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REGULATORY T CELLS AND VASECTOMY

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Abstract

CD4+CD25+ regulatory T cells (Tregs) strongly influence the early and late autoimmune responses to meiotic germ cell antigens (MGCA) and the gonadal immunopathology in vasectomized mice. This is supported by the published and recently acquired information presented here. Within 24 hours of unilateral vasectomy (uni-vx) the ipsilateral epididymis undergoes epithelial cell apoptosis followed by necrosis, severe inflammation, and granuloma formation. Unexpectedly, vasectomy alone induced MGCA-specific tolerance. In contrast, uni-vx plus simultaneous Treg depletion resulted in MGCA-specific autoimmune response and bilateral autoimmune orchitis. Both tolerance and autoimmunity were strictly linked to the early epididymal injury. We now discovered that testicular autoimmunity in uni-vx mice did not occur when Treg depletion was delayed by one week. Remarkably, this delayed Treg depletion also prevented tolerance induction. Therefore, tolerance depends on a rapid de novo Treg response to MGCA exposed after vasectomy. Moreover, tolerance was blunted in mice genetically deficient in PD-1 ligand, suggesting the involvement of induced Treg. We conclude that pre-existing natural Treg prevents post-vasectomy autoimmunity, whereas vasectomy-induced Treg maintains post-vasectomy tolerance. We further discovered that vasectomized mice were still resistant to autoimmune orchitis induction for at least 12–16 months; thus, tolerance is long-lasting. Although significant sperm autoantibodies of low titers became detectable in uni-vx mice at seven months, the antibody titers fluctuated over time, suggesting a dynamic “balance” between the autoimmune and tolerance states. Finally, we observed severe epididymal fibrosis and hypo-spermatogenesis at 12 months after uni-vx: findings of highly critical clinical significance.

Introduction

Vasectomy is a world-wide male contraceptive approach used by over 0.5 million men in the United States annually. Because over 50% of vasectomized men develop sperm autoantibody response after six to seven months, this is arguably the most common human

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autoimmune state. Yet, the mechanism of the post-vasectomy autoimmune response is unknown. Epididymal granuloma formation and focal orchitis are reported in vasectomized men, but the etiology and precise frequencies are not defined (Adams and Wald, 2009). Also undetermined are the precise mechanism for post-vasectomy pain syndrome (Horovitz et al., 2012) and the mechanism of infertility in vasovasostomy subjects, despite restoration of sperm count (van Dingen et al., 2012). Post-vasectomy autoantibody to sperm and autoimmune orchitis are well documented in all animal species, including humans (Tung and Menge, 1985; Alexander and Anderson, 1979), associated with deposition of meiotic germ cell antigen (MGCA) antibody complexes outside the Sertoli cell barrier (Bigazzi et al., 1976; Alexander and Tung, 1977). Post-vasectomy orchitis is adoptively transferred from vasectomized animals to naïve recipients; therefore, effector T cells are operative (Tung, 1978; Wheeler et al., 2011). Besides local testicular complications, systemic sequelae, including cardiovascular disease and neoplasms, have been reported (Kaufman et al., 1995; Mettlin et al., 1990; Rosenberg et al., 1990). Although they were not confirmed in subsequent studies (Lesko et al., 1999; Köhler et al., 2009), the importance of these serious long-term complications remains controversial and they influence clinical practice. More mechanistic understanding of the basic immune response to vasectomy is required to provide clarity to the post-vasectomy sequelae.

Since the earlier years of vasectomy research, there have been impressive gains in the basic knowledge germane to post-vasectomy immune response. In a recently published study, we applied new approaches to investigating the pathological, cellular, and genetic mechanisms responsible for the immune responses to vasectomy in inbred mice (Wheeler et al., 2011). We focused on the early events between day 1 and week 10, and this contrasts with most previous studies that focused on the long-term detection of serum sperm antibodies and testicular changes – end-products of the autoimmune response. In Part 1 of this paper, we review our published findings. In Part 2, we present new data that extend our findings in Part 1, including: 1) the mechanism of post-vasectomy tolerance, 2) the emergence of the post-vasectomy sperm antibody response, and 3) the long term persistence of the tolerance and autoimmune states. Finally, we have reported the unexpected findings of severe epididymal and testis pathology that are linked specifically to the vasectomy operation.

Part 1. Review of the early immune response of vasectomy mice with or without concomitant regulatory T cell (Treg) depletion

Vasectomy rapidly injures the epididymis, induces granulomatous inflammation, and exposes meiotic germ cell antigens—We studied unilateral vasectomized (uni-vx) mice, in which one vas deferens was cut and the two ends ligated. Thus, the contralateral testis and epididymis served as a control to monitor the systemic effects of vasectomy. The first observable change occurred in the ipsilateral epididymis within 24 hours (Fig. 1). Patches of apoptotic epithelial cells of the caudal epididymal ducts were replaced by dividing basal epithelial cells (Fig. 1 A). In the next two weeks, increasing interstitial inflammation and epithelial cell necrosis led to extrusion of sperm into the interstitial space (Fig. 1B). By three weeks, neutrophil, T cell, macrophage, and dendritic cell accumulation led to the formation of sperm granuloma of varying sizes, and evidence of sperm phagocytosis by dendritic cells and macrophages (Fig. 1C, D). Our preliminary data further indicate that MGCA are presented to antigen-specific T cells in the epididymis-draining lymph nodes (LN) (Rival, personal observation). However, despite the presentation of MGCA to T cells in an inflamed tissue environment, we detected no evidence of MGCA-specific autoimmune response in several of the inbred mouse strains studied, and their testes were normal even at ten weeks after vasectomy (Table 1, Study A, Wheeler et al., 2011).

Sperm granuloma was detected in over 80% of uni-vx mice, frequently in the cauda epididymis. Notably, many epididymal granulomata were microscopic in size, suggesting that epididymal granulomata are unlikely to be detected by gross examination in vasectomized men, and are therefore under-reported. Significantly, the release of MGCA from the epididymis after uni-vx is causally linked to the autoimmune orchitis and tolerance to MGCA since both responses were prevented by surgical ablation of the vasectomized epididymis and testis at two weeks (but not at three weeks) post-vasectomy (Wheeler et al., 2011).

Concomitant Treg depletion and unilateral vasectomy resulted in bilateral autoimmune orchitis—Physiological peripheral tolerance is controlled by multiple mechanisms, requiring continuous interaction between endogenous tissue autoantigen and antigen-specific T and B cells (Garza et al., 2000). One mechanism of physiological tolerance depends on antigen-specific Tregs (Wing and Sakaguchi, 2010). Treg continuously circulate through the LN, but it is in the regional LN that drains an organ where Treg encounter and respond to tissue-specific antigens of the organ (Walker et al 2003; Fisson et al., 2003, Lathrop et al., 2008). As a result, Tregs from a regional LN are 15- to 50-fold more potent in suppressing an autoimmune disease of the organ that drains that specific regional LN (Samy et al., 2005; Wheeler et al., 2009). Regional LN-specific enrichment of antigen-specific Tregs depends on tissue antigen stimulation, since organ ablation abolishes the enhanced Treg activity (Garza et al., 2000; Setiady et al., 2006).

We hypothesized that antigen-specific natural Treg are strategically positioned in the LN to prevent autoreactive T cell activation in response to local danger signal (Matzinger, 1994) coming from the draining organs (Wheeler et al., 2009). Post-vasectomy epididymitis with cell necrosis presents a persistent endogenous “danger” signal, or innate response to damage-associated molecular pattern (DAMP) (Kono and Rock, 2008; Kawai and Akira, 2010); we therefore tested our hypothesis by depleting the Tregs in mice with uni-vx.

At the same time as uni-vx, mice were injected with anti-CD25 monoclonal antibody (PC61). This depleted 60% of Tregs for five weeks. Treg-depleted uni-vx mice produced antibody to MGCA four weeks later. The titer rapidly rose from five to eight weeks to reach a plateau that lasted for 16 months (Wheeler et al., 2011). In parallel, testicular inflammation appeared at five to six weeks and reached a peak intensity and incidence (87%) by eight weeks. Importantly, the testis pathology was bilateral (Table 1, Study A). It resembled changes in experimental autoimmune orchitis (Imm-EAO) induced by testis homogenate (TH) immunization with adjuvants (Kohno et al 1983). Mechanistically, post-vasectomy EAO (pv-EAO) depended on both interferon gamma-producing CD4 Th1 cells and autoantibody. Accordingly:

1. Most testis infiltrating T cells produced interferon gamma rather than interleukin (IL) 17,
2. Orchitis was inhibited by late CD4 T cell depletion in vivo,
3. CD4+ T cells from testis-draining (but not from non-draining) LN adoptively transferred pv-EAO to naive recipients, and
4. Testis disease transfer by T cells was enhanced by co-transfer of serum MGCA autoantibody. Because both uni-vx and Treg depletion were required for pv-EAO induction, our findings support the hypothesis that natural Tregs normally prevent the induction of MGCA autoimmune response by uni-vx as a danger signal.

Genetic control of the Treg strength-influenced post-vasectomy autoimmune orchitis—The above studies were conducted in highly susceptible (C57BL/6xJ) F1

(B6AF1) mice. To determine genetic control of post-vasectomy autoimmunity, we compared B6AF1 mice with their parenteral C57BL/6 (B6) and A/J mice. Although they developed similar epididymal granuloma, and were equally tolerized to MGCA, pv-EAO occurred only in the uni-vx B6AF1 and A/J mice and not in B6 mice after partial (60%) Treg depletion. Notably, a similar mouse strain response profile has been reported for other models of testis and ovarian autoimmune diseases (Tung et al., 2005). In fact, many testis and ovarian disease-associated gene loci are co-mapped with genetic loci assigned to other autoimmune diseases, including type I diabetes, sialoadenitis and experimental autoimmune encephalomyelitis (EAE) (Yamanouchi et al., 2007; Butterfield et al., 1998). An example is the IL-2 locus in mouse chromosome 3. Because IL-2 is required to maintain Treg function (Yamanouchi et al., 2007; Wing and Sakaguchi, 2010), T cells from B6 mice produce a higher level of IL-2, and allelic variance of the IL2 locus in chromosome 3 exists between A/J and B6 mice (Del Rio et al., 2008), we hypothesized that resistance to post-vasectomy autoimmunity in B6 mice is due to high IL-2, which strengthens intrinsic Treg function. This hypothesis was supported by the finding that B6 mice developed post-vasectomy autoimmune orchitis after 98% (not 60%) Treg depletion. The 98% Treg depletion was achieved by the administration of diphtheria toxin (DT) to B6 DERE mice expressing DT receptor under the Foxp3 promoter (Lahl et al., 2007). Notably, DT injection alone does not cause autoimmune orchitis (Harakal and Tung, unpublished).

Therefore, Treg prevents induction of MGCA autoimmunity and pv-EAO in vasectomized mice. Autoimmune disease is a complex trait and diminished Treg function is considered an important predisposing factor (Wing and Sakaguchi, 2010). Indeed, ongoing clinical trials are attempting to enhance Treg function as a therapy for human autoimmune disease (Bluestone et al., 2010.). In this context, it will be interesting to determine whether the individual variations in the sperm antibody response observed in vasectomized men can be a useful predictor for an overall susceptibility to clinical autoimmunity.

Vasectomized mice developed MGCA-specific tolerance—The lack of responsiveness of uni-vx mice to MGCA prompted the hypothesis that uni-vx mice might develop resistance to EAO induction and become tolerant to MGCA after vasectomy. To test this, we immunized mice at three weeks after uni-vx or sham operation, with TH and adjuvants (Kohno et al., 1983). This led to an MGCA-specific T cell response and antibody responses, and severe pv-EAO in sham-operated mice. In stark contrast, the uni-vx mice produced no detectable serum antibody to MGCA. They had a minimal T cell response to MGCA and developed mild and focal pv-EAO. Importantly, the sham vasectomized and uni-vx mice developed equivalent EAE (Wheeler et al., 2011; Butterfield et al., 1998); therefore, the tolerance of uni-vx mice to MGCA is antigen-specific. We conclude that MGCA exposure in vasectomy induces profound tolerance to MGCA.

The vasectomy-induced tolerance to MGCA is consistent with the divergent nature of the responses to inflammatory signals elicited by danger (Medzhitov, 2010). Different local environments and different forms of cell death determine the nature of an innate response, and the antigen-presenting dendritic cells and macrophages elicited can preferentially elicit either immunity or tolerance (Steinman et al., 2003; Martinez et al., 2009). Importantly, a tolerogenic arm of the divergent response has been shown to stimulate Treg, which in turn promotes tolerance (Maloy et al., 2003; Watanabe et al., 2008).

The occurrence of tolerance to MGCA in uni-vx mice also raises the question of its possible effects on immune surveillance in the context of carcinogenesis. Immune surveillance requires adequate innate and adaptive responses against neo-antigens and autoantigens expressed in newly arising “pre-neoplastic cells” (Vesely et al., 2011), including the cancer/testis antigens expressed in normal meiotic germ cells (Simpson et al., 2005). Thus,

tolerance to MGCA might interfere with immune surveillance for tumors bearing cancer/testis antigens and favors cancer development, leaving vasectomized individuals more susceptible to cancer. In fact, Anderson and colleagues (1983) documented a significant increase in tumor burden in (BALB/c x DBA/2) F1 mice 2 years after vasectomy. It is therefore critical to clarify the mechanism of post-vasectomy tolerance and determine whether it persists over time.

Part 2. Mechanisms of post-vasectomy tolerance and long-term post-vasectomy immunopathological sequelae

Post-vasectomy tolerance required the expansion and/or induction of Treg after uni-vx—Autoimmunity to MGCA required concomitant uni-vx and Treg depletion, and did not occur when CD25 antibody treatment was delayed by one, two or four weeks after uni-vx (Fig. 2A). Thus, some early events must manifest in the first week post-vasectomy to confer resistance to MGCA autoimmunity. While CD25 antibody might have depleted activated CD25+ effector T cells required for MGCA autoimmunity, this event should also apply to mice with concomitant Treg depletion; yet, they are not resistant to pv-EAO. A more likely explanation is the rapid induction of Treg activity by MGCA released from the injured epididymis that confers immune resistance. If true, this might also provide an answer on the mechanism of MGCA tolerance in the uni-vx mice. We therefore investigated whether delayed Treg depletion would abrogate post-vasectomy tolerance to MGCA.

Uni-vx mice, treated with CD25 antibody or control rat IgG at one week post-vasectomy, were immunized with TH and adjuvants at week 3. Additional controls included: sham-operated mice treated with CD25 antibody, and non-operated mice. Three weeks later, we analyzed testicular pathology and MGCA antibody (Fig. 2B, C). Consistent with our previous finding, uni-vx mice were resistant to imm-EAO induction. Consistently, the uni-vx mice treated with rat IgG had milder and infrequent imm-EAO and minimal antibody response to MGCA, supporting tolerance to MGCA (Fig. 2B, C). In contrast, mice treated with CD25 antibody one week after uni-vx, developed severe imm-EAO and strong MGCA antibody response (Fig. 2B, C), to the same extent as the sham-operated mice with Treg depletion (Fig. 2B, C). Thus, Treg depletion at 1 week post-vasectomy can rescue the uni-vx mice from post-vasectomy tolerance. We conclude that tolerance to MGCA induced by uni-vx is due to rapid expansion of the Treg activity that follows vasectomy.

Overall, our new findings suggest that Treg plays two critical roles in controlling post-vasectomy MGCA autoimmunity. The first is prevention of post-vasectomy testicular autoimmunity, and the second is induction and maintenance of tolerance by preventing future induction of imm-EAO. We speculate that the Treg that operate at these two check points might have different origins and different MGCA specificities. Before uni-vx, the thymus-derived natural Tregs (nTregs) in normal mice maintain physiological tolerance to the MGCA that are not sequestered by the Sertoli cell barrier (Harakal and Tung, unpublished data); they are critical for preventing post-vasectomy autoimmunity to MGCA and their depletion at the time of uni-vx can lead to orchitis. After uni-vx, exposure of the normally sequestered “foreign” MGCA during the first week is sufficient to stimulate nTreg expansion, but also convert the Foxp3-negative conventional CD4+ T cells into induced-Tregs (iTregs). Together, they control the tolerance state of uni-vx mice. Our speculation on the emergence of iTreg is consistent with the current consensus that nTreg respond to accessible autoantigens that are expressed in thymus and peripheral LN (Wheeler et al., 2009), whereas iTreg predominantly respond to foreign antigens present in mucosal sites and fetal alloantigens in placenta. Although specific markers that distinguish nTreg and iTreg are lacking, preempting a precise analysis of this issue, our hypothesis has received

indirect support from the following observations (described below): our published data on MGCA targeted by autoantibody in uni-vx mice with EAO, and our new study on the role of the programmed death 1 (PD-1) ligand (PD1-L) inhibitory molecule on tolerance post-vasectomy.

Unilaterally vasectomized mice with Treg depletion produce MGCA autoantibodies with limited antigenic specificities—

Most investigators believe that the MGCA expressed in testis and epididymal sperm are completely sequestered behind tissue barriers, including the Sertoli cell or blood-testis barrier. Thus we had expected a highly diversified antibody response induced by the large number of “foreign” antigens exposed in uni-vx. Contrary to expectation, we detected MGCA antibodies with restricted specificities. In fact, most (>85%) of the Treg depleted uni-vx B6AF1 mice produced antibody that targeted the zonadhesin, a MGCA that binds the zona pellucida (Tardif et al., 2010; Wheeler et al., 2011). The unexpected oligo-specific MGCA response strongly suggests that not all MGCA are sequestered, and that zonadhesin is a dominant sequestered MGCA and therefore resemble a foreign antigen with capacity to generate iTreg.

The PD-L1 inhibitory costimulatory molecules is involved in vasectomy-induced tolerance—

The PD-1 receptor and its cognate PD1-L are one of the major inhibitory ligand-receptor pairs expressed on Tregs, activated T cells, and antigen-presenting cells (reviewed in Riella et al., 2012). PD-1 binds to two ligands: PD-L1 and PD-L2. While PD-L2 has a restricted cell distribution, PD-L1 is expressed on T cells, B cells, and myeloid cells. The interaction of PD-1 and PD-1L on T cells and antigen-presenting dendritic cells is critical for peripheral tolerance, autoimmunity prevention, prolonged allograft survival, and maternal acceptance of allogeneic fetus. It is important to appreciate that PD-L1 binds to two receptors: PD-1 and B7-1 (CD80), and delivers inhibitory signals to the PD-L1+ T cells in both cases (Riella et al., 2012). Tregs express high levels of both PD-1 and PD-L1; thus, we hypothesized that they might participate in tolerance induction after uni-vx.

Similar to the wt B6 mice, the uni-vx PD-1 ko B6 mice immunized with TH in adjuvants developed a weaker MCGA Ab response and milder imm-EAO (Fig. 3A, B). This indicates that PD-1 deficiency did not affect induction of post-vasectomy tolerance. In contrast, when PD-L1 was absent, the uni-vx mice developed a strong MGCA antibody response after TH immunization (Fig. 3A). In addition, they showed a trend toward an increment in EAO incidence and severity, although the differences from control mice did not reach statistical significance (Fig. 3B; $p=0.058$ and $p=0.07$ respectively). These findings indicate that PD-L1 is required, at least partially, for maintaining tolerance to MGCA in uni-vx mice. The fact that PD-L1 deficiency, but not PD-1 deficiency, negates tolerance suggests that PD-L1 interaction with B7-1 might be operative. Since several studies have documented that PD-L1 is required for the generation of iTreg and maintenance of Foxp3 expression (Riella et al., 2012); therefore, the findings support the involvement of iTreg in post-vasectomy tolerance.

Long-term effects of vasectomy: low and spontaneous MGCA antibody response at seven months post-vasectomy, while mice remain tolerant at 12 months—

Meiotic germ cell antigen antibodies were undetectable in uni-vx mice at early time points (<10 weeks) (Wheeler et al., 2011); however, most studies have detected serum sperm autoantibody in vasectomized animals and humans beyond 6 months (Tung and Menge, 1985; Alexander and Anderson, 1979). We therefore performed a long-term study in uni-vx B6AF1 mice and determined whether tolerance persists 12 months post-vasectomy. We discovered that 70–90% of the uni-vx mice also produced a low but detectable MGCA response at seven months post-vasectomy (Fig. 4A). In general, the antibody titers were much lower than those detected in mice with uni-vx and Treg depletion (Wheeler et al., 2011). When we performed a more detailed analysis by grouping the mice into low

responders (<160 antibody units) and very low responders (<30 antibody units), we observed a consistent pattern of waxing and waning in antibody titers in both groups (Fig. 4B, C), while some mice remained nonresponders (filled circles, Fig. 4B).

To determine whether uni-vx mice with low serum sperm antibody titers were still tolerant to MGCA, we studied B6AF1 mice that were uni-vx or sham operated at two months of age and TH-immunized 10 months later. Since the immune response tends to decline with age, we included a group of young non-operated mice (two months old) that were also immunized with TH. In fact, we observed a reduced EAO severity in 12-month-old sham operated mice compared with two-month-old mice (Fig. 5A). Nonetheless, when we compared the 12-month-old uni-vx and age-matched sham operated mice three weeks after TH immunization, we observed significant reduction in severity and incidence of imm-EAO in the vasectomized mice. Even more strikingly, while MGCA antibody titers were increased after immunization in sham-operated mice, no changes were detected in the uni-vx mice (Fig. 5B). We therefore conclude that vasectomy has long-term effects and that 12-month-old uni-vx mice remain tolerant to MGCA.

Why do mice tolerant to MGCA develop sperm antibody response? It is clear from our data that the MGCA released from the epididymis rapidly activates an effector response with the capacity to elicit orchitogenic T cells and MGCA-specific B cells that produce autoantibodies. However, these effector mechanisms are normally regulated by a concomitant Treg response. Since ipsilateral epididymal granuloma are still detectable in 50% of uni-vx mice at 12 months (Table 2), one can envisage a scenario where persistent MGCA stimulation would create both effector response and Treg response that co-exist, with an outcome that depends on the balance of the two responses. We have documented genetic regulation of this balanced state in Part 1, and it is very likely that the balance is also influenced by external and internal environmental factors that change over time. Thus, a transient event that leads to reduction of Treg responses, enhanced effector responses, or effector resistance to suppression might lead to escape from tolerance, resulting in low titers of sperm antibodies that fluctuate over time. We consider the hypothesis reasonable and interesting; however, a more definitive answer will require future studies that track the effector and regulatory T cell functions over time after vasectomy. Nevertheless, our study from the immunological perspective has clearly indicated that the immune sequelae of vasectomy can no longer be viewed as merely simple autoantibody responses to sequestered sperm antigens.

Long-term effects of vasectomy

1. Persistence of autoimmune orchitis in the contralateral testes,
2. Severe vasoligation-related epididymal fibrosis and severe hypospermatogenesis in the ipsilateral gonads, and
3. Regression of post-vasectomy sperm granuloma from the epididymis.

B6AF1 mice with uni-vx and Treg depletion at two months of age retained high titers of MGCA autoantibodies in their sera at 16 months (data not shown). To determine the persistence of pv-EAO, we focused on the contralateral testes of uni-vx mice. As shown in Table 2 (Study C), 3 out of 3 uni-vx mice with Treg depletion at two months still showed severe pv-EAO in the contralateral testis at 16 months. Most seminiferous tubules and the epididymal lumen were devoid of spermatogenic cells and sperm respectively. In contrast, none of the uni-vx mice without Treg depletion developed pathology in the contralateral gonads at 16 months (Table 2, Study B). Therefore, the pv-EAO that began within a few weeks post-vasectomy can persist for at least 16 months.

To determine the pathological changes associated with vasoligation per se, and probably of non-immune etiology, we focused on the ipsilateral testes of uni-vx mice with or without Treg depletion (Table 2). We made three important observations. First, the incidence of sperm granuloma in the ipsilateral epididymides was reduced from >80% at ten weeks (Wheeler et al., 2011) to 50% at 12 months, and it was undetectable by 16 months (Table 2, Studies A, B, and C). Second, the masson trichrome-hematoxylin staining revealed severe and diffuse interstitial fibrosis in 100% of the ipsilateral epididymides evaluated at 12 and 16 months (Fig. 6C and D; Table 2). The changes were observed in the caput (Fig. 6A), the corpus (Fig. 6B), and in the cauda (not shown) of the epididymis. This was accompanied by distortion and dilatation of the epididymal ducts that were devoid of spermatozoa (Fig. 6 C and D). Importantly, epididymal fibrosis was not found in the non-vasectomized gonads of the same mice (Fig. 6A and B; Table 2). Moreover, epididymal fibrosis occurred in 90–100% of the vasectomized mice, regardless of the depletion of Treg or TH immunization; therefore, the fibrosis is caused by vasoligation and is unrelated to other treatments (Table 2). Third, several orchitis and aspermatogenesis were also observed in the ipsilateral testes of uni-vx mice. Importantly, we detected the changes in the ipsilateral testes of TH-immunized mice with post-vasectomy tolerance that had normal contralateral gonads (Table 2, Studies A and B).

The severe epididymal fibrosis and major disruption of spermatogenesis, if they also occur in humans, could certainly explain the infertility after vasovasostomy, and the persistent groin pain reported by vasectomized men. These findings therefore represent post-vasectomy sequelae of major potential clinical significance, and should be confirmed by detailed clinico-pathological investigations.

Concluding remarks and clinical relevance

1. The common procedure of vasectomy can lead to several major immunopathological and nonimmunological consequences that are potentially of long-term clinical significance.
2. Epididymitis and sperm granuloma occur rapidly after vasectomy, exposing sperm and MGCA. The situation represents autoantigenic stimulation in the face of persist innate response or danger signals; they are responsible for all the subsequent immune and non-immune sequelae observed in the vasectomized mice.
3. Meiotic germ cell antigens from epididymal sperm trigger an autoimmune response with orchitogenic potential, as indicated by pv-EAO that occurs in uni-vx mice with Treg depletion. However, MGCA also trigger concomitant Treg response that controls pathogenic T and B cells, and confers resistance to future testis autoimmunity induced by TH immunization that lasts for at least 12 months. This prolonged tolerance state is MGCA-specific. Whether this tolerance state will interfere with immune surveillance against cancers/testis antigen is an important question to be addressed.
4. Despite tolerance, vasectomized mice produce low titers of sperm antibody after seven months with titers that fluctuate over time. We interpret the findings to reflect a dynamic and concomitant response of both effector and regulatory T cells initially triggered by MGCA. However, the balance is impermanent, allows escapes, and leads to low-level and fluctuating antibody responses. The balance is likely influenced by genetic and non-genetic factors.
5. By far the most serious non-immune sequela of vasectomy is the severe fibrosis in the epididymis and aspermatogenesis in the testes at 12 months post-vasectomy. These changes are tightly associated with vasoligation, occurring only on the side

of vasectomy, and not related to any other experimental manipulations. The fibrosis is very severe and likely irreversible. It will be critical to determine whether similar changes also occur in human vasectomy, and whether they are the basis for the clinical complications of vasectomy and vasovasostomy.

6. Our study has also addressed a number of critical issues related to basic immunology and fundamental reproductive biology. First, nTreg control autoimmune induction triggered by local danger signals as exemplified by the epididymal granuloma. Second, autoantigenic stimulation in the context of endogenous danger can lead to antigen-specific tolerance rather than autoimmunity, and this is likely due to induced-Treg. Third, our findings raise the possibility that only some MGCA are normally sequestered, but they dominate the post-vasectomy immune response. Fourth, we show that autoreactive Th1 cells and autoantibody synergize to promote post-vasectomy autoimmune orchitis. It is hoped that the many questions raised in this study will stimulate and guide future research on the mechanism of immune and non-immune sequelae of this important and common male contraceptive approach.

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Figure 1.

Early immunopathological changes of the ipsilateral cauda epididymis in uni-vx B6AF1 mice. (A) Sheets of apoptotic epithelial cells detected after 24 hours by TUNEL (x400). (B) Sperm leaking from ruptured epididymal epithelial ducts surrounded by inflammatory cells at two weeks post-vasectomy (hematoxylin and eosin, x100). (C and D) Numerous CD11c+ dendritic cells (C, x100) and CD4+ T cells (D, x50) in sperm granuloma surrounding a core of dead sperm at three weeks post-vasectomy. [Materials and methods: The TUNEL assay was carried out using FITC-dUTP and TdT enzyme from the APO-Direct kit (BD Biosciences). For detailed methodology and reagents, see Wheeler et al. (2011).

Figure 2.

Delayed Treg depletion (at one week post-vasectomy) does not lead to meiotic germ cell antigen (MGCA) autoimmunity and abrogates MGCA tolerance in uni-vx B6AF1 mice. (A) Experimental autoimmune orchitis (EAO) developed in the contralateral testes of uni-vx mice with Treg depletion by CD25 antibody (PC61) at the time of uni-vx (day [d] 0), but not when Treg depletion was delayed after one, two or four weeks (wks). (B and C) Post-vasectomy tolerance depends on Treg. Mice were uni-vx or sham operated on day 0, injected with PC61 or rat IgG (rIgG) on day 7, and TH-immunized on day 21. Three weeks after immunization, testicular pathology (B) and autoantibody response (C) were examined. (B) Severe EAO occurs in uni-vx mice treated with CD25 antibody at one week post-vasectomy, but not in uni-vx mice treated with control rat IgG (rIgG). (C) MGCA antibody (Ab) response is restored in uni-vx mice after CD25 Ab, but not by rIgG administration on day 7 post-vasectomy. (See text and Table 1 footnote for details of experimental design.)

Figure 3.

PD-L1 is required for the maintenance of post-vasectomy tolerance to MGCA. The MGCA antibody response (A) and contralateral EAO (B) induced by TH immunization were significantly reduced in uni-vx wt and PD-1 ko B6 mice (columns 1–3, open circles). However, the MGCA antibody (Ab) response is rescued in PD-1L ko B6 mice (A, filled circles). Note a trend toward enhanced contralateral EAO in PD-1L ko mice compared with the uni-vx wt mice (B, columns 2 and 4). The PD-1 ko B6 mice and PDL-1 B6 ko mice were kindly provided by Dr. Tasuku Honjo (Kyoto University, Japan) and Dr. Lieping Chen (Johns Hopkins University) respectively. (See text and Table 1 footnote for details of experimental design.)

Figure 4.

Low and fluctuating sperm antibody titers in uni-vx mice after seven months. Uni-vx B6AF1 mice developed significant sperm autoantibodies of low titers after seven months and the titers fluctuate over time. (A) Time course of sperm antibody (Ab) titers from sequential serum samples between cohorts of uni-vx versus sham operated mice. (B and C) Sperm antibody titers of individual mice with low titers (B) and very low titers (C) were plotted over time. (See text and Table 1 footnote for details of experimental design.)

Figure 5.

The uni-vx B6AF1 mice remain tolerant to MGCA at 12 months post-vasectomy. They show a significant reduction in contralateral EAO (A, column 3 with filled circles compared with column 2), and no increase in MGCA antibody response (columns 3 and 4, filled circles) after TH immunization. (See text and Table 1 footnote for details of experimental design.)

Figure 6.

Severe fibrosis develops in the ipsilateral epididymis at 12 months after uni-vx. The contralateral (A and B) and ipsilateral (C and D) epididymis of uni-vx mice were analyzed at 12 months. Note the minimal amount of fibrosis in the capsule of the caput (A) and body (B) of the contralateral epididymis and the numerous spermatozoa (B). A similar change is observed in the cauda. In contrast, the caput (A) and body (B) ipsilateral epididymis has empty lumen and massive interstitial fibrosis. Fibrotic tissue is in blue. The same extent of fibrosis is observed in uni-vx mice at 12 or 16 months with or without any additional treatment (Table 2). (Masson trichrome hematoxylin stain, x100).

Early analysis (<10 weeks) of the Autoimmune Response To MGCA After Unilateral Vasectomy With Or Without Treg Depletion And In Different Inbred Mouse Strains

Table 1

Study	Mouse Strain	Uni-vx or sham	% Treg depletion	TH/adjuvant immunization	Sperm antibody	T cell response	Bilateral orchitis	Epididymal fibrosis
A	B6AF1	Uni-vx	60%	-	+++	+++	+++	0
		Uni-vx	0%	-	0	0	0	0
		Sham	60%	-	0	0	0	0
B	B6AF1	Sham	60%	-	0	0	0	0
		Uni-vx	60%	+	0	+	+	0
		Sham	60%	+	+++	+++	+++	0
C	A/J	Uni-vx	60%	-	+++	+++	+++	0
	C57BL/6	Uni-vx	60%	-	0	0	0	0
	DEREG C57BL/6	Uni-vx	>95%	-	++	++	++	0

Footnote: To induce post-vasectomy meiotic germ cell antigen (MGCA) autoimmune response and autoimmune orchitis, B6AF1 adult mice were unilaterally vasectomized by vas ligation (day 0) and injected with a monoclonal antibody to CD25 (interleukin 2 receptor alpha chain) (PC61) at a dose of 250 µg on days -3, 0, and +3. Sperm autoantibodies are both detected at three to four weeks and peak at seven to eight weeks. To evaluate tolerance to MGCA, uni-vx mice were immunized with TH in adjuvants (CFA and pertussis toxin) at three weeks post-vasectomy. Three weeks post-immunization, mice were evaluated for experimental autoimmune orchitis (EAO) development (histopathology) and autoantibodies (ELISA). 0 = no change, + = mild and infrequent changes, +++ very frequent and severe changes; ++ is between + and +++.

Table 2

Long term effects of vasectomy (>12 months): Autoimmune Testis And Epididymal Pathology After Unilateral Vasectomy, With Or Without Additional Experimental Manipulations:

Study	Time	Treg depletion	TH-immunization	Operation (Number of testes)	% EAO	Mean EAO severity(0 - 15)	Empty Epididymides	Epididymal Granuloma	Epididymal fibrosis
A	12 m	No	Yes	Uni-vx (10)	80%	7.0	80%	50%	90%
				None (10)	10%	1.0	10%	0%	0%
B	16 m	No	No	Uni-vx (4)	100%	6.5	100%	0%	100%
				None (4)	0%	0	0%	0%	0%
C	16 m	Yes	No	Uni-vx (3)	100%	9.3	100%	0%	100%
				None (3)	100%	9.0	100%	0%	0%

Footnote: m = months.