Supporting Information

Wheeler et al. 10.1073/pnas.1017615108

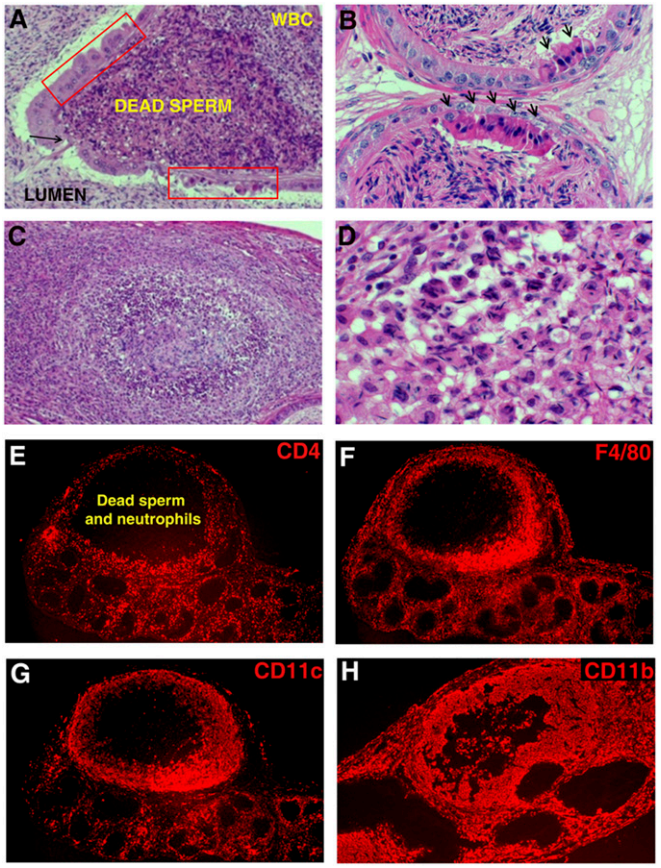

Fig. S1. Ipsilateral sperm granulomas in uni-vx mice have intense inflammation. Epididymis at 1 to 2 wk after vasectomy: (*A*) sperm extravasate (arrow) from duct with necrotic epithelial cells (red boxes); dead sperm and leukocytes (WBC) accumulate outside duct. (*B*) Focal apoptotic epithelial cells in cauda epididymis 24 h after vasectomy; note cells with red cytoplasm and pyknotic nuclei (arrows). Epididymis 8 to 10 wk after unilateral vasectomy: (*C*) organized sperm granuloma and (*D*) phagocytosis of sperm by macrophages. (H&E stain; magnification: *A*, 100×; *B*, 40×; *C* and *D*, 400×.) (*E*–*H*) Accumulation of $CD4^+$ T cells, F4/80^+ macrophages, $CD11c^+$ dendritic cells, and CD11b+ cells, respectively, in epididymal granuloma at 5 wk by fluorescence microscopy. (Magnification of 40×.) The TSA Biotin System (PerkinElmer) was used for immunohistology, with primary antibodies to the following: CD11c (HL3), CD11b (M1/70), F4/80 (C1:A3-1), and CD4 (L3T4).

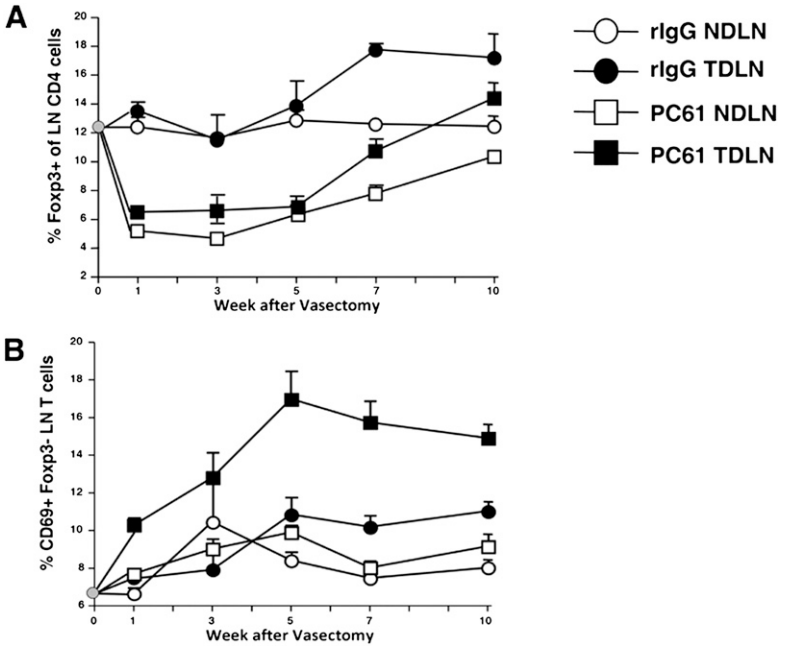

Fig. S2. Unilateral vasectomy and Treg depletion lead to regional LN-specific T-cell activation. For 5 wk after vasectomy and Treg depletion, the Foxp3$^+$ Tregs are reduced by 60% in all LNs (*A*). In the same period, a steady increase of CD69$^+$ Foxp3$^-$ effector T cells is confined to the testis-draining LNs (*B*). The filled gray circles at time 0 in *A* and *C* denote the average percentage of Treg and CD69$^+$ Foxp3$^-$ T cells in LN of untreated adult mice.

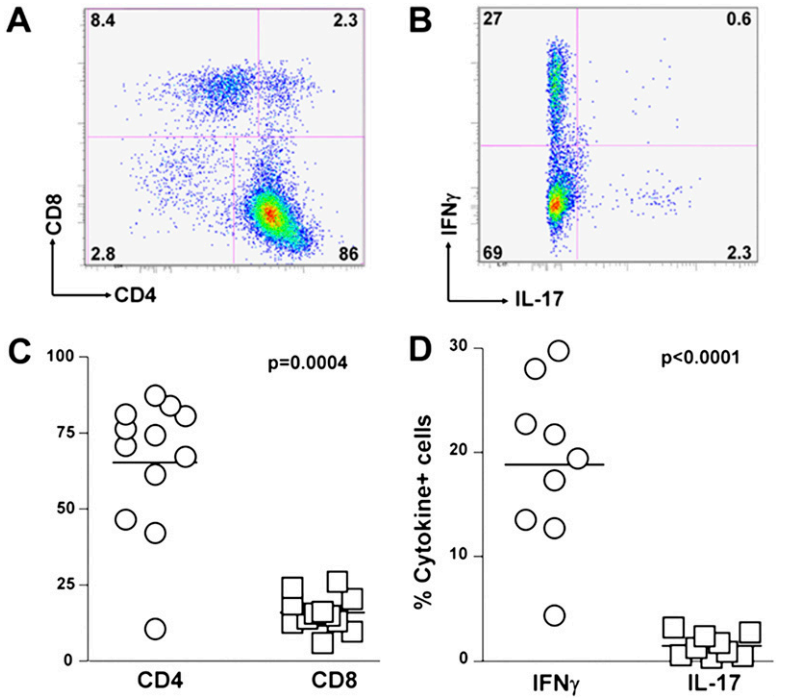

Fig. S3. IFN-γ–producing CD4 T cells dominate T cells infiltrating orchitic testes of uni-vx mice with Treg depletion. (*A* and *C*) Percentage of CD4$^+$ and CD8+ cells (gated on CD45$^+$CD3$^+$ cells). (*B* and *D*) Production of IFN-γ and IL-17 by testicular CD4$^+$ cells after in vitro stimulation and Golgi block. For flow cytometry, single testicular cells from decapsulated testes (1) were stained with antibodies to the following: CD45 (30-F11), CD4 (RM4-5; RM4-4), CD8 (53-6.7), Foxp3 (FJK-16s), IFN-γ (XMG1.2), IL-17 (TC11-18H10), CD11b (M1/70), and CD11c (HL3), plus live/dead fixable cell stain (Invitrogen). Intracellular cytokines were detected in CD4$^+$ T cells stimulated with 12-O-tetradecanoylphorbol-13-acetate/ionomycin, and treated with brefeldin A (leukocyte activation mixture with GolgiPlug; BD Pharmingen).

1. Yule TD, Montoya GD, Russell LD, Williams TM, Tung KS (1988) Autoantigenic germ cells exist outside the blood testis barrier. *J Immunol* 141:1161–1167.

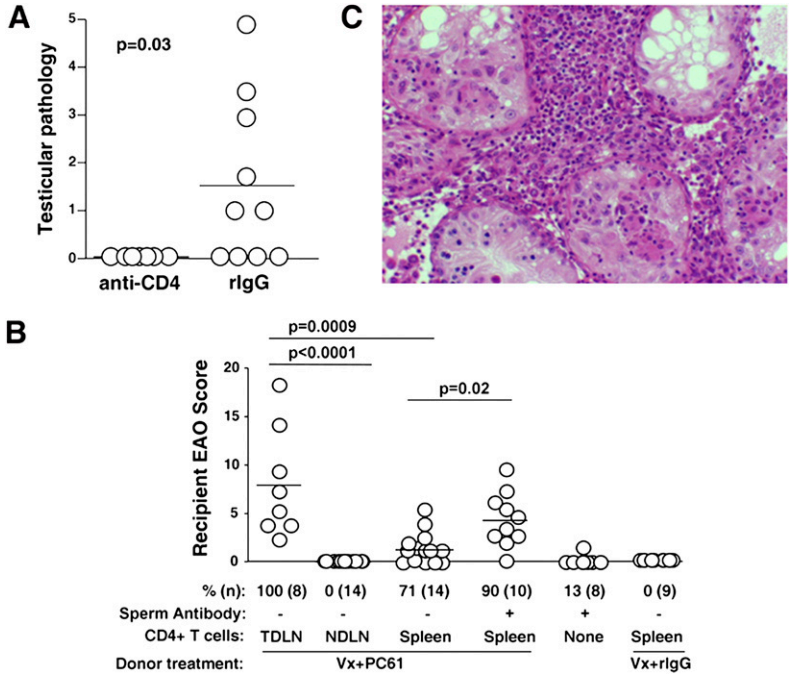

Fig. S4. CD4$^+$ T cells are necessary and sufficient for EAO induction, whereas autoantibody enhances pathology. (*A*) CD4 antibody inhibits orchitis development in uni-vx mice with Treg depletion. (*B*) Severe EAO is adoptively transferred only by CD4 T cells from testis-draining LNs (TDLN); and the mild EAO induced by spleen T cells is enhanced by cotransfer of serum sperm antibody IgG (NDLN, nondraining LN). (*C*) Severe EAO in TDLN CD4 T-cell recipient. (H&E stain, magnification of 400×.) CD4 T cells were depleted in vivo by GK1.1 (0.5 mg dose) from 4 to 7 wk. CD4 T cells were isolated magnetically from TDLNs (renal), NDLNs (axillary and brachial), or spleen, stimulated by CD3 antibody, and transferred at 2×10^7 cells per mouse. Testis-shielded recipients were irradiated with 650 rad (Varian 2300 Linear Accelerator) twice, 7 d apart, and received T cells 1 d later. Testis pathology was studied at 5 wk. IgG isolated by protein G column from sera of Treg-depleted uni-vx mice was transferred i.p.

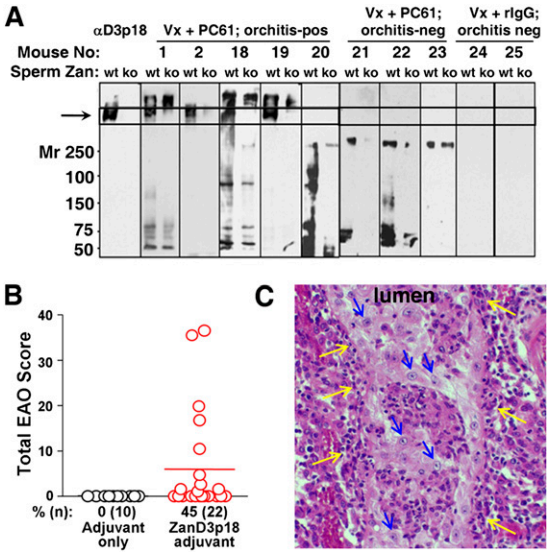

Fig. S5. Orchitogenic D3p18 polypeptide of Zan is a major antigen targeted by serum autoantibodies of uni-vx and Treg-depleted B6AF1 mice with EAO. (*A*) Zan-specific serum antibody binds to WT but not Zan-KO sperm antigens in adjacent lanes (arrow, 340-kDa band). As a result, in uni-vx mice with Treg depletion, Zan antibody is detected in EAO-positive (mice 1, 2, 5, 18, and 19), but not EAO-negative (mice 21, 22, and 23) mice. Control mice 24 and 25 are negative for Zan antibody. (*B*) EAO in B6AF1 mice immunized with Zan D3p18 in complete Freund's adjuvant. (*C*) Histopathology of a seminiferous tubule with severe EAO in a ZanD3p18-immunized mouse shows activated mononuclear cells at the BTB (yellow arrows). Inside seminiferous tubular lumen, leukocyte accumulation, germ cell depletion, and central displacement of Sertoli cells in the seminiferous tubule are evident (blue arrows). (H&E stain, magnification of 400×.)

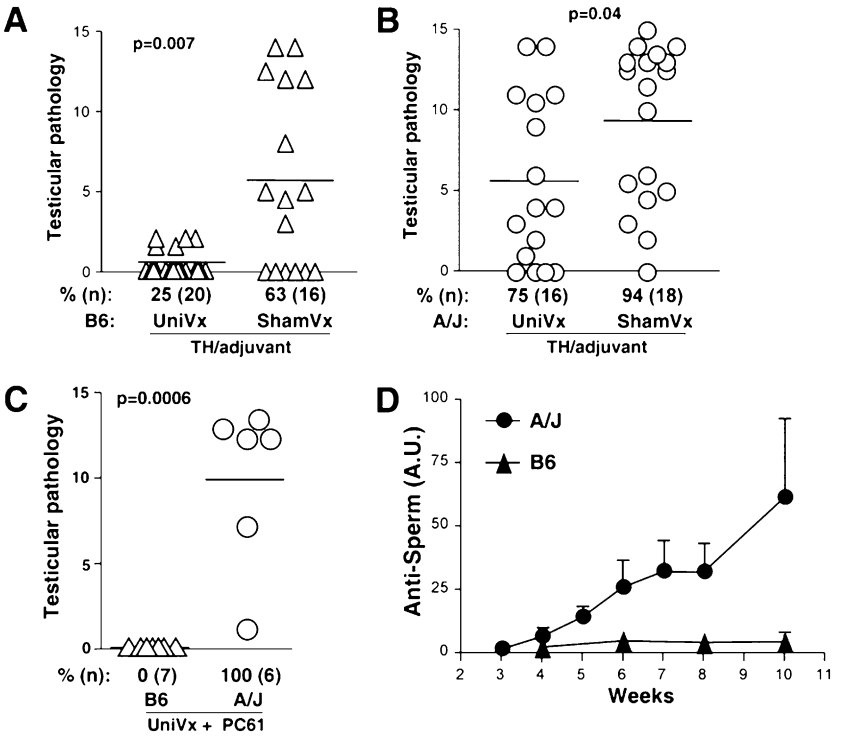

Fig. S6. Mouse strain variation in the response to unilateral vasectomy and Treg depletion by CD25 antibody. (*A* and *B*) To test for tolerance, uni-vx mice were immunized with TH and adjuvant 3 wk later, and their testicular pathology and serum sperm antibody were determined after another 3 wk. Note profound reduction of testicular pathology in uni-vx B6 mice (*A*) and only partial, although significant, reduction in uni-vx A/J mice (*B*). (*C* and *D*) A/J mice developed comparable testicular pathology and sperm antibody as B6AF1 mice (Fig. 2*A*). In contrast, B6 mice had no orchitis (*C*) or serum sperm antibodies (*D*) after unilateral vasectomy and Treg depletion.

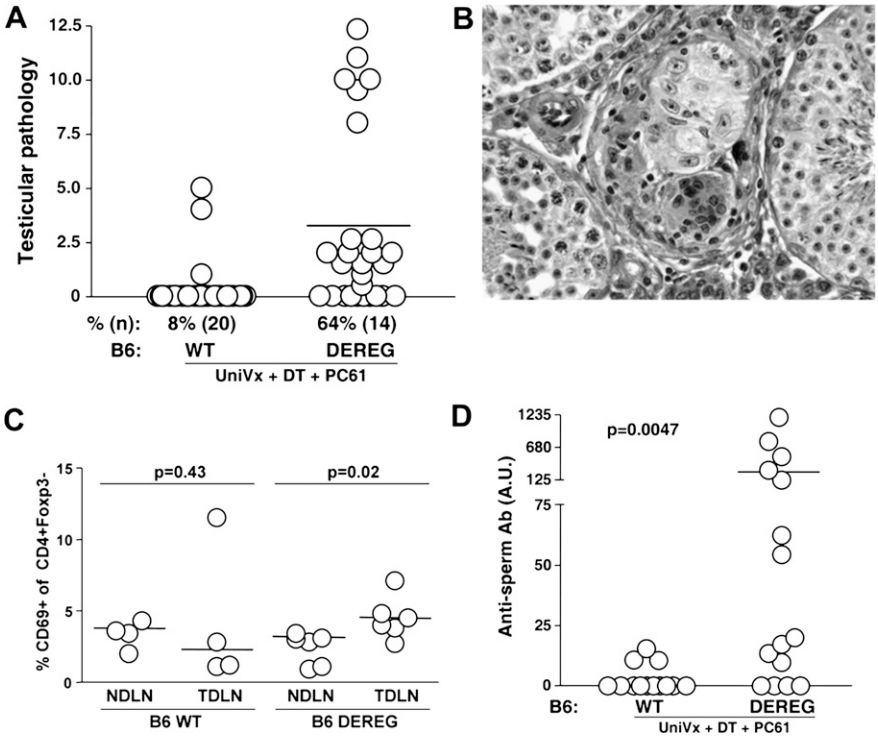

Fig. S7. Testicular pathology in uni-vx DEREG B6 mice after Treg depletion by diphtheria toxin (DT) and PC61 treatment. (*A*) Increased testicular pathology severity ($P = 0.0006$) and incidence were seen ($P = 0.00005$). (*B*) Orchitis pathology in DEREG B6 mice from *A*. (H&E stain, magnification of 400×.) (*C* and *D*) Increase in activated T cells in regional LNs ($P = 0.02$) (*C*) and sperm-specific antibodies (*D*) in uni-vx and Treg-depleted DEREG B6 mice.

Table S1. Summary of pathology scoring of experimental autoimmune orchitis

Location	Region	Type	Grade
Testicular pathology	Seminiferous tubules, straight tubules, rete testis	Inflammation (orchitis)	1–5 of 15
	Seminiferous tubules	Germ cell loss (aspermatogenesis)	1–10 of 15
Epididymal and vasal pathology	Caput, corpus, cauda, vas deferens	Inflammation (epididymitis)	0–8 of 16
	Caput, corpus, cauda, vas deferens	Sperm depletion	0–8 of 16
Total EAO score	All of the above	All of the above	Maximum, 31

Epididymal pathology can be induced by vasectomy alone. To exclude this from the evaluation of autoimmune testicular pathology, two different criteria are used to semiquantify the pathology in uni-vx versus nonvasectomized mice. In uni-vx mice, only the testicular pathologic process was graded. In nonvasectomized mice, testis pathology and epididymal pathology are combined to give a total EAO score. Testis and epididymal inflammation scores are as follows: 1, focal; 5, diffuse inflammation with necrosis; and 2–4, range of incremental inflammation. Aspermatogenesis: 1–10 represent the percentage of seminiferous tubules with reduction or absence of germ cells.