS-1. Importantly, *Aire*-deficient mice have a significantly altered thymic self-antigen expression profile, which causes inappropriate escape of self-reactive T cells and culminates in development of autoreactive CD4+ T cell and serum autoantibodies (Anderson et al., 2002; Derbinski et al., 2005; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002). In addition, disease onset and severity is highly variable both within and between genetic backgrounds (Jiang et al., 2005). Interestingly, several reports comment on decreased fertility in male *Aire*-deficient mice as well as the frequent occurrence of immune mediated prostatitis (Anderson et al., 2002; Hou et al., 2009; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002). However, neither a detailed evaluation of the autoimmune disease occurring within the reproductive track, nor the causal influences leading to infertility in male *Aire*-deficient mice have been examined. In the present study we explore the consequence of a disruption of AIRE-mediated gene expression on fertility and examine which reproductive organs are the targets of autoimmune disease in male Balb/c *Aire*-deficient mice.

METHODS AND MATERIALS

Animals

Aire-deficient mice on the Balb/cJ genetic background (>8 generations) (Anderson et al., 2002; Jiang et al., 2005) were a generous donation to our laboratory by C. Benoist and D. Mathis (Harvard Medical School, Boston, MA). Mice were housed under pathogen-free condition at the University of Kansas Medical Center (KUMC). All experiments both complied with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at KUMC. The *Aire*-KO male mice used in these experiments were generated using heterozygous breeders. Wild type (WT) Balb/cJ males and females were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Genotypes were confirmed by PCR using the following primer set: FWD 5' GTCCCTGAGGACAAGTTCCA 3'; REV 5' GGGACTGGTTTAGGTCCACA 3'. WT mice generate a 758 bp product, *Aire*-KO mice yield a 297 bp product and heterozygous animals produce both.

Fertility Assessment

Fertility was evaluated in 6-week-old *Aire*-KO males (n=11) and wild type controls (n=12). Male mice were cohabitated with a single Balb/c WT female and checked daily for the presence of a copulatory plug. If a plug was detected, the male was removed and euthanized for blood and tissue collection. The females were allowed 23 days post-mating to deliver pups and the offspring were counted if a viable litter was produced. If a plug was not visually confirmed over a period of 20 days (approximately four ovarian

cycles), the male was euthanized and the female was permitted 21 days of isolation to verify absence of pregnancy before being mated to a proven WT Balb/c male to confirm the female's fertility.

Blood and Tissue collection

Wild type and *Aire*-deficient males were sacrificed for the collection of blood and male reproductive tract tissues. Mice were anesthetized with a sterile solution of Avertin (2,2,2-Tribromoethanol dissolved in 2-methyl-2-butanol) (250mg/kg intraperitoneal (IP)) (Sigma-Aldrich, St. Louis, MO). Serum was collected under anesthesia via cardiac puncture and stored at -20°C. Mice were then euthanized via cervical dislocation and opening of the cardiac cavity, following which the testis, epididymis, seminal vesicle, prostate gland and vomeronasal organ (VNO) were removed, weighed, fixed in 4% paraformaldehyde overnight, dehydrated in 70% ethanol, embedded in paraffin and stored at 4°C. The vomeronasal organ samples required an additional seven day incubation in 0.25% EDTA at 4°C (changed every 48 hours) to decalcify the nasal cavity bones before paraformaldehyde fixation.

Determination of serum FSH, LH and Testosterone

Mouse serum FSH and testosterone were measured by radioimmunoassay at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA). The sensitivities of the assays are 2.0 ng/ml and 10ng/dL with a range of 2.0-25 ng/mL and 10-1000 ng/dL respectively. Mouse serum LH was

also measured at the University of Virginia by sandwich immunoradiometric assay. The sensitivity of the assay is 0.07 ng/mL with a range of 0.07-37.4 ng/mL.

Caudal Sperm Count

To determine epididymal sperm counts, the caudal region of epididymis from WT and *Aire*-KO mice were isolated, weighed and placed into 750µl of Human Tubal Fluid (HTF) media (EMD Millipore, Billerica, MA). The tissue was bisected and the sperm were allowed to swim out and complete capacitation for one hour at 37 °C. Finally, the number of sperm from a 10µl aliquot diluted in 990µl of PBS, was determined by a hemocytometer and normalized to the weight of the tissue.

Histopathologic evaluation and immunohistochemistry

For histopathologic examination, paraformaldehyde fixed and paraffin embedded reproductive tract tissues were sectioned at 5µm thickness and stained with hematoxylin and eosin (Dako, Carpinteria, CA). Tissues from *Aire*-KO males were compared to WT controls for morphologic differences and immune cell infiltrates. Immunohistochemistry was used for detection of CD3, ZO-1 and Ki67 positive cells within the tissues of the male reproductive tract of *Aire*-KO (n=6-10) and WT controls (n=3-5). Sections were rehydrated through decreasing concentrations of ethanol. Antigen retrieval was performed using a heated citrate buffer (Reveal buffer, BioCare Medical, Walnut Creek, CA) according to the manufacturer's instructions. Tissues were then blocked in 10% goat serum (Sigma-Aldrich, St. Louis, MO) prior to a 16 hour 4°C incubation with a monocolonal rabbit anti-mouse CD3 antibody at 4°C (5µg/ml, clone

SP7; Abcam Cambrige, MA). Rabbit IgG (5µg/ml, chromPure, Jackson ImmunoResearch, West Grove, PA) was used as a negative control. Sections were then incubated with a biotyinylated anti-rabbit secondary antibody (goat anti-rabbit IgG BA-1000, Vector Laboratories, Burlingame, CA), followed by depletion of endogenous peroxidases and addition of streptavidin peroxidase substrate (Invitrogen, Carlsbad, CA). Colorimetric detection was visualized using aminoethyl carbazole (AEC substrate kit, Invitrogen). Finally, sections were counterstained with hematoxylin and viewed by light microscopy on a Nikon 80i microscope (KUMC KIDDRC image core). For detection of Ki67 (1/200) (Thermo, SP6 clone, Pittsburg, PA) and ZO-1 (2mg/ml) (Life Technologies, 617300, Grand Island, NY), identical procedures were used, except that antigen retrieval for the ZO-1 staining required a five minute incubation with 0.1% proteinase kinase at 37°C in place of the citrate buffer.

To assess the mean number of mitotically active germ cells per tubule, 5µm thick testis sections were stained for Ki67. The numbers of positive nuclei were quantified from and normalized to the number of tubules within five random 20x fields per section.

Immunoblotting

WT and *Aire*-KO mouse serum was used to probe testis, caput, cadua, seminal vesicle, prostate and sperm lysates from *Rag2*-deficient mice (C.129S6(B6)-Rag2 ^{tm1Fwa}, Taconic) for the presence of autoantibodies. The tissue was homogenized on ice in RIPA buffer and then boiled for five minutes in a buffer containing 10% glycerol, 40mM Tris (pH 6.8), 1% SDS, 10% 2-mercaptoethanol and 0.01% bromophenol blue. 400µg of lysate was run on a 4-20% TGX stain-free curtain gel (BioRad, cat# 567-8091,

Hercules, CA) and transferred to a Whatman Protran nitrocellulose membrane (GE Healthcare Life Sciences) at 100 V for 20 minutes. The membrane was blocked for 3 hours at room temperature with a 3.5% solution of BSA (Sigma-Aldrich) in PBS (pH 7.4) before being placed inside a Mini-Protean II Multiscreen apparatus (BioRad). Serum (1:750 dilution) from WT (n=5) and *Aire*-KO (n=15) mice was loaded into the slots and incubated at 4˚C for 18 hours. The membrane was washed several times with PBS containing 0.1% Tween 20 and then incubated with an HRP conjugated anti-mouse secondary antibody (Sigma-Aldrich). Immunoreactivity was visualized with an ECL detection kit (GE Healthcare Life Sciences) per the manufacturer's instructions.

Immunoflorescence

Cellular reactivity of serum autoantibodies was determined by immunofluorescence using sera from *Aire*-KO mice to probe testis, epididymis, seminal vesicle and prostate gland. Briefly, male reproductive tract tissues from *Rag2*-deficient mice were flash frozen in dry ice-cooled 2-methylbutane (Sigma-Aldrich) for five minutes prior to embedding in OCT cryoprotective medium (Sakura, Torrance, CA). Ten-μm thick sections where cryosectioned and fixed in 100% cold acetone for five minutes. Sera (1:40 dilution) from WT (n=3) and *Aire*-KO (n=6) mice were used as probes, and bound antibodies were visualized with a goat anti-mouse IgG fluorescent secondary antibody (Jackson ImmunoResearch). To determine whether endogenous autoantibodies were bound within the cauda of the epididymis, FITC-labeled goat anti-mouse IgG antibody was applied directly onto cryosections from WT and *Aire*-deficient mice. All sections

were coverslipped usingProlong Gold containing DAPI (Invitrogen) and viewed on a Nikon 80i fluorescent microscope.

In vitro fertilization

Sperm from wild type (n=7) and *Aire*-KO (n=8) male mice was used to fertilize oocytes obtained from superovulated WT females *in vitro*. Briefly, female mice were given pregnant mare's serum gonadotropin (PMSG; 5IU/ml) IP at 9pm on day 1, followed by human chorionic gonadotropin (hCG;5 IU/ml, IP) at 9pm on day 3. Female mice were sacrificed 12 hours later and the oocytes were flushed from the ampulla region of the oviduct, washed through three microdrops of FHM-Hepes (EMD Millipore, Billerica, MA) and placed in a culture dish with HTF media. To collect sperm, males were sacrificed on the same day, and caudal epididymis was dissected and incubated at 37°C in 1ml of HTF for at least 90 minutes. Capacitated sperm ($1\text{-}2x10^6$) was incubated with collected oocytes at 37°C for an additional 4-6 hours. Potentially fertilized oocytes were washed and stored in microdrops of KSOM with amino acids and D-glucose (EMD Millipore, Billerica, MA). Embryos were visualized on an inverted light microscope 24 and 96 hours post fertilization, and the numbers and stages of embryos recorded.

Statistical Analysis

Hormone values, caudal sperm count, $Ki67^+$ cells per seminiferous tubule and testis weights between wild type controls and *Aire*-deficient mice were analyzed by two-tailed Student's T test. Percentages of mice that were capable of mating, producing viable

offspring, having normal VNO-associated glands and IVF results were compared by Chi-square analysis. Results were considered significantly different with a $P \leq 0.05$.

RESULTS

Assessment of fertility in male Aire-deficient mice.

Initial studies describing the *Aire*-deficient mice suggested that males are either subfertile or infertile (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002). Here, we examined the detailed fertility parameters of *Aire*-KO male mice as compared to WT control mice on the same genetic background. *Aire*-KO males demonstrated a significant reduction in the ability to produce a copulation plug and the ability to produce viable offspring compared to controls (**Figure 3.1A, B**). In addition, when offspring were observed, litter sizes from *Aire*-KO males are possibly reduced compared to WT controls, but not significant as only a small number could be evaluated (**Figure 3.1C**).

Evaluation of gonadotropins, testosterone and caudal sperm quantity in male Aire-deficient mice.

To assess possible endocrine disruptions related to infertility, we measured serum concentrations of LH, FSH and testosterone from *Aire*-KO males and wild type controls. *Aire*-KO male mice maintain levels of serum LH and FSH comparable to controls (**Figure 3.2A, B**); however, they have a significant reduction in circulating testosterone (**Figure 3.2C**). In a second set of animals, the numbers of sperm recovered from the caudal section of the epididymis was comparable between genotypes (**Figure 3.2D**).

Figure 3.1: *Aire* deficiency impairs male fertility. (A) Mean percentage of WT controls and *Aire*-KO males that produced a copulatory plug. **(B)** Mean percentage of WT and *Aire*-KO males that generated a viable litter. **(C)** Mean number of offspring resulting from successful pregnancies. WT, wild type; KO, AIRE-deficient mice. *, $P < 0.05$ by chi-square analysis.

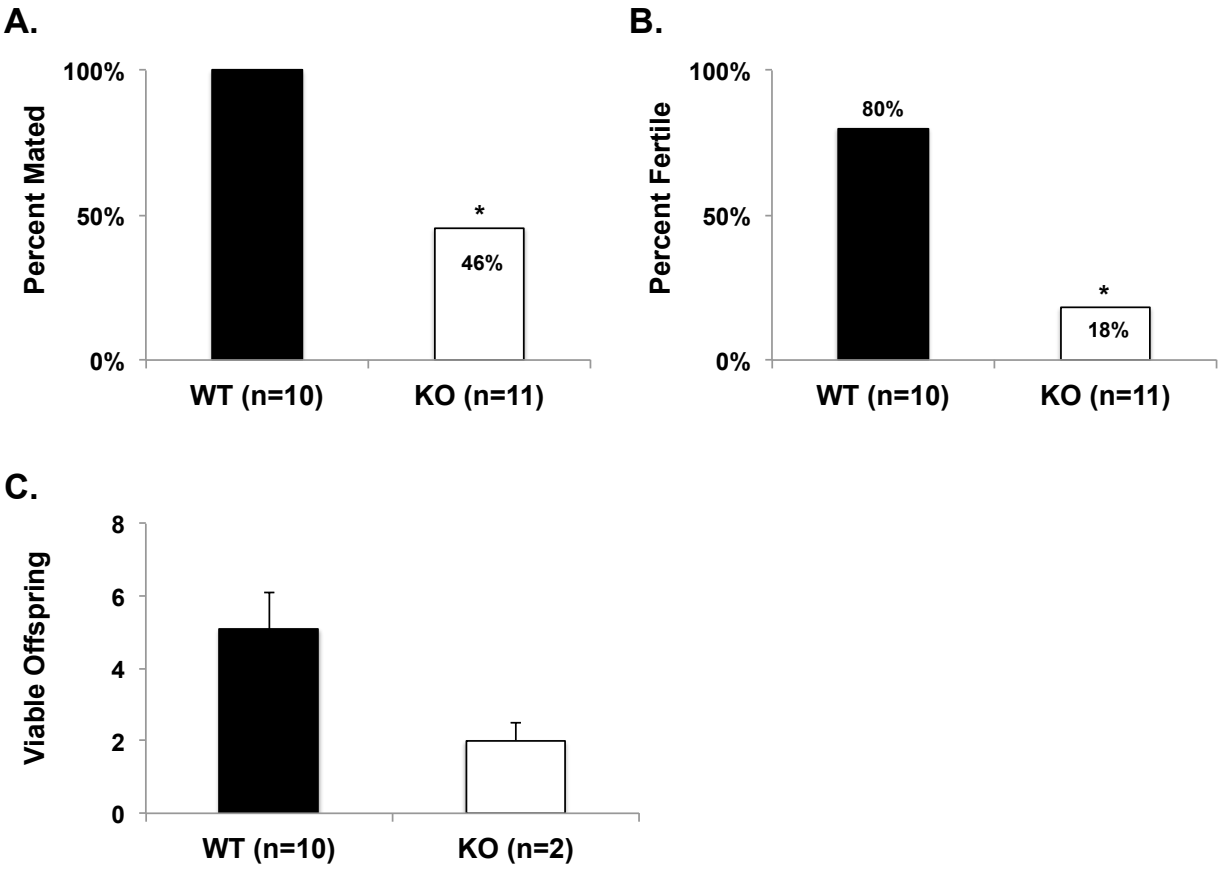
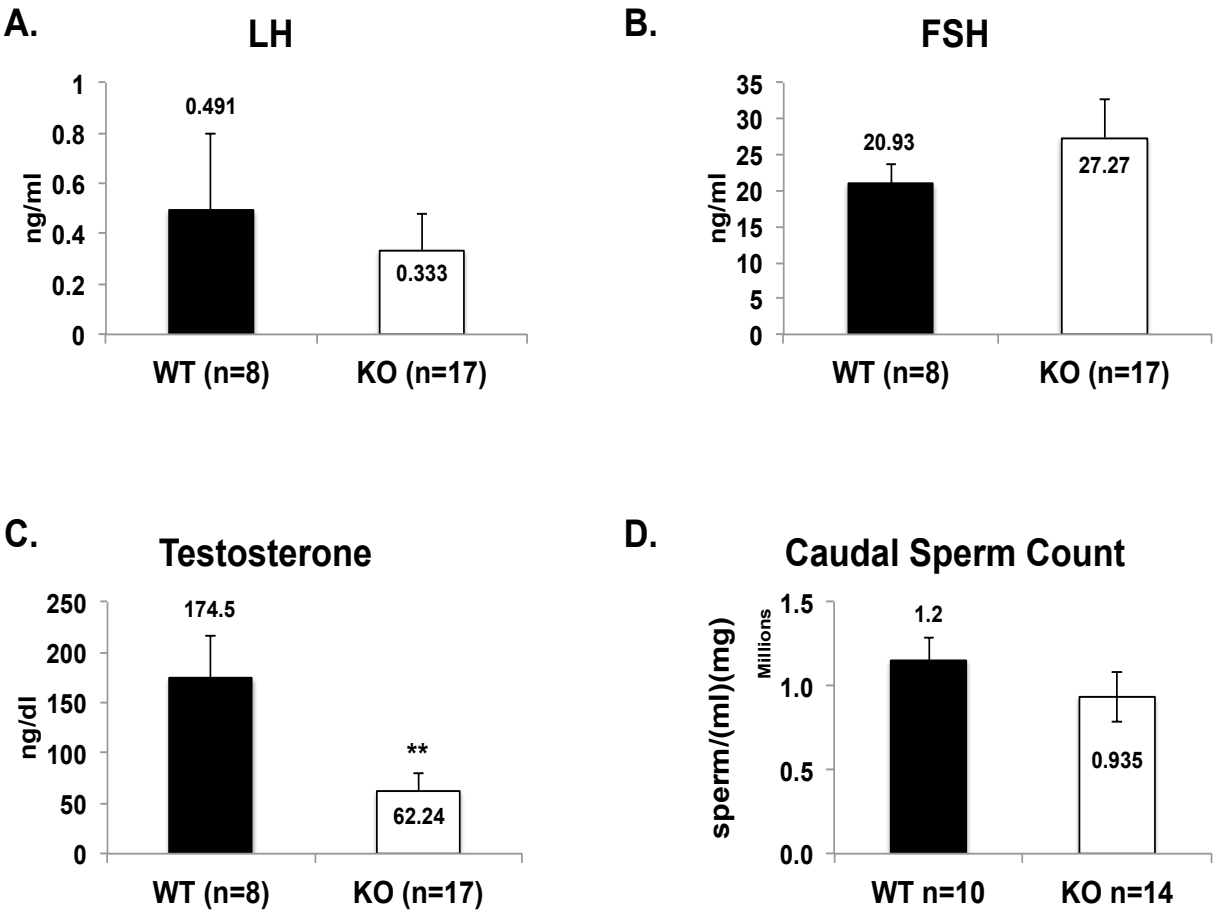

A.
100%
50%
0%
Percent Mated
*
46%
WT (n=10)
KO (n=11)
B.
100%
50%
0%
Percent Fertile
80%
*
18%
WT (n=10)
KO (n=11)
C.
8
6
4
2
0
Viable Offspring
WT (n=10)
KO (n=2)

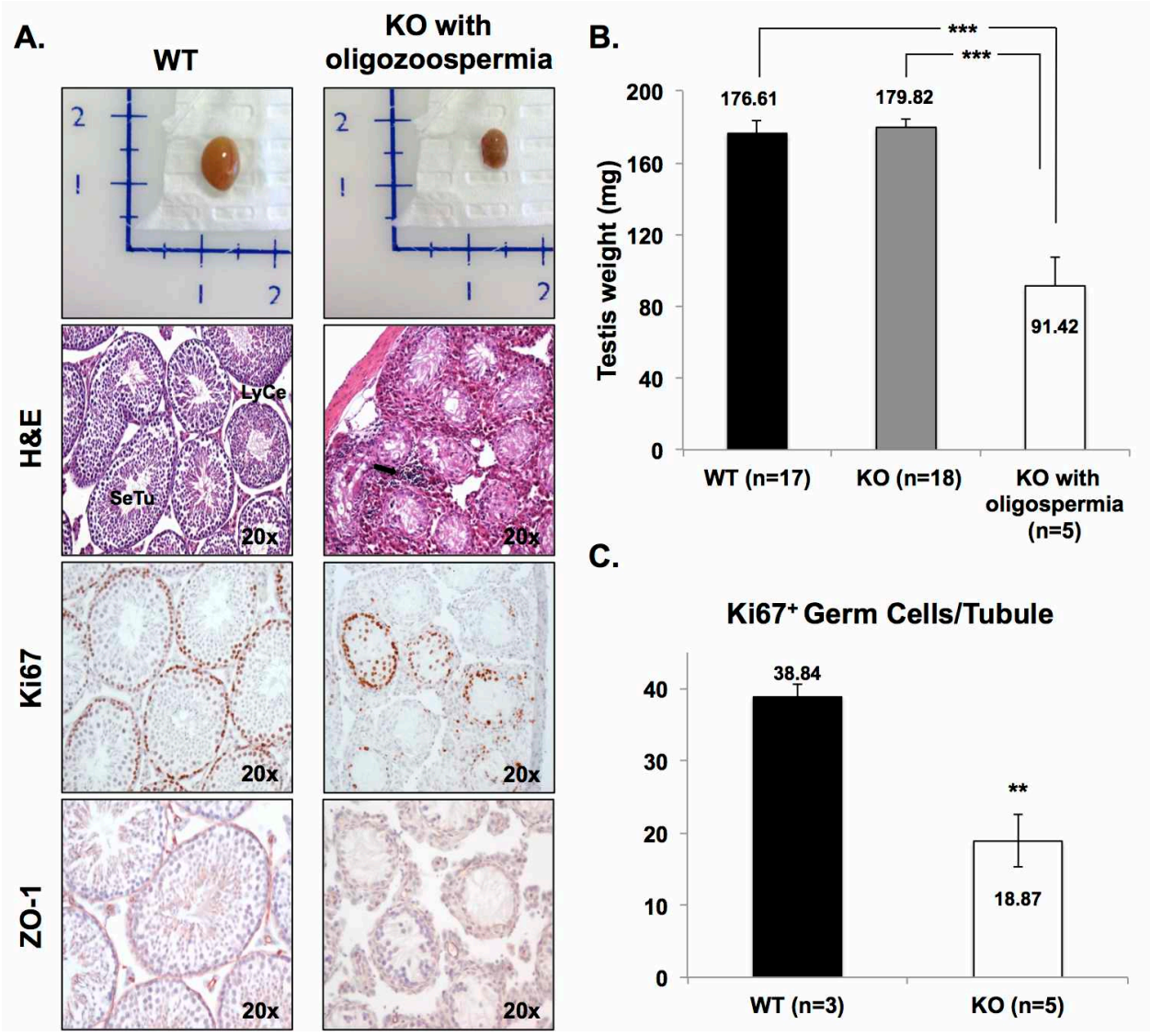
Figure 3.2: Evaluation of gonadotropins, testosterone and caudal sperm quantity in male Aire-deficient mice. (A) Mean values for serum luteinizing hormone. **(B)** Mean values for serum follicle stimulating hormone. **(C)** Mean values for free serum testosterone. **(D)** Mean values for capacitated sperm adjusted by caudal weight. WT, wild type; KO, AIRE-deficient mice; LH, Luteinizing hormone; FSH, Follicle Stimulating hormone. **, $P < 0.01$ by Student's t-test.


A.
LH
ng/ml
1
0.8
0.6
0.4
0.2
0
0.491
0.333
WT (n=8)
KO (n=17)
B.
FSH
ng/ml
35
30
25
20
15
10
5
0
20.93
27.27
WT (n=8)
KO (n=17)
C.
Testosterone
ng/dl
250
200
150
100
50
0
174.5
**
62.24
WT (n=8)
KO (n=17)
D.
Caudal Sperm Count
sperm/(ml)(mg)
Millions
1.5
1.0
0.5
0.0
1.2
0.935
WT n=10
KO n=14

Reproductive tract abnormalities within male Aire-KO mice.

We next performed histopathological evaluation of male reproductive tract tissues in 7-16 week old males. Gross morphology and tissue weight was comparable between genotypes, with the exception that five of 23 *Aire*-KO males (22%) had combined testicular weight that was significantly reduced as compared to both WT controls and remaining 78% of *Aire*-KO males (**Figure 3.3A, B**). Histologic evaluation revealed severe oligozoospermia and in two of the five animals, a mild intertubular lymphocytic infiltrate (**Figure. 3.3A**). The few remaining germ cells had a rounded morphology and demonstrated Ki67 positive nuclei, but were displaced from the basal regions of the seminiferous tubules (**Figure 3.3A**). This was confirmed by quantifying the $Ki67^+$ cells within the tubules: the mean number of $Ki67^+$ germ cells/tubule was significantly reduced in *Aire*-KO males compared to controls (**Figure 3.3C**). Interestingly, even in the testis sections from *Aire*-KO mice without overt loss of germ cells, there were still fewer Ki67+ cells per tubule. Finally the blood-testis-barrier was disrupted in oligozoospermic testis samples, as evidenced by sporadic detection of the tight junction marker ZO-1 along the basement membrane of the seminiferous epithelium, as compared to the continuous expression pattern in controls (**Figure 3.3A**).

Figure 3.3: Oligospermia in a subpopulation of *Aire*-KO male mice. (A) Representative images comparing whole testis, H&E sections, Ki67 staining and ZO-1 staining between WT controls and *Aire*-KO males with oligozoospermia. **(B)** Mean values for the combined testis weight of WT controls, *Aire*-KO mice without clear oligospermia and *Aire*-KO mice with oligospermia. **(C)** Mean value of Ki67 positive germ cells per tubule from five random 20x fields per section from WT controls (n=3) and *Aire*-KO (n=5) mice. All images are 200x. Arrow head, inflammatory cells; WT, wild type; KO, AIRE-deficient mice; H&E, Hematoxylin and Eosin; LyCe, Leydig cells; SeTu, Seminiferous tubules; ZO-1, Zona Occludens protein 1; **, $P < 0.01$; ***, $P<0.001$ by students t-test.


A.
WT
KO with oligozoospermia
H&E
Ki67
ZO-1
LyCe
SeTu
20x
B.
***
***
176.61
179.82
91.42
Testis weight (mg)
200
160
120
80
40
0
WT (n=17)
KO (n=18)
KO with oligospermia (n=5)
C.
Ki67+ Germ Cells/Tubule
38.84
18.87
**
40
30
20
10
0
WT (n=3)
KO (n=5)

In 15 of 22 mice *Aire*-KO mice (68%), epididymal sections revealed an increase in interstitial lymphocytic aggregates, whereas this was not detected in any WT samples. This held true even if epididymal gross morphology appeared normal, and if epididymal sperm was present (**Figure 3.4 A, B**). A complete absence of epididymal sperm was observed in six mice, all of whom displayed an abnormal interstitial morphology (**Figure 3.4 A, B**) characterized by an increase in intertubule space and fibrosis within the caudal region (**Figure 3.4B**). Morphology of the seminal vesicle was unaltered and a mild lymphocytic infiltrate within the surrounding connective tissue was document in 16 of 19 *Aire*-KO mice (84%) (**Figure 3.4C**). Finally, we confirmed the presence of pronounced prostatitis in 17 of 19 (89%) of our *Aire*-KO mice (**Figure 3.4D**), as reported previously (Gavanescu et al., 2008; Hou et al., 2009).

Loss of vomeronasal organ-associated glands in male Aire-KO mice.

Autoimmune disruption of the vomeronasal organ (VNO) could inhibit pheromone detection in male *Aire*-KO mice and explain the absence of normal mating behavior seen in 6 of 11 (54%) mice. To that end we evaluated the histopathology of the VNO and its associated glands (**Figure 3.5A**). Although we did not detect any morphologic alteration or lymphocytic infiltration within the VNO, we did observe a pronounced alteration to the mucus secreting glands associated with this organ in six of seven (86%) of *Aire*-KO mice (**Figure 3.5B, C**).

Figure 3.4: Male *Aire*-KO mice can develop reproductive tract abnormalities. (A-B) Histopathology of the morphologic changes in the caput and cauda of the epididymis. **(C-D)** Histopathology of the seminal vesicle and prostate. All images are 200x. Arrow head, inflammatory cells; *, Epididymal tubules devoid of spermatozoa; WT, wild type; KO, AIRE-deficient mice; A, Ampulla; AP, Anterior Prostate lobe; EpTu, Epididymal tubules; H&E, Hematoxylin and Eosin; LoCo, loose connective tissue; MuFo, mucosal folds; Se, secretions.

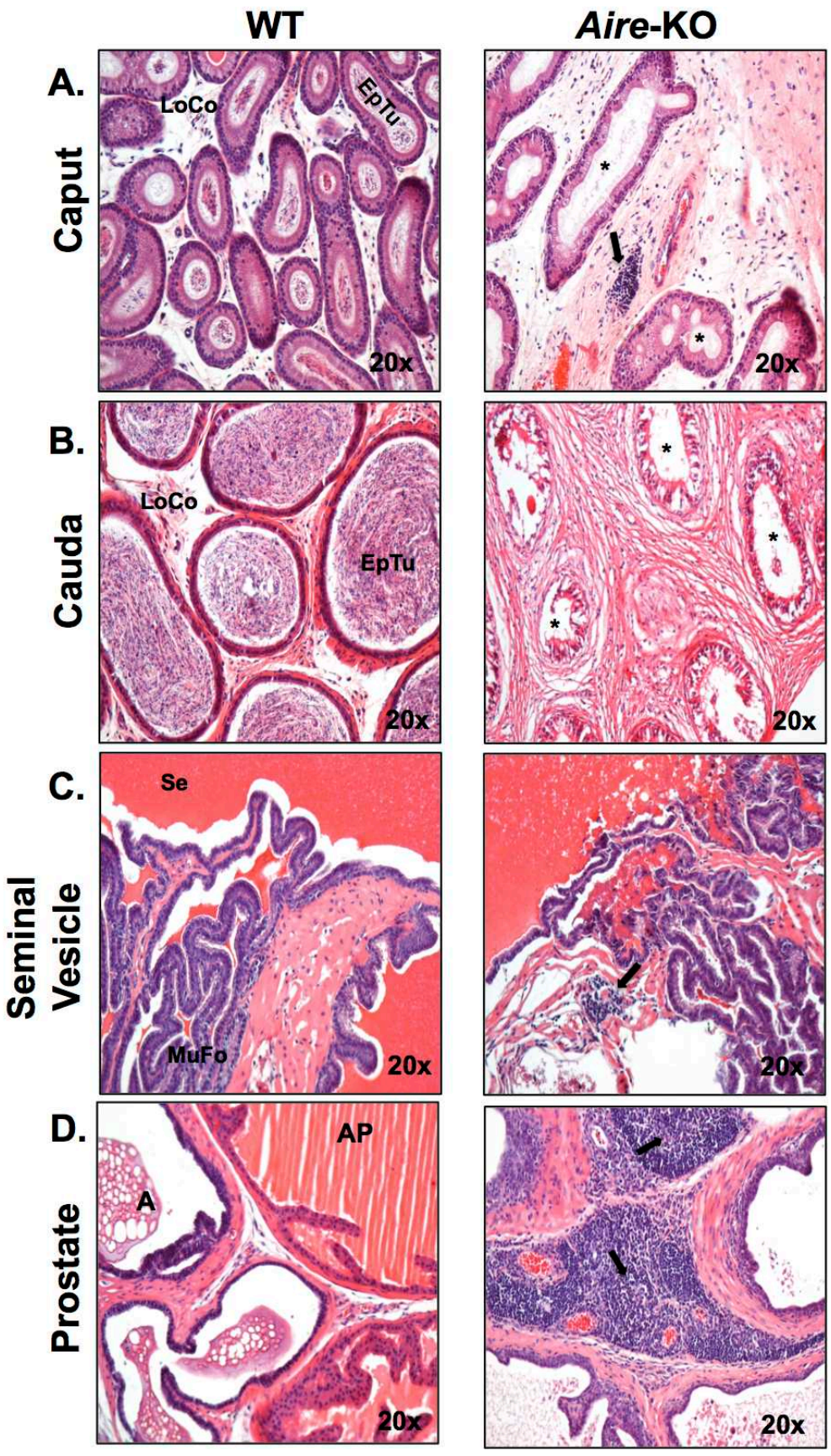
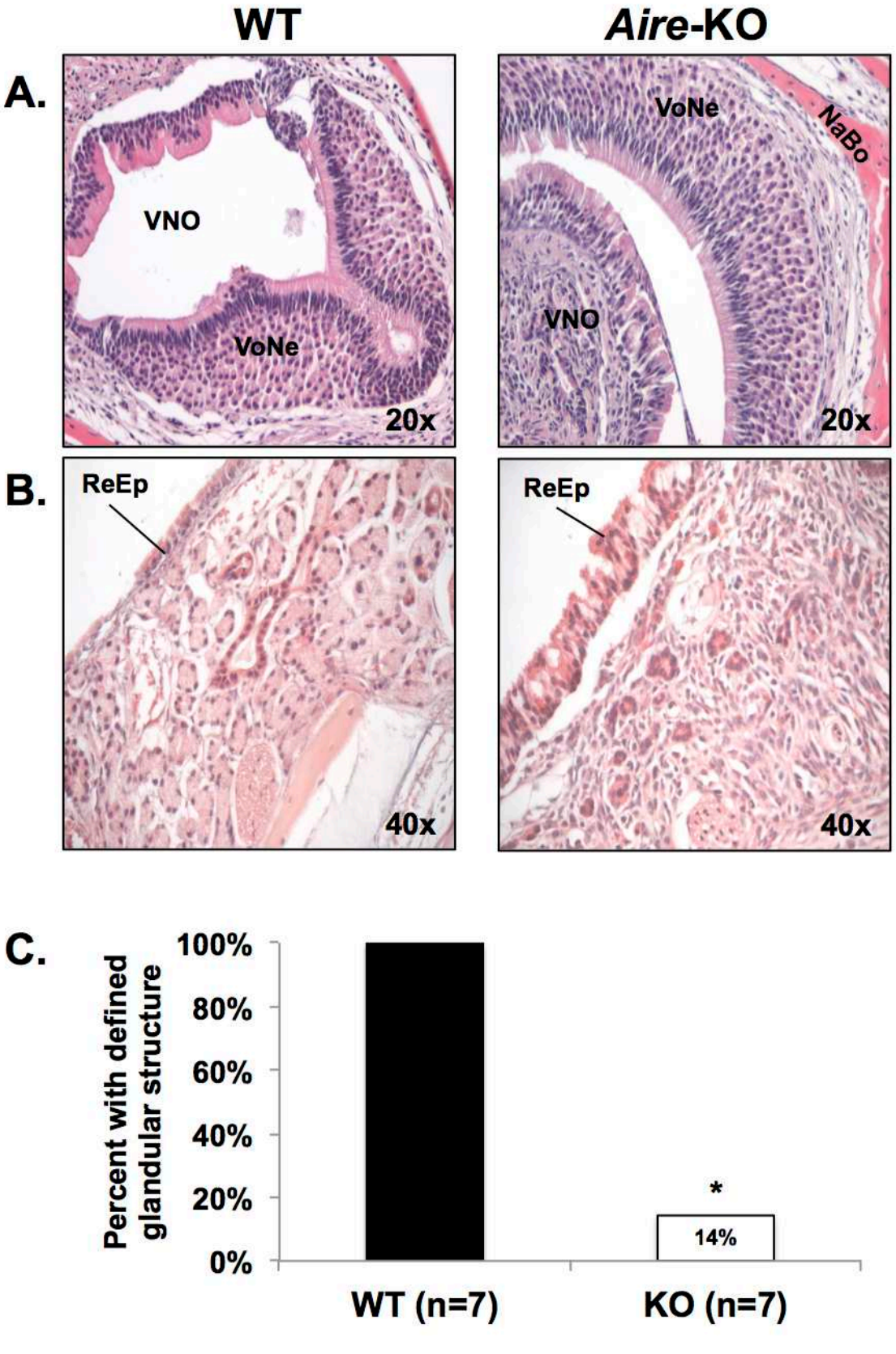

WT
Aire-KO
A.
Caput
LoCo
EpTu
20x
*
B.
Cauda
LoCo
EpTu
20x
*
C.
Seminal Vesicle
Se
MuFo
20x
D.
Prostate
AP
A
20x

Figure 3.5: Loss of Vomeronasal organ-associated glands in male *Aire*-KO mice. **(A)** Histology of the vomeronasal organ from WT and *Aire*-KO male mice at 200x magnification. **(B)** Histology of the VNO-associated glands at 40x magnification. **(C)** Percent of mice with intact VNO-associated glandular architecture. WT, wild type; KO, AIRE-deficient mice; H&E, Hematoxylin and Eosin; NaBo, Nasal bone; ReEp, Respiratory epithelium; VNO, Vomeronasal organ; VoNe, Vomeronasal neuroepithelium; *, $P < 0.05$ by chi-square analysis.

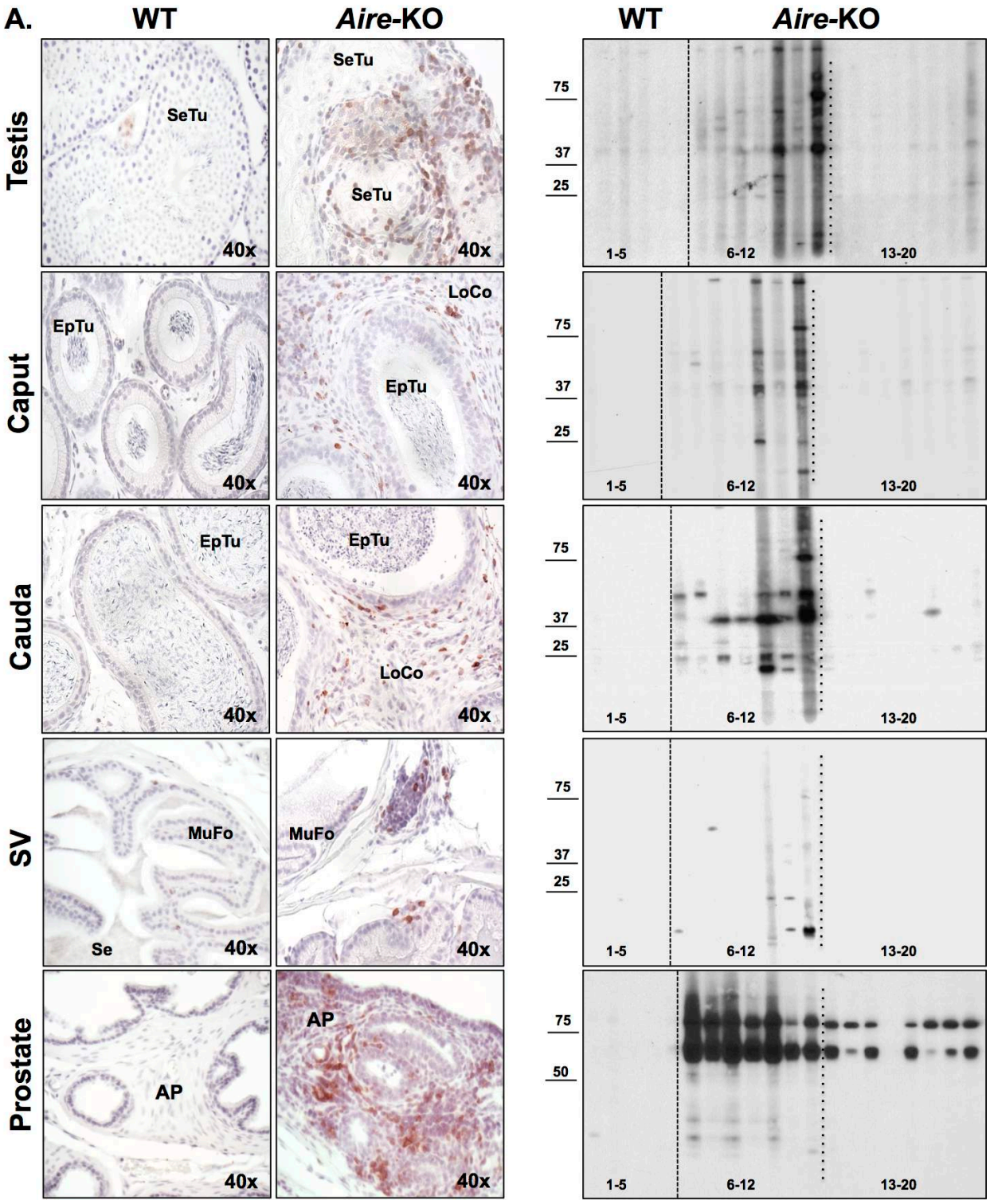

WT
Aire-KO
A.
VNO
VoNe
20x
VoNe
NaBo
VNO
20x
B.
ReEp
40x
ReEp
40x
C.
Percent with defined glandular structure
100%
80%
60%
40%
20%
0%
*
14%
WT (n=7)
KO (n=7)

Autoimmune recognition of the male Aire-KO mouse reproductive tract.

We confirmed the presence of T cells within the immune infiltrates identified by hematoxylin and eosin staining (**Figure 3.3A, Figure 3.4**) by probing sections of testis, epididymis, seminal vesicle and prostate with an anti-CD3 antibody. T cells density, while variable both between individual mice and differing organs, was more abundant in *Aire*-KO animals. In testis of Aire-KO mice, T cells were relatively sparse compared to other tissues examined, and were predominantly contained within the intertubular space. However, occasional $CD3^+$ T cells were identified within the lumens of seminiferous tubules that were devoid of germ cells (**Figure 3.6A**). Epididymal T cell populations in *Aire*-KO mice were detected within all organ segments and had infiltrated both the interstitial space, often in aggregates, as well as the ductal epithelium (**Figure 3.6A**). T cell aggregates within the seminal vesicle of *Aire*-KO mice were contained to the loose connective tissue that supports the mucosal folds (**Figure 3.6A**). Finally, our *Aire*-KO mice displayed a pronounced T cell infiltration into the glandular epithelium of the prostate comparable to what was shown by Hou et al (**Figure 3.6A**).

To determine the spectrum of autoantibodies targeting the male reproductive tract produced as a result of *Aire* deficiency, we probed immunoblots of reproductive tract tissue lysates with the serum of *Aire*-KO or WT mice (**Figure 3.6B**). The pattern and strength of reactivity among animals as well as different tissues was varied. However, serum from Aire-KO animals consistently revealed target bands of ~42, 50 and 58 kDa within both the testis and the epididymal lysate.

Figure 3.6: CD3+ T cell distribution and the identification of autoantibodies throughout the reproductive tract of *Aire*-KO males. The presence of an immune response within the reproductive tract of male *Aire*-KO mice was confirmed by detecting both CD3+ T cells and serum autoantibodies. **(A)** Immunohistochemical staining for the presence of $CD3^+$ T cells within the male reproductive tract and associated sex glands. **(B)** From left to right: *Rag*-KO protein lysate from testis, caput, cauda, seminal vesicle and the prostate gland were probed with serum from WT and *Aire*-KO mice. In each blot, columns 1-5 contain WT serum, 6-12 contain *Aire*-KO serum from mice >20 weeks of age and 13-20 contain *Aire*-KO serum from mice 7-8 weeks of age. All images are 400x. WT, wild type; KO, AIRE-deficient mice; *Rag*-KO, Recombinase Activation gene-1 deficient mice; AP, Anterior Prostate lobe; EpTu, Epididymal tubules; LoCo, loose connective tissue; MuFo, mucosal folds; Se, secretions; SeTu, Seminiferous tubules; SV, Seminal vesicle.

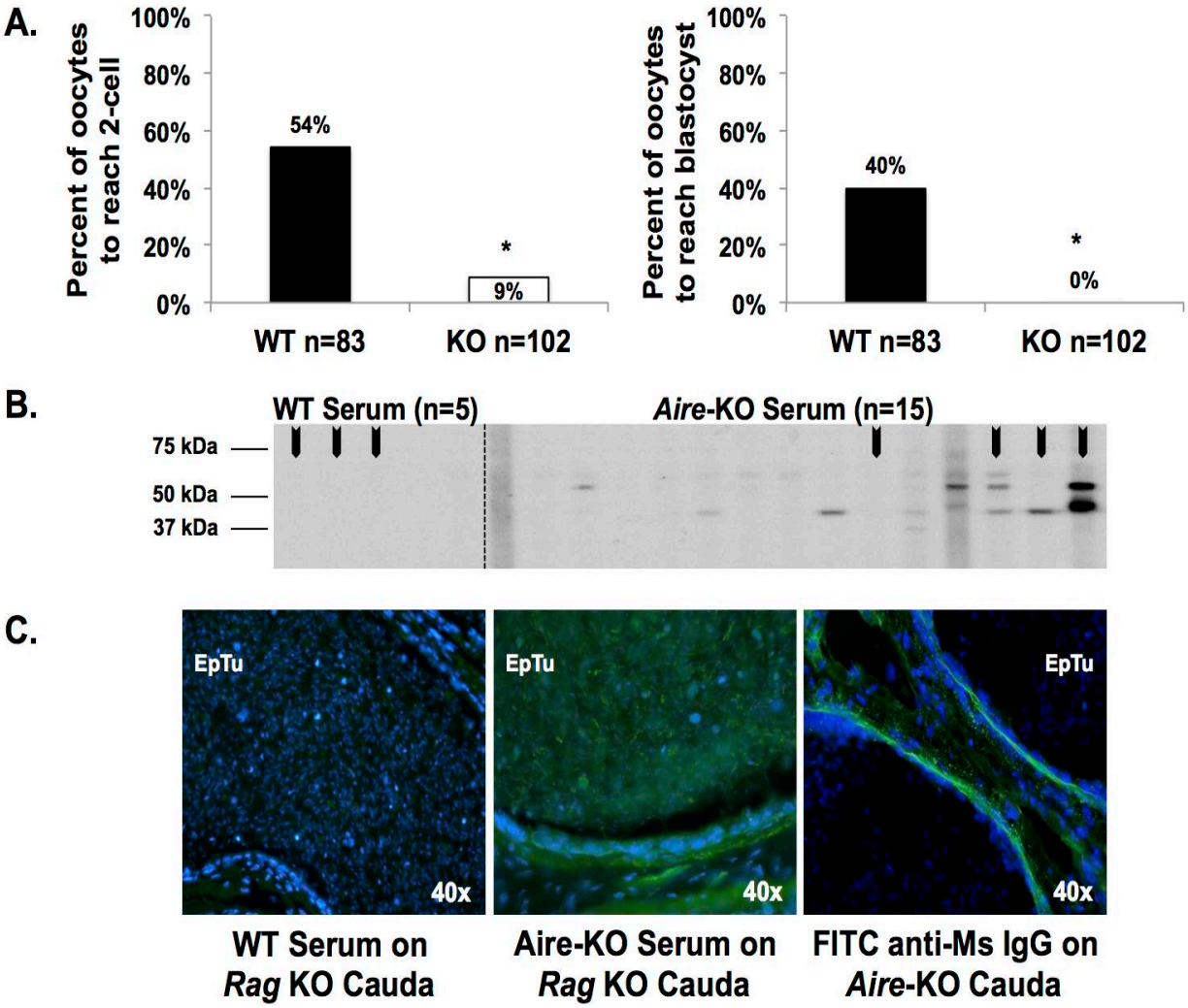

A.
WT
Aire-KO
WT
Aire-KO
Testis
Caput
Cauda
SV
Prostate
SeTu
SeTu
SeTu
40x
40x
EpTu
LoCo
EpTu
40x
40x
EpTu
EpTu
LoCo
40x
40x
MuFo
MuFo
Se
40x
40x
AP
AP
40x
40x
75
37
25
75
50
1-5
6-12
13-20

Additionally, age of the animals had a clear effect on autoantibody production, as both the number and intensity of bands was greater when probing with the serum from mice older than 20 weeks. Reactivity against the prostate was the strongest, had limited variability and was present in nearly all serum samples regardless of age. In comparison, reactivity against the testis, epididymis and seminal vesicle displayed weaker reactivity, was rare in young animals (37.5% for testis and epididymis and 25% for seminal vesicle), but often displayed a broader spectrum of epitope recognition.

Aire-KO mice generate sperm-specific antibodies and demonstrate low in vitro fertilization success.

We investigated the possibility that anti-sperm antibodies were contributing to the infertility in *Aire*-KO mice. To confirm a fertility defect at the level of the sperm, we used isolated and capacitated caudal sperm to fertilized oocytes from superovulated WT females. Fertilization with WT sperm produced 2-cell and blastocyst stage embryos with a success rate of 54% and 40%, respectively. However, sperm from Aire-KO males had only a 9% success rate in creating 2-cell embryos, and embryos never progressed to the blastocyst stage (**Figure 3.7A**). Next anti-sperm antibodies were detected in serum from *Aire*-KO mice by probing a lysate of caudal sperm by western blot. Of 15 serum samples from *Aire*-KO mice, 11 (73%) contained sperm-specific antibodies that recognized autoantigens of comparable size to those detected in whole testis and epididymal lysates (**Figure 3.6B, 3.7B**). Binding of autoantibodies to sperm was confirmed by probing frozen tissue sections of caudal epididymis from a *Rag*-deficient

Figure 3.7: *In vitro* fertilization with *Aire*-KO male sperm and localization of anti-sperm/anti-epididymal autoantibodies. (A) Percent of oocytes incubated with sperm from WT and *Aire*-KO mice to reach both 2-cell and blastocyst stage. **(B)** *Rag*-KO sperm protein lysate probed with individual serum samples from five WT and 15 *Aire*-KO mice. **(C)** From left to right: WT serum on a frozen *Rag*-KO caudal section, *Aire*-KO serum on a frozen *Rag*-KO caudal section, FITC anti-Mouse IgG on frozen *Aire*-KO caudal section. All images are 40x. Arrows indicate selected samples used for Fig. 2.7C. WT, wild type; KO, AIRE-deficient mice; EpTu, Epididymal tubules; *Rag*-KO, Recombinase Activation gene-1 deficient mice; *, $P < 0.05$ by chi-square analysis.


A.
Percent of oocytes to reach 2-cell
100%
80%
60%
40%
20%
0%
54%
*
9%
WT n=83
KO n=102
Percent of oocytes to reach blastocyst
40%
*
0%
WT n=83
KO n=102
B.
WT Serum (n=5)
Aire-KO Serum (n=15)
75 kDa
50 kDa
37 kDa
C.
EpTu
40x
WT Serum on Rag KO Cauda
Aire-KO Serum on Rag KO Cauda
FITC anti-Ms IgG on Aire-KO Cauda

mouse with selected serum samples (arrow heads) (**Figure 3.7C**). Compared to WT mice, autoantibodies within *Aire*-KO serum localized to the epithelium and connective tissue surrounding the epididymal tubules (all samples) as well as with caudal sperm (three of four). Finally, to determine whether endogenous antibodies bound tissue in situ, caual sections were subjected to direct immunofluorescence using a FITC-conjugated anti-mouse IgG antibody (**Figure 3.7C**). While the presence of anti-sperm autoantibodies was not detected, prominent IgG deposits marking the epithelium and basement membrane of epididymal tubules were observed.

DISCUSSION

Disruption of AIRE in both humans and mice impedes the establishment of central immune tolerance and produces a multi-organ autoimmunity characterized by the presence of autoreactive T cells and antibodies (Aaltonen et al., 1997; Anderson et al., 2002). While discrepancies in both the disease severity as well as the profile of target organs exist between *APS*-1 patients and *Aire*-deficient mouse models, a shared pathology is premature gonadal insufficiency (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Perheentupa, 2006; Ramsey et al., 2002). We have previously demonstrated that one cause of early reproductive senescence in female mice on the Balb/c genetic background is an age-dependent loss of ovarian follicular reserves beginning at three months of age (Jasti et al., 2012). However, information on fertility and reproductive immune targets in male *Aire*-deficient mice remains scattered and exiguous. In this study we demonstrate that the majority of male Balb/c *Aire*-KO mice are infertile, produce low levels of testosterone, develop autoimmune recognition against the male reproductive tract, have structurally abnormal glands associated with the vomeronasal organ and generate sperm with a severely reduced potential to fertilize an oocyte *in vitro*. Additionally, roughly one fifth of *Aire*-KO male mice are oligozoospermic with an apparent disruption of the blood-testis-barrier.

Gonadal insufficiency occurs in approximately 12-14% of male *APS-1* patients (Ahonen et al., 1990) with the vast majority of cases being diagnosed with primary hypogonadism due to low testosterone levels. Only one case of hypogonadotropic hypogonadism (Ahonen et al., 1990), which is common in female patients, has been reported in male APS-1 patients, suggesting that the autoimmune lesions of infertility

differ between sexes. However, the majority of both males and females develop anti-steroid cell antibodies specific for 17α–hydroxylase (Krohn et al., 1992), 21-hydroxlase (Uibo et al., 1994) and P450 side-chain cleavage (Winqvist et al., 1995).

Our analysis of the infertility in male Balb/c *Aire*-KO mice mirrors the constellation of symptoms observed in infertile APS-1 males. These mice are almost completely infertile, have low testosterone, and normal production of gonadotropins. Preliminary observations show histologically normal pituitary glands with no lymphocytic infiltration (unpublished). Interestingly, over half of the animals did not mate over a three-week period (**Figure 3.1**). The low testosterone (**Figure 3.2**) might be one potential cause, but another possibility is a disruption of vomeronasal organ function. The VNO is a component of the accessory olfactory system whose sensory neruoepithelium is responsible for detecting pheromones and is one of only two locations that express the odorant binding protein 1a (OBP1a). OBP1a is expressed in an *Aire*-dependent manner within the thymus presumably to provide antigen tolerance to the presumptive pheromone transporter (Bocskei et al., 1992; J. J. DeVoss et al., 2010; Pes et al., 1998). An autoimmune response against OBP1a, along with the ubiquitous antigen α-fodrin, has been shown to induce autoimmune dry eye and the destruction of the salivary and lacrimal glands of *Aire*-deficient mice resulting in condition similar to Sjögren's Syndrome (J. J. DeVoss et al., 2010). Histopathologic evaluation of the VNO in our mice revealed no direct inflammation; however, we did discover an alteration to the associated glands (**Figure 3.5**). Many of the sub-epithelial tubuloacinar glands secrete a mucous directly into the lumen of the VNO (Salazar, Quinteiro, & Cifuentes, 1997). The glandular secretions are responsible for dissolving various chemical

pheromone stimuli, transporting them to the VNO and removing them following chemoreception (Mendoza & Breipohl, 1983; Mendoza & Kuhnel, 1987; Roslinski, Bhatnagar, Burrows, & Smith, 2000). In humans the function of the VNO as a sensory organ is unclear (Trotier et al., 2000). However, pheromone detection through the VNO has been shown to be an essential component of mating behavior for sexually inexperience hamsters (Meredith, 1986) and its surgical removal, particularly in young mice, results in major behavioral changes to courtship, sexual behavior, aggression and the onset of puberty (Wysocki & Lepri, 1991). Further, male old world primates have been shown to demonstrate a behavior similar to the flehman response (Estes, 1972) when exposed to odorants from cycling females (Charpentier, Mboumba, Ditsoga, & Drea, 2013). It is possible that the changes to the VNO-associated glands results in a secondary disruption of the VNO in our sexually inexperienced male mice that impairs their mating behavior resulting in the presence of fewer copualtory plugs.

Aire-deficient mice have been shown to develop immune autoreactivity against multiple organs including, but not limited to, the eye, stomach, pancreas, ovaries and salivary glands (Anderson et al., 2002; Jasti et al., 2012; Jiang et al., 2005). With regards to the male reproductive tract, the existence of anti-testis and anti-prostate autoantibodies has been documented (Hou et al., 2009; Hubert et al., 2009; Ramsey et al., 2002). However an analysis of the inflammation occurring within the testis, epididymis or seminal vesicle has not been completed. Inflammation, both systemic (Kalyani et al., 2007) and local, can impair male fertility. In general, epididymitis is the most common male reproductive tract inflammatory disease (Luzzi & O'Brien, 2001); impacting over 600,000 US males annually (Krieger, 1984), and can lead to secondary

involvement of the testis. This paradigm is replicated in our *Aire*-KO mice in which 22% (**Figure 3.3**) of the male mice evaluated were oligozoospermic with evidence of acute testicular inflammation. However, 68% of animals had either ongoing inflammation (**Figure 3.6**) or increase fibrosis suggesting a previous inflammatory event (**Figure 3.4**) within the epididymis, suggesting that the epididymis is the initial target of the immune system in *Aire*-deficient mice.

Interestingly, the histopathology of the oligozoospermic animals resembled a Sertoli cell only syndrome. First described in 1947 (Del Castillo, Trabucco, & FA, 1947), the syndrome is characterized by a testis biopsy that demonstrates a complete absence of germ cells with an otherwise normal seminiferous epithelium. The syndrome is the leading cause of non-obstructive azoospermia in infertile males and believed to derive from microdeletions within the azoospermia factor (AZF) region of the Y-chromosome (Foresta et al., 1998; Kamp et al., 2001; Reijo et al., 1995). Here, we show for the first time that an immune response can also cause a total depletion of germ cells. While the molecular mechanism leading to the Steroli cell only phenotype in our *Aire*-KO mice remains unclear, one possibility is disruption of the blood-testis-barrier secondary to inflammation (Koksal et al., 2007). Sertoli cells respond to both autocrine and paracrine cytokine signaling to alter their adhesive proprieties to facilitate the transport of preleptotene/leptotene spermatocytes past the blood-testis barrier (BTB) (Xia, Mruk, Lee, & Cheng, 2006). It is possible that inflammatory cytokine signaling in our azoospermic mice alters the Sertoli cells, as suggested by the loss of consistent ZO-1 expression (**Figure 3.3**), such that they are no longer able to retain developing

spermatogonia behind the BTB and consequently exposing highly immunogenic sperm antigens to the immune system.

Chronic prostatitis and chronic pelvic pain syndrome (CP/CPPS) in men is a common condition and unfortunately not well understood. Patients present urologic symptoms, nonspecific discomfort in the pelvic region and sexual dysfunction (R. B. Alexander & Trissel, 1996). Despite initial speculation that these disorders had a bacterial etiology, it is now believed that the majority of CP/CPPS cases have noninfectious origins (de la Rosette, Hubregtse, Meuleman, Stolk-Engelaar, & Debruyne, 1993; Krieger & Egan, 1991). Importantly, the presence of prostatic immune aggregates is common (Kohnen & Drach, 1979; Theyer et al., 1992) and further, it has been demonstrated that a percentage of CP/CPPS patients have the ability to generate a proliferative T cell recall response when challenged with seminal plasma (Alexander, Brady, & Ponniah, 1997), both of which suggest a potential autoimmune component to CP/CPPS.

Aire-deficient mice evaluated on a mixed (129/Sv x C57BL/6) F2 genetic background have been shown to develop spontaneous immune response against the prostate autoantigen seminal vesicle secretory protein 2 (SVS2) (Hou et al., 2009). The result was a moderate to severe prostatitis in over 70% of the mice. We have confirmed this results in nearly 90% of our animals on a congenic Balb/c background (**Figure 3.4D, 3.6A**). Background genetics has a significant influence on the severity of disease and the range of autoantigens targeted in mouse models of *Aire*-deficiency (Jiang et al., 2005). However, some target organs, as is the case for the prostate, are consistent across strains. Interestingly, the dominant prostate antigens in Aire-deficient Balb/c

mice were not SVS2, but instead a higher molecular weight protein, identification of which is currently underway (**Figure 3.6B** and work in progress). Nevertheless, we contend that a deficiency in *Aire*, along with day-3 thymectomy (Taguchi et al., 1985), are good models for the study of chronic autoimmune prostatitis.

Mature spermatozoa contain numerous specific proteins (Suri, 2004) that are potentially immunogenenic in both men and women. However, the consequence of an immune response resulting in the production of anti-sperm antibodies its contribution to infertility remains unclear. It is estimated the sperm autoantibodies, which can form following trauma, inflammation or vasectomy (Meinertz, Linnet, Fogh-Andersen, & Hjort, 1990), are present in seven to 28 percent of infertile males compared to less than two percent of healthy men (Haas, Cines, & Schreiber, 1980; Heidenreich et al., 1994). These antibodies are considered clinically important when over 50% of the spermatozoa are coated with IgG and IgA antibodies which can block sperm penetration (Eggert-Kruse, Leinhos, Gerhard, Tilgen, & Runnebaum, 1989) and decrease *in vitro* fertilization rates (Clarke, Lopata, McBain, Baker, & Johnston, 1985).

Hubert et al. (2009) speculated that anti-sperm autibodies are a causative agent for the decreased fertility *Aire*-deficient males on the B6 background (Hubert et al., 2009). In our evaluation we demonstrated significantly lower IVF success rates, with only 9% of wild-type oocytes developing to the two-cell stage, and none reaching the blastocyst stage, after being incubated with epididymal sperm from Balb/c *Aire*-deficient mice (**Figure 3.7A**). It is possible that the observed orchido-epididymitis is impacting the health and quality of the developing sperm such that they have a reduced potential to fertilize an oocyte. While we documented serum antibody reactivity against sperm

protein in the majority of our animals (**Figure 3.7B, C**), we did not detect the presence of these autoantibodies in situ within the caudal region of the epididymis (**Figure 3.7C**). We did, however, note considerable autoantibody binding to the basal epithelium of the epididymal tubules (**Figure 3.7C**). The secretory products of the epididymal epithelium contribute to the completion of sperm development, enhancement of motility and the ability to bind the zona pellucida (Kirchhoff, Osterhoff, Pera, & Schroter, 1998; Moore, Curry, Penfold, & Pryor, 1992). It is possible that the abundant antibody deposition within the epididymis is altering the ability of the tubule epithelial cells to transport and secrete the requisite seminal plasma proteins for complete spermatozoa development and thus impairing their fertilization potential.

The discovery of *Aire* and its ability to regulate expression of tissue specific antigens within the thymus has shifted our understanding of the mechanisms behind immune tolerance. In this study, we show that a deletion of *Aire* results in wide spread recognition and inflammation within the male reproductive tract that is associated with decreased fertility. The correlation between impaired central immune tolerance and fertility has potential implications for not only male APS-1 patients but may also provide important insights into both male autoimmune and unexplained cases of infertility.

CHAPTER 4:

GENERAL DISCUSSION

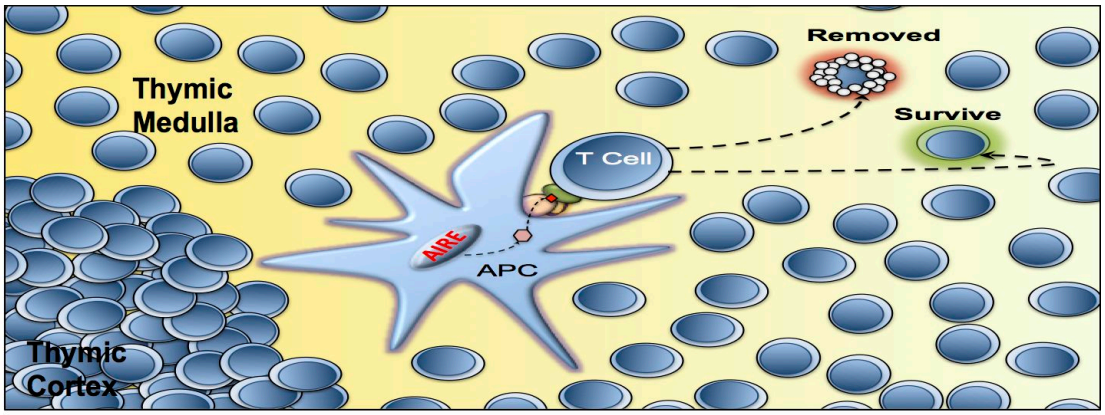
MODEL

The original dogma concerning the mechanisms of central immune tolerance for self-antigens has been forever changed by the discovery of the Autoimmune Regulator (AIRE) gene (Nagamine et al., 1997). AIRE-regulated gene expression within the medullary compartment of the thymus results in the expression of several thousand genes against which autoreactive thymocytes are negatively selected (Anderson et al., 2002; Derbinski et al., 2001). Importantly, an estimated 25% of the AIRE-regulated genes are highly restricted in their peripheral tissue distribution, suggesting that central tolerance controls autoimmune disease against both ubiquitous and tissue specific antigens (Anderson et al., 2002; Derbinski et al., 2005).

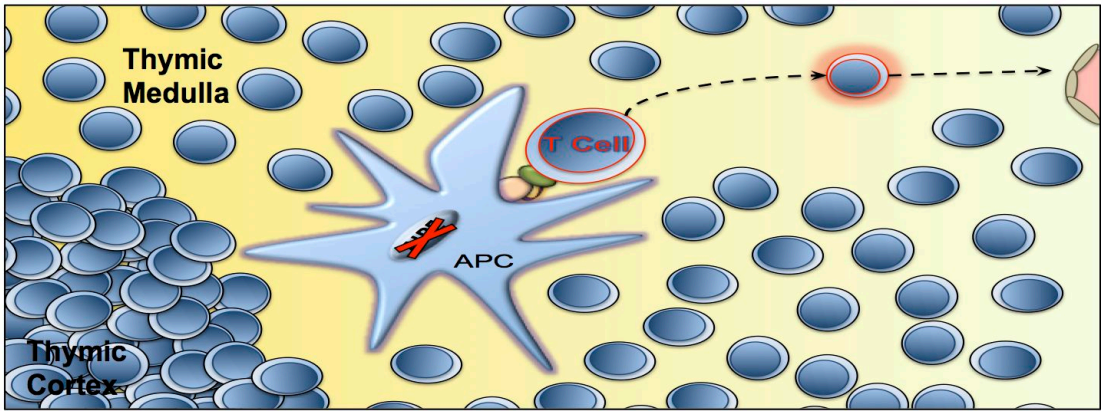
Our current understanding of the function of AIRE is modeled in **Figure 4.1**. AIRE induced gene expression provides a pool of antigens that are presented to developing T cells within the medulla of the thymus. T cells that demonstrate a high affinity for both restricted and ubiquitous self-antigens are negatively selected to prevent the release of self-specific naive T cells. Alternatively, non-self specific T cells are allowed to complete their maturation and provide immune surveillance within the periphery (**Figure 4.1A)**. Mutations to AIRE prevent this selection process and as a consequence allow the escape of self-antigen specific T cells that can incite numerous autoimmune diseases (**Figure 4.1B)**.

Figure 4.1: Model of AIRE-regulated gene expression for central immune tolerance. (A) AIRE-regulated gene expression within the thymic medulla provides an antigen source against which self-reactive T cells are negatively selected and removed to prevent autoimmune disease. **(B)** A disruption of the AIRE gene prevents the expression of numerous self-antigens. Autoreactive T cells that would otherwise have been removed are now allowed to escape the thymus and instigate multiple autoimmune disorders.

A.


Removed
Thymic Medulla
Survive
T Cell
AIRE
APC
Thymic Cortex

B.


Thymic Medulla
T Cell
APC
Thymic Cortex

The collection of AIRE regulated antigens includes several that are expressed within the male and female reproductive tracts. Expectedly, many of these genes are abundantly expressed throughout most tissues, but there are several proteins that are highly specific for the reproductive system. Selected female reproductive system specific genes include: aldehyde dehydrogenase family 1 (ALDH1A7), angiopoietin 4 (Ang4), amphiregulin (AREG), AU020206, branched chain aminotransferase 1 (BCAT1), cyclin dependent kinase inhibitor 1C (CDKN1C), embryonic hemoglobin Y (HBB-Y), Keratin 16 (KRT1-16) and zona pellucida 3 (ZP3) (Derbinski et al., 2005). Selected male reproductive tract specific examples include: DNA binding protein Obfc2a, Rab GTPase Tbc1d3, CD55 antigen (DAF1), glutathione S transferase mu 5 (GSTM3) and lactate dehydrogenase C (LDH3). Regardless of the inciting antigen, AIRE-mediated central immune tolerance is essential for fertility perseveration in both men and women (Aaltonen et al., 1997; Ahonen et al., 1990; Anderson et al., 2002; Nagamine et al., 1997). Our study of the specific immune targets leading to infertility in *Aire*-deficient mice advances our understanding of autoimmune infertility.

AUTOIMMUNE PRIMARY OVARIAN INSUFFICIENCY

Primary ovarian insufficiency (POI), previously known as “premature menopause” or “premature ovarian failure”, is the development of hypergonadotropic hypogonadism in women with a normal karyotype under the age of 40 (Nelson, 2009). The overall incidence of spontaneous POI is roughly 1 in 250 for females under 35 and 1 in 100 for women under 40 (Coulam, Adamson, & Annegers, 1986). POI is characterized by low serum estrogen, gonadotropin levels typically associated with menopause and either

oligomenorrhea (infrequent menstruation) or amenorrhea (absence of menstruation). In approximately 4% of women with spontaneous POI, the pathogenesis is thought to derive from autoimmune oophoritis (Bakalov et al., 2005; Hoek et al., 1997). Autoimmune POI was first confirmed in 1968 and often presents in association with autoimmune adrenal insufficiency (Irvine et al., 1968). The co-existence of ovarian and adrenal disease is likely due to their overlapping function as steroidogenic organs. Indeed, testing for anti-steroid cell antibodies is often done in women suspected for autoimmune POI (S. Chen et al., 1996; Falorni et al., 2002; Sotsiou, Bottazzo, & Doniach, 1980). Additionally, autoimmune POI can present in combination with the Autoimmune Polyglandular Syndrome Type I (APS-I). Patients with APS-I, due to a deficiency in AIRE, develop a multi-organ autoimmune syndrome that preferentially targets endocrine organs, including the ovary. Oophoritis secondary to APS-I occurs in approximately 60% of female patients by the age of 30 (Ahonen et al., 1990). Unfortunately, the precise mechanisms driving the immune response against the ovary remain unknown (Nelson, 2001).

Previous work from our lab has shown that the ovaries of *Aire*-deficient mice are highly immunogenic, resulting in complete follicular loss in nearly 60% of animals by 12 weeks of age (Jasti et al., 2012). Additionally, the primed immune systems of *Aire*-KO mice demonstrate a powerful memory response when challenged with a grafted wild-type ovary (**Figure 2.1)**. We have now shown that the abnormal trafficking of leukocytes to the ovaries, which can occur as early as three-weeks of age, is likely responsible for impaired fertility in mice prior to complete follicular senescence. Our data suggests that the consequence of this early immune response is an increase in degenerated oocytes

at ovulation **(Figure 2.7B)**, smaller implantation sites at embryonic day (ED) 5.5 **(Figure 2.6A, C)**, delayed *in vitro* and *in vivo* embryo development **(Figure 2.7C,D and Figure 2.8A, B)**, reduced trophoblast outgrowth **(Figure 2.8C)** and complete embryo loss in 50% of animals by ED7.5 **(Figure 2.3A)**.

I have previous discussed our current hypothesis; that the early inflammatory events within the ovary result in low quality oocytes that can be fertilized but fail to progress through development on a synchronized timeline with the uterine environment. The result is a significantly elevated rate of post-implantation embryonic loss. But what drives the oophoritis and what are the changes occurring within these doomed oocytes that prevent them from maintaining a viable pregnancy?

Initially we hypothesized that hormonal defects and autoantibody depended pathology were responsible for the fertility lesion in *Aire*-KO female mice. Support for this derived from that fact that women with autoimmune POI, as well as APS-I preferentially produced antibodies that are steroid cell specific (Dal Pra et al., 2003; Soderbergh et al., 2004; Welt, 2008). Additionally, autoantibodies can result in fetal demise. The best-documented example of this comes from the anti-phospholipid syndrome (APS), in which women develop anti-phospholipid antibodies leading to vascular thrombosis and several pregnancy morbidities (Branch & Khamashta, 2003; Lima et al., 1996; Wilson et al., 1999). Interestingly, we found that progesterone production was unaffected **(Figure 2.3C)** and were unable to detect the presence of unique antibodies specific for the uterus, embryo or placenta **(Figure 2.4)**. These negative results suggest that a hormonal deficiency and female reproductive autoantibodies are not respsonbile for the infertility in *Aire*-KO mice. However, additional

experiments including progesterone supplementation in previously oophorectomized animals and serum transfer from *Aire*-KO mice into pregnant WT controls may be required to completely exclude these possibilities.

Isolating the inciting antigen is an essential step in uncovering the etiology of an autoimmune disorder. Unfortunately the pathogenesis of spontaneous autoimmune POI remains unknown. The most common speculation is molecular mimicry (Garza & Tung, 1995; Luo et al., 1993). A prior immune response against a viral antigen that is structurally similar to an ovarian antigen could result in subsequent oophoritis. One significant aspect of the *Aire*-deficient mouse model is that it provides an opportunity to discover the ovarian antigen(s) capable of driving an immune response. Current unpublished work from Dr. Cheng at the University of California, San Francisco has suggested that the antigen has a high molecular weight. Similarly, we have demonstrated the presence of autoantibody reactivity against ovarian antigens > 75 kDa in 60% (9 of 15) of animals tested **(Figure 2A).** An important next step will be to identify these antigens by either mass spectrometry or sequencing the T cell receptors from a clonal population of *Aire*-KO T cells stimulated with ovarian lysate.

Once the instigating antigen has been determined, it will be important to assess what inflammatory-mediated changes are occurring within the oocyte that results in developmental delay of the embryo and peri-implantation infertility. We are currently developing a mating strategy that will allow us to identify infertile *Aire*-KO female mice. This design will allow us to selectively isolate RNA for sequencing from oocytes that are otherwise incapable of sustaining a successful pregnancy. Importantly, this effort should allow us to identify global gene changes downstream of ovarian inflammation. This will

provide a significant contribution to our understanding of the mechanisms behind autoimmune primary ovarian insufficiency.

IDIOPATHIC MALE INFERTILITY

Nearly one couple in five will seek medical advice for infertility (Chandra, 2013; Mahmoud, 2000; Slama et al., 2012). Often the etiology is related to an ovulatory disorder or semen abnormality. Unfortunately, 15% of infertility cases remain unexplained (Collins & Crosignani, 1992; Templeton & Penney, 1982). This is particularly important for male patients. The biology governing male fertility, beginning with spermatogenesis and concluding with timed intercourse, is very complex and many components are still not fully understood. As a result, in 40% of male infertility cases, even when a dysfunction can be identified, the cause remains unclear (de Kretser, 1997; Dubin & Amelar, 1971). Recent advances in our understanding of the genetics behind male fertility have improved these odds. Two important examples include the discovery of mutation to the cystic fibrosis transmembrane conductance regulator (CFTR) gene and Y chromosome microdeletions impacting the “deleted in azoospermia” (DAZ) gene (Reijo et al., 1995; Riordan et al., 1989). These genetic abnormalities are present in 1-2% and 10-20% of male infertility patients respectively (Jequier, Ansell, & Bullimore, 1985; Najmabadi et al., 1996). However, it remains clear that more work needs to be done.

Perhaps one hindrance preventing a greater drive to uncover the causes of unexplained male infertility is the success of two assisted reproductive techniques: *in vitro* fertilization (IVF) and intra cytoplasmic sperm injection (ICSI). These two

interventions provide the highest success rate in the shortest amount of time. The first live birth from IVF was recorded in 1978 (Steptoe & Edwards, 1978). Since then more than 4 million pregnancies have resulted from IVF. Similarly, the first report of a clinical pregnancy resulting from the injection of a single sperm from an infertile male into a female gamete occurred in 1993 (Van Steirteghem et al., 1993). While the success of ICSI can vary depending on the protocols used and the specific male disorder being treated (De Croo, Van der Elst, Everaert, De Sutter, & Dhont, 2000; Mantikou et al., 2013; Schiff et al., 2005), the reported live birth rates per treatment cycle and per patient are 24% and 60% respectively (Witsenburg, Dieben, Van der Westerlaken, Verburg, & Naaktgeboren, 2005). Further, compared to other assisted fertility protocols, IVF has the greatest documented success rates for males with unexplained infertility (Goldman et al., 2014; Pandian et al., 2012).

However, it should be noted that these options are not without consequence. Two primary issues regarding IVF/ICSI include their substantial cost and ethics. IVF and ICSI are the most financially prohibitive assisted reproductive interventions, with average cost between 10,000 and 15,000 dollars per treatment cycle. Additionally, concerns have been raised regarding the speed at which these techniques have advanced, often without accompanying progress in the knowledge of the underlying disorders (Cummins & Jequier, 1995). At the forefront of these ethical debates are topics concerning the increased frequency of sex chromosome abnormalities in children born from ICSI (Tournaye et al., 1995) as well as how to handle unused or abandoned embryos following a successful pregnancy from these interventions ("Disposition of abandoned embryos: a committee opinion," 2013).

Our investigation into the cause of infertility in male *Aire*-deficient mice has yielded an opportunity to advance our understanding of the impact of *several* autoimmune disorders on male fertility. We have shown that *Aire*-KO male mice have a significant reduction in both their frequency of mating **(Figure 3.1A)** as well as their fertility **(Figure 3.1B)**. Unlike *Aire*-deficient female mice, whose infertility appears to derive from the effects of autoimmune oophoritis, the infertility in males appears to be multi-factorial. The potential sources of male infertility include low testosterone **(Figure 3.2C)**, lack of female pheromone detection **(Figure 3.5)**, reproductive tract inflammation **(Figure 3.4, 3.6)**, oligozoospermia **(Figure 3.3)** and impaired fertilization (**Figure 3.7A)**.

Chemosensory detection of female pheromones is an important aspect of initiating male mating behaviors in many animal species (Fiber, Adames, & Swann, 1993; Meredith, 1986; Wysocki & Lepri, 1991). In rodents the accessory olfactory system, which includes the vomeronasal organ (VNO), is responsible for recognizing these environmental cues and initiating a neuronal response leading to mating behavior (Halpern, 1987; Stowers, Holy, Meister, Dulac, & Koentges, 2002). It is very possible that the destruction of the VNO-associated mucus glands seen in the majority of *Aire*-KO male mice **(Figure 3.5)** inhibits this behavioral pathway resulting in decreased mating frequency.

A second unexplored possibility is that the extensive inflammation within the region of the male reproductive tract containing the prostate, the ampullary gland and the vas deferens is somehow altering the non-sperm components of the male ejaculate. In internally fertilizing species, the various proteins of seminal plasma have been shown to alter several aspects of reproduction including the female immune response to both

male and female gametes, female receptivity and embryo implantation (Chen et al., 1988; Robertson, 2007; Rodriguez-Martinez, Kvist, Ernerudh, Sanz, & Calvete, 2011). Further, in many species the seminal proteins will coagulate and harden into a copulatory plug within the female cervix (Dixson & Anderson, 2002; Ramm, Parker, & Stockley, 2005). While the biology of plug formation is still being investigated, it has been shown that a prostate-specific transglutaminase is required for coagulation (Williams-Ashman, Notides, Pabalan, & Lorand, 1972). Importantly, evaluation of male fertility in our experiments relied on the detection of a copualtory plug. It is very possible that our male *Aire*-KO mice were in fact mating, but were unable to effectively fertilize a female due to autoimmune mediated alteration of the seminal components required for copulatory plug formation. In order to distinguish between a behavioral versus copulatory plug formation defect, it may prove necessary to record our animals when paired for breeding.

However, the severe autoimmune prostatitis seen in nearly all our male *Aire*-deficient mice **(Figure 3.4, 3.6)** might be resulting in enough pain and discomfort to dissuade any mating events from occurring. Indeed, chronic prostatitis/chronic pelvic pain syndrome, which affects approximately 2-10% of all adult males worldwide, is associated with male infertility (Krieger, Riley, Cheah, Liong, & Yuen, 2003; Rusz et al., 2012). Although poorly understood, one proposed mechanism is that inflammatory molecules present within prostatic fluid induce oxidative stress that impairs sperm motility and function (Diemer, Huwe, Ludwig, Hauck, & Weidner, 2003; Pasqualotto et al., 2000). Future efforts to evaluate the extent of pelvic pain as well as the cytokine and

leukocyte concentrations within prostatic fluid from *Aire*-KO mice could help advance our understanding of chronic autoimmune prostatitis on male fertility.

Beyond pain syndromes and behavioral disorders, our *Aire*-deficient male mice clearly develop a fertility lesion at the level of their sperm **(Figure 3.7A)**. Indeed, prostatitis and epididymitis have been shown to alter sperm morphology and function (Haidl, Allam, & Schuppe, 2008; Henkel et al., 2006). Further, we have detected anti-sperm antibodies in 73% (11 of 15) of male mice **(Figure 3.7B, C)**. As discussed above, antibodies reactive to sperm can prevent sperm-egg interaction and correlate with reduced IVF rates (Clarke et al., 1985; Eggert-Kruse et al., 1989). A vital next step in uncovering the cause of male infertility due to a deficiency in AIRE will be a detailed analysis of the mature sperm. This will include both descriptive measures (motility, morphology and intra-testicular sperm quantification) as well as functional evaluations (cervical mucosa penetration, acrosome reaction and capacitation) (Aitken, 2006).

An autoimmune component to male infertility occurs in approximately 12-18% of patients (Menge, Medley, Mangione, & Dietrich, 1982; Sinisi et al., 1993). Unfortunately our understanding of the mechanisms that impair male fertility have not maintained pace with the advances in treatment options. As mentioned previously in this section, assisted reproductive technologies have provided new hope for males coping with infertility. But this does not mean that we should forgo efforts to discern the underlying biology. In the specific case of male hypogonadal APS-1 patients, a discovery of effective treatment options will likely require an understanding of how the male gamete has been altered by the autoimmune events occurring within the reproductive tract. In a broad sense, male *Aire*-deficient mice provide a model system from which the

autoimmune involvement leading to chronic prostatitis, epididymitis, olfactory defects, the Steroli cell only syndrome and male infertility can be studied.

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APPENDIX

Bryce David Warren

Curriculum Vitae

PERSONAL DATA

First Name	Bryce
Last Name	Warren
Academic position	Graduate Student
Department	Anatomy and Cell Biology
Address	3010 Hemenway Life Science Innovation Center
Phone	(913) 588-9969
Cell	(503) 956-4755
Fax	(913) 588-7180
Email	bwarren@kumc.edu
Undergraduate GPA	3.67
Undergraduate Science GPA	3.58
Graduate GPA	3.92
MCAT Score	32 (Sept 2013)

PROFESSIONAL DEVELOPMENT

Undergraduate Education

Years (Inclusive)	Degree	Institution
2002-2006	B.S. in Biological Science	George Fox University

Graduate Education

Years (Inclusive)	Degree	Institution
2009-Present	Ph.D (Anatomy and Cell Biology) Mentor: Margaret G. Petroff	University of Kansas Medical Center

Honors and Awards

Year	Award
2006	George Fox University, *Duke Scholarship*
2006	George Fox University, *Magna Cum Laude*
2006	George Fox University, *National Dean's List*
2011	American Society for Reproductive Immunology, *Outstanding New Investigator Award*
2012	University of Kansas Medical Center, *Student Research Forum: 2nd basic science II*
2012	University of Kansas Medical Center, *Biomedical Research Training Program Fellowship*
2012	Society for the Study of Reproduction, *Larry Ewing Memorial Trainee Travel Fund*
2013	University of Kansas Medical Center, *Paul B. Freeburg Cell Biology Award*
2013	University of Kansas Medical Center, *Graduate Student School Of Medicine Award*
2013	University of Kansas Medical Center, *Biomedical Research Training Program Fellowship*

RESEARCH AND SCHOLARLY ACTIVITIES:

Scholarly Publications

1. Jasti S, **Warren BD**, McGinnis LK, Kinsey WH, Petroff BK, Petroff MG. 2012. The autoimmune regulator prevents premature reproductive senescence in female mice. *Biol. Reprod.* 86(4):110.

Published abstracts (denotes first author as recipient of award or selected as oral presentation):**

1. **Warren BD**, F. S. J. De Souza, V. Otero-Corchon, R. Kruse-Bend, M. Rubinstein, *M. J. Low. 2007. Reversible neuron-specific silencing of proopiomelanocortin (POMC) expression by targeting a loxP-flanked neo cassette to the distal upstream enhancer region of the Pomc gene. *Society for Neuroscience.*

2. A. L. Sharpe, **Warren BD,** V. Otero-Corchon, E. J. Kremer, F. S. J. De Souza, M. Rubinstein, M. J. Low. 2007. Neuroanatomical and functional mapping of hypothalamic proopiomelanocortin neurons in mice with a reversibly silenced Pomc gene allele by the retrograde axonal transport of a canine adenoviral vector expressing Cre recombinase. *Society for Neuroscience.*

3. **Warren BD****, Susmita Jasti, Brian K Petroff, and Margaret G Petroff. 2011. Auto immune regulator (AIRE) deficiency results in infertility involving embryonic loss in mice. *American Journal of Reproductive Immunology.*

4. **Warren BD**, Susmita Jasti, Brian K Petroff, and Margaret G Petroff. 2012. Autoimmune Regulator (AIRE) deficiency is associated with embryonic loss and generation of autoantibodies against the uterus, placenta and embryo. *Society for the Study of Reproduction.*

5. **Warren BD**, Leslie L Heckert, Timothy A Fields, Brian K Petroff, and Margaret G Petroff. 2013. The Autoimmune Regulator (AIRE) protects against infertility, reproductive tract inflammation and germ cell loss in male Balb/c mice. *American Journal of Reproductive Immunology.*

Presentations

1. **Warren BD**, Susmita Jasti, Brian K Petroff, and Margaret G Petroff. *Auto immune regulator (AIRE) deficiency results in infertility involving embryonic loss in mice*. Platform Presentation. 31st annual meeting of the American Journal of Reproductive Immunology, Salt Lake City, Utah; 05/2011.

2. **Warren BD**, Susmita Jasti, Brian K Petroff, and Margaret G Petroff. *Autoimmune Regulator (AIRE) deficiency is associated with embryonic loss and generation of autoantibodies against the uterus, placenta and embryo*. Platform Presentation. 45th annual meeting of the Society for the Study of Reproduction, State College, Pennsylvania; 08/2012.

EMPLOYMENT HISTORY

Years (Inclusive)	Title	Employer
2009-Present	Ph.D Candidate	University of Kansas Medical Center
Jan 2009 – Sept 2009	Research Associate	University of Kansas Medical Center
May 2006 – Dec 2009	Research Assistant	Oregon Health and Science University
Aug 2007 – Dec 2008	Varsity Women's Assistant Soccer Coach	George Fox University
April 2005 – May 2006	Server	Outback Steakhouse
Sept 2004 – April 2006	Biology Teaching Assistant	George Fox University
June 2002 – Aug 2004	Laboratory Technician	Pathology Associates Medical Laboratories (PAML)

VOLUNTEER EXPERIENCE

Years (Inclusive)	Position	Organization	Hours
March 2014	Student Research Forum: Presentation workshop invited speaker	University of Kansas Medical Center	1 hour
2011 – 2014	IGPBS recruitment ambassador	University of Kansas Medical Center	25 hours
July 2011 – Present	Usher Captain	Antioch Bible Baptist	56 hours
April 2011 – Oct 2011	Lawn Care	Antioch Bible Baptist	24 hours
Feb 2011 – Aug 2012	Youth Ministry	Antioch Bible Baptist	72 hours
April 2010	Student Research Forum Volunteer	University of Kansas Medical Center	4 hours
April 2009	Volunteer	American Lung Association	5 hours
April 2007 – Dec 2008	Volunteer Care Giver	Private Community Service	75 hours
Oct 2007 – April 2009	Big Brother	Big Brothers and Big Sisters of America	156 hours
Sept 2003 – April 2006	Biology Tutor	George Fox University	200 hours
Sept 2002 – Sept 2003	Community Service	George Fox University	16 hours
Aug 2001 – July 2002	Emergency Room Volunteer	Deaconess Medical Center	220 hours

PROFESSIONAL SOCIETIES AND AFFILIATIONS

Years (Inclusive)	Organization
2010 – Present	American Society for Reproductive Immunology
2011 – Present	Society for the Study of Reproduction

PHYSICIAN SHADOWING

Years (Inclusive)	Department/Organization	Physician	Hours
2013 – Present	Pathology and Laboratory Medicine; University of Kansas Medical Center	Dr. Maura F O'Neil	40 hours
2013 – Present	General Internal Medicine – Inpatient; University of Kansas Medical Center	Dr. David Naylor	10 hours

TEACHING

Date	Course	Instruction Type/Hours	Students
2012 – 2013	Prematriculation Program; KU-SOM	Lecture: Cells of the Immune system (2 x 4 hours)	20 student/year entering the University of Kansas School of Medicine
May 28 – May 31 2013	Frontiers in Reproduction; Marine Biological Laboratory, Woods Hole, MA	Teaching Assistant (20 hours)	15 students including graduate candidates, post-doctoral researchers and clinical fellows