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Regulatory T cells control tolerogenic versus autoimmune response to sperm in vasectomy

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REGULATORY T CELLS CONTROL TOLEROGENIC VERSUS AUTOIMMUNE RESPONSE TO SPERM IN VASECTOMY

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ABSTRACT (183 words)

Vasectomy is a well-accepted global contraceptive approach frequently associated with epididymal granuloma and sperm autoantibody formation. To understand the long-term sequelae of vasectomy, we investigated the early immune response in vasectomized mice. Vasectomy leads to rapid epithelial cell apoptosis and necrosis, persistent inflammation, and sperm granuloma formation in the epididymis. Vasectomized B6AF1 mice did not mount autoimmune response but developed instead sperm antigen-specific tolerance, documented as resistance to immunization-induced experimental autoimmune orchitis (EAO) but not experimental autoimmune encephalomyelitis (EAE). Strikingly, tolerance switches over to pathologic autoimmune state following concomitant CD4+CD25+Foxp3+ regulatory T cell (Treg) depletion: Unilaterally-vasectomized mice produce dominant autoantibodies to an orchitogenic antigen (zonadhesin), and develop CD4 T cell- and antibody-dependent bilateral autoimmune orchitis. Therefore: 1) Treg normally prevents spontaneous organ-specific autoimmunity induction by persistent endogenous danger signal; and 2) autoantigenic stimulation with sterile auto-inflammation can lead to tolerance. Finally, post-vasectomy tolerance occurs in B6AF1, C57BL/6 and A/J strains. However C57BL/6 mice resisted EAO after 60% Treg depletion, but developed EAO after 97% Treg reduction. Therefore variance in intrinsic Treg function - a possible genetic trait - can influence the divergent tolerogenic versus autoimmune response to vasectomy.

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INTRODUCTION

Vasectomy is a global contraceptive approach with an annual rate of over 0.5 million men in the US (1), therefore any significantly harmful effect attendant to vasectomy would pose serious health hazard. Well-documented are the development of sperm antibody response in vasectomized men and animals (2,3), and autoimmune orchitis in vasectomized animals (4). More alarming is a positive statistical correlation with prostate cancer in long-term vasectomized humans (5;6). Although not confirmed (4), the initial findings has continued to influence clinical decision on vasectomy (7). Despite the uncertainties, studies on the basic mechanism of response to vasectomy have only been proposed (4) but not investigated (1). We now initiate a study with the premise that early immunological changes before sperm antibody detection can modify late events, and may unravel mechanistic link to human systemic disease.

In animals and humans, sperm epididymal granuloma occurs commonly due to extravasated sperm (1), creating a localized endogenous danger signal (8;9). The post-vasectomy autoimmune response has likely resulted from continuous stimulation by exposed sperm antigens coming from the inflamed epididymis. However, the magnitude and incidence of the response vary greatly (30% to >80%), and the onset is often late (6-9 months) (2;3). We hypothesize that vasectomy may trigger an immunoregulatory process concomitant with an adaptive immune response to sperm antigens. This process may be T cell

intrinsic (10) or extrinsic, including the CD4+CD25+ Foxp3+ regulatory T cells (Treg) (11).

Treg is critical for peripheral tolerance (12). For internal organs, Treg may control tolerance in the regional lymph node (LN) where antigen-specific Treg continuously encounter tissue antigens (13-15). This idea has been advanced by two recent observations. The Hsieh group reported distinct TCR repertoire among the Treg in individual LN, but shared TCR repertoire among the naive CD4 Foxp3-negative T cells (16). We showed that the Treg from normal regional LN were 15 to 50 times more potent than those from non-draining LN in controlling autoimmune disease of the relevant target organs; in contrast, the CD4+CD25- naïve T cells from different LN induce tissue inflammation in the same organs (11). We postulate that LN-specific Treg maintain tolerance by anticipating local danger signals, and prevent organ-specific autoimmunity. To test this hypothesis, we have investigated vasectomy as a clinically-relevant, localized danger signal.

The study has two goals: 1) To elucidate the early immunopathologic events in the first three months post-vasectomy that may explain the long term effects of vasectomy; and 2) To investigate the hypothesis that Treg control pathogenic autoimmune response to localized endogenous danger signal.

RESULTS

Vasectomy rapidly induces epithelial cell necrosis and tissue inflammation in the epididymis without sperm antibody response.

After vasectomy, sperm production in testis is unperturbed (17), allowing millions of sperm per day to enter the epididymis, where dramatic changes were detectable (Fig S1). Within hours, extensive epithelial cell apoptosis and necrosis were followed by sperm extravasation and sperm phagocytosis. By day 7, activated macrophages, dendritic cells, neutrophils and T cells accumulated to form granuloma. Despite sperm antigen exposure and tissue inflammation, sperm antibodies were undetected in vasectomized (B6 x A/J)F1 (B6AF1), A/J, C57BL/6 (B6) or BALB/c mice for up to six months. Although they are known responders to EAO induced by testis antigens in adjuvant (2), we question whether in vasectomy, stimulation by epididymal sperm antigens without adjuvant may elicit immune tolerance instead.

Vasectomy induces testis antigen-specific tolerance despite sperm antigen exposure in the context of sterile tissue inflammation.

Indeed, the unilaterally-vasectomized (uni-vx) B6AF1 mice became highly resistant to EAO induction by immunization with testis antigen in adjuvant 3 weeks later (Fig 1A). Compared with control, the orchitis in contralateral testes were mild and infrequent (Fig 1A, C, D); the serum antibody and T cell response to testis antigens were profoundly reduced (Fig 1E, F). Tolerance is testis antigen-specific as EAE induction was not affected (Fig 1B). Tolerance is

maintained by continuously sperm antigen exposure in inflamed epididymis as it was terminated by ipsilateral testis and epididymis ablation at 3 weeks (Fig 1A).

Concomitant Treg depletion terminates the tolerance state and induces severe bilateral testicular autoimmune disease in unilateral vasectomy.

Treg from the normal mice prevent EAO that develops in day 3-thymectomized mice in an antigen-dependent and organ-specific manner (18). To investigate Treg in post-vasectomy tolerance, we depleted Treg from uni-vx B6AF1 mice by CD25 monoclonal antibody at vasectomy. This led to 60% reduction in Foxp3+ Treg in all LN for 5 weeks, and concomitant rise in activated Foxp3-negative effector T cells (Fig S2A). That T cell activation occurs exclusively in regional LN supports a testis antigen-specific effector T cell response (Fig S2B).

Serum antibodies to sperm and testis antigens were detected by 4 weeks (Fig 2A). Strikingly, left or right uni-vx was followed by severe bilateral orchitis 2 weeks later (Fig 2B, C). This requires stimulation by endogenous antigens from the epididymis and testis in the first 3 to 4 weeks since their removal at 2 weeks (but not 4 weeks) prevented the response (Fig 2D). Testicular pathology was characterized by: multi-focal infiltration of lymphocytes, granulocytes, dendritic cells, macrophages and multinucleated giant cells that surrounded seminiferous tubules, penetrated the blood-testis barrier (BTB), and infiltrated tubular lumen (Fig 2E, left). In 85% of mice with orchitis, sufficient germ cell loss led to sperm depletion in the epididymis - a finding predictive of infertility (Fig 2E, right).

Pathogenic CD4+ T cells are sufficient to induce post-vasectomy autoimmune orchitis and autoantibody has a supportive role.

About 65% of the testis-infiltrating T cells expressed CD4 (Fig S3A, C); among them, 20% had potential to produce IFNγ, and less than 2% produced IL-17 (Fig S3B, D). Orchitis was completely inhibited by depletion of CD4 T cells given after sperm autoantibody detection (Fig S4A). Importantly, CD4+ T cells from mice with orchitis transferred severe EAO to syngeneic recipients, and no other pathology. Notably, only the CD4+ T cells from the testis-draining lymph nodes were pathogenic (Fig S4B, C). Thus regional LN are unique locations where the pathogenic T cell respond to sperm antigen and preferentially accumulation.

In addition to immune cells, peri-tubular immune complexes were detected as patches of granular IgG and C3 (Fig 2G,H). Importantly, over 70% of them were co-localized with cluster of CD11c+ dendritic cells and CD4 T cells, at the BTB (Fig 2G-I). Indeed, serum antibody from uni-vx mice with Treg-depletion, co-transferred with splenic CD4+ T cells, enhanced orchitis pathology (Fig S4B). Therefore, while CD4+ T cells are necessary and sufficient to trigger post-vasectomy autoimmune orchitis, orchitis severity is enhanced by autoantibody as complement-activating immune complexes.

Sperm-specific zonadhesin is a target autoantigen with an orchitogenic polypeptide domain.

By western blot on a 4-8% SDS-PAGE and short chemiluminescence, the serum antibody from many uni-vx B6AF1 mice with Treg depletion recognized a prominent, high M_r (340 kD) antigenic band (Fig 3A, arrow). To verify our suspicion that this was zonadhesin (Zan) (19) (Fig 3B), we showed that the serum antibody reacted with the 340 kD protein in sperm of wild type but not *zan* null mice. In addition, reactivity of mouse serum antibody against the 340 kD Zan antigen was blocked by pre-incubation with an affinity-purified rabbit antibody to ZanD3p18: a partial region of the D3p domain of Zan with B cell epitope (20) (Fig 3B and Fig S5A). By detecting antibody binding to wild type but not *zan*-null sperm extract, Zan antibody was detected in 5 of 6 (83%) Treg-depleted and uni-vx mice with EAO, but not in 3 mice without EAO or 7 control mice (Fig S5A). Therefore uni-vx B6AF1 mice with Treg depletion mounted a dominant autoimmune response to Zan. We next showed that B6AF1 mice immunized with recombinant ZanD3p18 in adjuvant developed EAO (p=0.01) (Fig S5B,C), therefore, response to Zan is a likely mechanism of post-vasectomy orchitis. Zan is expressed on acrosomal membrane of spermatid and sperm, can bind to zona pellucida and inhibit interspecies gamete interaction (20). Notably, Zan is the first murine orchitogenic antigen identified.

Mouse strain variation in immune response to vasectomy is due to variance in natural Treg function.

To determine genetic control of post-vasectomy response, we investigated the parenteral strains of B6AF1 mice. A/J and B6 mice developed epididymal

inflammation and granuloma. While both strains exhibited post-vasectomy resistance to testis antigen immunization, the reduction in B6 mice was profound (Fig S6 A,B). Also, after Treg depletion by CD25 antibody treatment, the uni-vx A/J mice responded but uni-vx B6 did not (Fig S6 C,D). To determine whether this is explicable by a stronger Treg function in B6 mice, we studied the DEREG B6 mice with linked expression of diphtheria toxin receptor and Foxp3 (21). Indeed, 7 weeks after 97% Treg were depleted by combined diphtheria toxin and CD25 antibody treatment, most DEREG B6 mice developed antibody response, severe EAO (Fig S7 A,B,D), and T cell activation in the regional LN (Fig S7C). Thus the genetic variance is likely due to the unique Treg function of B6 mice.

DISCUSSION

We have investigated the mechanism of post-vasectomy sperm-specific autoimmune response in mice with uni-vx. Unlike other studies, we focused on the immunological events in the first 10 weeks – long before sperm antibodies were detectable. We obtained new and unexpected results germane to the mechanism of Treg function and immune sequele of vasectomy. First, vasectomized mice develop sperm-specific systemic tolerance despite sperm antigen presentation from an inflamed epididymis. Second, Treg depletion in vasectomy leads to spontaneous testis-specific autoimmune disease, invoked by the synergic effect between pathogenic CD4 T cell and autoantibody. Third, the antibody in B6AF1 mice dominantly targets the sperm-specific zonadhesin: the first murine orchitogenic antigen identified. Fourth, the post-vasectomy immune response is under genetic control, possibly dependent on intrinsic Treg function. We have shown that the well-documented late post-vasectomy autoimmune response is preceded by an early and Treg-controlled tolerance state, triggered by sperm antigens exposed in the inflamed epididymis soon after vasectomy.

Uni-vx alone does not cause autoimmunity unless Treg are depleted. This supports the contention that a natural Treg function is the prevention of autoimmune disease induction by persistent endogenous danger. The CD4 T cells are pivotal in the pathogenesis of post-vasectomy EAO: They respond to sperm antigens in the regional LN of the epididymis where they accumulate, and they synergize with immune complexes in the testis adjacent to the BTB, to induce maximal orchitis.

Mice with vasectomy alone are resistant to immunization-induced EAO. The tolerance state is testis-specific, maintained by sperm antigens produced in the testis and released into interstitial tissue space of the inflamed epididymis. Therefore, tolerance can be induced by sperm antigens released from tissue with persistent inflammation. This finding is new and unexpected for vasectomy, but it is less unexpected from the viewpoint of the known divergent inflammatory responses to danger signals (22). Different local environment and different forms of cell death can determine the nature of an innate response. In turn, antigen presenting dendritic cells (23) and macrophages (24) with disparate function are generated that may preferentially promote adaptive immunity or immune tolerance. Importantly, this divergent response can be regulated by Treg that foster a tolerance state (25-28). Therefore, as a plausible mechanism, post-vasectomy tolerance may depend on the feedback interaction of sperm antigen-specific Treg with tolerogenic dendritic cells (27). Relevant to this consideration is the reported immune suppressive property of sperm (29).

Vasectomized mice are more resistant to EAO induced by testis antigen immunization than sham-vasectomized mice, therefore “post-vasectomy” tolerance actually exceeds physiological level (Fig 1A). This could be due to a rapid response of sperm-specific Treg normally positioned in the testis-draining LN, and the subsequent expansion of Treg that surpass effector T cells. However, this is possible only if the normal meiotic germ cell autoantigens can access the regional LN (11).

According to prevailing dogma, the BTB formed by Sertoli cells in normal testis completely sequesters male meiotic germ cell antigens from immune recognition (2,30) and these antigens are therefore more “foreign” than “self”. However, the validity of this paradigm is in dispute for the following reasons. The preleptotene spermatocytes that express auto-immunogenic antigens are located outside the BTB (31). To transfer orchitis, CD4 T cells and autoantibody would have to recognize cognate antigens outside the BTB. The Treg of normal male surpass female donors in suppressing EAO induction (32). The female surpass male mice in their response to immunization with testis-specific lactate dehydrogenase (LDH) 3 (a sperm-specific antigen behind BTB) (33). Circulating LDH3 antibody was found to preferentially localize to the testis (34). Finally, with complete sequestration, the expectant antibody response to vasectomy should be highly diversified targeting many “foreign” sperm antigens; instead, our finding is a dominant antibody response to Zan. Based on existing experimental evidence, we propose a new “selective” antigen sequestration model: Non-sequestered germ cell antigens (LDH3) are protected by systemic tolerance in normal mice, and sequestered and immunogenic antigens (Zan) would dominate the post-vasectomy autoimmune response.

The post-vasectomy EAO in mice with Treg depletion is under genetic control. According to our data, the resistance state of the B6 mice is a reflection of their strong intrinsic Treg function. This mechanism has been documented in autoimmune diabetes (35), and subsequently in autoimmune ovarian disease (36), autoimmune dacryoadenitis (37), and EAE (38). In all cases, disease

resistance was mapped to the interleukin 2 locus in chromosome 3 (35), associated with enhanced production of interleukin 2 by activated T cells (35,39).

The genetic data can explain the variable detection of sperm antibody response in humans and outbred animals, and are beginning to provide insight into the mechanism of systemic post-vasectomy sequele. Vasectomized individuals with stronger intrinsic Treg function (like B6 mice) may develop tolerance and be less responsive to future sperm antigen challenge. While this would minimize EAO development and undesirable outcome in vasovasostomy, it could impair testis antigen-specific immune surveillance. Along with persistent local tissue inflammation, this could favor the emergence of tumors expressing neo-antigens shared with male germ cells (the cancer/testis antigens) (40). This consideration is consistent with a report on the significant increase incidence of malignant tumors in vasectomized BDF1 mice after 15-24 months (41). To directly extrapolate this chain of events to humans is premature; nonetheless, our study has provided a framework for further investigation of immune perturbation associated with vasectomy.

Methods

Mice, uni-vx, and Treg depletion: Mice were purchased or bred in house. The *zan* null B6 mouse (20) and B6-DEREG mice (21) were produced as previously described. Vasectomy was by occluding and bisecting the vas. To deplete Treg, 250 μg of CD25 antibody (clone PC61) was injected on days -3, +3, and +7 (Uni-vx, day 0); and 1 μg of diphtheria toxin (Calbiochem, 322326) was injected in DEREG mice on days -1 and +1. Experiments followed guidelines of the Animal Care and Use Committees of University of Virginia, University of Vermont, and Texas Tech University.

Expression of recombinant Zanp18 and production of rabbit antibody: Recombinant D3p18 domain (Accession AAC26680) inclusive of amino acid Cys^{4502}-Lys^{4621} of mouse Zan transcript (Accession U97068: nucleotides 13669 -14050) were expressed and purified; the rabbit antibody against recombinant D3p18-glutathione S-transferase (GST) was affinity-purified, devoid of GST reactivity (20).

EAO and EAE induction, CD4 T cell depletion and adoptive transfer: EAO (31) and EAE (38) were induced as described, using 100 μg ZanD3p18 fusion protein and 100 μg myelin oligodendrocyte glycoprotein peptide 35-55 ($pMOG_{35-55}$) per dose respectively.

Sperm and testis cell antibody and T cell proliferation assays: Antibody was detected by ELISA or indirect immunofluorescence (31). Pooled LN and splenic T cells were stimulated by testis cell in the presence of irradiated splenocytes, and detected by cell-associated H^3T (31).

SDS-Polyacrylamide gel electrophoresis and Western blot: Epididymal sperm proteins, extracted by Lammeli sample buffer (18), were analyzed in 4-8% gradient gels (disulphide-unreduced). After primary antibody (40 ng/mL), bound antibody was visualized by peroxidase-labeled antiserum to mouse or rabbit IgG, detected by chemiluminescence (Bio-Rad).

Statistical analyses: The Fischer exact test was used to compare incidences and unpaired Mann-Whitney tests were used at all other times.

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incidence and association with antisperm immunity. *Am J Pathol* 111:129-139.

FIGURE LEGENDS

Figure 1. Testis antigen-specific tolerance in uni-vx mice. (A): Testicular pathology in mice immunized with testis antigen and adjuvant (TH); tolerance is terminated by ipsi-lateral testis and epididymis ablation at 3 week (UniOx = unilateral orchiectomy). (B): Comparable EAE by pMOG-immunization in vasectomized or sham vasectomized mice. Focal orchitis in TH-immunized vasectomized mice (C, arrow), and severe diffuse orchitis in TH-immunized, non-vasectomized mice (D) (H & E stain, x200). Testis antibody (E) and testis antigen-specific T cell proliferation (F) were determined in TH-immunized uni-vx and non-vasectomized mice (Data of 3 independent studies; *$p<0.02$). EAO was graded as detailed in Table S1; and EAE clinical score was determined as in (38).

Figure 2. Bilateral EAO in uni-vx mice with Treg depletion. Sperm antibody response (A) and EAO progression (B) in uni-vx mice with Treg depletion ($p<0.04$ from 4 to 10 weeks; n = 4 to 10 mice per time point). (C): EAO in uni-vx mice with Treg depletion is bilateral (at 10 weeks). (D): EAO is prevented by ablation of the vasectomized epididymis and testis at 2 but not 4 or 6 weeks post-vasectomy. (E): Orchitis in uni-vx and Treg-depleted mice shows peritubular leukocytic infiltration inside aspermatogenic tubule (left) and epididiymal ducts without sperm (Right) (H and E, x400); (F) Uni-vx and rIgG treated mice have normal testis (Left) and sperm-filled epididymis (Right) (H & E, x400). (G-I): Peri-tubuliar immune complexes (complement C3, green) co-

localize with CD4+ T cells (red) (G) and CD11c+ dendritic cells (red) (H) (x400) in testis. (I): Semi-quantification by dual-color immunofluorescence microscopy shows co-localization of immune complex and CD11c+ cell clusters (*p=0.008, n=5). Polyclonal antibody to mouse IgG, kappa light chain and complement C3 were used.

Figure 3. Serum antibody of uni-vx B6AF1 mice with Treg depletion targets the dominant sperm-specific Zan antigen. (A): Many sera from uni-vx (Vx) mice with Treg-depletion react with a 340 kD sperm protein band. (B): Serum antibody from mouse No. 5 (from frame A) reacts with the 340 kD band of wild type (wt) but not *zan* -/- (ko) sperm; and it inhibits the binding of D3p18-specific rabbit antibody to the D3p18 B cell epitope of Zan (Reproducible in 3 independent studies using sera from three mice).