Received: 14 April 2018 | Revised: 6 June 2018 | Accepted: 13 June 2018

DOI: 10.1111/and.13092

INVITED REVIEW

WILEY ANDROLOGIA

The role of regulatory T Cells in autoimmune orchitis

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Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas; Universidad de Buenos Aires; Agencia Nacional de Promoción Científica y Tecnológica

Abstract

Regulatory T cells (Tregs) mediate tolerance to self-antigens maintaining immune homeostasis. Defects in the number and function of Tregs lead to aberrant immune responses to autologous components, thereby causing autoimmune diseases. Male infertility as a result of immune testicular damage follows through auto-reactive T-cell activation by antigens or pathogens that disrupt testis tolerance mechanisms. In this review we summarise the main evidence on Treg behaviour in inflammatory testicular pathologies focusing on reports on experimental autoimmune orchitis. Increased numbers of different Treg phenotypes are observed in the chronically inflamed testis and in lymph nodes draining to it; however these cells are outnumbered by effector T cells. Distortion of the effector/regulatory cell balance in favour of a pro-inflammatory response is suspected to contribute to exacerbation of autoimmune disease. Under inflammatory conditions, effector T-cell subsets can overwhelm the inhibitory effect of Tregs, and pro-inflammatory cytokines may directly or indirectly affect the ability of Tregs to control autoimmunity. Therefore, Tregs alone may not be sufficient to limit excessive T-cell activation in autoimmune settings. Treg immunotherapy for autoimmune disease treatment aims to restore the normal balance of effector and Tregs in the inflamed tissue. Therapies combining the transfer of Tregs with Treg-stabilising drugs are expected to be the most effective to restrain autoimmune diseases.

KEYWORDS
autoimmunity, inflammation, testis, Treg

1 | INTRODUCTION

Tolerance to self-antigens is a critical process to avoid autoimmune diseases. Tolerance begins in the thymus before maturation and circulation of T cells (central tolerance) and continues in the peripheral lymphoid organs (peripheral tolerance) where T cells constantly recognise auto-antigens expressed extrathymically. Regulatory T cells (Tregs) constitute a phenotypic and functionally heterogeneous lymphocyte subset which has a central role in regulating tolerance to auto-antigens, maintaining tissue homoeostasis (Wing & Sakaguchi, 2012). The Treg family is composed mainly of CD4+ cell subsets: natural CD4+CD25+ Tregs of thymic origin and adaptive CD4+CD25+ Tregs generated from conventional CD4+CD25− T cells in peripheral lymphoid organs. CD4+CD25+ Treg function is critically dependent on expression of the transcriptional repressor Foxp3, the most reliable marker to identify this T-cell subset at the present. Besides these cells, some reports show that CD8+T cells can also express Foxp3 and have immunosuppressive functions (Singh, La Cava, Wong, Ebling, & Hahn, 2007; Tao et al., 2017). Mechanisms used by Tregs to suppress immune responses involve: (a) modulation of dendritic cell maturation and function; (b) cytolysis of target cells by a granzyme- and perforin-dependent mechanism; (c) disruption of metabolic pathways on effector T cells; and (d) production of inhibitory cytokines including IL-10, IL-35 and TGF-β (Vignali, Collison, & Workman, 2008). Continuous auto-antigen recognition and signalling through the T-cell receptor in tissue regional lymph nodes

(LNs) is essential for maintenance of Treg function in the periphery. Each regional LN is enriched by tissue-antigen-dependent Tregs that preferentially inhibit auto-pathogenic responses against antigens generated in the drained organ (Samy, Wheeler, Roper, Teuscher, & Tung, 2008; Setiady et al., 2006; Tung et al., 2017; Wheeler, Samy, & Tung, 2009).

Based on its ability to tolerate auto-antigens expressed by developing germ cells at puberty following the constitution of immune competence, the testis is called an immune privileged organ (Jacobo, Guazzone, Theas, & Lustig, 2011; Lustig & Tung, 2016). During spermatogenesis, novel self-antigens are expressed behind the Sertoli cell barrier in meiotic germ cells. As antibodies and immune cells are effectively blocked from arriving to the adluminal compartment of seminiferous tubules by the Sertoli cell barrier, this barrier is also presumed to prevent the passage of meiotic germ cell antigens into the interstitial compartment, thereby avoiding activation of auto-reactive immune cells. However, a weak blood–testis barrier that allows antigen leakage at *tubuli recti* and *rete testis* appears to be a relevant factor for establishment of testicular self-tolerance (Tung, Yule, Mahi-Brown, & Listrom, 1987; Takahashi et al., 2007). In a recent work, Tung et al. (2017) demonstrated that only some antigens are sequestered behind the blood–testis barrier in seminiferous tubules. Nonsequestered antigens discarded during spermiation by spermatids continuously egress from seminiferous tubules in residual bodies via a physiological pathway important for testis function and maintenance of systemic tolerance. In contrast, other antigens are sequestered and highly immunogenic. Importantly, systemic tolerance to nonsequestered antigens was also reported to be critically dependent on Foxp3+ Tregs. Many studies in recent decades largely established that Sertoli cells are relevant for maintenance of a tolerogenic testicular microenvironment. Sertoli cell immune privilege is attributed to several factors secreted by these cells, such as complement inhibitors, immunoregulatory cytokines, apoptosis inhibitors and anti-inflammatory prostanoids which can control immune response (Doyle et al., 2012). Interestingly, Campese et al. (2014) described a novel mechanism by which Sertoli cells promote the in vitro induction of functional CD4+CD25+Foxp3+ Tregs via Notch pathway signalling by JAGGED-1.

Immune dysregulation is a frequent cause of autoimmune diseases. Despite the fact that testis is an immunoprivileged organ, infection, trauma and immune-mediated inflammation are often causes or co-factors of fertility disorders in men (Li, Wang, & Han, 2012; Schuppe & Meinhardt, 2005). Defects in Treg function lead to abnormal immune responses to self-antigens (Grant, Liberal, Mieli-Vergani, Vergani, & Longhi, 2015). In particular, chronic testicular inflammation (orchitis) occurs in patients with impaired Treg function due to mutations in the gene AIRE which cause autoimmune polyendocrine syndrome-1 (Kekäläinen, 2007). The aim of this presentation is to review experimental evidence supporting our current knowledge of Tregs and their role in inflammatory testicular pathology, focusing on our data on experimental autoimmune orchitis (EAO).

2 | TESTICULAR IMMUNOPATHOLOGY AND T-CELL INVOLVEMENT

Infection and inflammation of the reproductive tract account for up to 15% of the male infertility or subfertility cases (Schuppe et al., 2008). In men, testicular inflammation frequently evolves as a complication of acute epididymitis produced by ascending infections with uropathogens such as *Escherichia coli* or *Chlamydia trachomatis*, approximately 40/10,000 persons/year being affected according to data from UK general practice (Loveland et al., 2017). A primary inflammatory process may occur in the testis as a complication of systemic infections caused by mumps, Coxsackie, Epstein–Barr, influenza and human immunodeficiency viruses. A more severe granulomatous orchitis may also develop as a manifestation of infections with bacterial agents that cause tuberculosis, syphilis, lepromatous leprosy and brucellosis (Schuppe et al., 2008). More recently, oligospermia together with a reduction in testosterone and inhibin B levels following Zika virus infection have been reported by Govero et al. (2016) in male mouse. However, the extent to which these results are predictive for humans remains to be clarified.

Noninfectious causes of testicular inflammation may also lead to infertility. In fact, testis may be involved in systemic or organ-specific autoimmune diseases (Schuppe et al., 2008). Male infertility as a result of testicular immune damage ensues through T-cell activation by antigens or pathogens that disrupt the testicular tolerance mechanism to self-antigens (Lustig & Tung, 2016). In fact, perivascular and peritubular inflammatory infiltrates were detected in 50% of testis biopsies analysed in a systemic evaluation of infertile patients (Schuppe et al., 2008). The presence of inflammatory infiltrates is also observed in other pathologies in which tissue injury due to trauma, infections, torsion, tumours, cryptorchidia or vasectomy facilitates the release of germ cell antigens (Pérez et al., 2013). Besides the increase of pro-inflammatory CD4+ T helper (Th)-1 cells, an imbalance of the Th17/Treg ratio in favour of Th17 cells producing IL-17, IL-21 and IL-22 was reported by Duan et al. (2011) in the interstitium of azoospermic patients with chronic inflammation. Moreover, IL-23-producing antigen presenting cells (Th17-inducing cells) were also detected in these patients.

Experimental models of autoimmune orchitis have contributed greatly to clarify immune mechanism compromise when immune tolerance in the testis is disrupted. EAO is characterised by immune cell infiltrates and damaged seminiferous epithelium exhibiting apoptotic germ cells and cell sloughing, thereby mimicking most of the major alterations observed in human testicular pathology. At the onset of disease in rats, mild immune cell infiltrate and few foci of seminiferous tubules with a variable and progressive level of damage occurs. At EAO chronic phase, increased interstitial cell infiltrate and severe damage to seminiferous tubules extending to the whole organ is observed (Jacobo, Guazzone, et al., 2011; Jacobo, Pérez, Theas, Guazzone, & Lustig, 2011). At this disease stage, seminiferous tubules become atrophic, granulomas are frequently observed and fibrosis and infertility ensue. Concomitantly, impairment of adherens

and gap cell junction and blood–testis barrier functionality also occur (Pérez et al., 2013). Quantification of immune infiltrating cells shows an increase in the number of pro-inflammatory macrophages (Rival et al., 2008), mature dendritic cells (Rival et al., 2007) and T cells, including effector and Treg subsets (Jacobo, Guazzone, Jarazo-Dietrich, Theas, & Lustig, 2009). In contrast with murine and human orchitis, immune cells were never observed inside seminiferous tubules in rats with EAO. Temporal variations were detected in the composition of effector T-cell population during EAO development. CD4+ Th1 and Th17 subsets co-govern EAO onset, whereas a central role is attributed to CD8+ T cells able to produce Th1- and Th17-type cytokines in establishment of chronic inflammation (Jacobo, Guazzone, et al., 2011; Jacobo, Pérez, et al., 2011). Pro-inflammatory cytokines such as TNF-α, IFN-ɣ, IL-6, IL-17, IL-23 and Fas ligand released by immune cells in the interstitium disrupt the immunosuppressive milieu promoting a severe imbalance in fine testicular immune regulation. Deleterious effects of these cytokines on germ cell viability and on blood–testis barrier permeability facilitate release of commonly sequestered and highly immunogenic antigens to the testicular interstitium, thereby amplifying local autoimmune response (Guazzone, Jacobo, Theas, & Lustig, 2009; Jacobo et al., 2012).

3 | ROLE OF TREGS IN TESTICULAR INFLAMMATION

Together with the influx of pro-inflammatory cells, CD4+ and CD8+ T cells expressing Foxp3 actively accumulate within the testis of EAO rats. At EAO onset, the number of CD4+ Tregs increases dramatically, but decreases as the disease progresses. However, the number of CD8+ Tregs which also increases at EAO onset remains stable through the chronic phase. CD25+ Tregs peripherally generated in vivo were the most abundant cells in both Treg subsets (Jacobo, Guazzone, Jarazo-Dietrich, Theas, & Lustig, 2009). In contrast to effector T cells, widespread in the interstitium, groups of Tregs were restricted to subalbuginea and peritubular areas near the seminiferous tubules damaged first at EAO onset.

During EAO development, the percentage of CD4+CD25+Foxp3+ Tregs also increase in draining LNs from testis (TLN) but not in draining LNs from the immunisation site (ILN). Most of these cells show an activated/memory phenotype. Foxp3+ cells are frequently localised in primary follicles of TLN from EAO rats but not from normal rats and are only occasionally localised in primary follicles of ILN from EAO rats. Similar distribution is observed for cells expressing Helios, a marker for an activated Foxp3+ Treg subset with enhanced suppressive capacity (Elkord & Al-Ramadi, 2012). Functional studies indicate that spermatic antigens may induce a strong T-cell proliferative response specifically in TLN where cells are continuously challenged by tissue antigens in both normal and inflammatory conditions. Cell suppression assays show that CD4+CD25^{bright}Foxp3+ Tregs from TLN of normal and EAO rats can suppress conventional T-cell proliferation. However, cells from EAO rats are more suppressive than those from normal rats, suggesting that these cells are over-activated by testicular inflammatory microenvironment. Moreover, Tregs express TGF-β which might have a key role in suppressing immune response (Jacobo, Guazzone, Pérez, & Lustig, 2015). These results agree with the notion that physiological status of each regional LN modulates the behaviour and functional capacity of Tregs. Overall results show that TLN are enriched by sperm-specific Tregs able to suppress auto-pathogenic T cells.

As observed in other autoimmune diseases (Dejaco, Duftner, Grubeck-Loebenstein, & Schirmer, 2006; Tao et al., 2017), in EAO functionally competent Tregs accumulate within the testis and LNs draining into it. Nevertheless, these cells fail to effectively suppress the advance of inflammation and tissue destruction. Although some progress has been made in ascertaining the cause of this deficiency, its precise mechanisms remain unknown. However, some hints emerge from numerous studies in several autoimmune settings: (a) inadequate numbers of Tregs; (b) intrinsic defects in Treg function; (c) decreased susceptibility of pathogenic T cells to suppression by Tregs; and (d) detrimental effects of pro-inflammatory cytokines on Treg function. In EAO, the first option offers a plausible explanation for los of testicular self-tolerance. Although Tregs increase in number in testis and TLN of EAO rats, these cells are outnumbered by auto-pathogenic T-cell subsets. This could explain why the increase in Treg number was unable to restrain exaggerated T-cell activation preventing disease outbreak. Deficiencies in the suppressive capacity of Tregs have been proposed to trigger autoimmune disease (Tao et al., 2017). Our results on cell functionality argue against the existence of intrinsic defects in CD4+CD25+Foxp3+ Tregs; however, as we only studied Tregs confined to the CD4+CD25^{bright} compartment, we cannot rule out the possibility that CD25− Tregs and Tregs expressing CD25 at low/intermediate levels might have some kind of deficiency. The relative proportion between each type of effector and regulatory cell subset is essential to understand why immunosuppressive actions of Tregs are insufficient. In this regard, Th17 cells appear to be a critical subset for EAO establishment, having been reported as a cell subset possibly refractory to Treg suppression. Several authors argued in favour of an inhibitory effect of pro-inflammatory cytokines from the inflammation site on Treg suppressive ability (Leipe, Skapenko, Lipsky, & Schulze-Koops, 2005; Pasare & Medzhitov, 2003). TNF-α and IL-6, cytokines relevant for EAO development, may affect Tregs in vivo, compromising their function locally. In addition, continuous activation of T cells by TCR stimulation and aberrant expression of costimulatory molecules, and stimulation of CD4+ T cells by GITR ligand have been proposed as mechanisms involved in Treg inhibition at inflammatory sites (Dejaco, Duftner, Grubeck-Loebenstein, & Schirmer, 2006).

Evidence of the immunosuppressive role of gonadal steroid hormones has been provided by numerous experimental and clinical studies (Fijak et al., 2015; Lee, Lydon, & Kim, 2012; Lee, Ulrich, Cho, Park, & Kim, 2011). Testosterone supplementation in rats during EAO development results in a protective effect both at local and systemic levels, mediated by expansion of competent CD4+CD25+Foxp3+ Tregs in the testis and by inhibition of Th1-type cytokine production in TLN (Fijak et al., 2011). The

importance of Tregs in the control of tolerogenic versus autoimmune response was also reported by Wheeler et al (2011) in a model of unilateral vasectomy characterised by apoptosis and necrosis of epithelial cells, severe inflammation and formation of granuloma in the epididymis. As tolerance maintenance depends on rapid response of Tregs to meiotic germ cell antigens released after vasectomy, Treg depletion simultaneous with unilateral vasectomy promotes development of spontaneous autoimmune orchitis induced by auto-pathogenic CD4+ T cells and autoantibody. Moreover, tolerance to testis antigens was locally compromised in mice genetically deficient in PD-L1, a co-inhibitory molecule involved in mediation of immunosuppression, suggesting a central role of induced Tregs (Rival et al., 2013).

4 | FUTURE PERSPECTIVES

Defects in the number and function of Tregs lead to aberrant immune responses to self-tissue components, thereby resulting in development of autoimmune diseases. Pathogenesis of many autoimmune diseases in humans, including rheumatoid arthritis, autoimmune diabetes, systemic lupus erythematosus and others, involves Tregs. Based on their immunosuppressive properties, many researchers try to treat autoimmune pathologies by transferring Tregs in vivo (Rakebrandt, Littringer, & Joller, 2016). However, progress of these approaches was limited thus far by technical difficulties associated with Treg isolation and ex vivo amplification protocols. The lack of a reliable surface marker enabling isolation of viable and highly purified Tregs without contamination with activated conventional T cells is perhaps the greatest obstacle. Dependence on Foxp3 expression for Treg function has made it the most specific marker for these cells (Azimi et al., 2016; Tao et al., 2017). However, as Foxp3 is an intracellular marker, current techniques cannot isolate live Foxp3+ cells. Also, the stability of Tregs is controversial. Reports show that human conventional CD4+CD25- T cells may transiently upregulate Foxp3 upon activation (Harbuz et al., 2010; Tran, Ramsey, & Shevach, 2007). Foxp3 expression in inducible Tregs is considerably less stable than in natural Tregs. As the expression level of Foxp3 is closely linked to Treg function, natural Tregs are a more stable subset and thus desirable for use in Treg-based therapy (Lal et al., 2009; Sakaguchi, Miyara, Costantino, & Hafler, 2010; Tran et al., 2007). Regardless of its origin, Foxp3 expression by Tregs could be lost in specific conditions. Natural CD4+Foxp3+ Tregs are converted into CD4+Foxp3– T cells when adoptively transferred in vivo into lymphopenic mice. Tregs become effector T cells when prompted by environmental inflammatory characteristics. If IL-6 is present, which occurs in most inflamed tissues, Tregs can down-regulate Foxp3 expression, thereby losing their inhibitory function. Moreover, they can even become Th17 cells that may also secrete IL-17, consequently amplifying autoimmune inflammation and tissue destruction (Bailey-Bucktrout et al., 2013; Komatsu et al., 2009; Yang et al., 2008). We clearly need detailed and comprehensive studies of inflammatory factors that might promote phenotypic changes in Tregs to increase the success rate and biosecurity of Treg-based therapies.

5 | CLINICAL IMPLICATIONS

Data indicate the high degree of plasticity of differentiated CD4+ T cells. This phenomenon could enable the immune system to adapt functionally to many physiological conditions during immune response. The plasticity of Tregs and Th17 cells is a critical factor to be considered in autoimmune disease treatment (Kleinewietfeld & Hafler, 2013). Relevance of Th17-like Tregs was reported in psoriasis (Bovenschen et al., 2011; Singh et al., 2013), autoimmune hepatitis (Longhi et al., 2012; Muratori & Longhi, 2013) and systemic sclerosis (Liu et al., 2013). However, no direct identification of these cells exists in patients with testicular inflammation or in animal models of autoimmune orchitis. Therefore, an important issue is clarification of the potential of the inflammatory milieu in testicular pathology to mediate conversion of Tregs into Th1-, Th17-like Tregs and the role of these cells throughout disease development. To derive full benefit from Treg-based therapy, the Tregs milieu must be considered. As proposed by Tao et al. (2017), neutralisation of inflammatory cytokines (e. g., TNF-α, IL-6) either before or after Treg administration may enhance their function, increasing their ability to recover lost immune tolerance. Also, drugs that stabilise Treg cells (e.g. glucocorticoids, nonsteroidal anti-inflammatory drugs) or drugs to stimulate Treg differentiation (TGF-β, all-trans retinoic acids) may also be utilised to maximise the benefits of Treg-based therapies in autoimmune settings.

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How to cite this article: Jacobo P. The role of regulatory T Cells in autoimmune orchitis. *Andrologia*. 2018;50:e13092. https://doi.org/10.1111/and.13092