Regulatory T Cells in Tolerance and Autoimmunity

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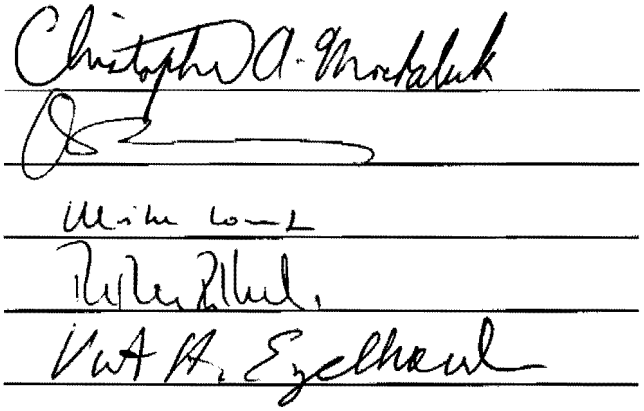

ABSTRACT

The purpose of this research was to understand the mechanisms by which regulatory T cells (Treg) prevent the induction of autoimmune disease. First, we wished to elucidate whether regulatory T cells were present in day 3 thymectomized (d3tx) mice, which develop strain- and organ-specific autoimmune disease. It was thought that a paucity of Treg secondary to their late ontogeny relative to effector T cells (Teff) was central to disease induction. Contrary to this paradigm, we found that functional Treg were present in d3tx mice as well as normal 3 day old mice. Importantly, they dampened the severity of autoimmune ovarian disease (AOD) and dacryoadenitis (DA) in the endogenous host. Treg present in d3tx mice were distributed such that Treg that suppress AOD were preferentially found in the ovarian draining lymph node (LN). This disease-specific accumulation could belie a mechanism by which Treg suppress disease in a normal individual, but could also reflect the active autoimmune disease in the host.

We next determined whether LN-specific accumulation was present in normal mice. We took advantage of the organ specificity of d3tx disease and ability of Treg to suppress disease if given before 7 days of age. Using Treg isolated from the ovarian-draining LN (OLN), prostate-draining LN (PLN), or lacrimal gland draining LN (cervical LN; CLN), we tested the efficiency of LN-specific Treg to suppress disease by titrating down the dose from 0.5×10^6 to 0.003×10^6. Indeed, Treg from the OLN, PLN, and CLN preferentially suppressed AOD, prostatitis, and DA, respectively. OLN Treg were enriched 15 times more than Treg from pooled LN, and PLN Treg 50 times more than CLN Treg. This disease-specific Treg accumulation was dependent on endogenous

antigen. Effector T cells (Teff) isolated from the PLN, CLN, or mesenteric LN all elicited a similar patter of disease in Rag-/- recipients, indicating that Treg were unique in their LN-specific accumulation.

To rationalize the significance of regional LN enrichment of antigen-specific Treg, we hypothesized that, in the face of a "danger" signal (infectious or sterile inflammation), antigen-specific Treg in the organ draining LN were poised to recognize self-antigens presented by antigen presenting cells (APC) and prevent a subsequent autoimmune response. To test this, we exploited vasectomy, a widely used method of contraception that induces inflammatory granulomas, as a source of organ-specific sterile inflammation.

When Treg were depleted (CD25 Ab) from unilaterally vasectomized (UniVx) mice, frequent and bilateral EAO developed. A reduction of Treg from all LN was accompanied by T cell activation in the regional LN and a robust testis antibody response. CD4+ T cells that produced IFNγ predominated in the diseased testes. CD4+ T cells are sufficient for disease induction as in vivo depletion of CD4+ cells blocked the emergence of EAO and CD4+ cells from UniVx mice were able to adoptively transfer disease. We also identified sperm Zonadhesin (Zan) as the major orchitogenic autoantigen. By immunoblot, autoantibodies from UniVx mice prominently recognized Zan, and immunization with Zan in adjuvant induced EAO. As EAO induction in Vx men would be devastating, we depleted Treg two weeks following UniVx and found they were free of EAO. To test whether UniVx in fact induces tolerance to testicular antigens, we compared UniVx and Sham-vasectomized mice in their response to EAO induction by immunization with testis homogenate in CFA. Indeed, UniVx mice developed

significantly lower EAO scores and autoantibody and T cell proliferative responses. Thus, Treg protect vasectomized mice from post-vasectomy EAO by blocking propagation of danger signals to pathogenic autoimmunity while granting heightened tolerance to testis antigens.

I believe the results of my research have uncovered new information that will contribute to the understanding of how Treg prevent autoimmune disease in the autoimmune and normal host. 1) Contrary to popular belief, functional Treg are present in d3tx mice with concurrent autoimmune disease, they temper ongoing disease, and accumulate in a regional LN- and organ-specific manner. 2) Regional LN specific accumulation of Treg can also be found in normal mice and is dependent on organ-antigens. 3) This distribution of Treg is unique as effector T cells do not have a similar distribution. 4) These organ-specific Treg function to prevent the propagation of autoimmune disease in the face of vasectomy, a model of organ-specific danger. And (5) vasectomy alone, which generates large epididymal granulomas, induces a state of "super-physiologic" tolerance to sperm and testicular antigens.

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LIST OF ABBREVIATIONS

Aire	Autoimmune regulator/autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
AOD	Autoimmune ovarian disease
AU	Arbitrary units
B6AF1	(C57BL/6 x A/J) F1
CFA	Complete Freud's adjuvant
CLN	Cervical lymph node
CTLA-4	Cytotoxic T lymphocyte antigen-4
DA	Dacryoadenitis
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DS-Treg	Disease-specific regulatory T cells
D3tx	Day 3 thymectomy
EAO	Experimental autoimmune orchitis
EAP	Experimental autoimmune prostatitis
Foxp3	Forkhead box P3
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
LN	Lymph node
NDLN	Non-draining lymph node
OLN	Ovarian-draining lymph node
PAMP	Pathogen-associated molecular pattern
PLN	Prostate-draining lymph node
rIgG	Rat IgG
TCR	T cell receptor
TDLN	Testis-draining lymph node
Teff	Effector T cells
TLR	Toll-like receptors
Treg	Regulatory T cells
UniVx	Unilateral vasectomy

OVERVIEW OF THESIS

The immune system must constantly deal with inflammatory insults, including pathogens, allergens, and cellular damage. As such, the immune system has evolved several mechanisms to recognize harmful stimuli and prevent reactivity to "self." These include a set of receptors specific for pathogenic microbes and the culling of self-reactive T and B cells. Autoimmune disease represents a breakdown in this tolerance to self and has been associated with environmental and genetic factors that elicit immune dysregulation. In my thesis research, I will try to discern a better understanding of how regulatory T cells function to control and prevent organ-specific autoimmune disease. This thesis is divided into 6 chapters.

Chapter I will provide an overview of the literature pertinent to my research, including regulatory T cells, the danger hypothesis, and vasectomy. The markers that define regulatory T cells, their mechanisms of suppression, and the uniqueness of their TCR repertoire will be reviewed. A synopsis of the "danger signal" hypothesis and the known sequelae of vasectomy will also be presented.

In chapter II, I will detail the experimental procedures used in my research.

Chapter III will describe finding of functional regulatory T cells in day 3 thymectomized (d3tx) mice with autoimmune disease. Disease in d3tx mice was thought to arise due to a paucity of regulatory T cells. Contrary to this paradigm, the Treg in d3tx mice function to dampen on-going autoimmune disease. This result may help to explain the difference in disease susceptibility among diverse strains of mice.

In Chapter IV, I document the existence of disease-specific regulatory T cells that preferentially suppress one autoimmune disease over another and accumulate in the

draining lymph nodes. We hypothesize that this lymph node-specific accumulation, which is not found in effector T cells, allows the regulatory T cells to anticipate and prevent activation of autoreactive T cells in the presence of organ-specific danger.

Chapter V will describe testicular autoimmune disease (orchitis) development in mice with organ-specific sterile inflammation (from vasectomy) and depleted of regulatory T cells, supporting the hypothesis put forth in chapter IV. Additionally, we have identified a novel orchitic autoantigen and describe the induction of tolerance when vasectomy alone is performed. This study represents the first comprehensive study on the short-term vasectomy sequelae in mice, and demands further studies on the dichotomous role that vasectomy may play in the development of disease and tolerance.

I will conclude with chapter VI, in which I present a summary of my thesis and discussion of the significance of the regulatory T cells in the prevention of autoimmune disease and the implications of tolerance induction in the presence of sterile inflammation.

Chapter I. Topic Review

I.A. Regulatory T cells

I.A.1. Introduction

Tolerance is a physiologic process by which we are protected from immune reactivity against self antigens. There are two major mechanisms of tolerance: central and peripheral. Central tolerance refers to the deletion of autoreactive T and B cells in the thymus and bone marrow, respectively. In the thymus, all CD4+ T cells must express T cell receptors (TCR) that are able to bind to self MHC class II molecules with low affinity in order to be positively selected, while TCR of high affinity for self antigens are negatively selected and die by neglect [1]. Because CD4+ T cells are positively selected against self MHC II and deletion is not perfect, it is no surprise that some autoreactive cells escape to the peripheral lymphoid tissues [2, 3]. Thus, mechanisms must exist in the periphery to prevent these autoreactive cells from initiating autoimmune disease. These mechanisms include deletion or anergy of Teff, which can be mediated by lymphoid epithelial cells[4] in the normal LN and Treg, which can dampen or prevent the activation of other immune effector cells.

The term "regulatory cell" refers to several cell types, including: myeloid derived suppressor cells [5], Tr1 cells [6], and a subset of CD4+ cells expressing constitutive CD25 (IL-2Rα) and the transcription factor *foxp3*. The CD4+CD25+Foxp3+ regulatory T cells (Treg) subset is the focus of this thesis. Treg were originally identified as the CD4+CD25+ population [7]. Since then, their aberrant presence and function has been

associated with tumor persistence and chronic survival of parasites and other microbes [8, 9]. This also explains the well known capacity of CD4+ cells to suppress autoimmune disease, including in d3tx mice[10].

Treg can arise from the thymus and are known as natural Treg or can be induced from naïve Teff in the periphery. Induced Treg (iTreg), often arise at sites of contact with the external environment (gut, skin, lung, etc.), where there is constant exposure to foreign antigens. Retinoic acid, a metabolite of Vitamin A, in the intestine[11], and Vitamin D3 in the skin[12] have been shown to generate immunomodulatory dendritic cells, which in turn preferentially induce Treg.

I.A.2. Foxp3

Foxp3 is a Treg-specific transcription factor. Located on the X chromosome, mutations in the forkhead box P3 (*foxp3*) gene were originally described responsible for the scurfy phenotype in mice[13] and the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans[14]. Scurfy mice die 3-4 weeks after birth with a lymphoproliferative disorder that includes enlarged lymph nodes (LN) and spleens and rampant lymphocyte infiltration in the lung, liver, skin, and other organs[15]. In similar fashion, humans with IPEX syndrome present with a range of disorders including diabetes, enteropathy, and eczema within a few days of birth[16]. Patients with IPEX rarely survive more than a year, and those that do are maintained on heavy doses of immunosuppressants[16]. Scurfy mice are rescued by Foxp3+Treg[17], and bone marrow transplantation as treatment for IPEX patients is under investigation[18].

Foxp3 is a family member of transcription factors that contain a winged helix-forkhead DNA-binding domain and also contains a zinc finger domain and leucine zipper motif[13]. Despite the plethora of DNA binding domains, it is believed that Foxp3 physically interacts with NFAT and NF-kB, blocking their ability to bind DNA, and thus functions as a transcriptional repressor for a variety of genes, including IL-2, IL-4, and TNF[19].

Foxp3 is highly expressed in the CD4+CD25+ subset of cells in mice[20] and Foxp3-/- bone marrow cannot give rise to CD4+CD25+ cells when mixed with wild type bone marrow and transferred to irradiated hosts[17]. Specific deletion of Foxp3+ positive cells in healthy animals also recapitulates the lymphoproliferative syndrome seen in scurfy mice[21, 22]. Moreover, when Foxp3 is retrovirally transduced into CD4+CD25- cells, they gain suppressive capacity and the expression of CD25, CTLA-4, and other Treg markers[17, 20]. Thus, Foxp3 appears to be necessary for the induction of Treg-specific functional molecules that equip Treg with their capacity to suppress other cells.

I.A.3. TCR Repertoire

There is significant controversy as to the repertoire of TCR that Treg express, with data suggesting that Treg have a unique, highly self-reactive TCR repertoire, and, conversely, that the TCR repertoire of Treg overlaps significantly with those of Teff. Favoring the first scenario, TCR derived from Treg and retrovirally transduced into Teff cells show increased avidity for self antigen/MHCII[23]. Additionally, a more diverse and non-overlapping TCR repertoire was found in Treg compared to Tcon after the detailed analysis of individual TCR-VDJ segments from mice containing a restricted TCR-VDJ

repertoire[24]. This same group, however, later showed a significant overlap in the TCR repertoire between Treg and Teff[25]; while Wong et.al. reported that Treg contained both unique TCR and those that overlapped with Teff[26]. All of these studies may be confounded by the use of pooled LN or spleen as a source of Treg. Lathrop et. al. showed, by individual TCR-VDJ analysis in mice with a fixed Vβ chain, that Treg from individual LN have a unique set of TCR when compared to Treg from a different LN or to Teff, while naïve Teff do not show such LN-specificity[27].

I.A.4. IL-2 and Treg

Treg constitutively express the surface marker CD25, the high affinity subunit of the IL-2 receptor, suggesting a role for IL-2 in the development or survival of Treg. Indeed, mice deficient for IL-2, IL-2Ra, and IL-2Rb die between 4 – 12 weeks of age with a lymphoproliferative disorder [28-30] that is less severe, but resembles, the scurfy mouse. Early studies in these mice showed decreased percentages of Treg in the thymus and periphery [31]and the lymphoproliferative disorder seen in IL-2RB-/- could be rescued when IL-2R was expressed only in thymic T cells[32]. These studies were hindered by the fact that CD25 was used to define Treg. Later, however, studies using the Foxp3-gfp reporter mouse showed that Treg from IL-2-/- and IL-2Ra-/- mice are reduced in percentage in the thymus and LN, have lower levels of Foxp3 and CD25 expression in the thymus, and have decreased fitness in the periphery when compared to wild type Treg [33]. These studies imply that IL-2 plays a major role in Treg development in the thymus and survival in the periphery. However, IL-2 is not necessary for Treg to suppress other

cells as Treg from IL2-/- and IL2Ra-/- mice are able to suppress Teff proliferation in vitro similarly to wild type Treg [33].

I.A.5. Mechanism of suppression

I.A.5.a. Cell-contact dependent mechanisms.

Treg are known to inhibit the effector function of a variety of immune cells, including: T cells, B cells, NK cells, DC, etc[34]. Originally Treg were shown to suppress the in vitro proliferation of Teff in a cell contact-dependent manner as separation of Treg from Teff by a semi-permeable membrane abrogates Treg-mediated suppression[35]. Indeed, Treg can increase intracellular cAMP levels in effector cells through gap junctions made between Treg and Teff[36] and can increase pericellular concentrations of adenosine through conversion of ATP by the hydrolase CD39 and nucleotidase CD73[37]. Moreover, Treg can indirectly inhibit Teff activation by killing APC through granzyme B- and perforin- dependent mechanisms[38]. Additionally, several reports have shown that, in vivo, Treg are able to interact with APC for extended periods of time, effectively competing-out effector T cells, and blocking their ability to recognize antigen and become activated[39, 40].

I.A.5.b. CTLA-4

Cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) is a homologue of the positive costimulatory molecule CD28 and binds to CD80 and CD86[41]. Mice deficient for CTLA-4 develop an extensive lymphoproliferative disorder reminiscent of scurfy

mice[42], suggesting a role for CTLA-4 in maintaining peripheral tolerance. CTLA-4 is constitutively expressed by Treg and is upregulated on activated Teff[41]. CTLA-4 may compete with CD28 for CD80/86 binding[43], and signaling through CTLA-4 downregulates IL-2 production and induces cell cycle arrest in CD4+ T cells[44]. Thus, disease in CTLA-4 deficient mice may be due to loss of CTLA-4 on the Teff, resulting in decreased inhibitory signals, or Treg, abrogating their ability to suppress APC and T cell activation. Recently, however, Wing et.al showed that CTLA-4 deficiency specific to Treg recapitulated the disease seen in the global knockout[45]. Moreover, these Treg are unable to suppress T cell proliferation in vitro or in vivo or inhibit the upregulation of CD80/86 on activated APC[45].

I.A.5.c. Cytokine dependent mechanisms

Although initial in vitro analyses indicated Treg suppression was mediated only through cell-contact, work in mouse models of immune regulation of mucosal sites, including the intestine and the lung indicated critical roles for the cytokines IL-10 and TGF-B. In both diseases, Treg are unable to suppress disease when they lack the ability to produce IL-10 or TGF-B[46-48]. Furthermore, Treg-specific IL-10 deficient mice develop pathology primarily in the lung, colon, and skin[49].

Thus, Treg can suppress activation of a variety of immune cells through multiple, non-exclusive mechanisms, which may also depend on the organ and disease model being studied.

I.B. Day 3 Thymectomy

I.B.1. Disease model

Thymectomy on day 3 of life (d3tx) results in spontaneous, organ-specific autoimmune disease[50]. Disease induction is strictly dependent on the time of thymectomy, which must occur between the second and fifth day of life[51]. D3tx autoimmunity is characterized by T cell infiltration into affected organs, autoantibody production, and is mediated by CD4+ T cells, which can transfer disease to neonatal recipients[51, 52]. Disease can be prevented by early infusion of normal adult CD4+ cells[10].

D3tx-induced autoimmune disease can affect the ovary, stomach, thyroid, prostate, lacrimal gland and testis and the repertoire of diseases is highly strain dependent. Oophoritis (autoimmune ovarian disease; AOD), prostatitis (experimental autoimmune prostatitis; EAP), and dacryoadenitis (DA) dominate in (C57BL/6xA/J)F1 (B6AF1) female mice, whereas C57BL/6 mice develop only mild EAP[53]. Studies on F1 mice and further backcrossing showed that multiple genetic loci are involved in disease susceptibility[53, 54].

I.B.2. Mechanism of d3tx-induced disease

An intriguing feature of autoimmune disease induction is the requirement for thymectomy on neonatal days 1 to 4[51]. Furthermore, CD4+ splenic cells from neonatal but not adult mice are able to induce disease in athymic mice[55], supporting the idea that neonatal CD4+ cells are more pathogenic than adults. We proposed two mutually nonexclusive explanations for this increased pathogenicity: 1) d3tx enriches for

autoreactive, pathogenic CD4+ T cells that escape deletion in the neonatal thymus, and 2) d3tx deprives mice of Treg of late ontogeny [56].

I.B.2.a. Enrichment of autoreactive T cells

The first hypothesis was supported by the finding of T cell responses to endogenous Mouse Mammary Tumor Virus (MMTV) superantigen [57]. Thymic expression of endogenous MMTV superantigen in B6AF1 mice appeared late in ontogeny, and the deletion of cognate Vβ11+CD4+CD8- thymic or peripheral T cells was not observed until neonatal day 10. In the LN of adult d3tx B6AF1 mice, there was a 10-fold increase in Vβ11+CD4+ T cells. Additionally, sequencing of neonatal thymic CD4+CD8+ cells showed a significant differences from adult mice, stemming from a decrease in N region diversity[58], which was later shown to mediate more efficient positive selection[59, 60]. These findings support the paradigm that autoreactive T cells that escape thymic deletion in the neonatal period expand following d3tx and might elicit autoimmune disease. The validity of this mechanism remains unresolved.

I.B.2.b. Depletion of Treg of late ontogeny

The Treg-deficiency hypothesis was supported by the findings that autoimmune disease in d3tx mice is suppressed by CD4+CD25+ Treg, and that these Treg are undetectable in neonatal thymuses or spleens until after day 5 [7, 61]. Later studies using the Foxp3gfp reporter mouse corroborated these findings and suggested that Treg's late ontogeny is due to decreased thymic medulla organization[62]. This paradigm, however, is not supported by our studies below.

I.C. The danger hypothesis

Janeway originally hypothesized that the immune system was able to survey "self" versus "non-self" through recognition of pathogens through receptors specific for molecules commonly displayed by pathogens, but not by one's own body[63]. This hypothesis proved correct with the discovery of the toll-like receptors (TLR), which are able to recognize a host of unique pathogen-associated molecular patters (PAMPs), which include microbial carbohydrates, proteins, and nucleic acids[64]. Ligation of TLRs has been shown to induce activation of a variety of cells, including macrophages, dendritic cells, and T cells[65]. Activation of innate cells through TLR induces the direct clearance of pathogens[66] as well as the presentation of microbial antigens to the adaptive immune system[67].

Finding the self vs non-self model to be inadequate to explain how individuals can be exposed to "non-self" and not respond (as during puberty, being vaccinated without adjuvants, or pregnancy), or conversely why some people respond to "self" (as in autoimmunity), Matzinger proposed the "danger" hypothesis in 1994[68]. This hypothesis suggests that the immune system may respond to the damage of endogenous cells rather than to the invading microorganisms. Thus, factors released by apoptotic and/or necrotic cells may elicit activation of innate cells and, in turn, adaptive cells. This hypothesis was supported by studies showing that cytosolic components of necrotic cells were able activate DC and induce potent T cell responses[69, 70] . Independent studies by A. Marshak-Rothstein have shown that endogenous DNA and RNA are able to activate B cells to produce autoantibodies[71]. To date, a variety of cellular components are known to activate

immune cells, including heat shock proteins, uric acid, HMGB1, and double-stranded DNA[72].

I.D. Vasectomy

I.D.1. Sequelae of human vasectomy

Vasectomy is one of the most common contraceptive procedures performed world wide; and over 500,000 are performed per year in the U.S. alone. It is largely considered to be a safe and benign procedure, save the risk of hematoma. There are, however, reports linking vasectomy to an increased risk of cardiovascular disease[73], prostate cancer[74, 75], and testicular cancer[76, 77]. Shortly after these studies were released, better controlled studies were released that showed no correlation between vasectomy and these negative outcomes and argued that there was no mechanistic link between them[78]. As such, the prevailing view is that vasectomy has no long-term negative sequelae.

Vasectomy is not completely without consequence, however. Approximately 50% of men develop anti-sperm antibodies within a year following vasectomy[79]. These antibodies are able to immobilize sperm in vitro[80] and are suggested to play a role in fertility failure following vasovasostomy. However, the accuracy of sperm antibody is questionable due to the use of ejaculated sperm as a source of antigen, which can differ depending on donor, and are contaminated with prostatic secretions. Low serum titers (1:10-1:20) are also often used.

In addition to autoantibodies, 15-30% of vasectomized men are reported to develop vasal or epididymal granulomas based on biopsies at the time of

vasovasostomy[81]. Despite the presence of epididymal inflammation, few studies have examined histologic changes in the testis, and only an increase in the testis capsule thickness has been reported[82]. Systematic studies on the presence of epididymal granuloma and changes in human testes have not been performed, and would add much to the understanding of vasectomy sequalae.

I.D.2. Immunologic sequelae in other species

Similar to humans, rabbits, guinea pigs, and mice are known to develop autoantibodies after vasectomy[83, 84], although the incidence (up to 50%) depends on outbred status and strain, if inbred[85, 86]. And, despite scant evidence in man of testicular and epididymal changes following vasectomy, several studies in guinea pigs and rabbits have revealed an epididymal granuloma incidence of 70-80% and orchitis incidence of up to 100% several months post vasectomy[87]. Interestingly, one study in (C57BL/6 x DBA/2)F1 mice are showed the development of spontaneous aspermatogenesis (but not orchitis), autoantibody production, and increased hepatic tumor burden a year after vasectomy[88].

In addition, long term studies of rhesus monkeys reported a 60% incidence of serum anti-sperm antibodies and orchitis, and a 30% incidence of epididymal granuloma[89]. Thus, it is clear from studies in rodents and non-human primates that there is potential for an extensive autoimmune response to vasectomy, including autoantibodies, epididymal granulomas, and orchitis.

Chapter II. Methods and Materials

II.A. Mice and surgery:

C57BL/6N and A/J adults were obtained from NCI and B6AF1 mice produced from mating these mice. Rag1-/- mice were obtained from Jackson and bred in house. *Foxp3-gfp* mice on a mixed (C57BL/6 x 129) background were a kind gift of Dr. A. Rudensky. To produce A/J nu/nu mice, $Fox1^{nu}$ was transferred from B6.Cg-$Foxn1^{nu}$/J heterozygotes to the A/J background through 10 generations of backcrossing and selection. $Fox1^{nu}$ heterozygotes were identified using single strand conformation polymorphism of the denatured sequence amplified by the 5' CAGACCCAGAGCAGATCCAG 3' and 5' AGGAGTGTCCATCAAGTGCC 3' primers. The Zonadhesin (Zan) knockout mouse was produced as previously described[90]. Briefly, the proximal promoter and first six exons of the Zan gene were replaced with a *Neo* cassette using the PGKneolox2DTA vector. The mice, originally in 129S1/Sv background, were backcrossed to C57BL/6.

Thymectomy and neonatal orchiectomy were performed under hypothermia[91]. Mice with incomplete thymectomy were excluded. 5α-dihydrotestosterone pellets (20mg/60 day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously. Vasectomy and adult orchiectomy were performed under general anesthesia and sterile conditions. Vasectomy was performed through an abdominal incision. The vas deferen was doubly ligated with 6.0 silk sutures and bissected in between. Abdominal wounds were closed in two layers. Sham vasectomy was a laporatomy without vasal ligation. For orchiectomy, the vasectomy incision was re-

opened, the testicular artery ligated by 6.0 silk sutures, and the testis and granulomatous epididymis were excised. Mice were kept in a pathogen-free facility; and experiments were performed under the guidelines of the Animal Care and Use Committee of University of Virginia, University of Vermont, and Texas Tech University.

II.B. Histology and Disease Scoring:

Tissues fixed in Bouin's were stained with hematoxylin and eosin and evaluated as blinded samples. AOD was graded as previously described[92], with additional scoring of the degree of ovarian atrophy (loss of primordial oocytes and loss of growing/mature oocytes), graded from 1-4. Total AOD scores were: ovarian inflammation + (1.5 x growing/mature follicle atrophy) + (2 x primordial follicle atrophy). EAP: 1 (focal monocytic inflammation), 4 (severe EAP with diffuse inflammation and loss of glands), 2 and 3 (increasing severity between 1 and 4). Inflammation in the following organs was similarly graded as EAP: lacrimal gland, lung, liver, colon, skin, kidney, stomach, thyroid, eye, small intestine. For EAO, inflammation was graded as: 1 (focal), 4 (severe and diffuse inflammation), 2 and 3 (intermediate and increasing monocytic inflammation). The seminiferous tubules, straight tubules, rete, ductus efferentes, vas deferens, and the caput, body, and cauda of the epididymis were graded separately for inflammation. Extent of loss of testis function was graded by the extent of testicular aspermatogenesis (1-10) and the sperm contents in epididymal ducts (full, partial, empty). The final orchitis score combines the inflammation score and the aspermatogenesis score. Epididymal granulomas were graded by adding the inflammatory score (1-4) for each region of the epididymis, including the caput, corpus and cauda, and the scorefor the vas

deferens. For the clinical EAE score, mice were scored daily starting at day 5 after injection as previously described[93].

II.C. Immunofluorescence:

Indirect immunofluorescence using ethanol-fixed frozen sections of normal tissue was used to detect serum ovarian, prostate, testicular, and epididymal antibodies at 1:50 serum dilution, followed by FITC-labeled anti mouse IgG (Jackson ImmunoResearch). The infiltrating leukocytes in diseased tissue were identified on ethanol-fixed frozen sections, using the TSA Biotin System (PerkinElmer), and antibodies to the following antigens: CD11c (HL3; BD Pharmingen), CD11b (M1/70; BD Pharmingen), F4/80 (C1:A3-1; Cedarlane), CD4 (L3T4; BD Pharmingen), kappa chain (polyclonal; Rockland), Foxp3 (FJK-16s; eBioscience) and C3 (polyclonal; MPBiomedical). Foxp3 was detected on sections permeabilized with 0.3% Triton X-100 (Sigma). CD4+ and Foxp3+ cells were counted on serial frozen sections of 3-4 week old d3tx ovaries, and the percentage of Foxp3+ CD4 cells computed.

II.D. Flow cytometry:

Cells infiltrating testes and epididymides were isolated by decapsulating testes or cutting epididymides into small pieces and digesting with collagenase type II (100U/mL; Worthington), collagenase type I (100U/mL; Worthington) and DNase (Sigma; 10ug/mL) for 15 or 45 minutes, respectively, at 34^{o}C. Enzymes were inactived with 0.1M EDTA and cells filtered. Cells from lymph nodes, testes, or epididymides were stained with the following antibodies for flow cytometry: CD45 (30-F11), CD4 (RM4-5; RM4-4), CD8

(53-6.7), CD25 (7D4; PC61), Foxp3 (FJK-16s), IFNg (XMG1.2), IL-17 (TC11-18H10), CD11b (M1/70), CD11c (HL3), and live/dead fixable cell stain (Invitrogen). Intracellular cytokine staining was performed on CD4+ T cell isolated via magnetic beads (negative selection; Miltenyi Biotec), cultured for 4 hours in complete medium with PMA/Ionomycin and Brefeldin A (Leukocyte Activation Cocktail with GolgiPlug; BD Pharmingen), and fixed and permeabilized for FACS staining (BD Bioscience).

II.E. Antibody inhibition:

Anti-CD4 (Gk1.1) and anti-CD25 (PC61) were produced by hybridoma with the bag method, isolated on protein G column, diluted in PBS and injected i.p. at designated times. D3tx mice received i.p. injections of 50ug, 100ug, 100ug, and 200ug anti-CD25 mAb (PC61) or rat IgG (ThermoScientific) control Ab on days 5, 7, 14, and 21, respectively. Adult mice received i.p injections of 250ug anti-CD25 mAb (PC61) at days -3, +3 and +7 around vasectomy (day 0).

II.F. Cell Transfer and Adoptive Transfer of EAO:

Ovary-draining (renal), lacrimal-gland draining (cervical), and prostate-draining (lumbar/sacral) LN were identified as regional LN of ovary, lacrimal gland, and prostate, respectively. CD4+CD25+ Treg or CD4+ CD25- Teff, were isolated to ~95% purity via magnetic beads (autoMACS; Miltenyi Biotec). Cells were transferred i.p. to 5 day-old d3tx mice, which were studied 8 weeks later or i.v. into syngeneic adult RAG knockout recipients that were studied 3-4 weeks later. For EAO, cells were isolated from the testis-draining LN (renal), non-draining LN (axillary and brachial), or spleen. CD4+ cells were

isolated via negative selection using magnetic beads (autoMACS; Miltenyi Biotec) and cultured with irradiated splenocytes, IL-2 (4000U/mL; BD Pharmingen) and anti-CD3 (1:4000; Cedarlane). Cells were harvested on day 3 and dead cells were eliminated through centrifugation on a Histopaque (Sigma) gradient. Recipients were irradiated with two doses of 650 rad (xray and type of irradiator?) one week apart, with the testes shielded. Cells were injected i.p. one day after the second irradiation, and testis pathology determined 5 weeks later.

II.G. ELISA for autoantibodies:

Normal testicular cell and epididymal sperm suspensions were obtained from normal adult testes and epididymides, and washed as described previously[94]. 10^5 cells were seeded into each well of a 96-well plate (Costar), spun down (500xg), fixed with 1%PFA for 1hr at room temperature. Serum, diluted 1:100, was added to each well, incubated for 1hr, and bound IgG was detected by goat-anti-mouse IgG conjugated to HRP (Southern Biotech; 1030-05), followed by o-Phenylenediamine dihydrochloride (Sigma; P8287), and stop buffer (2.5N H_2SO_4), and the O.D. at 490nm was determined in a photometer. Pooled sera from mice immunized with testis homogenate in CFA served as standards and were assigned a fixed number of arbitrary antibody units. Unknown serum samples were developed with the standard, and results expressed relative the standard as arbitrary antibody units.

II.H. Immunization:

Mice were immunized with testicular homogenate (TH), myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}), as previously described[95]. Briefly, each mouse was injected subcutaneously once with 10 mg TH or 100ug MOG_{35-55}, emulsified in complete Freund's adjuvant (CFA; Sigma). This was followed by one i.p. injection of a Bordetella pertussis extract. EAO was studied 3-4 weeks post immunization.

II.I. T cell proliferation assay:

T cells were isolated via nylon wool adherence exclusion from lymph nodes and spleens as previously described[96] or CD4+CD25+ and CD4+CD25- cells isolated via magnetic beads (autoMACS; Miltenyi Biotec). Cells were cultured in complete RPMI medium containing 10% fetal calf serum antibiotics and 0.01% B-mercaptoethanol, in 96-well plates at 37^{o}C and 5% CO_2 in air. T cells were stimulated with irradiated splenocytes and anti-CD3 (testicular cells as antigen in different concentrations. After 4 days, 0.05mCi of H^3-thymidine was added per well and H^3-thymidine uptake determined on a beta counter (Beckman).

II.J. Recombinant Zan D3p18 and polyclonal antibody to ZanD3p18:

The predicted antigenic regions of von Willebrand D3p18 (Cys4502-Lys4621) domains (Accession AKA28052), encoded by nts 6680-6989 and 13669-14050 respectively of the mouse zonadhesin transcript (Accession U97068) were expressed in *E. coli* strain BL21 as a recombinant glutathione S-transferase (GST) fusion proteins using pGEX-4T-1 (Amersham Pharmacia Biotech, Piscataway, NJ). Rabbit antibody to

mouse Zan D3p18-GST, and control rabbit antibody to GST, were produced as described by Tardif et. al[90]. Briefly, GST-D3p18 fusion proteins (1 mg/injection) were used to immunize female rabbits (New Zealand rabbits) in CFA. Antibodies were affinity purified from rabbit antisera as for preparation of antibodies to pig zonadhesin[97].

II.K. SDS-Polyacrylamide gel electrophoresis and Western blot:

Sperm proteins were extracted in Laemmli buffer; disulfides not reduced were separated by SDS-PAGE ($1x10^6$ sperm cells/lane; 4-8% or 8-15% linear acrylamide gradients), transferred to nitrocellulose, and antigens detected with antibody to zonadhesin or with autoantisera (1:10,000 dillution in TBST). Bound autoantibody was detected with HRP-conjugated goat antibody to mouse IgG.

Chapter III. In d3tx mice, functional, disease-specific Treg enrich in the corresponding organ draining LN and suppress endogenous autoimmune disease.

III.A. Introduction

Although Treg were undetectable in the spleens of 3 day old mice[7], studies have found Treg in 3 day-old LN [98] with the ability to suppress CD25- T cell proliferation *in vitro* [61]. In 2004, Dujardin et. al. [99] reported that the percentage of CD25+CD4+ T cells in the spleens of d3tx BALB/c mice was increased over untreated mice (>20% vs. <10%). The CD25+ T cell population expressed Foxp3 mRNA, suppressed *in vitro* polyclonal anti-CD3 stimulated CD25- T cell responses, and prevented colitis in *scid* recipients of CD25- Teff cells. Thus, there is a fractional increase in functional Treg in the CD4+ T cell compartment of d3tx mice [99]. However, Dujardin et. al. did not directly examine the capacity of Treg to suppress autoimmune diseases known to occur in d3tx mice (AOD and gastritis), thereby leaving open the question of whether Treg deficiency is the mechanism underlying d3Tx-induced autoimmune disease [7]. Also, colitis is not relevant to d3tx as it does not occur in d3tx mice [53]. Because colitis does not affect germ-free animals, even its autoimmune nature is in question [100].

Therefore, we attempt to obtain more direct evidence for the basis of Treg deficiency as causal to d3tx-induced autoimmune disease. Toward this end, we have addressed: 1) the status of Treg in d3tx mice; 2) the LN distribution of functional, organ-specific Treg; 3) the capacity of endogenous Treg to suppress autoimmune diseases induced by d3tx; and 4) the ability of Treg from normal 3 day-old neonatal mice to suppress the same autoimmune diseases.

III.B. Results

III.B.1. Foxp3+CD4+ T cells are increased in the LN and target organ of d3tx mice.

D3tx mice developed persistent T cell lymphopenia after the first week of life (Figure 1A). Simultaneously, the Treg fraction of CD4+ T cells rapidly expanded from 1 to 3 weeks to reach a plateau above that of control B6AF1 mice (Figure 1B). Twenty-five percent of CD4+ T cells in d3tx B6AF1 mice were Foxp3+ as compared to 5-10% in controls (Figure 1C). This expansion is likely due to the propensity of Treg to proliferate in lymphopenic environments [101]. Treg from the LN of d3tx mice suppressed the proliferation of CD25- T cells at a comparable dose response as Treg from untreated donors (Figure 2).

The enrichment of Treg in d3Tx mice was not confined to the LN, as they were also found in the target organ with early inflammation. At the onset of AOD (3-4 weeks) [91], an average of 13% (n=11) of the ovarian CD4+ T cells were Foxp3+, with some exceeding 35% (Figure 1D).

Our findings confirm the observations of Dujardin et. al. [99]: in d3tx mice, functional Treg, now identified as Foxp3+CD4+ T cells, are persistently increased relative to the CD25- effector T cells. To extend the study, we investigate the in vivo function of Treg in d3tx mice, in particular, their capacity to influence autoimmune diseases that develop in d3tx mice [92].

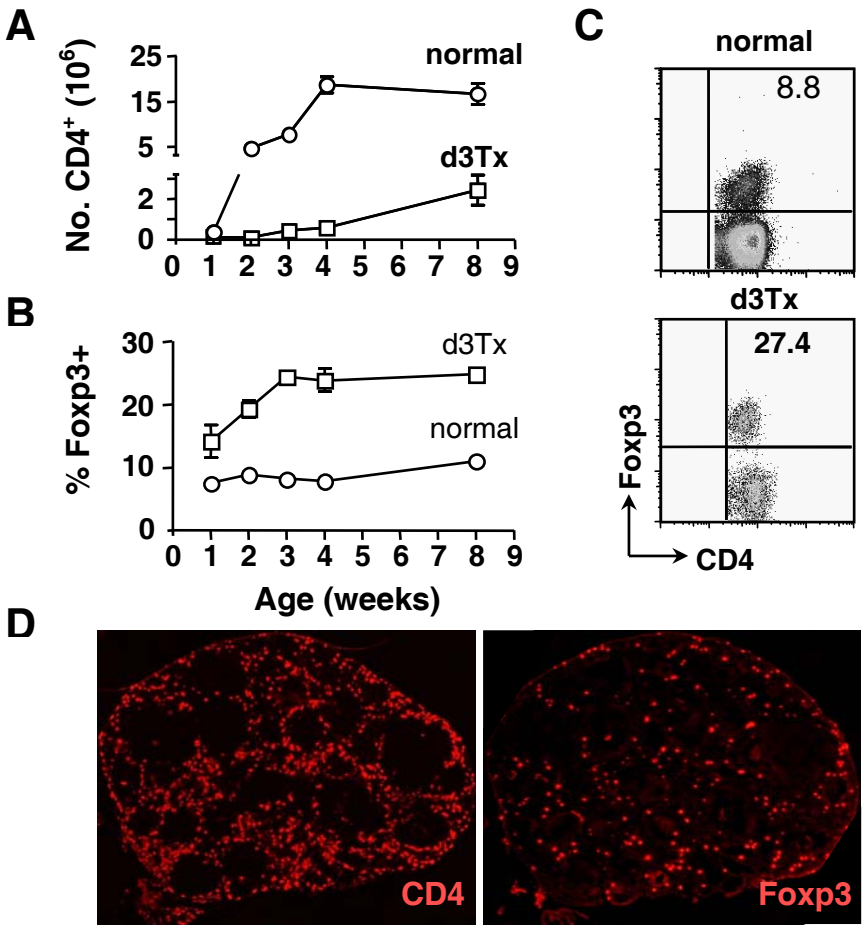

Figure 1: The Foxp3+ Treg among CD4 T cells are increased in the LN and diseased organ of d3tx mice. (A): Persistent CD4+ T lymphopenia in d3tx mice. (B and C): Persistent increase in %Foxp3+CD4+ Treg in LN. (D): CD4+ and Foxp3+ cells in ovaries of 3-4 week old d3tx mice.

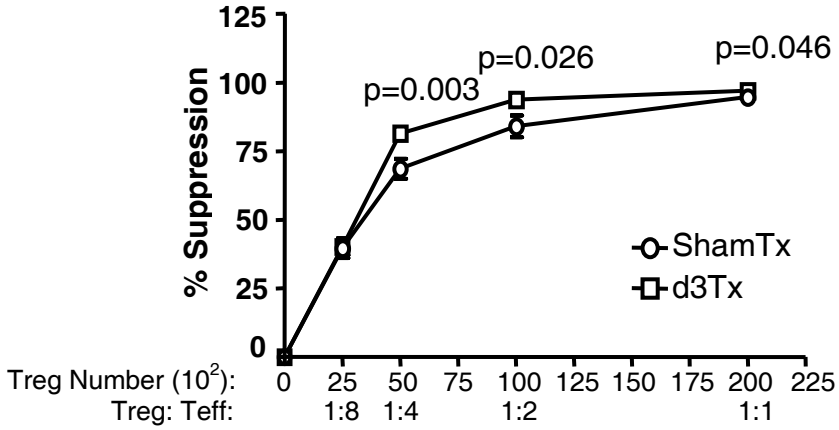

Figure 2: Treg from d3Tx mice have greater suppressive function in vitro compared to Treg from normal mice. 2.0 x 10^4 CD4+CD25- effector T cells (Teff) were cultured with anti-CD3 and 2.0 x 10^5 irradiated splenocytes with or without different ratios of CD4+CD25+ Treg from d3Tx or Sham-thymectomized (Sham Tx) mice. Results are a combination of 4 individual experiments. P-values calculated by Student's *t* test.

B.2. Disease-specific Treg suppress d3tx-induced autoimmune disease and accumulate in the regional LN of d3tx mice.

Antigen-specific polyclonal Treg cannot be identified due to low clonal frequency. We can, however, detect disease-specific Treg populations that preferentially suppress one autoimmune disease over another in the same d3tx host. This approach was used to investigate d3tx mice under suppression by normal Thy1.1+ Treg[92]. At the suboptimal dose of $0.1x10^6$ cells, Thy1.1+ donor Treg retrieved from ovarian LN (renal LN) suppressed AOD completely, but not DA. Conversely, the Treg obtained from the lacrimal gland LN (cervical LN) suppressed DA but not AOD. Because effector T cell suppression also occurred exclusively in the draining LN, the disease-specific Treg from normal donors must exert their effect in the regional LN of recipient d3tx mice during disease suppression[92].

CD25+ Treg were obtained from the ovarian LN, or were pooled from cervical, inguinal and axillary LN, of which about 50% of the Treg came from the lacrimal gland LN. Notably, AOD was completely suppressed by $0.1x10^6$ Treg from the ovarian LN but not by Treg from the non-ovarian LN (Figure 3A). Conversely, $0.1x10^6$ lacrimal gland LN-enriched Treg did not suppress AOD but reduced DA severity (Figure 3B). Therefore, the Treg obtained from d3tx mice at the peak of disease can adoptively suppress AOD and DA. Moreover, the endogenous disease-specific Treg of d3tx mice are enriched in the draining LN of relevant target organs, recapitulating the distribution of donor Treg in d3tx recipients under disease suppression [92].

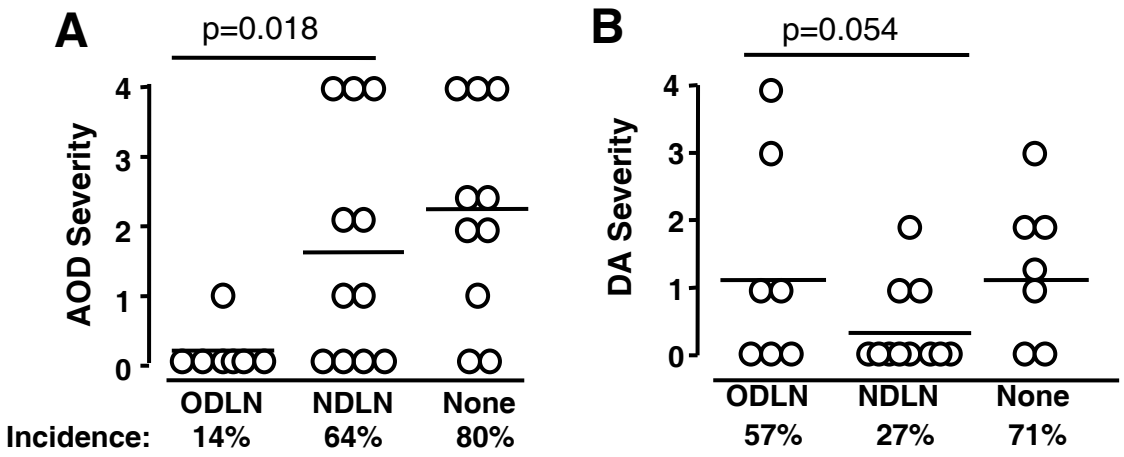

Figure 3: Functional and disease-specific Treg of d3tx mice are enriched in the regional LN of the target organ. (A): Complete AOD suppression by Treg from ovarian draining LN (ODLN), but not by Treg enriched from lacrimal gland draining LN (NDLN). (B): DA is more effectively suppressed by Treg from NDLN than Treg from ODLN. CD4+CD25+ Treg ($0.1x10^6$) from ODLN or NDLN, pooled from 6-8 wk-old d3tx mice, were transferred to 5 day-old d3tx recipients, and pathology evaluated 8 wks later. *p*-values: Student's *t* test.

III.B.3. Depletion of endogenous Treg from d3tx mice significantly enhances the incidence and severity of subsequent autoimmune disease.

In the preceding study we showed that the Treg in d3tx mice can traffic and respond to self antigens in appropriate locations, and when transferred to other d3tx mice, adoptively prevent autoimmune disease. While the experiment has defined the LN distribution of disease-specific Treg in d3tx mice and documented their ability to prevent autoimmune disease in new neonatal d3tx recipients, it does not elucidate whether the Treg can suppress disease in the autologous d3tx host. We therefore undertook a more direct approach to examine the endogenous Treg in the d3tx host itself.

Treg from d3tx mice were depleted during the first 3 weeks of life using anti-CD25 antibody and disease outcome was evaluated at 6 weeks. The timing of antibody treatment coincided with the duration of pathogenic T cell priming by endogenous ovarian antigens [91]. D3tx mice treated with anti-CD25 antibody had a dramatic increase in mean AOD severity ($p=0.011$) (Figure 4 A, C, and D). Treg depletion also increased the prevalence of DA from 58% to 100% ($p=0.007$), and enhanced the mean DA severity ($p<0.0001$) (Figure 4 B, E, and F). Therefore, the endogenous Treg in d3tx mice are actively restraining the full pathogenic autoimmune response and d3tx mice develop an attenuated pathology.

III.B.4. Three day-old neonatal mice possess functional CD4+CD25+Foxp3+ Treg capable of suppressing autoimmune disease in d3tx mice.

Pivotal support for the Treg-depletion hypothesis of d3tx disease was provided by the reported late ontogeny of Treg development [7, 61] but the supporting data is controversial [61, 99]. More importantly, there has not been an attempt to investigate the in

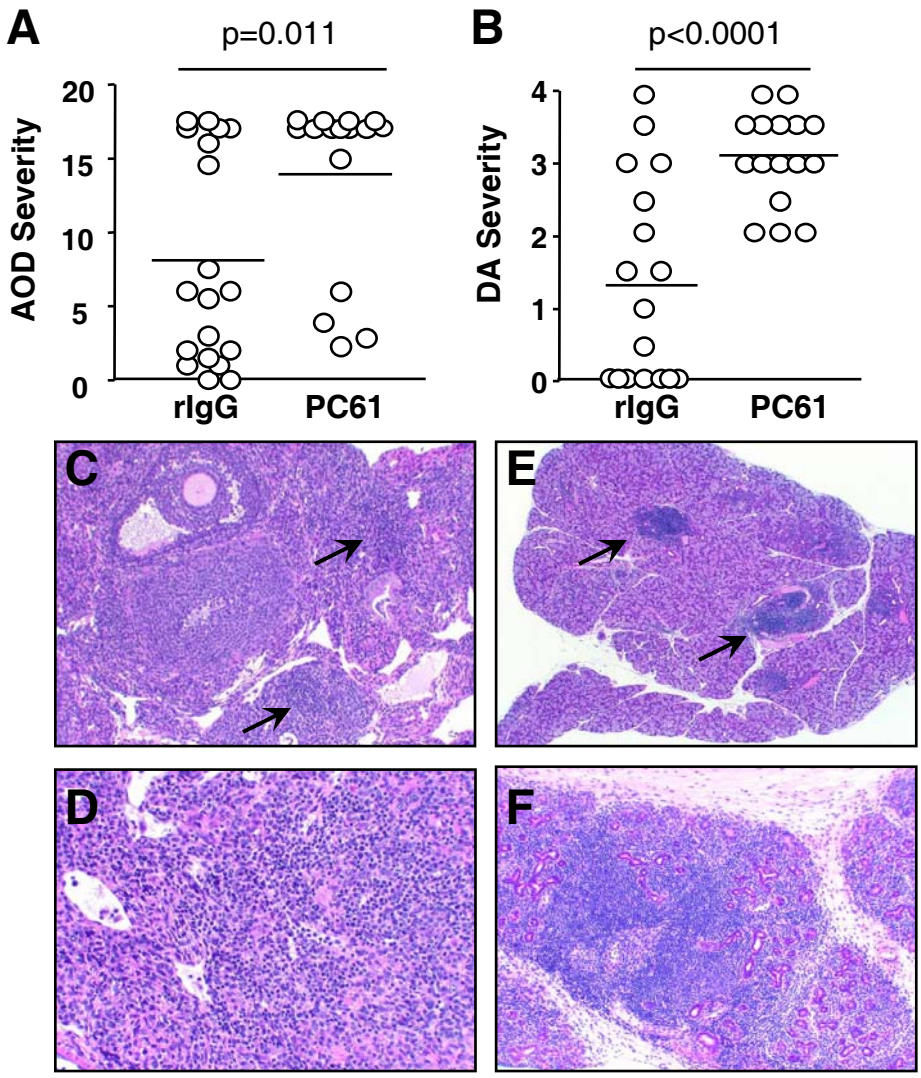

Figure 4: ***In vivo*** **depletion of endogenous CD25+ cells greatly enhances the autologous autoimmune disease of d3tx mice.** (A and B): The incidence and severity of AOD (A) and DA (B) in d3tx mice treated with anti-CD25 (PC61) antibody or control rat IgG. (D-F): In PC61-treated mice (D, F), heavy and diffuse tissue inflammation is associated with loss of oocytes (D) or loss of lacrimal glandular acini (F). The ovary (D) and lacrimal gland (E) of rat IgG treated d3tx mice had focal lymphocyte infiltrate (arrows) with intact oocytes or glandular acini (H and E, x100). *p*-values:Mann-Whitney U test.

vivo function of neonatal Treg, specifically their capacity to suppress the autoimmune disease of the d3tx mice.

Foxp3+ Treg, hardly detectable in the spleen, were readily detected in the LN and the thymus of 3 day-old B6AF1 mice. When the day of birth was counted as day 0, ~5% of the CD4+ T cells in day 3-old LN were Foxp3+ (Figure 5A), and ~3% of CD4+CD8- thymocytes were Foxp3+ (Figure 5A). Similar data were obtained in the 3 day-old Foxp3-gfp knockin mice (Figure 5A). This result is comparable to the report on day 4-old Foxp3-gfp knockin mice thymocytes by Fontenot et. al. [62] if we consider the fact that they counted the day of birth as day 1, and showed a significant rise in the percentage of Foxp3+ CD4+CD8- thymocytes from day 3 (0.74%) to day 4 (2.24%).

The percentages of LN Treg in B6AF1 mice increased from day 3 to day 5 (Figure 5B), reaching the normal plateau of 5-10% (Figure 1B). Neonatal LN Treg expressed adult Treg markers (CTLA4, GITR and high CD62L) and also suppressed in vitro CD25- T cell proliferation induced by CD3 antibody with comparable efficiency as adult Treg (data not shown).

To study in vivo function of the limited Treg obtainable from neonatal mice, we used a highly sensitive model of AOD and AIG, induced by the transfer of 10^5 CD25- effector T cells of adult euthymic B6AF1 donors into adult B6AF1 nu/nu recipients (Figure 5 C and D). Both diseases were completely suppressed by co-transfer of 10^5 adult or neonatal Treg (Figure 5 C and D). Thus, neonatal Treg prevent autoimmune diseases known to occur in d3tx mice.

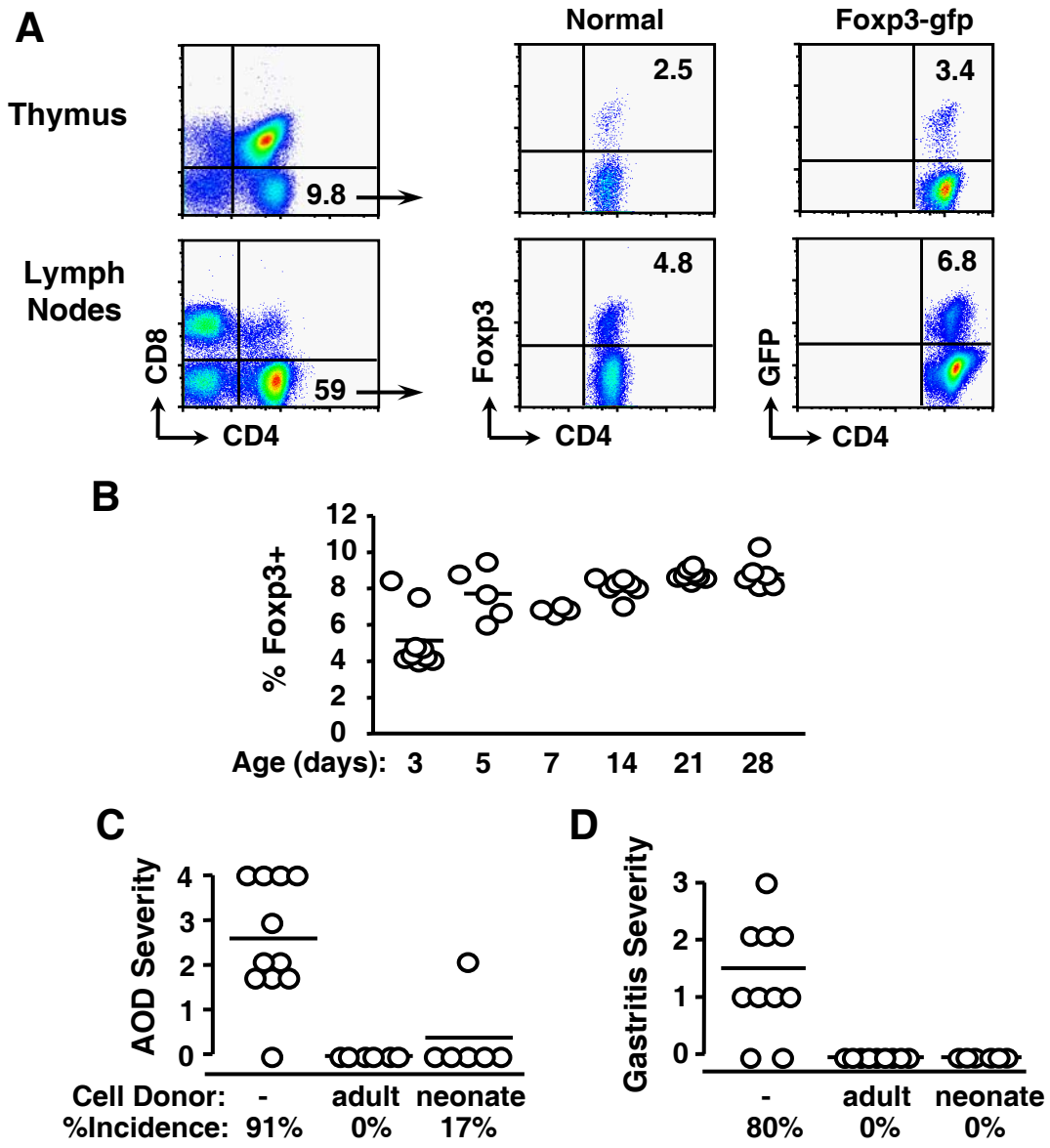

Figure 5: Treg that suppress disease of the d3tx autoimmune syndrome exist in the normal 3 day-old mice. (A): About 3% of CD4+CD8- thymocytes, and 5-6% of CD4+ LN cells express Foxp3 by flow cytometric analysis of B6AF1 and *Foxp3-gfp* knockin mice. (B): Age-dependent increase in % Foxp3+CD4+ LN cells of normal B6AF1 mice. (C and D): AOD and AIG in adult B6AF1 nu/nu recipients of $0.1x10^6$ adult CD25-CD4+ T cells are completely suppressed by $0.1x10^6$ Treg from adult or 3-5 day-old donors. Histopathology was determined 10-12 weeks after cell transfer.

III. C. Conclusions

Contrary to popular opinion, this study provides direct evidence for the existence of functional Treg in d3tx mice with progressive autoimmune disease. Prior to the identification of Treg, results from Smith, et.al. showed that d3tx mice harbor an increased number of neonatal repertoire Teff, which are deleted in normal adult mice[57]. Other data has shown that neonatal T cells have lower levels of N-region diversity in their TCR[58], which results in increased positive selection[59] and increased peptide promiscuity for the TCR[60]. It was hypothesized that this would help neonatal T cells to recognize and combat an increased number of pathogens despite lower overall TCR diversity[60]. By the same token, however, TCR may also recognize a wider variety of self-antigens. These data strongly indicate that the neonatal repertoire of Teff is fixed in d3tx mice and may be more pathogenic than adults, aiding in the development of autoimmune disease in d3tx mice. The discovery of Treg, however, coincided with the hypothesis that d3tx mice develop disease due to a Treg deficiency.

In 1995, Asano, et. al. found that CD4+CD25+ Treg were not present in spleens of 3 day old mice and that CD4+CD25+ cells could block the development of disease in d3tx mice[7]. The identification of Treg lead to a burst in research on Treg, including the discovery of the transcription factor *foxp3*[17] and the generation of foxp3-gfp[33] mouse. The foxp3-gfp mouse was, in fact, later used to corroborate Asano et.al.'s earlier finding that Treg were absent in 3 day old spleens and thymi[62]. Despite strong evidence pointing to the lack of Treg as the mechanism of d3tx-induced disease, one study showed the presence of CD4+CD25+ Treg in the LN of 2 day old mice, and also found an rapid increase in the percentage of CD4+CD25+ cells, which they attribute to activation of

Teff[98]. Still, a lack of Treg was overwhelmingly taken as the mechanism for d3tx-induced disease. Only after Dujardin, et.al. published results showing the presence of Foxp3 mRNA in LN of 3 day old mice and their ability to suppress colitis after transfer of Teff was the idea again put forward that a lack of Treg was not the sole cause for disease after d3tx[99]. Our findings of the presence of Treg in d3tx mice further overturn the "lack of Treg" mechanism of d3tx disease. In fact, we find that the Foxp3+ Treg expand quickly to reach an increased Treg:Teff ratio that persists in the face of d3Tx-induced disease progression. These endogenous Treg are fully functional as they prevent diseases in other d3tx mice that are representative of the d3tx-induced autoimmune syndrome, including AOD, DA and gastritis. Importantly, they restrain the diseases of the autologous d3tx host, and may do so by preferentially accumulating in the regional LN of the relevant target organs [92].

We also document the capacity of neonatal Treg to suppress autoimmune disease. Together, this study provides conclusive evidence that the Treg in d3tx mice can down-modulate concurrent autoimmune disease; and in the presence of putative highly pathogenic Teff, the co-existing Treg strongly influence autoimmune disease outcome. Bonomo et.al. found almost identical results in Balb/c mice, including a functional Treg compartment and increased Treg:Teff ratio[102]. Thus, it is clear that Treg are present and functional in d3tx mice, but are still insufficient to fully control the disease.

Why, then, do the endogenous Treg in d3tx mice fail to fully control their own autoimmune diseases? First, recent studies have indicated that Treg suppress Teff responses through interaction with dendritic cells [39]. In d3tx mice, their profound lymphopenia would reduce the T cell to dendritic cell ratio; and this would in turn reduce

the efficiency of Treg to compete against effector T cells for dendritic cell interaction [39]. Thus, lymphopenia can render Treg less functional, allowing for the activation of autoreactive (compared to normal mice) Teff and autoimmune disease induction.

Second, it is interesting to compare the d3tx mouse with the Aire (autoimmune regulator/autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) knockout mouse. The organ distribution, histopathology, and autoantibody specificity associated with diseases in Aire deficient mice are strikingly similar to those seen in d3tx mice[103-105]. Moreover, organs affected in different strains of Aire deficient mice, mirror those afflicted in d3tx mice. In comparison, Foxp3 deficient mice have a very different distribution of disease when compared to d3tx mice, affecting mostly the lung, liver, and gut regardless of strain[15]. The concordances of autoimmunity raise the intriguing possibility that d3tx mice and Aire knockout mice may share a common disease mechanism. The Aire transcription factor controls the ectopic thymic expression of peripheral autoantigens required for central deletion of autoreactive T cells [103, 104, 106]. In Aire knockout mice, thymic expression of many ectopic antigens is reduced, and thymic deletion of potentially pathogenic T cells is also significantly curtailed [106, 107]. Meanwhile, the Treg from these mice were found to function similarly to wild type mice ex vivo[106]. Thus, both d3tx mice and Aire deficient mice harbor highly pathogenic Teff and a functional subset of Treg. While lymphopenia may play a role in the pathogenesis of d3tx mice, it will be interesting to determine if the mechanisms by which Teff from Aire deficient mice overcome suppression by Treg also apply to d3tx mice.

Chapter IV. In normal mice, antigen-specific Treg are enriched in the draining LN whereas Teff are equally distributed through all LN.

IV.A. Introduction

While studies have implicated Treg in peripheral tolerance, it is unknown how Treg play a role in tolerance to internal organs, including: endocrine organs, gonads, pancreatic islets, thyroid, CNS, exocrine glands – all of which are major targets of human autoimmune disease. It has been shown that Ag specific Treg are continuously capacitated by responding to autoAg in normal regional LN [108]. Accordingly, Treg with transgenic TCR proliferate in regional LN of normal or lymphopenic hosts [109, 110], and regional LN are a location where the Treg control effector autoimmune T cell response [92]. Significantly, in mice with highly restricted TCR-VDJ expression, the Treg from individual LN showed distinctive TCR repertoires based on VDJ analysis [27]. Collectively, these findings support LN-specific distribution of antigen-specific Treg. However, the functional capacity of the Treg in autoimmune disease suppression was not investigated in these studies, therefore the relevance of the data to self tolerance has remained undefined.

The requisite to investigate tolerance mechanisms, based on detection and functional analysis of antigen specific polyclonal T cells, is hindered by their rare occurrence. As an alternative approach, we have investigated antigen-specific polyclonal Treg by querying whether there are Treg populations that suppress one organ-specific autoimmune disease over another in the day 3-thymectomized (d3tx) mice (disease-specific Treg [DS-Treg]) [51, 53].

IV.B. Results

IV.B.1. Normal ovarian-LN draining Treg have enhanced capacity to suppress AOD.

Disease in d3tx B6AF1 mice is fully suppressed by $0.5x10^6$ or more Treg pooled from all the LN of normal donors [105]. To compare the Treg from regional versus non-regional LN in AOD suppression, CD4+CD25+ Treg from ovarian LN, or pooled from non-draining LN of untreated B6AF1 female donors, were transferred to groups of d3tx female mice at doses of 0.003 to 0.5 x 10^6, and the ovarian pathology was determined 8 weeks later. AOD was suppressed only by $0.5x10^6$ Treg from non-draining LN (Figure 6B and E). In stark contrast, AOD was completely suppressed by $0.03x10^6$ Treg from the ovarian LN (Figure 6A and D). Thus a 15-fold difference in AOD suppression was detectable between the Treg from draining and non-draining LN (Figure 6C). Serum oocyte autoantibody was detected only in d3tx recipients of less than $0.01x10^6$ Treg from ovarian LN, and in d3tx recipients of less than $0.1.x10^6$ Treg from the non-draining LN (Figures 6A, B, and E).

IV.B.2. Prostate LN Treg and lacrimal gland LN Treg of normal mice preferentially suppress EAP and DA (respectively) in the same host.

To determine whether the finding on AOD was a general phenomenon, we investigated EAP and DA. EAP was completely prevented by $0.01x10^6$ Treg from

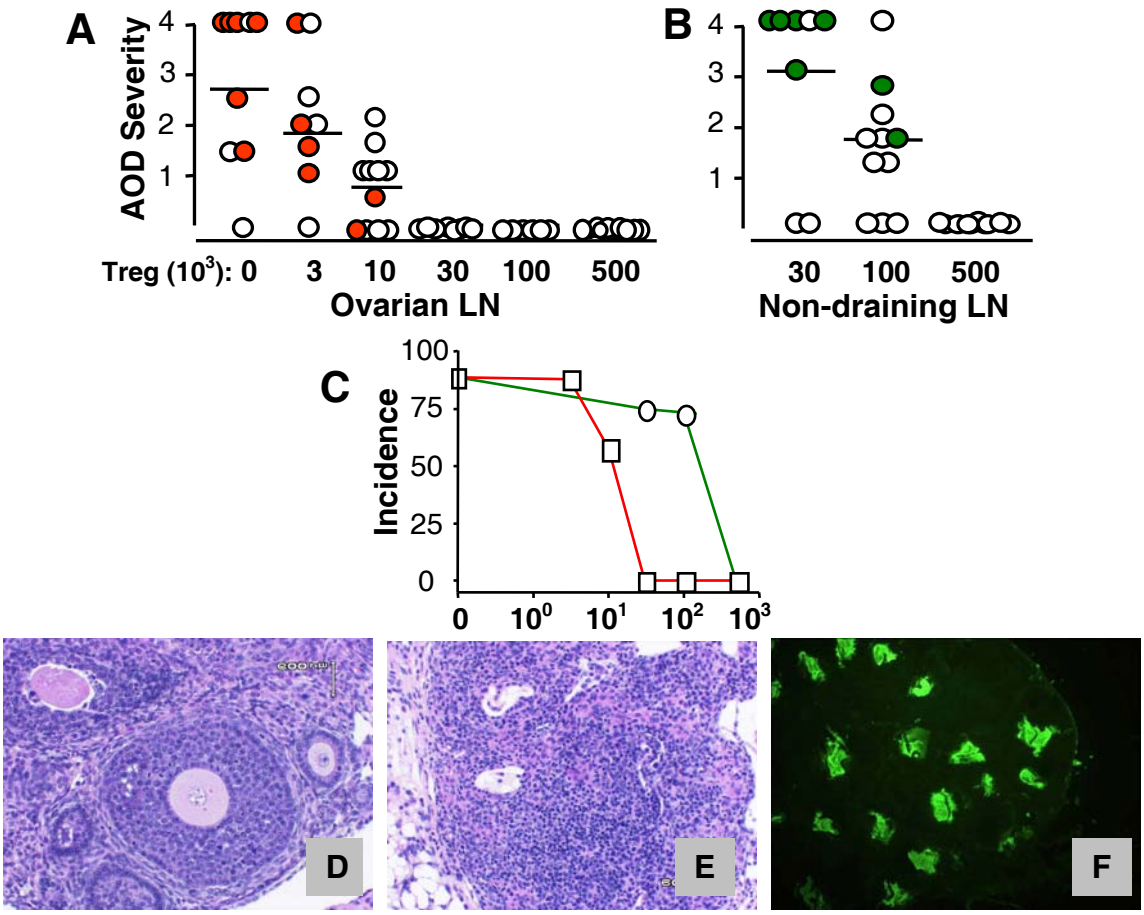

Figure 6. Treg from normal ovarian LN suppress AOD more efficiently than Treg from non-ovarian LN. (A and B): Severity of AOD in d3tx recipients of ovarian LN Treg (A) or non-ovarian LN Treg (B). (Filled circles = positive serum oocyte antibody.) (C): AOD incidences between recipients of ovarian LN Treg (squares) and recipients of non-ovarian LN Treg (circles) (n = 6-8). (D) Normal histology of the ovary from d3tx recipients of ovarian draining LN Treg. (E) Severe AOD, present in recipients of Treg from non-draining LN, includes loss of ovarian follicles and presence of ovarian lymphocytic infiltration. (H&E; x100). (F) Positive serum oocyte antibody by indirect immunofluorescence (x100).

prostate LN, whereas $0.5x10^6$ Treg from the same LN was required to completely suppress DA (Figures 7A and C; Figure 8A and E). In contrast, Treg from LN draining the lacrimal gland completely suppressed DA at a dose of $0.03x10^6$ Treg, whereas $0.5x10^6$ Treg were needed to prevent EAP (Figures 7B and D; Figure 8B and D). Serum autoantibody to the prostate gland was detected only in recipients of less than $0.003x10^6$ Treg from the prostate LN, and in recipients of less than $0.1x10^6$ Treg from lacrimal gland LN (Figures 7A and B; Figure 8C). Thus the Treg from prostate LN were 50 times more potent in suppressing EAP over DA, whereas Treg from the lacrimal gland LN were 15 times more potent in suppressing DA over EAP (Figures 7E and F).

This 50-fold enrichment of EAP-specific Treg in the prostate-draining LN is markedly greater than the 3-fold enrichment of the prostatitis-suppressing Treg present in the pooled LN cells of male mice over female mice determined earlier [105]. Despite this difference in functional capacity, the Treg from LN draining the prostate, ovary, and lacrimal gland have similar expression of Foxp3 and ability to suppress in vitro pan-T cell proliferation (Figure 9).

The new bioassay described herein is useful for semi-quantitation of functional Ag-specific Treg. Notably, the assay is not dependent on T cell recognition of a specific T cell epitope that may or may not be relevant to, or be exclusive, for a given autoimmune disease. Instead, it detects Treg that recognize all organ-derived autopeptides that are relevant to suppression of disease in that organ. Based on the assay, we have documented for the first time that Treg from normal regional LN are highly enriched in functional Treg with the capacity to suppress autoimmune disease of its

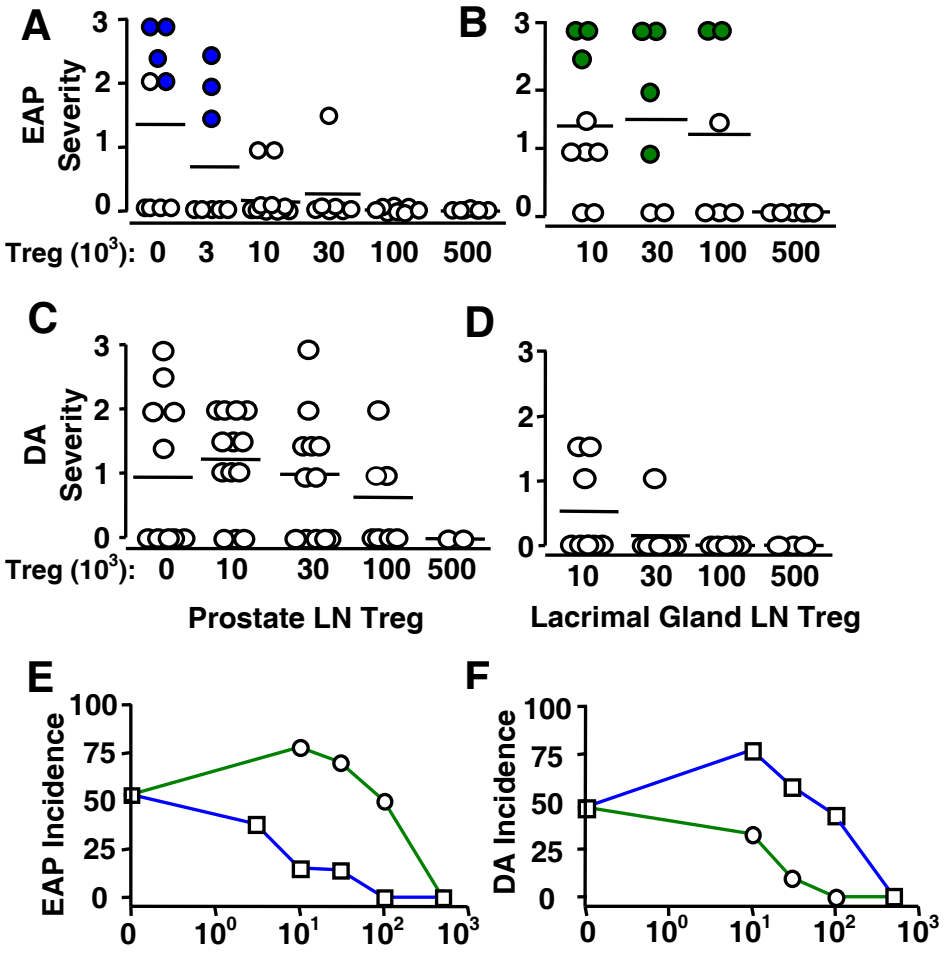

Figure 7. Selective enrichment of EAP-specific Treg and DA-specific Treg in prostate LN and lacrimal gland LN of normal mice. (A and B): EAP severity between d3tx recipients of Treg from prostate LN (A) and lacrimal gland LN (B). (C and D): DA severity between d3tx recipients of Treg from prostate LN (C) and lacrimal gland LN (D). (E and F): Incidences of EAP (E) and DA (F) between these Treg recipients (squares, prostate LN Treg recipients; circles, lacrimal gland LN Treg recipients). Filled circles denote positive prostate autoantibody.

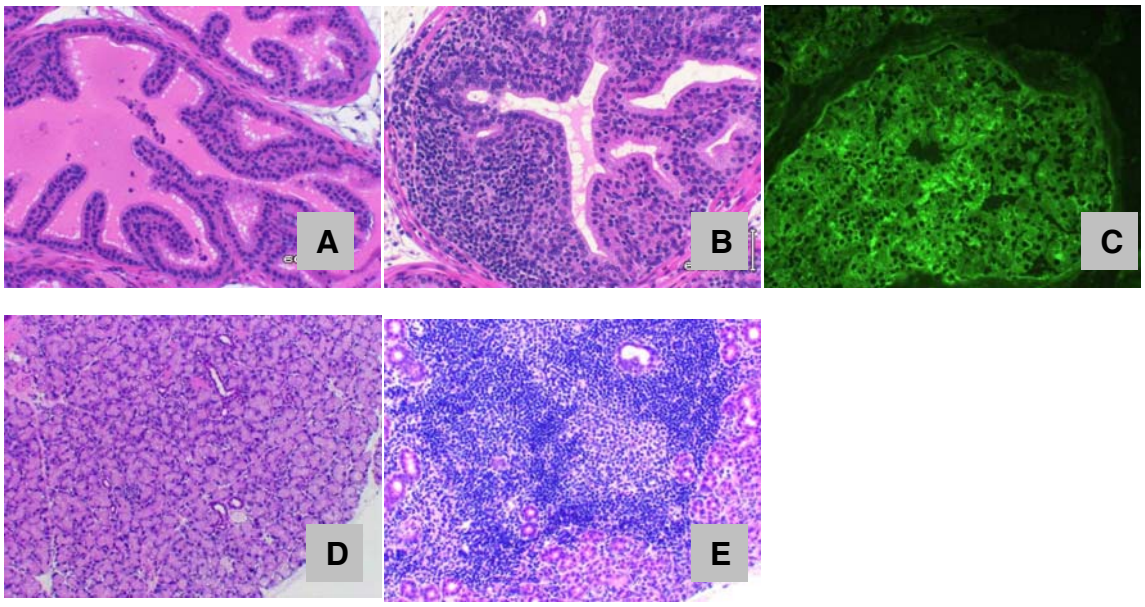

Figure 8. Histopathology and autoantibodies in d3tx recipients of Treg. (A and D): Normal histology of prostate (D) and lacrimal gland (G) from d3tx recipients of draining LN Treg. (B): Severe EAP, present in recipients of Treg from lacrimal gland LN, shows heavy inflammation and loss of glandular secretion. (E): Severe DA in recipients of Treg from prostate LN, shows germinal center formation within lymphoid infiltrate, and loss of lacrimal glands. (Hematoxylin and eosin stain, x100). (C) Positive serum prostate antibody by indirect immunofluorescence (x100).

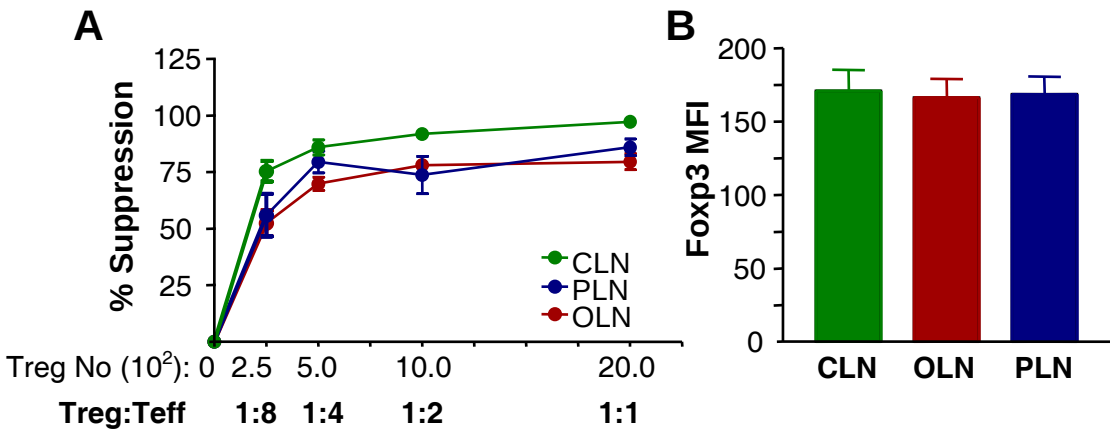

Figure 9: Treg from normal cervical LN (CLN), ovarian LN (OLN), and prostate LN (PLN) have comparable in vitro function and levels of Foxp3 expression. (A): CD4+ CD25+ Treg were used to suppress CD3 Ab-induced proliferation of pooled LN CD4+ CD25- at different Treg:T effector cell (Teff) ratios. (B): The MFI of Foxp3 of Treg from individual LN was determined by flow cytometry. Data are representative of the results of three experiments.

draining organ. In our earlier reports, we studied DS-Treg in d3tx mice with ongoing autoimmunity, therefore they are not pertinent to self tolerance [92, 111].

IV.B.3. Prostate-specific antigen expression influences LN enrichment of EAP-specific Treg.

The DS-Treg enrichment in regional LN could result from an increase in Treg number and/or an enhancement of intrinsic Treg activity; and this may depend on the response of Treg to autoAg, their preferential homing to the regional LN, or both. To address the requirement of autoAg, we determined whether prostate antigen expression influences regional LN enrichment of EAP-specific Treg. When prostate Ag expression was eliminated in mice with neonatal orchiectomy (NOX) [105], the dominance of Treg from the prostate LN to suppress EAP was lost. When prostate Ag expression resumed in Treg donors that underwent NOX and 5α- dihydrotestosterone (DHT) treatment [105], the EAP-specific Treg activity in prostate LN was restored (Figure 10). Thus in the steady state, presentation of organ-derived tissue Ag to Treg is required for regional LN-specific enrichment of DS-Treg. A caveat is that this study has not ruled out a potential sex hormone effect on Treg function, inherent in the experimental design.

IV.B.4. Normal regional LN have enriched DS-Treg but not enriched DS-pathogenic T cells, correlation with reported TCR repertoire data.

The antigen dependency or Ag specificity of polyclonal autoimmune T cell response can also be extrapolated from TCR repertoire analysis of LN T cells. Lathrop et. al. [27] recently reported a distinctive repertoire among the Treg in individual LN. This

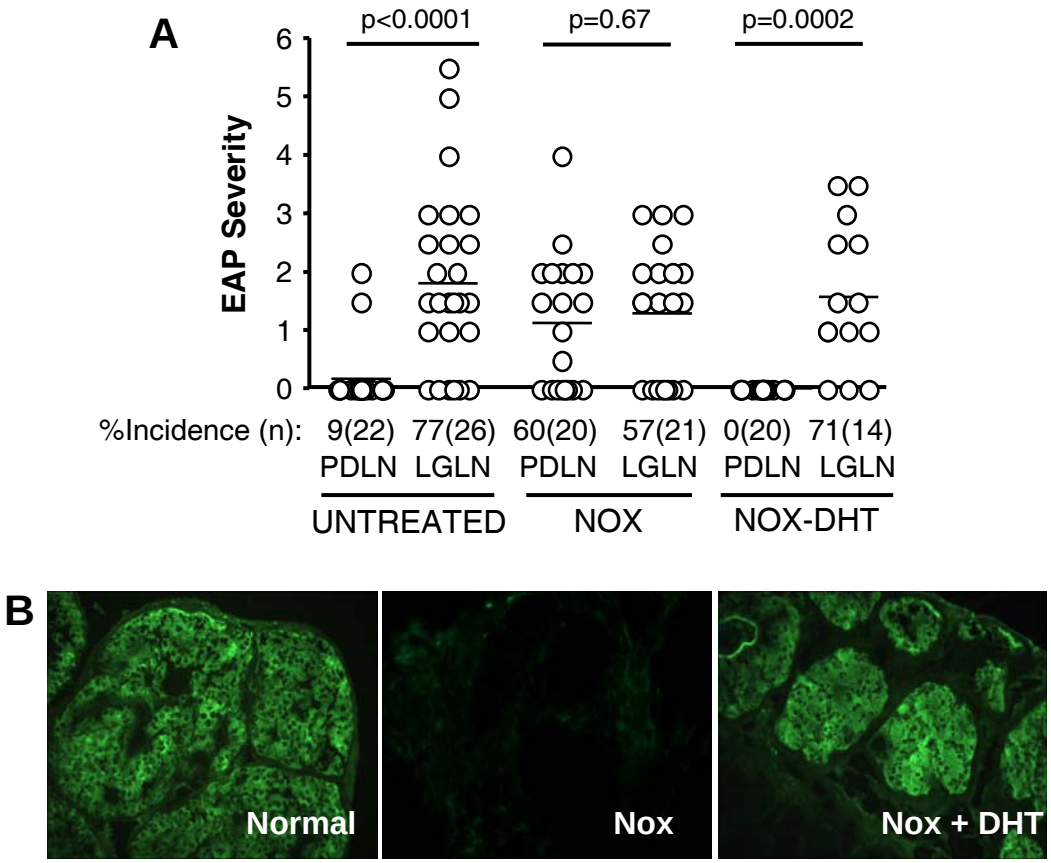

Figure 10. Loss and gain in prostate Ag expression influences EAP-specific Treg activity in the normal prostate LN. (A): EAP severity and incidence in mice that received Treg ($0.03 – 0.05x10^6$) from prostate LN (PDLN) or lacrimal gland LN (LGLN), obtained from: normal donors, NOX donors, or donors with NOX and DHT treatment (NOX+DHT). (B): Immunohistochemical detection of prostate antigens in normal mice, NOX mice, and NOX+DHT. P-values determined by Mann-Whitney test.

was a finding unique to Treg since comparable TCR repertoires were detected among the naïve CD4+Foxp3- T cells of individual LN. In order to establish an additional correlate between the two studies, and add functional support to the TCR repertoire study, we next determined the distribution of autoimmune inflammation in recipients of CD4+CD25- T cells from individual LN of normal donors [7, 55, 112] . As shown in Figures 11 and 12, when CD4+CD25- T cells from renal LN, mesenteric LN or cervical LN of normal mice,were transferred to syngeneic RAG knockout recipients, they elicited autoimmune inflammation with very similar organ distribution and severity. Monocytic inflammation and tissue destruction were noted maximally in the prostate, testis and epididymis, lung, liver, skin and colon; moderately in the kidney, lacrimal gland, stomach and thyroid, but spared the pancreatic islets, heart, eye and small bowels. The finding of such a striking correlation between TCR repertoire and T cell function for both LN-specific Treg and effector T cells, provides additional support for the conclusion that the normal LN enrichment of DS-Treg is Ag-dependent.

IV.C. Conclusions

Whether Treg suppress Teff in an antigen specific manner in vivo remains controversial. Non-specific suppression is suggested by the ability of activated, TCR-specific Treg to suppress Teff of a different TCR specificity in vitro[113], and the suggestion that their mechanism of suppression is to act as a "sink" for survival cytokines[114]. Other studies, however, found in vivo Treg suppression to be dependent on the presence of antigen[39]. Hindering the answer to the specificity of Treg suppression is the inability to identify antigen-specific Treg in unmanipulated, wild type mice. Indeed,

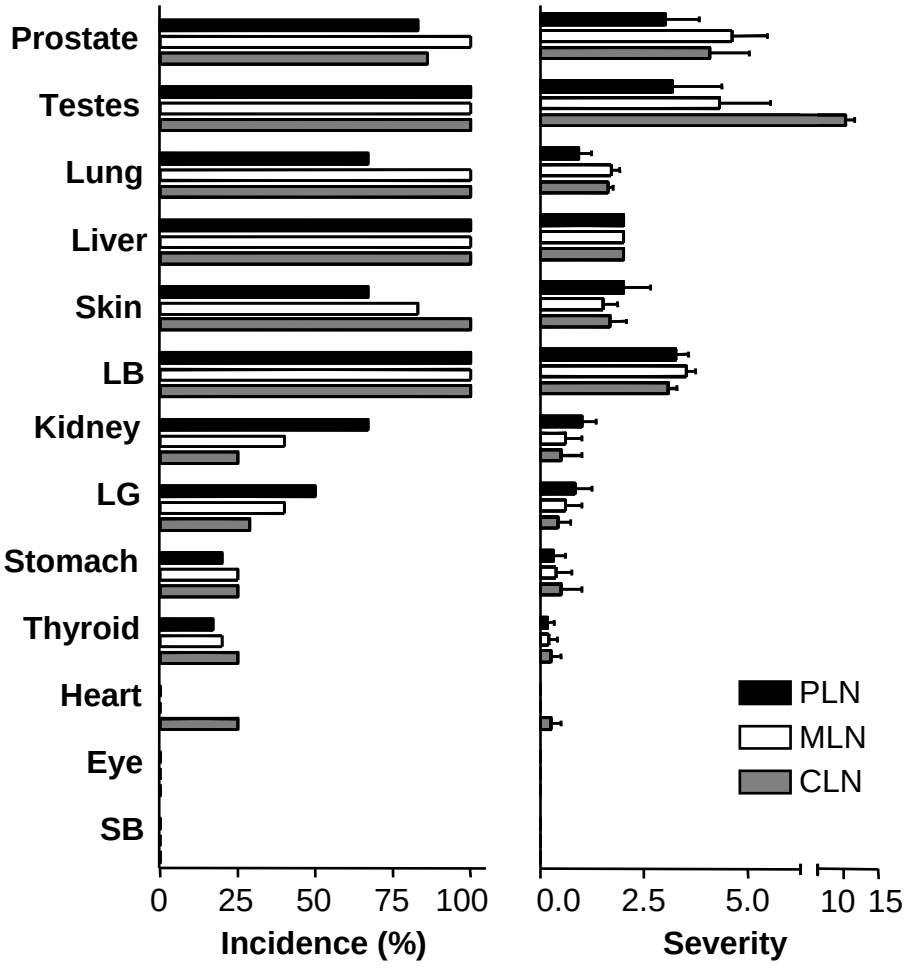

Figure 11. Organ distribution of tissue inflammation in B6 RAG-/- recipients of CD4+CD25- T cells from individual LN of wild type B6 donors. Data represent the incidence (%) and severity (mean ± SD) of tissue inflammation and destruction. Comparisons are made between pathology induced by lacrimal gland-draining cervical LN (gray bars), mesenteric LN (white bars) or prostate LN (black bars). Each recipient received $0.2x10^6$ CD4+CD25- T cells i.v., and were studied 3-4 weeks later. Data represents the summation of three independent experiments, with two mice per experiment. Abbrev: LB, large bowel; LG, lacrimal gland; SB, small bowel.

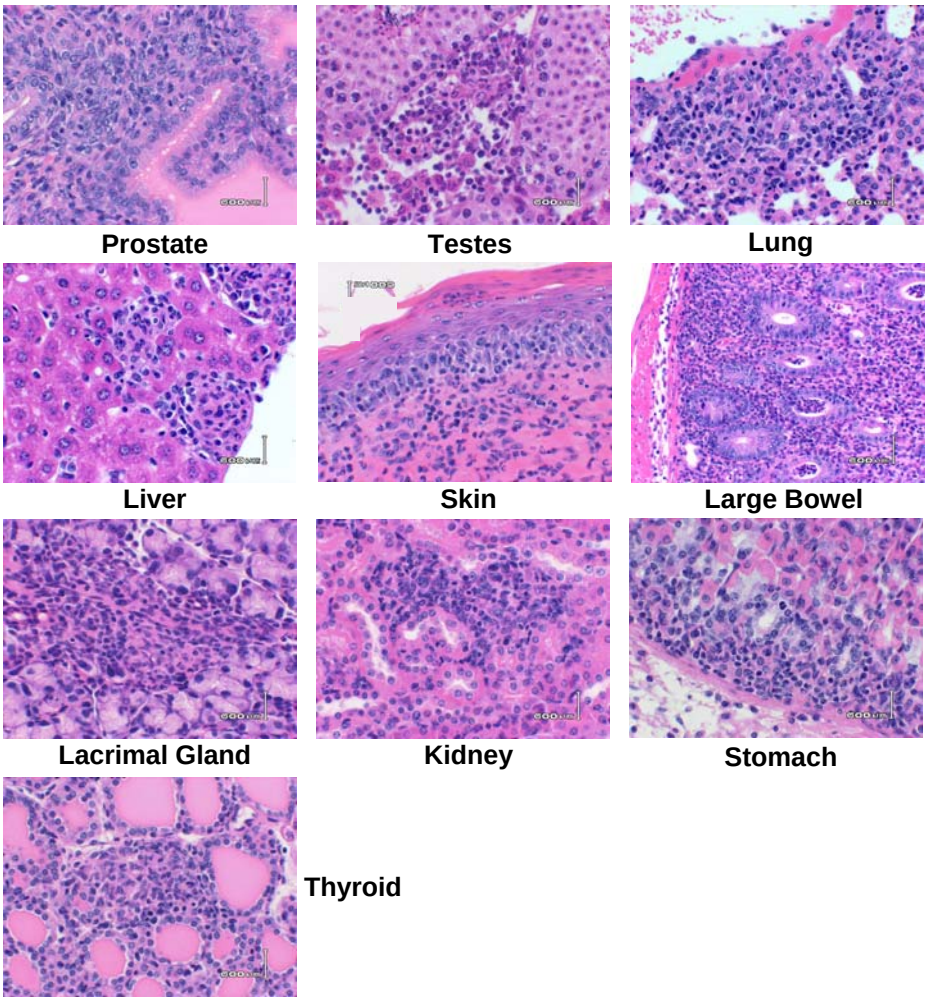

Figure 12: Illustration of histopathologic findings in B6 RAG-/- recipients of CD4+CD25- T cells from individual LN of wild type B6 donors. Prostate: Monocytic infiltration mainly in lateral and posterior lobes. Testes: Interstitial monocytic infiltration. Lung: peribronchial and perivascular monocytic infiltration. Liver: portal inflammation with granulomas. Skin: hyperkeratosis, epidermal and dermal inflammation. Large bowel: Heavy monocytic and neutrophilic infiltration with crypt abscesses. Kidney: Monocytic tubulitis. Lacrimal gland: Periductal inflammation. Stomach: Monocytic inflammation at base of gastric glands. Thyroid: Focal monocytic inflammation. All: hematoxylin and eosin, x400; large bowel x200.

much of the work on antigen-specific suppression by Treg has been accomplished with transgenic TCR mice. In a diabetes models where OVA is expressed under the rat insulin promoter, it was shown that Treg expressing an OVA-specific TCR preferentially home to and divide in the pancreas draining LN[109]. Almost identical results were found using HA-specific Treg and mice expressing beta cell-specific HA[110]. Thus, our results describe the first evidence for antigen-specific Treg in normal mice, and we hypothesize further that LN-enrichment allows them to prevent autoimmune disease in normal hosts.

The mechanism of LN specific antigen-specific Treg enrichment might involve factors regulating T cell homing to LN, encounter with self antigen, and their retention in the LN. Homing of naïve T cells and Treg to normal LN are known to involve CD62L, CCR7 and the chemokines CCL19 and CCL21 [115]. Autoimmune diseases occur in mice deficient in CD62L or CCR7 [116, 117], and we can now add loss of DS-Treg enrichment in the regional LN as a potential explanation.

Treg retention may result from up-regulation of CD69 on antigen-specific Treg that temporarily sequesters sphingosine 1-phosphate receptor type 1 ($S1P_1$), which is required for T cell egress from LN [118]. Additional mechanisms may involve Treg response to anti-apoptotic and/or cellular proliferative signals [119]. Constrained by T cell homeostatic mechanisms [120], the number or activity of DS-Treg in the regional LN would be maintained at a threshold 15-50 folds greater than those in the non-draining LN.

Additional mechanisms participate in antigen-specific Treg homing to regional LN and maintenance of abundant resident antigen-specific Treg in the normal skin, lung and mucosal sites[115, 121]. It is conceivable that they also participate in Treg homing to internal organs and their regional LN. However, unlike mucosa such as intestinal lamina

propria, very few Foxp3+ cells are detected in normal internal organs; for example, only 1% of normal prostate CD4+ T cells are Foxp3+ (data not shown).

There is significant controversy as to the repertoire of TCR utilized by Treg. Studies where several thousand CD3 regions of TCR were sequenced from mice with a limited diversity of TCR, have shown both significant similarities between Treg and Teff, and significant differences[24-26]. The most recent study, however, used Treg and Teff from individual LN, and found a unique set of Treg CD3 regions in each different LN, whereas naïve Teff showed no difference[27]. Our findings corroborate this idea by finding function differences amongst the Treg from individual LN, whereas Teff are not unique. These findings appear not to be consistent with the prevailing concept that common chemokine receptors allow for the co-localization of both Treg and Teff with shared TCR specificity [115, 122, 123], as their distribution amongst individual LN differs.

In summary, there are major differences in the LN distribution of autoimmune disease-relevant effector T cells and Treg in normal mice. In the normal steady state, LN distribution of antigen specific T cells is non-random, due to the enrichment of DS-Treg in regional LN. The results provide strong support for a critical role of antigen-specific Treg in peripheral tolerance. In addition, we hypothesize that DS-Treg, positioned strategically in the organ-draining LN, can control organ-specific tolerance by negating effector T cell response to self peptides, and prevent autoimmune disease induction in the event of infection, tissue necrosis and other forms of local danger signal. Our study also points out that results of studies based on pooled LN Treg may not be indicative of the physiological status of Treg.

Chapter V. Treg control the immune response to sperm antigens released after vasectomy: autoimmunity versus tolerance

V.A. Introduction

In order to test the hypothesis that Treg function to prevent autoimmune disease, the ideal experiment would include eliciting organ-specific danger (either via infection or tissue damage), depleting Treg in the corresponding draining LN, and querying for organ-specific autoimmune disease. Vasectomy, known to cause epididymal ductal rupture, the release of large amounts of sperm, and the induction of sperm granulomas[124], was used as an organ-specific danger signal. Additionally, by performing unilateral vasectomy (UniVx), the contralateral testis remains intact and can be used as a readout of a systemic autoimmune response.

LN-specific Treg depletion proved a difficult task. The two major ways of depleting Treg are through the use of either the anti-CD25 monoclonal antibody PC61 or diphtheria toxin (DT) treatment of a mouse with DT expressed under the Foxp3 promoter (DEREG mice)[22]. Targeting a specific LN would require delivery of these molecules (PC61 Ab or DT) to a specific LN and preventing the diffusion of the molecule into circulation. So, we instead decided to globally deplete Treg.

V.B. Results

V.B.1. Unilateral Vasectomy and Treg depletion lead to the development of bilateral autoimmune orchitis.

Treg depletion was achieved through PC61 treatment given 3 days prior to and 3 and 7 days after vasectomy. Although the depletion of total Treg (CD4+Foxp3+) was incomplete (~6% residual CD4+Foxp3+ cells), the % of Treg remained low in the LN and epididymis throughout the experimental period as compared to control mice (Figure 13A and B). The percentage of Treg did, however, begin to increase between 5 and 7 weeks.

In mice receiving control rat IgG (rIgG), UniVx induced severe granulomatous inflammation in the vasectomized epididymis, while the contralateral epididymis remained unaffected (data not shown). At 1-2 weeks post vasectomy, sperm could be found leaking out of the epdidymal ducts through ruptures of necrotic epithelium (Figure14A). Many macrophages containing phagocytosed dead sperm could be found at the edge of sperm rupture in the interstitium (Figure 14B). Later, at 10 weeks post vasectomy, clear granulomatous inflammation with a necrotic sperm core and giant cells be found (Figure 14C and D). CD4+ T cells, CD11c+ dendritic cells, and F4/80+ macrophages were also highly prevalent throughout the edge of the granuloma (Figure 14E). UniVx mice depleted of Treg or control had a comparable numbers and percentage of CD45+ cells in the granuloma, however, there was an increase in the absolute numbers of CD11c+ DC and CD11b+Siglec F+ eosinophils in the epididymis at 10 weeks (Figure 15). Thus, unilateral vasectomy, regardless of Treg depletion, begets sperm granulomas containing a host of innate and adaptive inflammatory cells which may be slightly increased in Treg depleted mice.

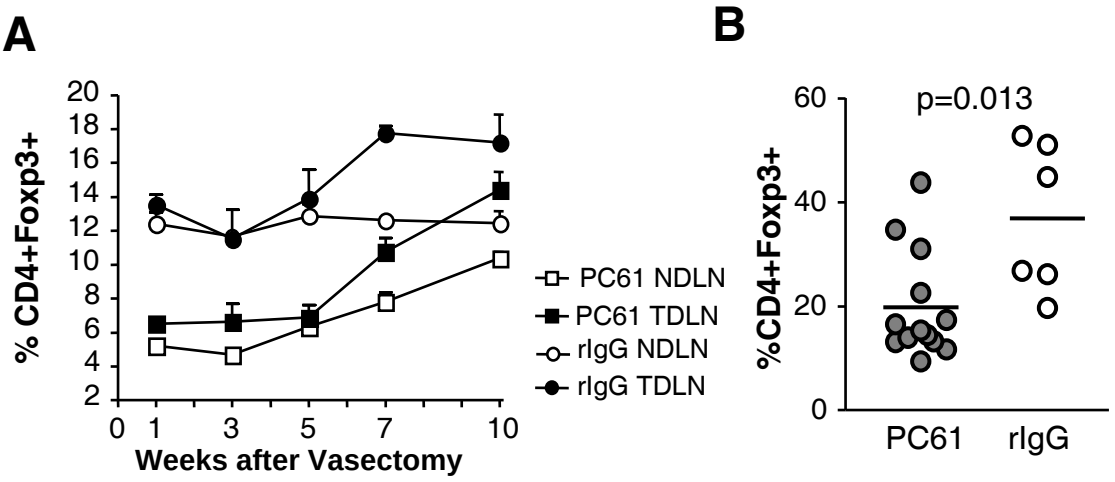

Figure 13: Treg depletion in vasectomized mice. UniVx mice were treated with PC61 or control rIgG and testis-draining (TDLN) or non-draining LN (NDLN) were studied over 10 weeks. (A-B): Percentage of CD4+Foxp3+ cells in the LN (A) or epididymis (B). P-values determined by Mann-Whitney test.

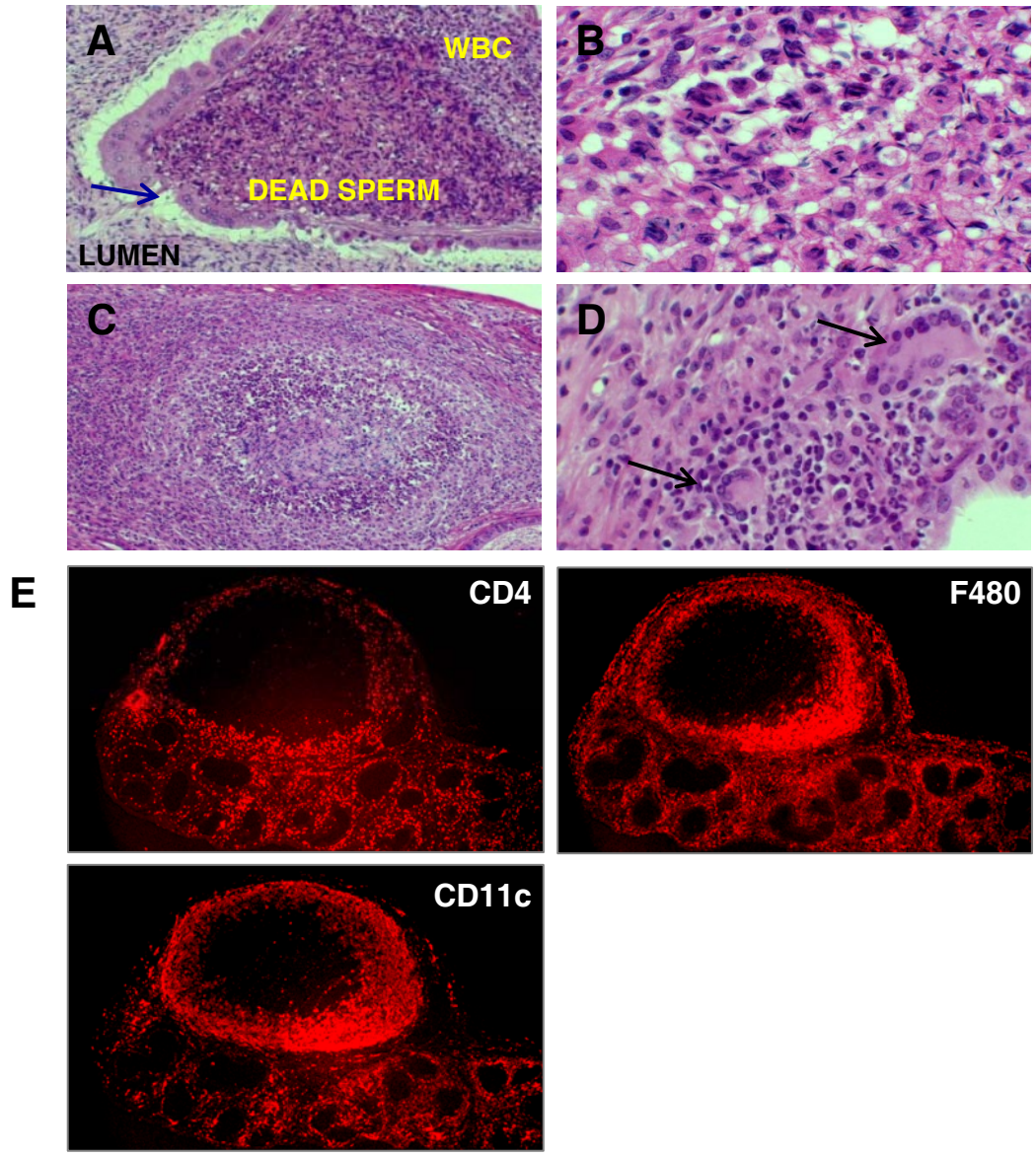

Figure 14: Sperm granulomas in unilaterally vasectomized mice are sites of extensive inflammation. (A – B): Histopathology of epididymides (epi) 1-2 wks after vasectomy illustrating (A) extravasations (arrow) of sperm through necrotic ductal epithelium and (B) phagocytosis of sperm by macrophages. (C – D): Histopathology of epi 8-10 wks after vasectomy illustrating sperm granuloma (C) and giant cells (D). E: Immunofluorescence of epididymal granulomas 5 weeks after Vx indicating infiltration with CD4+, F4/80+, and CD11c+ cells (x40). (A,C = H&E; x200; B,D = H&E; x400)

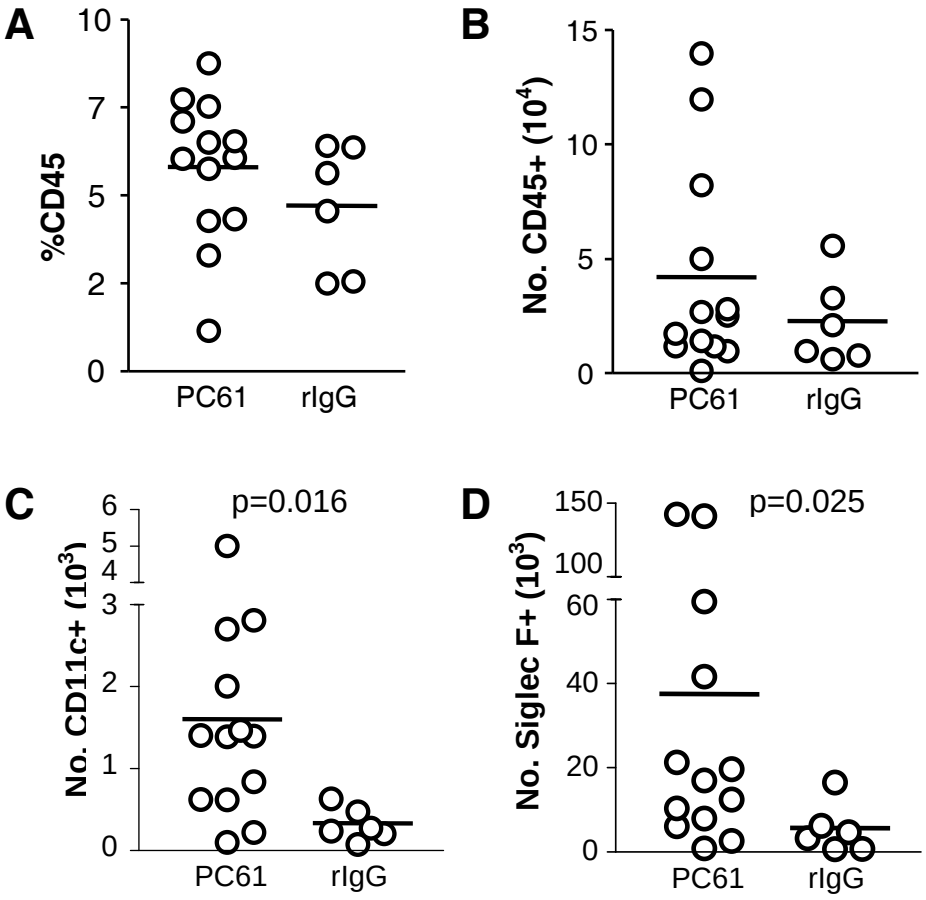

Figure 15: Numerous inflammatory cells found in granulomas of vasectomized mice. Vasectomized and Treg depleted (PC61) or control (rIgG) mice were studied at 10 weeks. (A and B): The percentage (A) and number (B) of CD45+ cells. (C and D): Absolute numbers of CD11c+ DC (C) and CD11b+SiglecF+ eosinophils (D). P-values determined by Mann-Whitney test.

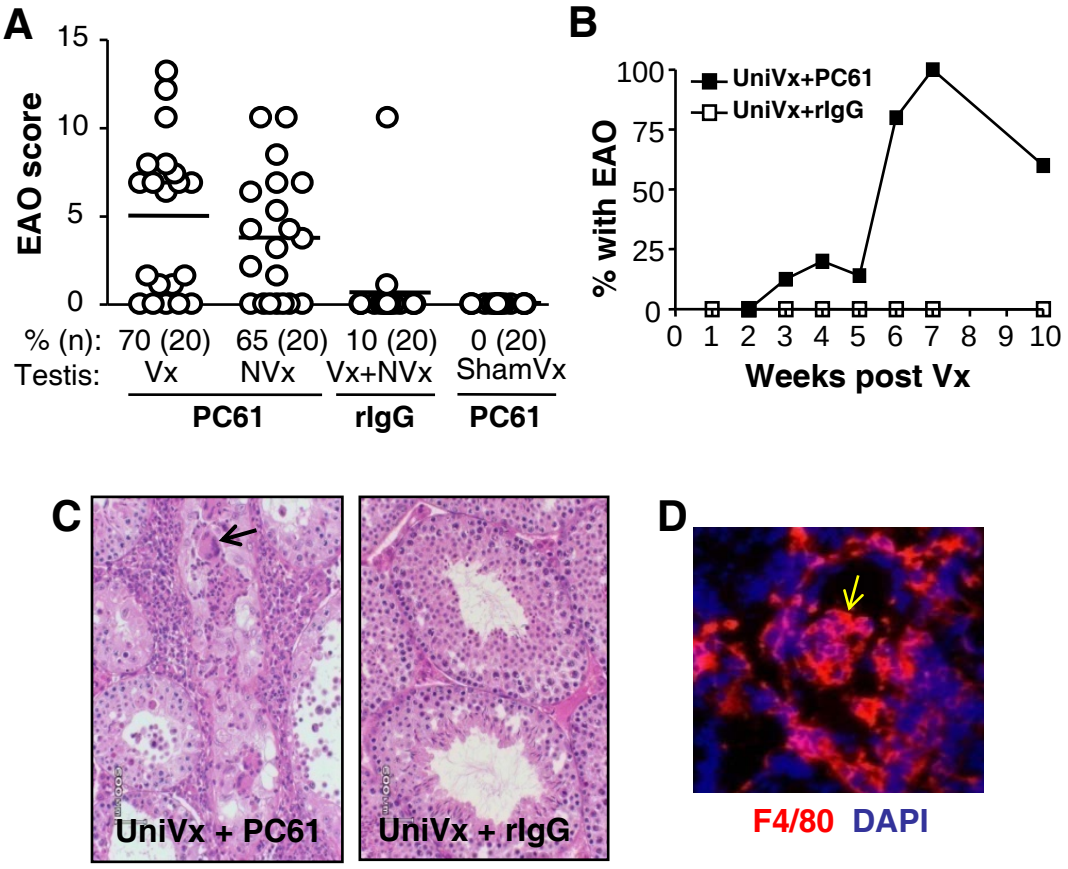

Figure 16: Unilateral vasectomized (UniVx) mice with Treg depletion develop bilateral EAO. (A): EAO in both Vx testes and non-Vx (NVx) testes of UniVx mice treated with CD25 Ab (PC61), but not in control mice ($p<0.002$ UniVx PC61 vs rIgG). (B): Kinetics of EAO development in UniVx mice treated with PC61 or rIgG (4-10 mice per time point). (C): Severe orchitis detectable only in UniVx mice treated with PC61 (H & E, x200). (D): F4/80+ giant cell (arrow) and macrophages in seminiferous tubules. P-values determined by Mann-Whitney test.

Despite the apparent similarities in the granulomas of control and Treg depleted mice, the later developed severe inflammation in both the testes of the vasectomized side as well as the non-vasectomized side (Figure 16A). Orchitis began to appear as early as six weeks in the Treg depleted mice, and while control mice failed to develop orchitis (Figure16C). The inflammatory infiltrate included substantial interstitial mononuclear cells, cells invading into the seminiferous tubules, and the presence of multinucleated giant cells (Figure 16B). Sham UniVx mice treated with PC61 or rIgG were devoid of both epididymal and testicular inflammation (Figure 16A). Thus, in the presence of a sperm granuloma, Treg depletion results in severe bilateral autoimmune orchitis. This proves that Treg indeed play a direct role in the prevention of autoimmune disease following a localized danger signal.

V.B.2. T cells from Treg depleted and UniVx mice develop a robust Teff and autoantibody response.

In order to further define the autoimmune response in UniVx mice, we assessed the level of activation in CD4+ T cells in testis-draining (renal; TDLN) and non-draining (axillary and brachial; NDLN) LN and the level of autoantibody responses. Effector CD4+Foxp3- cells from the TDLN of Treg depleted mice upregulated CD69 expression by 1 week post vasectomy ($p=0.001$) and continued out to 10 weeks as compared to the NDLN (Figure 17). Additionally, they were significantly increased as compared to TDLN of control mice ($p=0.007$ at all points). Similar results were found with CD4+CD44+CD62L- Teff (Figure17). Hence, although control mice develop a mild

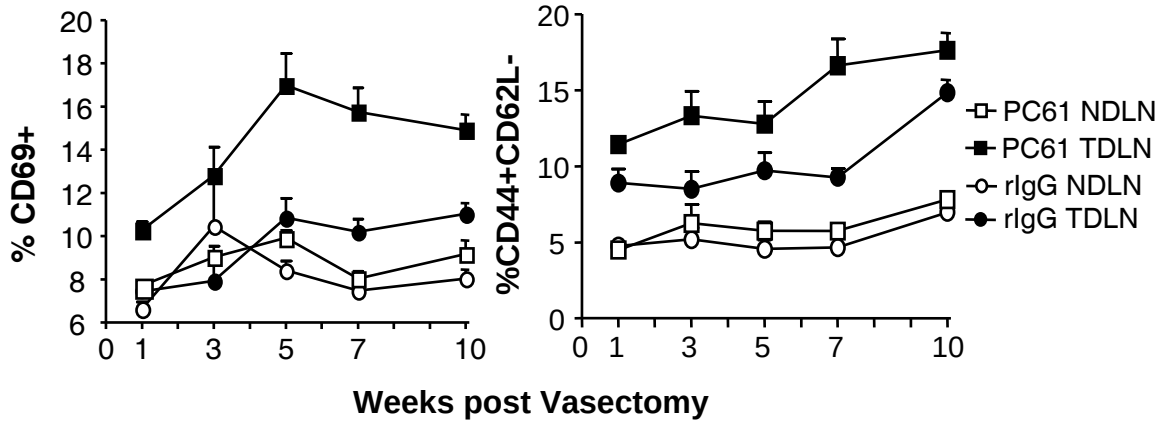

Figure 17: Effector T cell activation in vasectomized mice. UniVx mice treated with PC61 or control rIgG were studied over 10weeks for the % of CD69+ (left) or CD44+CD62L- (right) of CD4+Foxp3- Teff in the testis-draining (TDLN) or non-draining LN (NDLN).

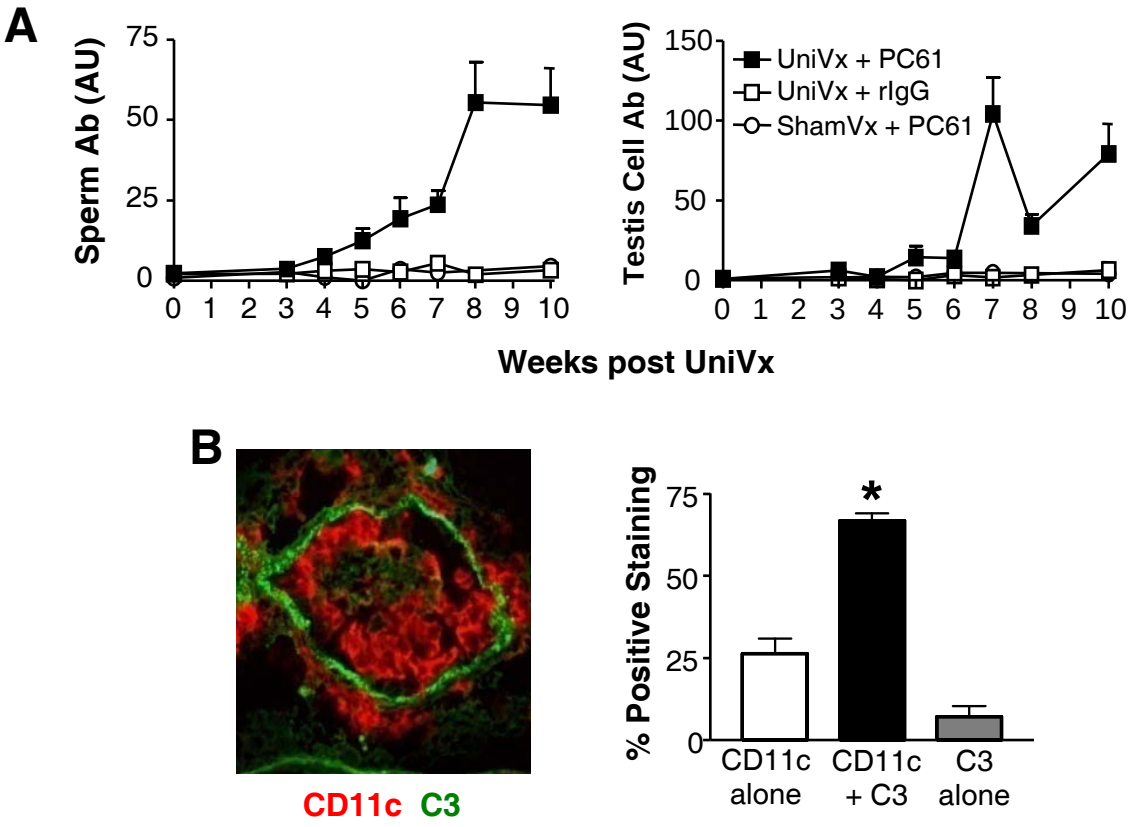

Figure 18: Autoantibodies in UniVx mice co-localize with DC. (A): Kinetics of anti-sperm (left) and anti-testis cell (right) antibodies in sham UniVx (ShamVx) or UniVx mice treated with PC61 or rIgG (AU = arbitrary units). (B): Co-localized CD11c+ dendritic cells and C3+ immune complex (*p=0.008, n=5). P-values determined by Mann-Whitney test.

effector T cell response in the draining LN, the onset of the effector T cell response as well as the magnitude of response in Treg depleted mice is considerably increased.

Serum autoantibodies directed to sperm and testicular cell antigens were followed by ELISA and were negative in both control rIgG treated UniVx and sham UniVx mice (Figure 18A). Treg depleted mice, however, developed a significantly high anti-sperm antibody response as early as 4 weeks post vasectomy that rose until 8 weeks and plateaued out to 10 weeks (Figure 18A). Similarly, the anti-testicular cell autoantibody response began to rise at 5 weeks and peaked at 7weeks (Figure 18A). Immune complexes could also be found in the testes of Treg depleted mice, as defined by complement C3 (Figure 18B) and were often co-localized with CD11c+ DC (Figure 18B), suggesting that immune complexes may play a role in targeting DC to the testes or activating endogenous testicular cells.

V.B.3. Post vasectomy orchitis is dependent on CD4+ T cells.

Although CD4+ cells were activated in the draining LN, the contribution of CD4+ cells to disease induction was unclear. Although the absolute number of live cells was similar between control rIgG and Treg depleted UniVx mice, the absolute number of CD45+ cells was higher in the Treg depleted mice (Figure 19A and B). CD3+ T cells in the testes of Treg depleted UniVx mice mostly CD4+ (CD4:CD8 ratio = 10:1) (Figure 19C). When these CD4+ cells were isolated and activated in vitro, about 20% produced IFNg and less than 2% IL-17 (Figure 19D).

The dominant Th1 inflammation in the testes and strong CD4+ Teff activation in the draining LN suggested that CD4+ cells play a significant role in disease induction. Indeed, when CD4+ cells were depleted in vasectomized and Treg depleted mice, disease

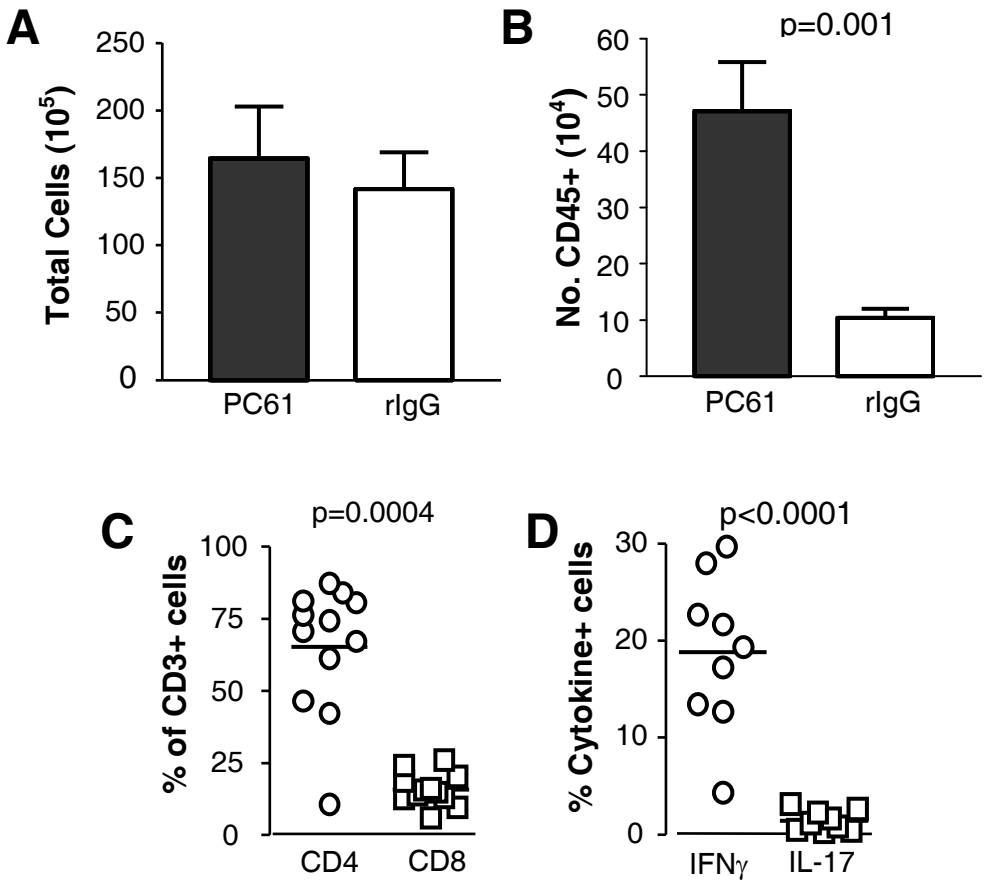

Figure 19: CD4+ cells producing IFNg in the testes of UniVx and Treg-depleted mice. (A and B): Total cell numbers (A) and number of CD45+ cells (B) in the testes of UniVx mice. (C): Percentage of CD4+ and CD8+ cells (gated on CD45+CD3+ cells). (D): Production of IFNg and IL-17 from in vitro stimulated CD4+ testis cells. P-values determined by Mann-Whitney test.

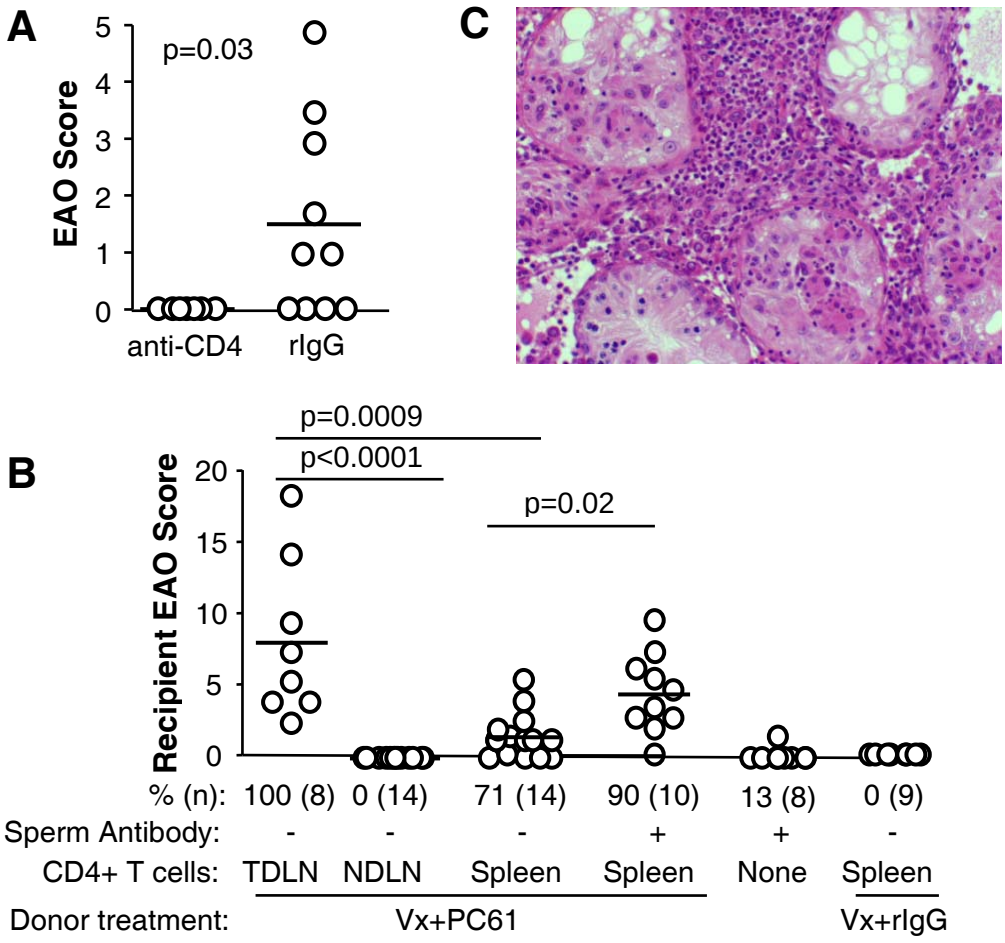

Figure 20: CD4+ pathogenic T cells are necessary and sufficient for EAO and are enhanced by sperm autoantibody. (A): In UniVx mice with Treg depletion, CD4 antibody treatment completely inhibits EAO. (B): Maximal EAO is induced by adoptive transfer of CD4 T cells from testis-draining LN (TDLN); and the low EAO severity induced by spleen T cells is enhanced by antibody co-transfer. Recipient EAO determined at 5 weeks. (NDLN = non-draining LN.) (C): Histopathology of TDLN adoptive transfer (H&E; x400).

was completely abrogated (Figure 20A), although a low level antibody response remained (data not shown). Furthermore, when in vitro activated CD4+ cells from Treg depleted UniVx mice were adoptively transferred to irradiated (testis shielded) recipients, mice developed orchitis (Figure 20B and C). Severe disease was only transferrable by CD4+ T cells isolated from the testis draining LN and, to a lesser extent, the spleen, while those from non-draining LN or control mice were non-pathogenic. Therefore, pathogenic CD4+ cells that are required and sufficient for disease induction reside primarily in the draining LN.

V.B.4. Antibody intensifies the adoptive transfer of orchitis.

The strong systemic antibody response and the presence of immune complexes co-localized with CD11c+ cells in the testes imply that antibody may also play a role in disease induction. IgG was isolated from Treg depleted and UniVx mice and injected into recipients of CD4+ splenic cells from similarly treated mice. Addition of disease-specific antibody enhanced the severity of orchitis from transferred splenic CD4+ cells (Figure 20B), indicating that antibody synergizes with CD4+ T cells to induce maximal disease.

V.B.5. Zonadhesin is a dominant and pathogenic autoantigen in post vasectomy orchitis. (These biochemical studies were done collaboratively with Dr. Dan Hardy and Dr. Steve Tardif in Texas Tech University, Lubbock Texas.)

The robust serum autoantibody response in UniVx and Treg-depleted mice and its pathogenic affect upon transfer spurred us to look for potential autoantigens that are

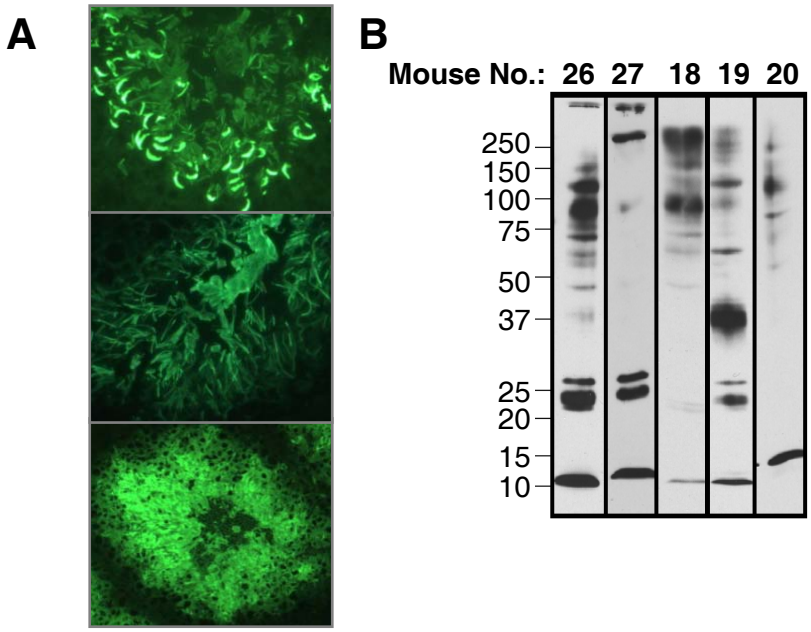

Figure 21: Serum autoantibodies from UniVx and Treg-depleted mice recognize multiple sperm antigens. (A): Indirect immunofluorescence of serum autoantibodies that recognize acrosome (top), tail (middle) and cytoplasmic (bottom) antigens on normal testicular tissue (x200). (B): Multiple sperm antigens are targets of serum autoantibodies when developed for a longer period of time on an 8-15% gradient gel.

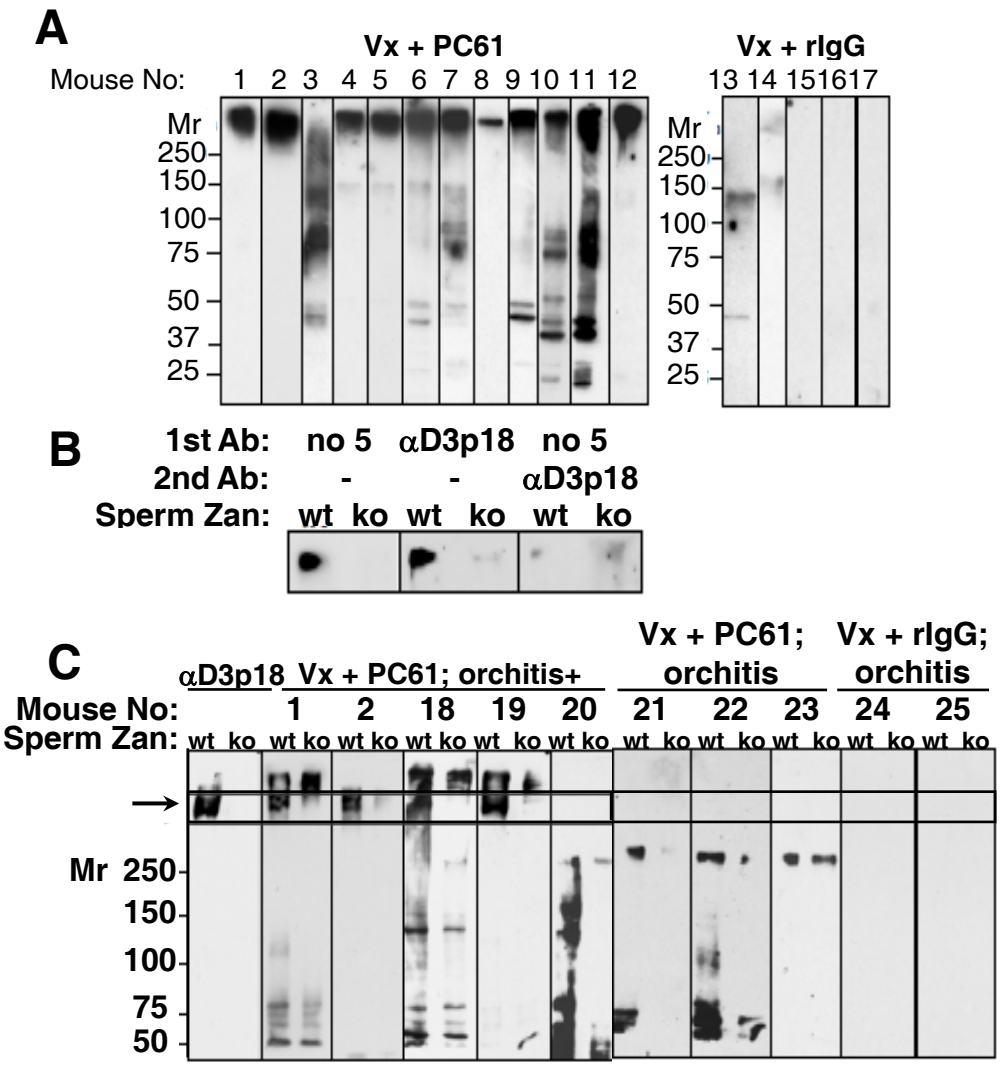

Figure 22: Sperm zonadhesin (Zan) is a dominant orchitogenic autoantigen targeted by autoantibodies of UniVx mice with Treg depletion. (A): Most sera from mice with EAO reacted with a 340 kD band. (B): Serum antibody from mouse 5 reacts with the Zan band of wild type but not Zan -/- sperm, and its binding to the Zan band is blocked by rabbit anti-D3p18 of Zan (□D3p18). (C): Detection of serum Zan-specific antibodies (arrow = *Mr*340). (Representative of 3 independent experiments; 4-8% gradient gels.)

targeted by the serum autoantibodies. Reactivity against sperm acrosomes, sperm tail, and spermatocyte cytoplasm in normal testes could be seen after indirect immunofluorescence with serum autoantibodies (Figure 21A). And initial immunoblots on sperm proteins using an 8-15% gradient gel also elucidated several patterns of serum antibody reactivity (Figure 21B). However, when a 4-8% gradient gel was used to separate sperm proteins, a band of 340kDa was recognized after very short chemiluminescence exposure (1-5 seconds) by a majority of sera from UniVx and Treg depleted mice (Figure 22A). Because this band had characteristics similar to Zonadhesin (Zan), a sperm acrosome protein under investigation by one of our collaborators (D. Hardy and S. Tardif), we set out to prove that the 340kDa band of dominant reactivity was, in fact, Zan. This was achieved by two methods. First, positive sera lost reactivity when sperm proteins from Zan deficient mice were used (Figure 22B). Second, pre-incubation with serum antibodies blocked the ability of a Zan-specific polyclonal rabbit antibody to recognize Zan (Figure 22B). Finally, the incidence of sera that is Zan-specific was determined after incubation with sperm proteins from wild type and Zan deficient mice. All together, 86% (5/6) mice had reactivity to Zan (Figure 22B and C) while Treg depleted but orchitis negative or control treated groups of mice were negative (0/12) (Figure 22A and C). These data indicate that Zan is a predominant, although not sole, autoantigen recognized by sera from vasectomized and Treg depleted mice.

To determine whether Zan is also a pathogenic autoantigen, mice were immunized with a 120mer of Zan (Figure 23A) conjugated to GST[90]. (This 120mer was also used to generate the rabbit antibody to Zan used in earlier experiments.) Forty-five percent of mice immunized with ZanD3p18 in CFA developed orchitis (Figure 23B and

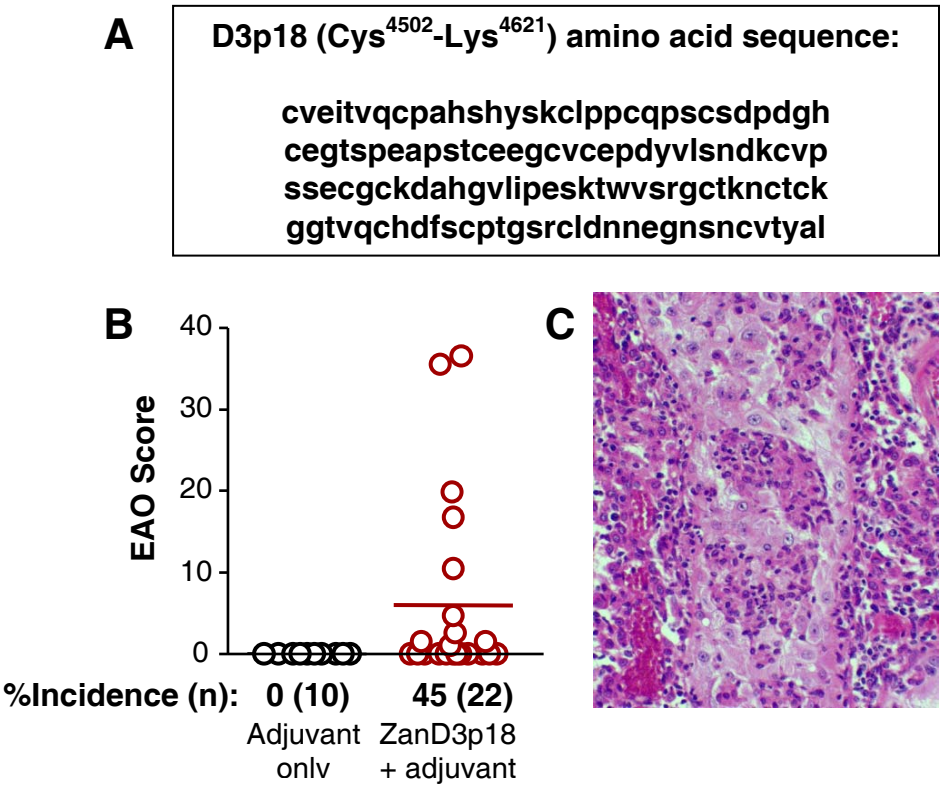

Figure 23: Zan D3p18 is a pathogenic autoantigen. (A): Amino acid sequence of the120mer D3p18 of Zan. (B): B6AF1 mice were immunized with Zan D3p18 in adjuvant or adjuvant only (Incidence p=0.01; Fischer-exact test). (C): Histopathology of ZanD3p18 immunized mice showing infiltration of the seminiferous tubules and gem cell loss (H&E; x400).

C), proving that Zan is, indeed, orchitogenic. Thus, T cell and antibody responses to Zan may play an important role in orchitis post-vasectomy and Treg depletion.

V.B.6. Susceptibility to vasectomy-induced orchitis is genetically dominant and orchitic autoantigens are different between susceptible strains.

Thus far we have studied B6AF1 mice, a strain known to be susceptible to other models of autoimmune orchitis, while the parental strains have varying disease susceptibilitis[53, 125]. Thus, we Treg-depleted B6 and A/J mice were UniVx to determine susceptibility. Both strains of mice developed similar incidences of epididymal granuloma as B6AF1 mice (B6: 86% (n=7) and A/J: 83% (n=6)). B6 mice, however, were completely resistant to orchitis development and did not have a measureable autoantibody response (Figure 24A and B). All of the UniVx and Treg depleted A/J mice developed orchitis and a robust antibody response, of a magnitude similar to or higher than B6AF1 mice (Figure 24A and B). These results are similar to published results on the propensity for B6 and A/J to develop EAO after d3tx[53]. Interestingly, western blot analysis revealed that serum autoantibodies from vasectomized A/J mice do not recognize p340 as a dominant autoantigen (Figure 24C). Thus, post-vasectomy orchitis is a genetically dominant phenomenon, although the major orchitic autoantigen may vary between strains.

V.B.7. Late depletion of Treg in vasectomized mice does not allow for orchitis development.

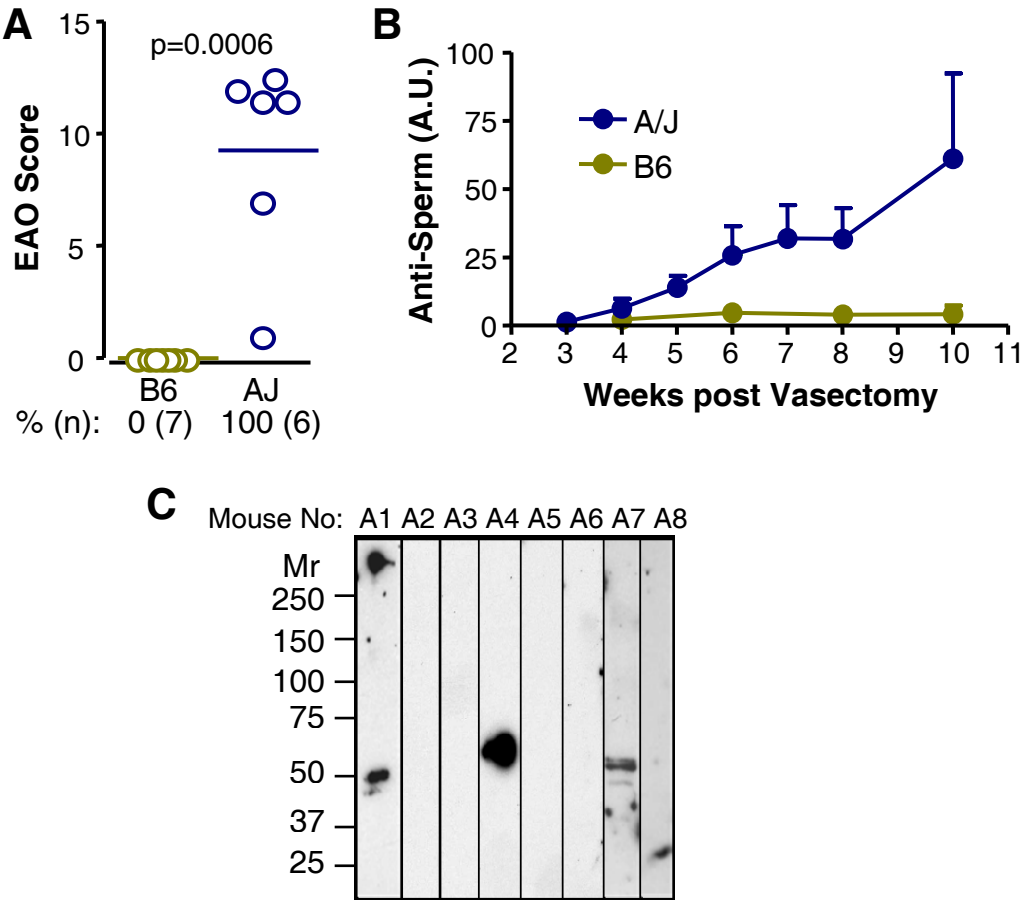

Figure 24: Genetically dominant susceptibility to orchitis after UniVx and Treg depletion. (A and B): Disease score (A) and anti-sperm autoantibody response (B) in Treg depleted UniVx C57BL/6 and A/J mice studied over 10 weeks. (C): Most sera from A/J mice did not react with a 340 kD band (4-8% gradient gel).

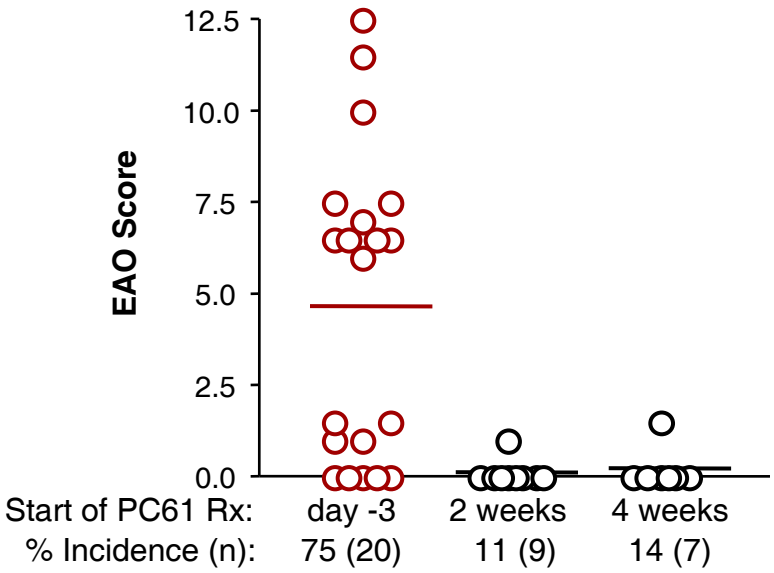

Figure 25: Depletion of Treg two weeks or more after UniVx does not induce EAO.

Mice were studied 10 weeks after beginning of PC61 treatment.

To assess whether orchitis would develop when Treg are depleted late after vasectomy (as would be most like in the human population), we delayed Treg depletion for 2-4 weeks. Orchitis failed to develop using this strategy (Figure 25). Thus, the timing of Treg depletion required to induce disease must occur at a precise time early during danger signal generation. This also suggested that vasectomy alone may not simply result in a lack of disease, but actually induce a state of unresponsiveness, or tolerance, to disease development.

V.B.8. Vasectomy leads to the development of tolerance to testicular antigens.

In order to test this hypothesis, unilaterally vasectomized mice were immunized with testis homogenate in CFA, which induces experimental autoimmune orchitis (EAO)[126]. UniVx mice were tolerant to disease induction as they had significantly reduced orchitis severity, with patchy, focal inflammatory lesions as compared to total testicular destruction seen in non-vasectomized and sham UniVx mice (Figure 26A and C). Antibody and T cell proliferative responses to testicular cells were also reduced in vasectomized mice (Figure 26). Tolerance is specific to testicular antigens vasectomized and sham vasectomized mice developed EAE of similar severity and timecourse after immunization with MOG35-44 (Figure 26B).

Furthermore, when the vasectomized testis and epididymis were removed 3 weeks after UniVx, and then immunized, mice developed EAO similar to non-vasectomized mice (Figure 26A). Thus, the granuloma is necessary to induce a state of testicular antigen-specific state of tolerance after vasectomy.

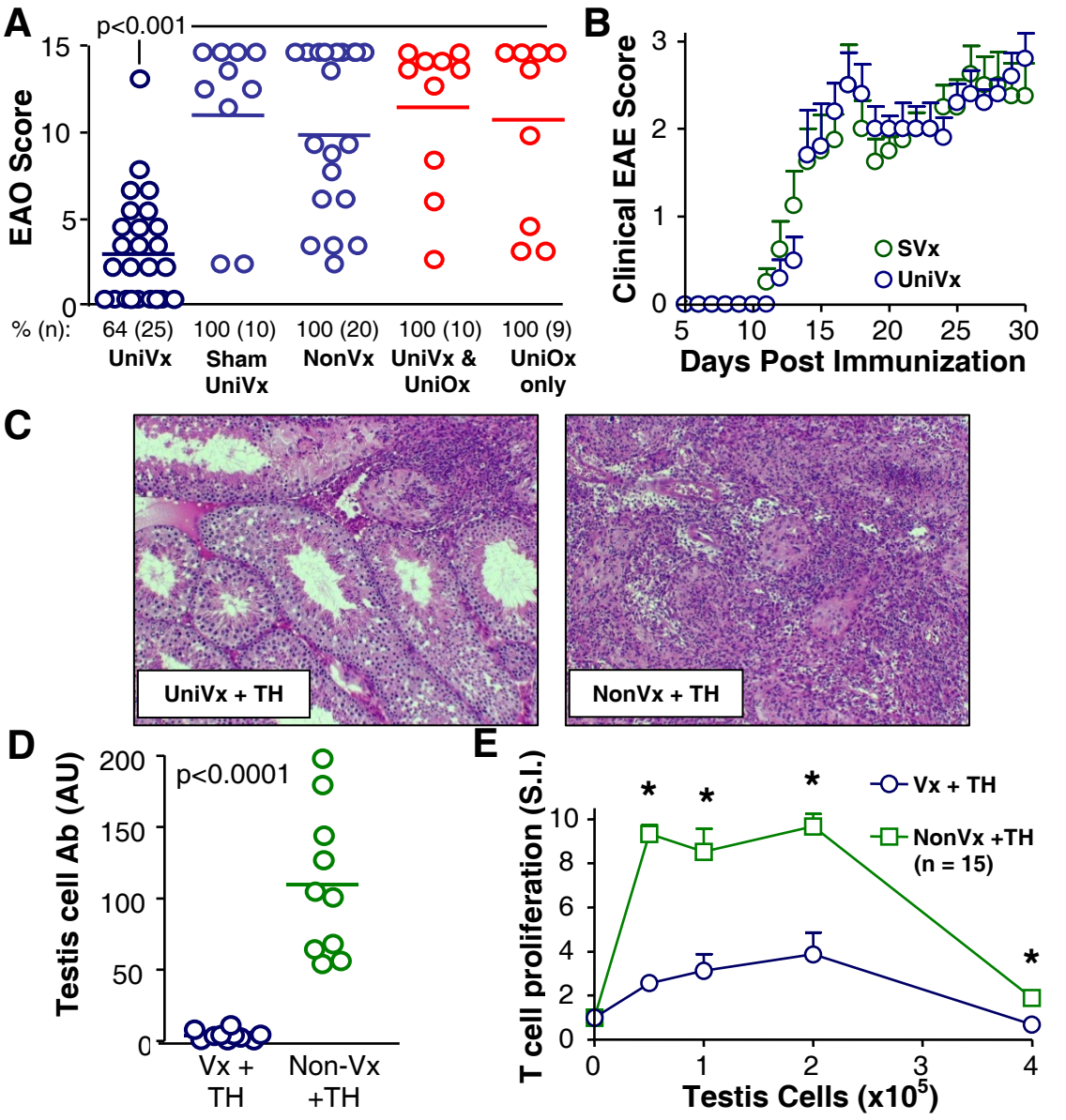

Figure 26: UniVx induces tolerance to testis (TH) but not brain antigen. (A): UniVx mice develop less severe EAO compared with controls; and the difference is eliminated by epididymo-orchiectomy on the Vx side (UniOx). (B): EAE progression. (C): Orchitis is focal in UniVx, and diffuse and severe in nonVx (H & E stain, x200). Significant reduction of testis-specific antibody (D) and T cell proliferative (E) responses in Vx mice (representative of 3 experiments; *p<0.02; p-values determined by student t-test).

V.C. Conclusions

In mice, as in humans, vasectomy induces severe epididymal granulomas, which may be induced through the release of cytoplasmic and nuclear contents from a constant supply of necrotic sperm (Figure 14), which have the potential to activate innate cells through a host of DAMP and PAMP receptors, including RAGE and TLR[72]. Despite the robust response in the epididymal granuloma, there is no induction of EAO or an autoantibody response. When Treg are depleted, however, a robust autoimmune response is induced, highlighted by severe EAO that leads to infertility. Autoimmune disease is dependent on CD4+ T cells, which are able to transfer severe EAO. Additionally, autoantibodies develop in vasectomized and Treg-depleted mice which form immune complexes and co-localize with DC in the testes. These autoantibodies synergize with CD4+ T cells in disease transfer, and may thus play a role in orchitis pathogenesis. Using these serum-derived autoantibodies, we have identified Zan as a major pathogenic target autoantigen. These results support the proposal that Treg normally function to avoid the propagation from a localized danger signal to pathogenic autoimmunity [127].

Interestingly, Treg depletion 2 weeks or more after UniVx does not induce EAO. Instead, vasectomy alone induces testis antigen-specific tolerance. This indicates that presence or absence of Treg at the time of vasectomy may control a checkpoint between tolerance maintenance and autoimmune disease induction; rather than simply between disease and lack of disease. There are several possible mechanisms by which Treg can control post-vasectomy tolerance versus autoimmunity. They may directly control the nature of Ag specific T cell responses incited by a common set of innate cellular responses. Additionally, Treg have recently been shown to induce an anti-inflammatory

(M2) phenotype in peritoneal macrophages, while Teff induce a pro-inflammatory (M1) phenotype[128]. Induction of anti-inflammatory macrophages may alone mediate tolerance, alternatively, they may also lead to the induction of anti-inflammatory granuloma formation [129].

While it is tempting to speculate that Treg are the lone modulator of tolerance versus autoimmunity, a single experiment in which Treg were partially depleted in vasectomized mice at the time of immunization failed to show a decrease in the tolerance status of vasectomized mice. Because Treg depletion was incomplete and vasectomy may skew Treg in the epididymis and TDLN towards testis antigen specificities, the residual Treg may still be able to control subsequent EAO induction. Additionally, Treg may act acutely (within days) to induce tolerance, for instance by inducing anti-inflammatory macrophages and/or granuloma. Thus, this preliminary study does not disprove that Treg are pivotal in tolerance induction, but it does suggest that we think about alternative modes of tolerance induction, including deletion or anergization of testis antigen-specific effector T cells, which will be elucidated in future studies.

Although sperm autoantibody development is the normal finding reported in the murine literature, inbred control mice in this study did not develop sperm antibodies. It is possible that the length of our study was too short to allow for antibody detection, as most other studies look at mice 4 months to a year after vasectomy. However, instead of mounting an autoimmune response, mice developed super-physiologic tolerance to EAO and autoantibody induction. In vasectomized outbred populations, including humans and guinea pigs, the antibody response is generally limited to about 50% [79]. Our unexpected

finding in inbred mice suggests that tolerance to sperm antigens may be the basis for the seronegative phenotype in the outbred population.

Chapter VI. Summary and significance

The results presented in this thesis shed light on the in vivo mechanisms by which Treg prevent autoimmune disease. In the first chapter, we look for the presence of Treg in d3tx mice, which develop organ-specific autoimmune disease. The hypothesis of late Treg ontogeny originally came about from Sakaguchi's group, which showed a lack of CD4+CD25+ cells detected in the spleen and the ability to suppress disease with CD4+CD25+ Treg[7]. Despite earlier reports that d3tx mice may harbor a predominantly neonatal and autoreactive repertoire of T cells, Sakaguchi's hypothesis became the principal view on the mechanism of d3tx-induced disease. Even later reports using the Foxp3-gfp reporter mouse corroborated a lack of Foxp3+ Treg in the spleens and thymi of 3 day old mice[62]. Still other reports showed the presence of CD4+ CD25+ Treg in the LN of 2 day old mice and even found an increase of CD4+CD25+ Treg in d3tx mice over time, but attributed this rise to an expansion of activated Teff[98]. Our findings that Treg are present and functional in d3tx mice corroborates previous and subsequent reports[99, 102] and argues against late ontogeny of Treg being the sole mechanism of d3tx-induced disease. Unique to our study, however, is the detection of disease-suppressing Treg from the LN of 3 day old mice and also the ability of Treg from d3tx mice to prevent AOD and gastritis, diseases relevant to d3tx autoimmune disease. Furthermore, we found that these disease relevant Treg function within the d3tx host to dampen ongoing disease. Altogether, these results suggest that enrichment of pathogenic Teff may be the culprit in disease induction in d3tx mice.

Indeed, it is known that the T cell repertoire in neonates differs from adult mice, and may be skewed towards an increase in autoreactivity[57, 58]. But the presence of

autoreactive T cells per se is not enough to induce autoimmune disease, as they are present in normal neonates and even adults. Other factors must exist that cause activation of Teff and prevent their ability to be suppressed by Treg. One possibility is that the severe lymphopenia created by d3tx increases the APC:T cell ratio, thus preventing Treg from competing out Teff for sites of cognate antigen presentation on APC. This hypothesis is fully testable in vitro by using standard Treg suppression assays and altering the Treg:Teff:APC ratio. We might expect that increasing the number of APC would decrease the suppressive capacity of Treg.

Whatever the mechanism of activation, the pathogenicity of Teff becomes fixed during the first 2 weeks of life because: 1) after 2 weeks, infusion of normal Treg to d3tx mice no longer prevents disease, and 2) depletion of Treg from d3tx mice after 2 weeks also does not enhance disease (appendix II). This permanent alteration in Teff may reflect a decrease in inhibition of TCR signaling. We have accrued preliminary data in collaboration with Dr. Ulrike Lorenz's lab showing that cbl-b, a non-functional mutation in which elicits autoimmune disease[130], is decreased in CD4+CD25- effector T cells from d3tx mice. If repeat experiments confirm these results, further in vitro studies assaying the ability of Teff from adult d3tx mice to be suppressed by Treg from normal mice could corroborate that Teff from d3tx mice are resistant to suppression.

The finding that Treg within the d3tx mouse dampen ongoing disease is an intriguing one. It suggests that endogenous Treg control the level, and perhaps the repertoire of diseases in d3tx mice. Could this then be a mechanism to explain the strain-dependent susceptibility to different organ-specific diseases after d3tx? Genetic analysis of AOD susceptibility in B6 and A/J mice and diabetes susceptibility in NOD mice

identified the same disease positive loci: AOD2[54] and Idd3[131], respectively. Within the AOD2/Idd3 loci is the IL-2 gene, which is known to play a role in the survival and fitness of Treg[33]. Studies have also shown that A/J mice have polymorphisms in the promoter region of the IL-2 gene that cause a reduced production of IL-2[132]. Thus, disease susceptible mice produce less IL-2 and subsequently have less fit Treg, resulting in disease and/or disease of higher severity. Indeed, preliminary studies in d3tx B6 mice show that severe AOD develops when Treg are depleted (appendix III). Furthermore the repertoire of organ-specific diseases in all strains of mice expands when Treg are depleted, and includes disease of the lung which histologically mimics human pulmonary vasculopathies (appendix IV).

In the future, it will be appealing to determine what, if any, differences exist in Treg from strains of mice that do (A/J and B6AF1) and do not (B6) develop disease after d3tx. Differences might exist in the inherent function of Treg from various strains, detectable by in vitro suppression assays. Additionally, it would be interesting to look at the levels of different molecules known to play a role in Treg function, including CTLA-4[45], or markers identifying a more function Treg subset, such as CD103[133].

The second and third chapters of this thesis examine the nature of disease-preventing Treg in normal mice. We find in chapter two the existence of organ-specific Treg, with the capacity to suppress autoimmune disease of one organ but not another. More importantly, we also showed that such organ specific Treg are highly enriched in the LN draining the organ. This accumulation is dependent on organ antigen, as depletion of prostate antigen abrogates, and addition of prostate antigen restores

accumulation. Effector T cells of different specificities, meanwhile, are found equally throughout the different LN.

In the future, it will be exciting to determine the precise mechanism that leads to Treg accumulation. Because specific chemokine receptors and integrins have been described for homing of Teff and some Treg to gut- and skin-associated LN, determining the cytokine and chemokine receptor and integrin profiles of Treg from ovary-, lacrimal gland- and prostate-draining LN would be a logical place to begin. If differences are found and confirmed by other methods (flow cytometry, western blot, or real time rtPCR), further evaluation of the specific cytokines/chemokines involved in LN-specific homing of Treg and their source within the LN is warranted.

We hypothesized that, when faced with organ-specific danger signals, these organ- and disease-specific Treg detect activated APC presenting self antigen and prevent the development of autoimmune disease. Proof of this hypothesis came from using vasectomy as a model of danger and globally depleting Treg, which resulted in development of autoimmune orchitis. Orchitis was accompanied by a robust antibody response, unseen in control vasectomized mice, and required CD4+ T cells. Although antibodies isolated from the sera of diseased mice were unable to induce disease per se, they did amplify suboptimal disease transfer with splenic CD4+ cells. The role of autoantibodies in autoimmune disease remains controversial as they are the hallmark of autoimmune diseases such as lupus, but are unable to transfer disease in and of themselves[134]. We have shown in previous work that autoantibodies can induce disease in neonates, however not in adult mice[135]. Thus, the role of pathogenic autoantibodies in

orchitis demands further work into what mechanisms are responsible for synergy of autoantibodies with T cell mediated disease.

Using these serum autoantibodies, we were also able to identify the first murine orchitic autoantigen, zonadhesin. The majority of Treg depleted and vasectomized mice had serum antibodies specific for Zan and a 120mer of Zan induced orchitis when combined with CFA to immunize mice.

Excitingly, we found that vasectomy alone induces a heightened state of tolerance specific for testicular antigens. Whether Treg are responsible for tolerance induction is not completely clear from these studies, but it remains highly probable that they are involved in some capacity. It is tempting, then, to conjecture that, much like d3tx mice, inherent differences in the strength, fitness, or TCR repertoire of Treg may influence the outcome of vasectomy – either autoimmune response or tolerance induction. Indeed, even when mice were partially depleted of Treg after vasectomy, some strains – B6AF1 and A/J – developed disease, while others (B6) did not, consistent with susceptibility to autoimmune disease after d3tx[53]. In addition, delayed Treg depletion in vasectomized mice also failed to induce orchitis. It will be interesting, then, to more completely deplete Treg from mice with these resistance states to see if orchitis will develop. This would provide evidence that Treg are pivotal in post-vasectomy tolerance induction. Whether the resistance state is due to expansion of antigen specific Treg will be investigated in mice bearing ovalbumin as surrogate sperm-specific autoantigen.

Another intriguing observation is that only 50% of the human population exhibits an autoantibody response after vasectomy[79]. If our results can be extrapolated to men, the other 50% may represent a population of men conditioned to remain hyporesponsive

to sperm antigens. While this would minimize testis autoimmune disease development, it might also impair the level of antigen-specific immune surveillance and favor susceptibility to neoplasia, particularly tumors expressing the cancer/testis antigens [136]. It could also render individuals less responsive to vaccines utilizing cancer/testis antigens. Epidemiologic studies correlating autoantibody response and cancer rates after vasectomy would shed more light on this potential association.

In total, the results presented in this thesis aid our understanding of how Treg function to prevent ongoing disease in the d3tx mouse and how they prevent disease in the face of organ-specific danger stimuli. In the later case, organ antigen specific Treg accumulate in organ-draining LN and act as sentinels, detecting the presence of activated APC bearing self-antigens. Using vasectomy as the organ-specific danger signal has also allowed us to identify unique post-vasectomy sequelae, including the identification of a unique autoantigen in the autoimmune disease state and, importantly, tolerance induction. While it is known that vasectomy potently induces epididymal sperm granuloma, it remains to be seen whether tolerance induction can be applied more broadly to cases of sterile inflammation or if it is related to unique factors present in the epididymal environment.

Chapter VII. References

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APPENDIX

I. Future studies

A. To determine whether effector T cells from d3tx mice are resistant to suppression by Treg via in vitro suppression assays.

B. Examine the mechanisms of LN-specific accumulation by Treg.

C. To look at the differences in vasectomy induced granuloma from normal and Treg-depleted mice.

D. Examine changes in macrophage and dendritic cell phenotype in early epididymal inflammation of normal and Treg depleted mice.

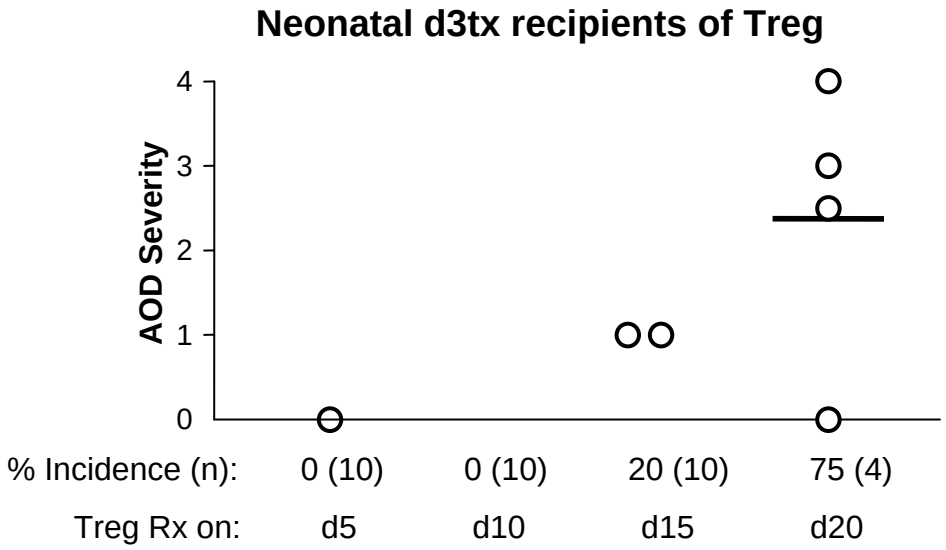
E. To elucidate the mechanisms by which autoantibodies synergize with CD4+ T cells to amplify disease transfer.

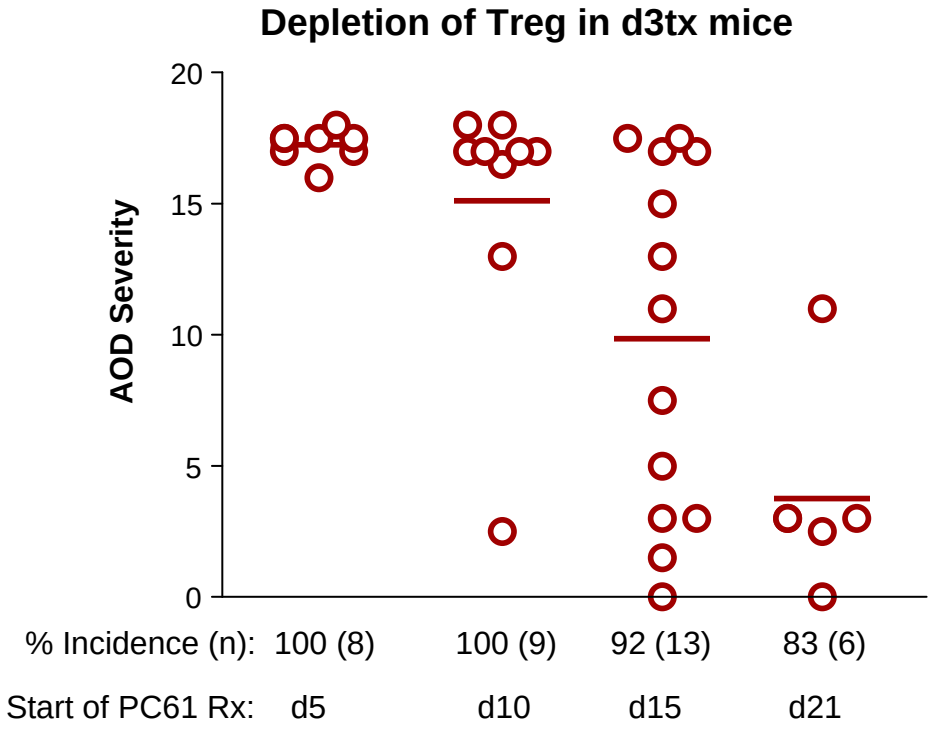
F. To determine if Treg are responsible for tolerance induction after vasectomy.

1. Depletion of Treg in B6 DEREG mice during vasectomy
2. Depletion of Treg at the time of immunization to induce EAO in vasectomized B6AF1 DEREG mice
3. To determine potential mechanism of Treg-dependent tolerance in vasectomized mice
    a. Timing of tolerance induction
    b. Expansion/accumulation of antigen-specific Treg (DO11.10) in vasectomized mice expressing a surrogate testicular antigen (ovalbumin).
    c. Induction of antigen-specific Treg

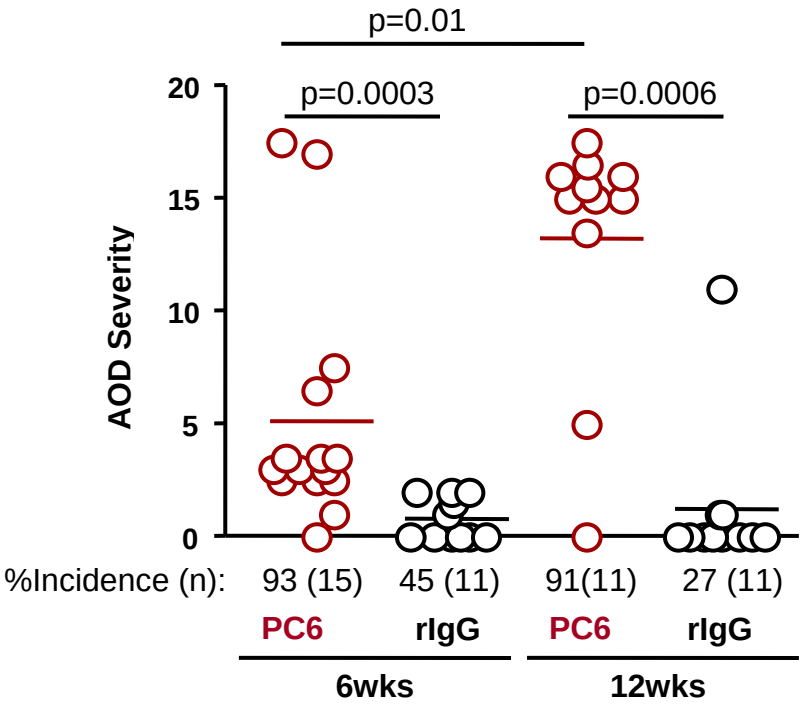
G. To see if vasectomy-induced tolerance is mediated by deletion or anergy of testis-specific effector T cells

II. Effector T cell function is fixed during the first two weeks after d3tx.





III. AOD develops in d3tx C57BL/6 mice after Treg depletion.



IV. Multiple organ-specific autoimmune diseases occur in d3tx and Treg depleted mice.

Organ	Strain (12wks)					
	B6		B6AF1		Balb/c	
	PC61	rIgG	PC61	rIgG	PC61	rIgG
n (M/F)	16/11	12/11	8/4	7/10	4/6	3/2
Ovary	91 *	27	100	67	100	67
Prostate	82	82	100	63	75	0
Testes	41	18	38	0	75	0
Epididymis	41	27	63	54	75	0
Lacrimal Gland	91 *	37	92	50	78	40
Salivary Gland	32	5	33	0	44	0
Lung	70 *	33	42	17	33	40
Trachea	50	13	55*	0	43	20
Stomach	20	5	42	0	89	20
Liver	12	6	8	0	11	
Thyroid	13	0	25	17	20	0
Pancreas	12	0	0	0	22	0
Adrenal Gland	0	0	0	17	0	0
Large Intestine	0	0	0	0	0	0
Small Intestine	0	0	0	0	0	0
Kidney	0	0	0	0	0	0
Heart	0	0	0	0	0	0
Skin	0	0	0	0	0	0
Brain	0	0	0	0	0	0
Eye	0	0	0	0	0	0

*p=0.05 (vs. rIgG)