Caput epididymitis but not orchitis was induced by vasectomy in a murine model of experimental autoimmune orchitis

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Abstract

Immunization of mice with viable syngeneic testicular germ cells (TGC) alone can induce autoimmune responses against autoantigens of both round and elongating spermatids, resulting in the development of experimental autoimmune orchitis (EAO). Histological lesions in this EAO model without an adjuvant are characterized by lymphocytic infiltration into the testes, spermatogenic disturbance, and a complete lack of epididymitis. In this study, we investigated the effects of vasectomy (Vx) on TGC-induced EAO expecting that Vx augments the severity of testicular inflammation in A/J mice. The results showed that mice receiving Vx alone exhibited no significant inflammatory cell response in either the testes or epididymides, and mice receiving shamVx+TGC immunization had EAO with no epididymitis. In sharp contrast, no EAO was found in the testes of any mice receiving Vx+TGC immunization. Instead, caput epididymitis involving CD4+T cells, CD8+T cells, B cells, and macrophages were induced in them with striking elevation of the tissue levels of both *IL6* and *IL10* mRNA. Furthermore, serum autoantibodies induced by shamVx+TGC immunization were reactive with both round (immature) and elongating (mature) spermatids; however, those induced by Vx+TGC immunization were specific to acrosomes of mature spermatids and spermatozoa. These unexpected results indicate that Vx may induce the mode by which autoreactive lymphocytes gain access to TGC autoantigens in the epididymides, leading to autoimmune responses against the autoantigens of mature rather than immature spermatids.

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Introduction

Vasectomy (Vx) is a simple, effective, and widely used method of male contraception. The nature and incidence of local and systemic changes induced by Vx continue to be of interest. Many studies have revealed the induction of spermatogenic disturbance following Vx whereas numerous others have not. Some studies revealed the induction of humoral and cell-mediated autoimmune responses directed against sperm antigens after Vx (Ansbacher 1973, Alexander & Tung 1977, Herr *et al.* 1987, Nashan *et al.* 1990, Flickinger *et al.* 1994, 1995).

Experimental autoimmune orchitis (EAO) is one of the models of immunologic male infertility characterized by lymphocytic infiltration into the testes, leading to spermatogenic disturbance. In most EAO models, epididymitis occurs prior to orchitis, and the inflammatory lesions of the epididymides usually become more severe than those in the testes (Sato *et al.* 1981, Taguchi & Nishizuka 1981, Tung *et al.* 1987, Roper *et al.* 1998). Taguchi & Nishizuka (1981) were the first to investigate the effects of Vx on EAO in mice. They found that EAO with epididymitis could spontaneously develop after neonatal thymectomy on day 3 (day 3 Tx) without the use of any adjuvant in

(C57Bl/6×A/J) F1 mice. In this disease model, the incidence of EAO was found to increase when day 3 Tx mice received Vx on day 60. Later, Kojima & Spencer (1983) also reported that Vx increased the incidence of testicular atrophy in day 3 Tx mice when compared with those without Vx in Balb/c strain.

We previously demonstrated that immunization of C3H/He and A/J mice with viable syngeneic testicular germ cells (TGC) alone induces EAO without an adjuvant (Itoh *et al.* 1991a, 1991b, 1995, Tokunaga *et al.* 2008). However, this EAO model is very unique in which orchitis can occur without epididymitis. This biological phenomenon promoted us to investigate the effect of Vx on EAO. In this study, we examined Vx in TGC-immunized mice expecting that Vx augments the severity of both testicular and epididymal inflammations of autoimmune origin.

Results

The incidence and severity of the testicular and epididymal lesions are summarized in Table 1. The shamVx+PBS group showed normal spermatogenesis

Table 1 The effects of vasectomy on testicular germ cells (TGC)-induced experimental autoimmune orchitis (EAO).

Treatments	n	Orchitis Incidence (%)	Epididymitis Incidence (%)	Autoantibodies		Spermatogenic disturbance		
				Incidence (%)	Ig class	Incidence (%)	^a Grade (mean ± S.E.M.)	^b Apoptosis (mean ± S.E.M.)
None	8	0	0	0	None	0	0	0.48 ± 0.11 ^h
ShamVx+PBS	8	0	0	0	None	0	0	0.52 ± 0.18 ⁱ
Vx+PBS	10	0	10 ^c	100	IgG	100	3.13 ± 0.07 ^e	0.78 ± 0.03 ^j
ShamVx+TGC	8	100	0	100	IgM	100	2.17 ± 0.08 ^f	0.03 ± 0.01 ^k
					IgG			
					IgA			
Vx+TGC	22	0	100 ^d	100	IgM	100	1.70 ± 0.29 ^g	0.28 ± 0.05 ^l
					IgG			
					IgM			

P < 0.05 = e versus g, i versus k, i versus l, j versus l, k versus l, h versus k and h versus l; not significant = f versus g, h versus j, i versus j and i versus h.
^aThe level of spermatogenic disturbance was graded, on a 0–4 scale, using the degree of percentage of the seminiferous tubules showing the disappearance of mature germ cells and desquamation of germinal epithelium in the prepared sections: grade 0, 0%; grade 1, 1–5%; grade 2, 6–25%; grade 3, 26–50%; and grade 4, 51–100%. ^bNumber of TUNEL-positive cells/round or oval-shape of seminiferous tubules. ^cInflammatory cells focally infiltrated into the corpus but not the caput epididymis. ^dInflammatory cells infiltrated into the caput epididymis.

with no inflammatory signs in either the testes or epididymides (Fig. 1a and e). In the Vx+PBS group, the testes were characterized by significant aspermatogenesis without inflammatory cell infiltration. In their epididymides, there were many retained spermatozoa in the epididymal ducts with no significant inflammatory cell infiltration (Fig. 1b and f). Only one out of the ten mice in the Vx+PBS group exhibited the focal lymphocytic infiltration into the interstitium of the corpus of epididymis (Table 1). In the shamVx+TGC group, orchitis characterized by lymphocytic infiltration and the

resultant aspermatogenesis was seen in all examined mice with no epididymitis (Fig. 1c and g). Apoptotic germ cells of this group were much lower in number because of removal of stem cells from the damaged seminiferous tubules (Table 1). In sharp contrast, unexpectedly, no lymphocytic infiltration was found in the testes of any of the Vx+TGC group (Fig. 1d). Furthermore, the spermatogenic disturbance was less severe than that in the Vx+PBS group, and the number of apoptotic germ cells in the Vx+TGC group was significantly higher than that in the shamVx+TGC group and lower than that in the Vx+PBS

Figure 1 Testicular and epididymal sections from (a and e) the shamVx+PBS (b and f) Vx+PBS, (c and g) shamVx+TGC and (d and h) Vx+TGC groups. Testis sections of (a) shamVx+PBS group showing intact seminiferous tubules exhibiting all stages of maturation of the germinal epithelium from spermatogonia to spermatozoa; (b) Vx+PBS group showing suppression of spermatogenesis without inflammatory cell infiltration; (c) shamVx+TGC group showing damaged seminiferous tubules surrounded by many inflammatory cells and (d) Vx+TGC group showing slight suppression of spermatogenesis without inflammatory cell infiltration. Sections of the caput of the epididymis from (e) shamVx+PBS group showing closely packed epididymal ducts with little interstitium; (f) Vx+PBS group showing enlarged ducts containing many spermatozoa; (g) shamVx+TGC group showing the lack of evidence of inflammatory cell response; and (h) Vx+TGC group showing the widened interstitium containing many lymphocytes. Asterisks indicate areas of lymphocytic inflammation. Bar, 20 µm.

group (Table 1). Alternatively, in the Vx+TGC group, extensive interstitial inflammation was found in the caput epididymides away from the vasectomy site (Fig. 1h; Table 1). In the shamVx+TGC group, many CD4+T cells with significant number of CD8+T and B220 cells infiltrated into the testes while no positive cells were found in testes of the Vx+TGC group (Fig. 2; Table 1). Instead, CD4+T, CD8+T, and B220 cells were found in the caput epididymides of the Vx+TGC group, although no or few lymphocytes could be observed in the caput epididymides of the other animal groups (Fig. 3; Table 1). A significant number of F4/80-positive macrophages, differing from lymphocytes, were detected in the epididymides of all animal groups (Fig. 4). However, the macrophages in the Vx+TGC group tended to infiltrate into the epithelial layers of the epididymal ducts when compared with the other four groups.

On reaction of sera with normal frozen sections of seminiferous tubules and epididymal ducts, anti-TGC autoantibodies were detected in the Vx+PBS, shamVx+TGC, and Vx+TGC groups but not in the untreated and shamVx+PBS groups (Fig. 5). The immune sera contained both IgG and IgM autoantibodies. In addition, IgA autoantibodies were further detected in the shamVx+TGC group (Table 1). Faint immunostains were detected in various types of germ cells in the seminiferous tubules in the Vx+PBS group.

Figure 2 Testicular sections from (a–c) shamVx+TGC and (d–f) Vx+TGC groups examined with the use of mAbs to (a and d) CD4, (b and e) CD8, and (c and f) B220. Note the infiltration of CD4-, CD8-, and B220-positive lymphocytes in the shamVx+TGC but not in Vx+TGC group. Bar, 50 μ m.

In the shamVx+TGC group, the serum autoantibodies preferentially reacted with immature spermatids in the seminiferous tubules and epididymal spermatozoa in the caput but not cauda of epididymis. By contrast, in the Vx+TGC group, the autoantibodies specifically reacted with the crescent-shaped acrosomes of mature spermatids and spermatozoa.

To determine why Vx treatment induced caput epididymitis but not orchitis after TGC immunization, the expression of inflammation-related genes was examined in both testicular and epididymal tissues in each animal group (Fig. 6). In the epididymides, Vx+PBS group showed strong expression of the IFN- γ (*Ifng*), IL-6 (*Il6*), and IL-10 (*Il10*) genes while no striking change was detected in the epididymal cytokines in the shamVx+TGC group. In the Vx+TGC group, the gene expression of epididymal cytokines was increased similarly to that in the Vx+PBS group. In particular, the IL-6 level was more significantly increased while both the IFN- γ and IL-10 levels tended to decrease when compared with the Vx+PBS group. In contrast, some testicular cytokines were significantly but slightly increased only in the shamVx+TGC group.

Discussion

In this study, we demonstrated that Vx did not augment the severity of TGC-induced EAO although it had been

Figure 3 Epididymal sections from (a–c) Vx+PBS and (d–f) Vx+TGC groups examined with the use of mAbs to (a and d) CD4, (b and e) CD8, and (c and f) B220. Note the infiltration of CD4-, CD8-, and B220-positive cells in the Vx+TGC but not in Vx+PBS group. Bar, 50 μ m.

Figure 4 F4/80 antigen-positive cells in epididymal sections obtained from mice in the (a) untreated (b) shamVx+PBS, (c) Vx+PBS, (d) shamVx+TGC, and (e) Vx+TGC groups. It should be noted that many macrophages had infiltrated into the epithelial layers of the epididymal ducts in Vx+TGC group. (f) Negative control without anti-F4/80 antibody. Bar, 20 μ m.

reported that Vx makes day 3 Tx-induced EAO more severe (Taguchi & Nishizuka 1981, Kojima & Spencer 1983). Conversely, Vx suppressed EAO and alternatively induced caput epididymitis in the TGC-induced model.

The lymphocytic population in the epididymitis in the Vx+TGC group was composed of CD4+T, CD8+T, and B cells and macrophages, like in EAO lesions in the shamVx+TGC group (Itoh *et al.* 1991b). Interestingly, the autoantibodies in the Vx+TGC group were found to be against the heads of mature spermatids and spermatozoa although those in the Vx+PBS or shamVx+TGC group were against more immature germ cells. Taguchi & Nishizuka (1981) have reported that autoimmune epididymitis had been induced prior to orchitis in day 3 Tx mice. Furthermore, their immunohistochemical examination of day 3 Tx mice demonstrated that the serum autoantibodies were against the crescent-shaped heads of elongated spermatids and spermatozoa. Therefore, the presence of autoantibodies against mature spermatozoa in both the day 3 Tx- and Vx+TGC-induced models may be related to the autoimmune inflammation affecting the epididymis rather than the testis. It has been reported that the ducts of the caput epididymis are the sites of absorption

of various materials leaving the testis (Hamilton 1972). Therefore, Vx may allow the epididymal ducts to absorb testis-secreting materials, including autoantigens of mature spermatids, more strongly and to leak or excrete some of the absorbed autoantigens to the outside of the ducts. Actually, Howards & Johnson (1979) reported the leakage of epididymal spermatozoa from the ducts of the caput epididymis after Vx in hamsters. In preliminary experiments, we tried to induce autoimmune epididymitis by means of immunization with epididymal spermatozoa (instead of TGC) with or without Vx. However, autoimmune epididymitis was never induced in them. This implies that the autoantigens of the mature spermatids in TGC rather than epididymal spermatozoa are important for Vx+TGC-induced caput epididymitis. Unfortunately, Taguchi & Nishizuka (1981), and Kojima & Spencer (1983) did not document the histopathological effects of Vx on epididymitis in day 3 Tx-induced EAO. Therefore, it remains unknown whether Vx affects the severity of the autoimmune epididymitis of this model. Furthermore, it is also unclear why Vx augmented day 3 Tx-induced EAO. We suppose that the epididymitis induced by day 3 Tx may become more severe with Vx, leading to spreading of more inflammation to the testes. It has been demonstrated that autoreactive lymphocytes could preferentially gain access to the tubuli recti and the rete testis where the blood–testis barrier is incomplete (Dym & Fawcett 1970, Aoki & Fawcett 1975, Itoh *et al.* 1995, 1998). However, Vx may increase the hydrostatic pressure in the epididymal ducts rather than the tubuli recti and the rete testis, resulting in possible leakage of more germ cell autoantigens into the epididymal interstitium. There may be other possibilities that blood flow or lymphatic drainage in the testis and epididymis is changed by Vx. These factors may induce the different inflammatory responses between shamVx+TGC- and Vx+TGC-induced autoimmunity.

In this study, it was also unexpectedly found that the levels of IFN- γ , IL-6, and IL-10 in the epididymides strikingly increased with Vx alone. These elevated cytokine levels may also have affected the induction of epididymitis in the Vx+TGC group. Although it remains unknown which cell population produces each cytokine after Vx, it was reported that epithelial and myoid cells of the epididymis produce IL-10 and IL-6 respectively, in cryptorchid cryptepididymis (Verajankorva *et al.* 2002). Clinically, the semen IL-6 level is increased in Vx reversal patients (Nandipati *et al.* 2005). We had showed that IL-6 administration reduced the incidence and severity of TGC-induced EAO (Li *et al.* 2002). On the other hand, an *in vitro* study demonstrated that IL-6 had promoted apoptosis of germ cells in isolated seminiferous tubules in rats (Rival *et al.* 2006). IL-10 is ordinarily known to be an immunosuppressive cytokine. In EAO, adeno-associated virus-mediated human IL-10 gene transfer suppressed the development of the disease

Figure 5 Normal frozen sections of seminiferous tubules and epididymal ducts reacted with diluted sera (50-fold) obtained from (a, f and k) untreated, (b, g and l) shamVx+PBS, (c, h and m) Vx+PBS, (d, i and n) shamVx+TGC, and (e, j and o) Vx+TGC groups followed by incubation with HRP-conjugated anti-mouse IgG. Note that the autoantibodies in the Vx+TGC group specifically reacted with the crescent-shaped acrosomes of (e) elongated (mature) spermatids in seminiferous tubules and (j and o) spermatozoa in ducts of both caput and cauda epididymis. Bar; 10 μ m.

(Watanabe *et al.* 2005). However, it exerts the opposite effect in some autoimmune diseases including EAO (Rosenbaum & Angell 1995, Hill & Sarvernick 2002, Kaneko *et al.* 2003, Tokunaga *et al.* 2008). Although pathophysiological effects of these two cytokines on the epididymal tissues remain unclear, the Vx-induced elevation of the epididymal cytokines may attract inflammatory cells to the epididymis rather than the testis. In our next study, we will attempt to identify the cell population that secretes each cytokine in Vx+TGC-induced epididymitis by means of both immunohistochemistry and *in situ* hybridization.

In other experiments involving donor lymphocytes obtained from TGC-immunized mice, we examined an adoptive transfer of EAO (passive EAO) into recipient mice and found that the histopathology of passive EAO in the recipients differed from that in TGC-immunized donors (active EAO; Itoh *et al.* 1991b, 1992). Namely, the lesions in active EAO showed severe orchitis with a complete lack of epididymitis; however, the passive EAO was consistently accompanied by autoimmune epididymitis. Moreover, in some recipients, it was found that

only autoimmune epididymitis had developed, i.e., without EAO. Although the reason for the different histopathologies of active and passive EAO remains unclear, this phenomenon may help us to elucidate the pathogenesis of Vx+TGC-induced epididymitis with suppression of EAO. There may be a possibility that lymphocyte traffic route is changed by Vx, leading to epididymitis rather than orchitis. Comparative studies on shamVx+TGC and Vx+TGC groups with regard to the histopathology of adoptive transferred inflammation with donor lymphocytes are currently in progress.

Materials and Methods

Animals

Experiments were conducted with 8-week-old A/J male mice, weighing 25–28 g. The animals were purchased from SLC (Shizuoka, Japan) and kept in the Laboratory Animal Center of Tokyo Medical University for 2 weeks before use. They were maintained at 22–24 °C and 50–60% relative humidity with a 12 h light:12 h darkness. The approval of the Tokyo Medical University Animal Committee was obtained for this study.

Figure 6 Expression of mRNAs of TNF- α , IFN- γ , IL-1 α , IL-6, and IL-10 in both testicular and epididymal tissues of the four experimental groups. Relative intensity was calculated after which the expression in the control (shamVx+PBS group) for each point had been normalized as 1. Each bar represents the mean $\pm$ s.d. ($n=3$). Asterisks indicate $P<0.05$ versus control group. Double asterisks indicate $P<0.01$ versus control group.

Vx treatment

The operation was performed under sodium pentobarbital (50 mg/kg body weight, i.p.) anesthesia via a lower mid-abdominal incision. The vas deferens on each side was exposed without causing injury to the adherent blood vessels and then occluding ligature comprising silk thread was applied to each vas deferens. Then the incision was closed in two layers. The procedure without the vasa deferentia being ligatured was performed as a shamVx for control mice. All operations were carried out under sterile conditions.

TGC preparation

Testes were excised from syngeneic mice, teased with scissors in cold Hanks' balanced salt solution (HBSS), and then passed through a stainless steel mesh. The TGC, including spermatogonia, spermatocytes, round spermatids, and elongating spermatids, were harvested by centrifugation at 400 g for 10 min and washed three times with cold HBSS, after determining their viability by means of trypan blue dye exclusion. The TGC suspension contained $>99\%$ germ cells at all stages of spermatogenesis; the remainder ($<1\%$) comprised Sertoli cells and Leydig cells [Itoh *et al.* 1991a].

Experimental design

The mice were divided into five groups as follows: untreated normal group, naive mice without any treatments; shamVx+PBS and shamVx+TGC group mice received the sham operation on day 0 and then were injected subcutaneously with 200 μ l PBS and 1×10^7 TGC in 200 μ l PBS respectively, on days 7 and 21; Vx+PBS and Vx+TGC group mice received bilateral Vx on day 0 and then were injected subcutaneously with 200 μ l PBS and 1×10^7 TGC in 200 μ l PBS respectively, on days 7 and 21. Each group consisted of 8–22 mice (Table 1). Previous studies had already shown that EAO fully develops within 40 days after the first TGC immunization [Itoh *et al.* 1995]. Therefore, on day 47, the mice were deeply anesthetized with pentobarbital (65 mg/kg body weight) and blood was collected from the hearts of all mice in the five groups. Serum samples from individual mice were stored at -80°C until used.

Histopathological assessment

The testes and epididymides were immediately removed from killed mice. For histological examination, the organs were fixed with Bouin's solution and embedded in plastic (Technovit 7100; Kulzer & Co., Wehrheim, Germany) without cutting them to avoid artificial damage to the testicular and epididymal tissues. Sections (5 μ m) were obtained at 40–45 μ m intervals and stained with Gill hematoxylin III and 2% eosin Y for light microscopical observation. The level of spermatogenic disturbance was graded on a 0–4 scale, using the degree of percentage of the seminiferous tubules showing the disappearance of mature germ cells and desquamation of germinal epithelium in the prepared sections: grade 0, 0%; grade 1, 1–5%; grade 2, 6–25%; grade 3, 26–50%; and grade 4, 51–100% [Itoh *et al.* 1991a]. More than 200 round or oval-shaped seminiferous tubules were counted in each mouse.

For immunohistochemical and histochemical examinations, the testes and epididymides of experimental mice were put in OCT compound (Miles Laboratories, Naperville, IL, USA), and then frozen in liquid nitrogen and stored at -80°C until used. Sections of 6 μ m were cut with a cryostat (CM1900; Leica, Wetzlar, Germany), dried in air, and then fixed in 95% ethanol for 10 min at -20°C . The sections were rinsed in Tween-buffered saline (TBS) and then incubated with Block Ace (Yukijirushi, Hokkaido, Japan) for 20 min at room temperature to inactivate endogenous peroxidase activity. After this treatment, serial sections were incubated for 2 h with rat anti-mouse CD4 monoclonal antibodies (1:100 dilution, class II MHC antigen-restricted T cells; BD Biosciences, San Jose, CA, USA), rat anti-mouse CD8 monoclonal antibodies (1:50 dilution, class I MHC antigen-restricted T cells; BD Biosciences), rat anti-mouse B220 monoclonal antibodies (1:100 dilution, B cells and some activated T cells; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rat anti-mouse F4/80 monoclonal antibodies (1:100 dilution, macrophages; Abcam, Cambridge, UK). After washing, the sections were incubated for 30 min with rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA, USA). Immunoreactive cells were visualized with a Vectastain ABC

Kit (Vector Laboratories) with 0.05% 3, 3'-diaminobenzidine 4HCl (DAB, Nickel Solution) and 0.01% H₂O₂ as the chromogen. The sections were finally counterstained with methyl green. Sections processed with PBS instead of the primary antibodies were used as negative controls.

For the detection of serum autoantibodies, frozen sections of testes and epididymides from normal mice were fixed with 95% ethanol for 10 min at -20 °C and then incubated with 50-fold serial dilutions of the collected sera for 60 min at room temperature. After rinsing in PBS, the sections were incubated for 60 min with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgA (with 1:500 dilution each; ZyMax, South San Francisco, CA, USA), or rabbit anti-mouse IgM (1:200 dilution, ZyMax) at room temperature. After washing with PBS, the HRP-binding sites were detected with 0.05% DAB and 0.01% H₂O₂ (Itoh *et al.* 1994).

For the detection of apoptotic cells, TUNEL was performed according to the method of Gavrieli *et al.* (1992) with a slight modification. Paraffin sections (5–6 µm) from experimental mice were cut onto silane-coated glass slides, dewaxed with toluene, and rehydrated in an ethanol series. After washing with PBS, the sections were treated with 5 µg/ml proteinase K in PBS at 37 °C for 15 min. The sections were then rinsed once with deionized distilled water, and the commercially available kit (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Serologicals Corporation, New York, NY, USA) was used for detection of the 3'-OH end of DNA. Endogenous peroxidase activity was blocked by treating the sections with 3% H₂O₂ in PBS for 5 min at room temperature. The sections were incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled dideoxy nucleotide in a humidified chamber at 37 °C for 1 h. After reaction with stop buffer for 10 min, the sections were incubated with an anti-digoxigenin peroxidase conjugate for 30 min. Peroxidase activity was detected by exposing the sections to a solution containing 0.05% DAB. The sections were finally counterstained with methyl green. Negative controls were performed using distilled water instead of the TdT enzyme. For statistical analysis, more than 200 round- or oval-shaped seminiferous tubules/animal were counted, and the number of TUNEL-positive cells/seminiferous tubule (mean ± s.d.) in each group was determined.

Analysis of mRNA expression of cytokines by real-time RT-PCR

To investigate the relationship between the histopathological alteration and cytokine levels in the testes and epididymides, we analyzed the expression of the IL-1 α (*Il1a*), IL-6 (*Il6*), IL-10 (*Il10*), IFN- γ (*Ifng*), and TNF- α (*Tnf*) genes, using real-time RT-PCR. Total RNA was purified from each fresh tissue sample using TRIzol reagent (Invitrogen Corp.) according to the manufacturer's protocol, and its concentration was calculated from the extinction at 260 nm as determined spectrophotometrically. For the first-strand cDNA synthesis, 10 µg total RNA was reverse transcribed with a High Capacity cDNA Archive Kit (PE Applied Biosystems, Foster City, CA, USA), according to the standard protocol. Subsequently, 5 µl cDNA encoding IL-1 α (ID: Mm00443258-m1), IL-6 (ID: Mm00446190-m1), IL-10

(ID: Mm00439616-m1), TNF- α (ID: Mm00443258-m1), IFN- γ (ID: Mm00801778-m1), and β -actin (ID: Mm00607939-s1) respectively with forward and reverse primers designed from TaqMan primers (PE Applied Biosystems) were amplified by 40 cycles of PCR, carried out with an iCycler thermal cycler (Bio-Rad). Real-time fluorescence-monitored PCR was performed with a PE Applied Biosystems model 7500 Sequence Detection System using TaqMan PCR Master Mix Reagents 2 $\times$ and TaqMan Gene Expression Assays of primers 20 $\times$, according to the manufacturer's instructions. The results are expressed relative to the amount of β -actin transcript used as an internal control. In preliminary experiments, testicular cytokines were found to temporally increase during the first 1 week after shamVx. To exclude the influence of stress such as abdominal incision and s.c. injection, cytokine data from shamVx+PBS group were used as controls in this study.

Statistical analysis

The significance of differences was analyzed by means of ANOVA. Statistical significance was concluded when $P < 0.05$.

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