Activation of PTH1R alleviates epididymitis and orchitis through Gq and β-arrestin-1 pathways

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Inflammation in the epididymis and testis contributes significantly to male infertility. Alternative therapeutic avenues treating epididymitis and orchitis are expected since current therapies using antibiotics have limitations associated to side effects and are commonly ineffective for inflammation due to nonbacterial causes. Here, we demonstrated that type 1 parathyroid hormone receptor (PTH1R) and its endogenous agonists, parathyroid hormone (PTH) and PTH-related protein (PTHrP), were mainly expressed in the Leydig cells of testis as well as epididymal epithelial cells. Screening the secretin family G protein–coupled receptor identified that PTH1R in the epididymis and testis was down-regulated in mumps virus (MuV)- or lipopolysaccharide (LPS)-induced inflammation. Remarkably, activation of PTH1R by abaloparatide (ABL), a Food and Drug Administration–approved treatment for postmenopausal osteoporosis, alleviated MuV- or LPS-induced inflammatory responses in both testis and epididymis and significantly improved sperm functions in both mouse model and human samples. The anti-inflammatory effects of ABL were shown to be regulated mainly through the Gq and β-arrestin-1 pathway downstream of PTH1R as supported by the application of ABL in *Gnaq*$^{\pm}$ and *Arrb1*$^{-/-}$ mouse models. Taken together, our results identified an important immunoregulatory role for PTH1R signaling in the epididymis and testis. Targeting to PTH1R might have a therapeutic effect for the treatment of epididymitis and orchitis or other inflammatory disease in the male reproductive system.

PTH1R | epididymitis | orchitis | mumps virus | abaloparatide

A total of ∼15% of childbearing–age couples are infertile, and male infertility accounts for nearly 50% of this sterility. Currently, male infertility is not only a personal problem but also becomes a public health issue (1–3). Epididymitis and orchitis, which are inflammatory diseases of the epididymis and testis, respectively, are estimated to account for 6 to 15% of male infertility and thus have attracted increasing attention in recent years (4–6). The current treatment of epididymitis and orchitis mainly relies on antibiotics since the inflammations were commonly caused by bacteria-induced infections. However, antibiotic therapies are known to cause undesired adverse effects. In addition, infection by viruses, such as mumps virus (MuV), cytomegalovirus, and herpes simplex virus, could also lead to epididymitis and orchitis, which are insensitive to regular antibiotic therapies (4, 7, 8). Therefore, novel therapeutic strategies for threating epididymitis and orchitis are needed.

The G protein–coupled receptors (GPCRs) are the largest family of membrane proteins with wide-ranging expression and physiologic functions (9–11). Currently, GPCRs account for ∼30% of drug targets with clinical usage, but the physiological and pathological functions of the majority of GPCRs in the male reproduction system are still undefined (3, 12). Therefore, the characterization of functions of GPCRs involved in the regulation of inflammatory responses in the epididymis and testis may provide novel clues for treatments of epididymitis and orchitis. Mammalian GPCR are grouped into five families including Rhodopsin (Class A), Secretin (Class B1), Adhesion (Class B2), Glutamate (Class C), and Frizzled (Class F) (12, 13). The secretin family GPCR, consisting of 15 members, are peptide receptors that bind important endogenous peptide hormones (14–16). The secretin family GPCR and their cognate peptide ligands are implicated as drug targets in many pathologies, such as migraine, cardiovascular disease, diabetes, psychiatric disorders, neurodegeneration, osteoporosis, and inflammation (15–17). Among them, the type 1 parathyroid hormone receptor (PTH1R) primarily mediates skeletal development, bone turnover, and calcium homeostasis through interacting with two endogenous polypeptide ligands, parathyroid hormone (PTH) and PTH-related protein (PTHrP) (18, 19). PTH1R has been found to play an important role in many pathologies, such as osteoporosis, hypoparathyroidism, and cancer-associated hypercalcemia and cachexia (20, 21). Recent RNA sequencing results suggested a high expression of PTH1R in spermatozoa, implying a regulatory role of this receptor

Significance

Currently, male infertility has become a public health issue. The inflammations in the epididymis and testis are estimated to account for 6 to 15% of male infertility and thus have attracted increasing attention. Conventional therapies for epididymitis and orchitis using antibiotics have limitations in terms of side effects and ineffectiveness for nonbacterial inflammations, thus highlighting an urgent need for novel therapeutic strategies. Here, the screening of secretin family G protein–coupled receptors (GPCRs) enabled us to identify that activation of PTH1R by a Food and Drug Administration–approved osteoporosis drug abaloparatide effectively counteracts epididymitis and orchitis through selective transducer-mediated pathways. Our results offer potential therapeutics to treat epididymitis and orchitis by targeting GPCRs other than traditional antibiotic therapies.

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in the male reproductive system (22). However, the detailed functions and potential underlying mechanisms still remain unknown.

In the present study, we found that PTH1R and its two endogenous ligands, PTHrP and PTH, were expressed in the Leydig cells of testis as well as epididymal epithelial cells. Both messenger RNA (mRNA) and protein levels of PTH1R and its ligands were down-regulated in the testis and epididymis upon MuV- or lipopolysaccharide (LPS)-induced inflammation. Interestingly, abaloparatide (ABL), which is an agonist of PTH1R and a Food and Drug Administration (FDA)–approved drug for postmenopausal osteoporosis (23), significantly alleviated MuV- or LPS-induced inflammation in the testis and epididymis and also enhanced sperm motility of mice. Further studies using *Gnaq*$^{\pm}$ and *Arrb1*$^{-/-}$ mice models and cellular approaches elucidated the mechanisms underlying the regulatory role of the PTH1R in the testis and epididymis. Our findings suggest that endogenous PTH1R signaling plays an important immunoregulatory role in the male reproduction system, and PTH1R is a potential therapeutic target for the treatment of epididymitis and orchitis.

Results

Expression of PTH1R and Its Endogenous Ligands Were Down-Regulated in Testis and Epididymis upon MuV-Induced Inflammation. To examine the potential functional roles of secretin family GPCR, a group of receptors serving as important drug targets, in the regulation of epididymitis and orchitis, we detected the expression patterns of secretin family GPCR in the cauda of the epididymis and the testis by qRT-PCR analysis at 24 h after orthotopic injection of MuV (1×10^7 plaque-forming units [PFU]) in the testis or cauda of the epididymis in mice. Intriguingly, only the expression of PTH1R in both the testis (Fig. 1*A*) and the cauda of the epididymis (Fig. 1*B*) of mice was significantly decreased. The mRNA level of PTH1R was relatively enriched in the cauda of the epididymis and testis as well as in the mouse TM3 Leydig cell line (*SI Appendix*, Fig. S1 *A* and *B*). Moreover, immunofluorescence (IF) analysis revealed that PTH1R was coexpressed with HSD3B2, a mouse Leydig cells marker, and with AQP9, an epididymal epithelial cells marker, in the testis and the cauda of the epididymis, respectively (Fig. 1*C*). Notably, the specificity of the PTH1R antibody was verified by Western blot analysis in the heterologous expression system using other Flag-tagged secretin family GPCRs, including PTH2R and CRHR1, as the negative control (*SI Appendix*, Fig. S1*C*). The marked decrease of PTH1R IF in both the testis and the cauda of the epididymis using anti-PTH1R small interfering RNA (siRNA) further supported the PTH1R antibody specificity (*SI Appendix*, Fig. S1*D*). In accordance with the screening data, both the mRNA and protein levels of PTH1R were reduced by more than 50% in the testis and the cauda of the epididymis after MuV injection (Fig. 1 *D–F*). Similar results were also obtained in mouse TM3 Leydig cells and epididymal primary epithelial cells after MuV injection (5 multiplicity of infection [MOI]) in vitro (Fig. 1 *G–I*). The PTH and PTHrP are two known endogenous ligands of PTH1R (18, 19). We therefore measured the expression levels of PTH and PTHrP in testis and the cauda of the epididymis and found that PTH and PTHrP mRNA levels were approximately a quarter of those in parathyroid (*SI Appendix*, Fig. S1*E*). IF analysis further revealed that, similar to PTH1R, PTH and PTHrP were primarily expressed in the Leydig cells and epididymal epithelial cells (*SI Appendix*, Fig. S1*F*). Intriguingly, whereas the mRNA levels of PTH and PTHrP in testis and the cauda of the epididymis were up-regulated at 6 h after MuV injection, their expression were dramatically decreased at 12 and 24 h post-MuV administration (Fig. 1 *J* and *K*).

Importantly, the expression of PTH1R, PTH, and PTHrP in the Leydig cells and epididymal epithelial cells were confirmed in normal human testis and epididymis samples and were significantly down-regulated in orchitis and epididymitis samples compared with normal human samples through immunohistochemistry (Fig. 1*L*). Similar to that of the mouse, the mRNA and protein levels of PTH1R, PTH, and PTHrP were significantly down-regulated in the testis and the cauda of the epididymis of human samples at 12 h after MuV stimulation in vitro (Fig. 1 *M–O*). Collectively, these results implied that the endogenous PTH/PTHrP-PTH1R signaling system might be functionally participated in MuV-induced inflammation in the testis and the cauda of the epididymis in both mice and humans, which needs to be further validated by examining their potential paracrine effects.

PTH1R Activation by ABL Alleviated MuV-Induced Inflammatory Responses in Testis. Different from the endogenous PTH and PTHrP, ABL is a 34–amino acid peptidic PTH1R agonist, which barely activates the PTH2R (24). Given its prominent anabolic effects in increasing bone mineral density and reducing fractures, ABL is currently an FDA-approved treatment for postmenopausal osteoporosis. We therefore used ABL to interrogate how PTH1R activation affected the MuV-induced inflammatory responses in testis. C57BL/6J mouse were given a single dose injection of ABL (5 mg/kg) at 6 h after MuV administration. We found that serum testosterone levels were improved after ABL injection compared to the control vehicle–treated group under MuV challenge (Fig. 2*A*). However, the ameliorative effects of ABL were significantly abrogated by PTH1R knockdown (Fig. 2*A*). Moreover, ABL significantly ameliorated MuV-induced testicular dysfunction of the mice as evidenced by improved morphologic injury, including the increased spermatogenic cells and reorganization of the germinal epithelium (Fig. 2*B*). The MuV-induced cell apoptosis was reduced upon ABL treatment as demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Fig. 2*C* and *SI Appendix*, Fig. S2*B*). Moreover, the MuV-induced expression of multiple inflammatory factors in the testis, such as tumor necrosis factor (TNF-α), interleukin (IL6), chemokine (C-C motif) ligand 2 (MCP1), and chemokine (C-X-C motif) ligand 10 (CXCL10), were significantly suppressed by ABL application (Fig. 2*D*). Similarly, in the TM3 cell line or human testicular tissue, ABL (40 nM) treatment after 3 h of stimulation with MuV (5 MOI) alleviated MuV-induced aberrant elevation of inflammatory factors (Fig. 2 *E* and *F*). Importantly, in vivo knocking down of PTH1R in testis or preincubation of the PTH1R antagonist PTHrP–(7–34) (10 μM) with mouse TM3 Leydig cells suppressed ABL-mediated anti-inflammatory responses, suggesting that the ABL improved the MuV-induced inflammation through the activation of PTH1R (*SI Appendix*, Fig. S2 *A* and *B* and Fig. 2 *A–E*).

PTH1R Activation by ABL Reduced MuV-Induced Inflammatory Responses in Epididymis. The ABL significantly ameliorated MuV-induced epididymitis of mice, supported by improved epithelium lining, decreased vacuolated cells, and degenerated germ cells (Fig. 3*A*). Moreover, the interstitial infiltration by LY6G$^+$ cells (a neutrophils marker) and CD11C$^+$ cells (a macrophages marker) was reduced by ABL (Fig. 3*B*). Similar to its effects in orchitis, ABL treatment effectively inhibited MuV-induced epithelial cell apoptosis as demonstrated by TUNEL staining (Fig. 3*C* and *SI Appendix*, Fig. S2*B*). Accordingly, the MuV-stimulated expression of inflammatory factors (Fig. 3*D*) in the cauda of the epididymis, such as TNF-α, IL6, MCP1, and CXCL10, were moderately inhibited by ABL. Similar results were also obtained using epididymal primary epithelial cells of mice and cultured human epididymal tissue, showing that ABL effectively alleviated MuV-induced abnormal elevation of inflammatory factors (Fig. 3

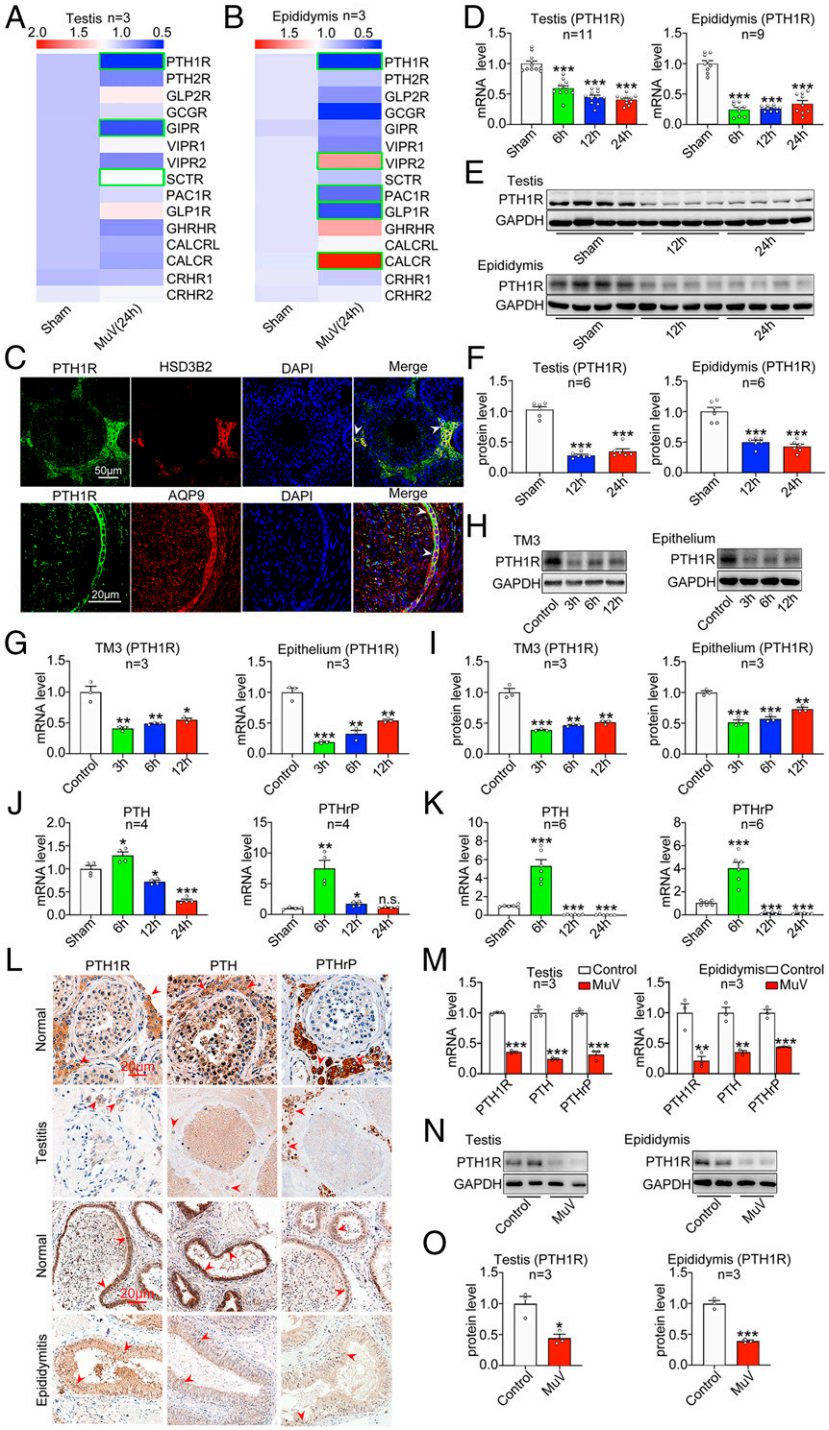

Fig. 1. The expression of PTH1R was reduced in the testis and epididymis upon MuV-induced inflammation. (*A* and *B*) Relative mRNA levels of the secretin family GPCR in the testis (*A*) or the cauda of the epididymis (*B*) of mouse treated with MuV (1×10^7 PFU) for 24 h ($n = 3$). (*C*) The expression profile of PTH1R in the testis and the cauda of the epididymis of mouse by IF staining (HSD3B2, mouse Leydig cells marker; AQP9, epididymal epithelial cells marker). (*D*) Relative *Pth1r* mRNA levels in the testis (*Left*, $n = 11$) or the cauda of the epididymis (*Right*, $n = 9$) of mouse treated with MuV (1×10^7 PFU) for different time. (*E* and *F*) Representative blots (*E*) and quantification (*F*) of PTH1R protein levels in the testis or the cauda of the epididymis of mouse treated with MuV (1×10^7 PFU) for different time ($n = 6$). (*G*) Relative *Pth1r* mRNA levels in mouse TM3 Leydig cells (*Left*) or epididymal primary epithelial cells (*Right*) treated with MuV (5 MOI) for different time ($n = 3$). (*H* and *I*) Representative blots (*H*) and quantification (*I*) of PTH1R protein levels in TM3 cells or epididymal primary epithelial cells treated with MuV (5 MOI) for different time ($n = 3$). (*J* and *K*) Relative mRNA levels of *Pth* and *Pthrp* in the testis ($n = 4$) (*J*) or in the cauda of the epididymis ($n = 6$) (*K*) of mice treated with MuV (1×10^7 PFU) for different time. (*L*) The expression profiles of PTH, PTHrP, and PTH1R in the testis and epididymis from human by immunohistochemistry staining. (*M*) Relative mRNA levels of *PTH*, *PTHrP*, and *PTH1R* in the testis (*Left*) and the cauda of the epididymis (*Right*) from human samples treated with MuV (5 MOI) for 12 h ($n = 3$). (*N* and *O*) Representative blots (*N*) and quantification (*O*) of PTH1R protein levels in testis (*Left*) and the cauda of the epididymis (*Right*) from human samples treated with MuV (5 MOI) for 12 h ($n = 3$). Data information: (*D*, *F*, *J*, and *K*) *n.s.* no significance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle-treated samples (sham). (*G* and *I*) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle-treated cells (control). (*M* and *O*) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle-treated samples (control).

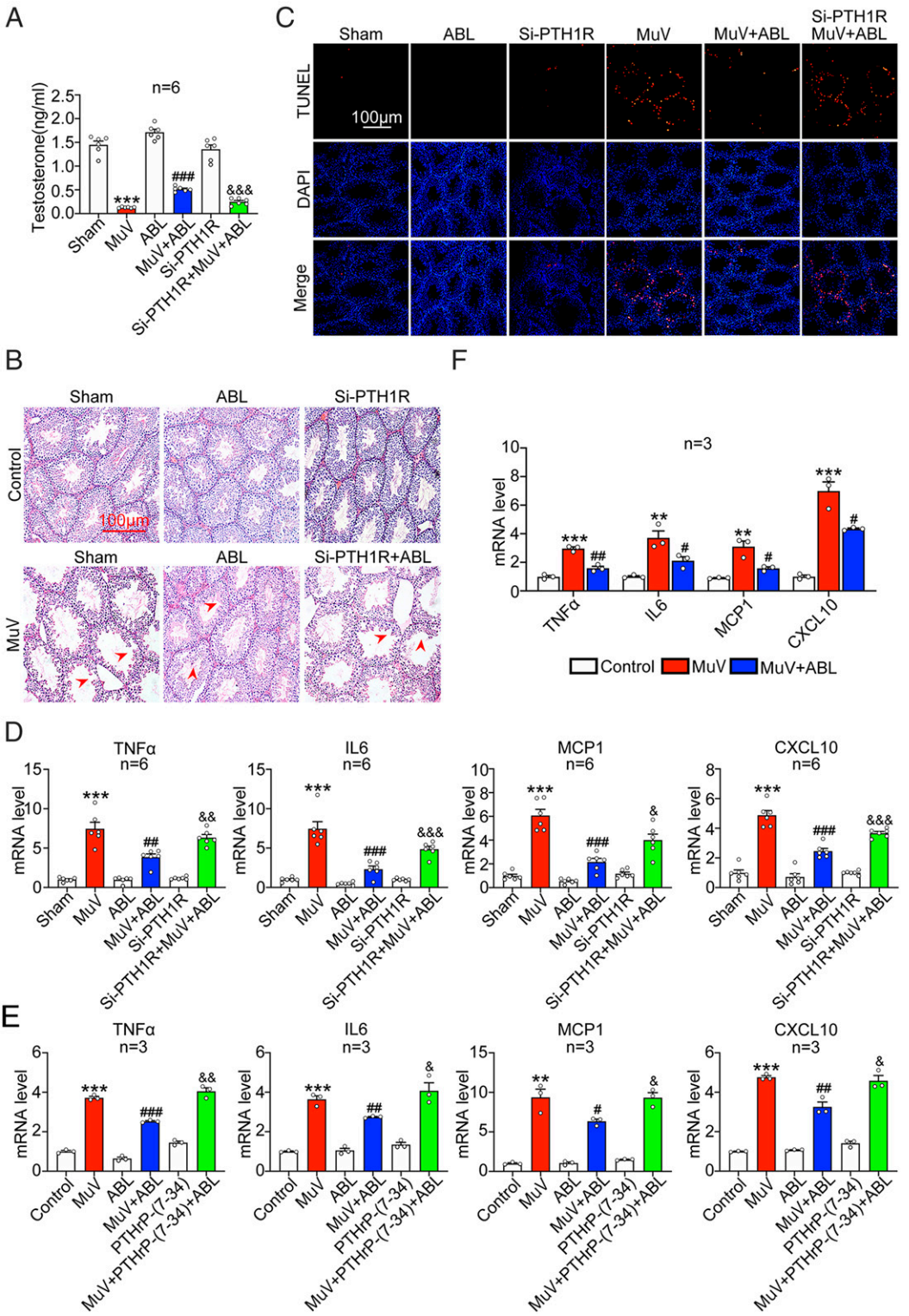

Fig. 2. PTH1R activation by ABL alleviated MuV-induced inflammatory responses in testis. (*A*) Effects of ABL on serum testosterone levels of mice without or with PTH1R knockdown in testis challenged with MuV (1×10^7 PFU) for 24 h. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after MuV injection ($n = 6$). (*B*) Hematoxylin and eosin staining of the testis of mouse. (*C*) The cell apoptosis revealed by TUNEL staining analysis in the testis of mouse. (*D*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the testis of mice from different groups ($n = 6$). (*E*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in TM3 cells 12 h after MuV (5 MOI) treatment. The cells were pretreated without or with PTH1R antagonist PTHrP– (7 to 34) (10 μM) for 30 min before the ABL (40 nM) was applied 3 h after MuV treatment ($n = 3$). (*F*) Relative mRNA levels of *TNF-α*, *IL6*, *MCP1*, and *CXCL10* in the testis of human samples from different groups. The testis samples were challenged with MuV (5 MOI) for 12 h, and the ABL (40 nM) was applied 3 h after MuV treatment ($n = 3$). Data information: (*A*, *D*, and *F*) $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-treated samples (sham). #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with samples treated only with MuV. &$P < 0.05$, &&$P < 0.01$, &&&$P < 0.001$ compared with samples treated with MuV+ABL. (*E*) $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-treated cells (control). #$P < 0.05$, ##$P < 0.01$ compared with cells treated only with MuV. &$P < 0.05$, &&$P < 0.01$ compared with cells treated with MuV+ABL.

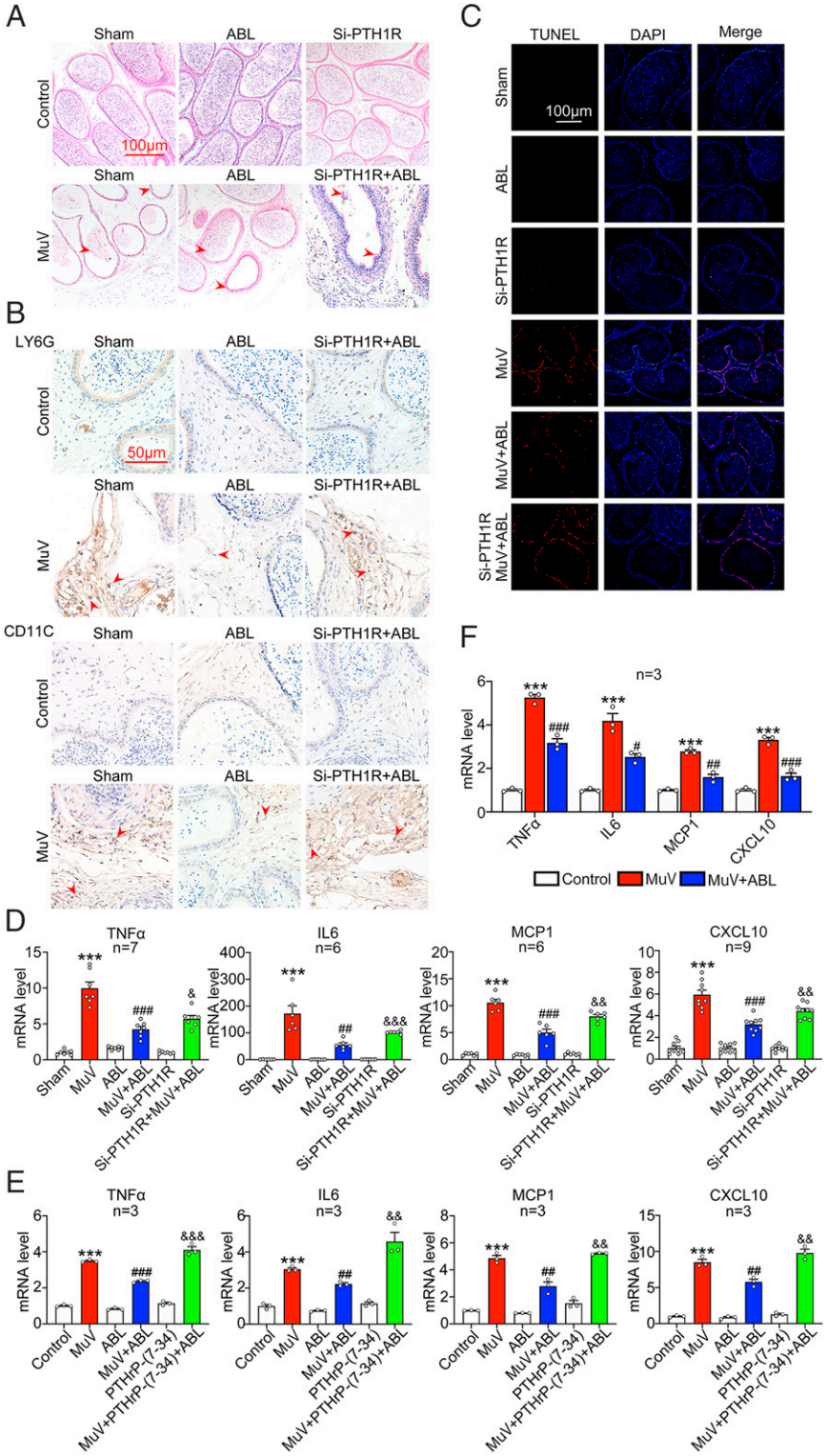

Fig. 3. PTH1R activation by ABL reduced MuV-induced inflammatory responses in epididymis. (*A*) Hematoxylin and eosin staining of the cauda of the epididymis of mice from different groups. The mice epididymis without or with PTH1R knockdown was challenged with MuV (1×10^7 PFU) for 24 h. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after MuV treatment. (*B*) Inflammatory infiltration of $LY6G^+$ cells (a neutrophils marker) and $CD11C^+$ cells (a macrophages marker) revealed by immunohistochemistry staining in the cauda of epididymis of mice from different groups. (*C*) The cell apoptosis revealed by TUNEL staining analysis in the cauda of the epididymis of mice. (*D*) Relative mRNA levels of *Tnf-α* ($n = 7$), *Il6* ($n = 6$), *Mcp1* ($n = 6$), and *Cxcl10* ($n = 9$) in the cauda of the epididymis of mice from different groups. (*E*) Relative mRNA levels of *Tnf[-α*, *Il6*, *Mcp1*, and *Cxcl10* in epididymal primary epithelial cells 12 h after MuV (5 MOI) treatment. The cells were pretreated without or with PTH1R antagonist PTHrP– (7 to 34) (10 μM) for 30 min before the ABL (40 nM) was applied 3 h after MuV treatment ($n = 3$). (*F*) Relative mRNA levels of *TNF-α*, *IL6*, *MCP1*, and *CXCL10* in the cauda of the epididymis of human samples from different groups. The epididymis samples were challenged with MuV (5 MOI) for 12 h and the ABL (40 nM) was applied 3 h after MuV treatment ($n = 3$). Data information: (*D–F*) ***$P < 0.001$ compared with vehicle-treated samples or cells. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with MuV-treated samples or cells. &$P < 0.05$, &&$P < 0.01$, &&&$P < 0.001$ compared with samples or cells treated with MuV+ABL.

E and *F*). Of note, the anti-inflammatory effects exerted by ABL were blunted by PTH1R knocking down in the epididymis or pre-incubation of the PTH1R-specific antagonist PTHrP− (7 to 34) (*SI Appendix*, Fig. S2 *A* and *B* and Fig. 3 *A*–*E*), thus confirming that ABL improved anti-inflammatory effects via the activation of PTH1R. Intriguingly, knocking down of PTH or PTHrP, the endogenous agonists of PTH1R, further deteriorated the MuV-induced inflammatory response in the absence of ABL treatment in either the epididymis or testis, as revealed by the significantly increased production of inflammatory factors, such as TNF-α and MCP1, suggesting a potential role of the local PTH/PTHrP-PTH1R signaling in the regulation of immune homeostasis (*SI Appendix*, Fig. S2 *C*–*F*).

Inflammatory responses in the epididymis were known to impair sperm maturation and motility (4). Given the protective effects of ABL in MuV-induced epididymitis, we next explored the potential role of ABL in the regulation of fertilization by analyzing the effects of ABL on sperm activity. A total of 24 h after the injection of MuV, the sperms were collected from the cauda of the epididymis and were subjected to computer-assisted semen analysis. Upon challenge with MuV, the sperm survival rate (SSR) and motility parameters including average path velocity, straight-line velocity, and curvilinear velocity were all decreased by 10 to 40% compared with the control vehicle–treated group (Table 1). Notably, ABL pretreatment for 18 h before sperm collection significantly improved the SSR as well as motility defects caused by MuV. Interestingly, ABL could even significantly enhance sperm motility under normal circumstances, suggesting that ABL might be used as a novel therapeutic for improving male fertility (Table 1).

Gq and β-arrestin-1 Signaling Was Required for ABL-Mediated Anti-Inflammatory Effects through PTH1R. PTH1R was known to signal through multiple G proteins as well as β-arrestins (21). To investigate the mechanism underlying anti-inflammatory effects after the engagement of PTH1R by ABL in the testis and the cauda of the epididymis, we examined the expression of Gs, Gq, β-arrestin-1, and β-arrestin-2 in the testis (*SI Appendix*, Fig. S3*A*) and the cauda of the epididymis (*SI Appendix*, Fig. S3*B*) after the injection of MuV. The expressions of Gs, Gq, β-arrestin-1, and β-arrestin-2 were not significantly affected (*SI Appendix*, Fig. S3 *A* and *B*). We next examined the potential roles of different G protein or arrestin subtypes in ABL-mediated anti-inflammatory effects in TM3 cell lines and epididymal primary epithelial cells by combined genetic and pharmacological approaches. In particular, the specific siRNAs targeting Gs, Gq, β-arrestin-1, or β-arrestin-2 were designed and functionally validated by Western blotting analysis (*SI Appendix*, Fig. S4 *A* and *B*). Moreover, NF449, YM-254890, and RO-318220, which are widely used inhibitors of Gs, Gq, and PKC, respectively, were employed (1, 25–30). The specificity of these inhibitors was confirmed by secondary messenger measurements and Western blot of the phosphorylation level of downstream signaling components. Notably, whereas the NF449 specifically inhibited the ABL-induced cAMP accumulation in both TM3 cells and epididymal primary epithelial cells, the YM-254890 selectively blocked the ABL-stimulated IP3 production (*SI Appendix*, Fig. S4 *C* and *D*). Despite showing no significant effect on secondary messenger production, the RO-318220 abrogated the ABL-induced phosphorylation of MARCKS, which is a typical cellular substrate of PKC (*SI Appendix*, Fig. S4 *C*–*E*) (31–33). These pharmacological inhibitors and knockdown strategies were shown to have no significant effects on the basal levels of inflammatory factors, including TNF-α, IL6, MCP1, and CXCL10 (*SI Appendix*, Figs. S5*A* and S6*A*). Intriguingly, whereas β-arrestin-2 knockdown showed no significant effects on the ABL-mediated reduction of MuV-stimulated inflammatory response, the application of NF449 or Gs knockdown led to slight but significant inhibition of ABL effects on certain parameters, including the TNF-α, IL6, and MCP1 in TM3 cells and CXCL10 in epididymal epithelial cells. In stark contrast, either knockdown of Gq or β-arrestin-1 or the application of YM-254890 or RO-318220 all significantly blocked the ABL-induced protective effects on any of the four inflammatory factors (*SI Appendix*, Figs. S5*B* and S6*B*). Furthermore, the beneficial effects of ABL, such as the enhanced serum testosterone levels and decreased histological damage in the testis and epididymis, were significantly diminished in the $Gnaq^{\pm}$ and $Arrb1^{-/-}$ mice compared to that of their wild-type (WT) littermates (*SI Appendix*, Fig. S7 *A* and *B* and Fig. 4*A*). Moreover, the inhibitory effects of ABL on MuV-induced inflammatory infiltration and inflammatory factor production were also blunted in $Gnaq^{\pm}$ and $Arrb1^{-/-}$ mice as revealed by immunohistochemical, mRNA, and enzyme-linked immunosorbent assay analysis (Fig. 4 *B*–*F* and *SI Appendix*, Fig. S7*C*). Collectively, the results indicated that the Gq and β-arrestin-1 signaling downstream of PTH1R play critical roles in regulating ABL-mediated anti-inflammatory responses in the testis and the cauda of the epididymis, whereas Gs also contributes to this regulatory process.

PTH1R Activation by ABL Alleviated LPS-Induced Inflammatory Responses in Testis and Epididymis. We next examined whether the activation of PTH1R-mediated beneficial effects in epididymitis and orchitis caused by nonviral infections, and so we employed LPS-induced inflammatory models. Similar to MuV-induced effects in the testis and epididymis, the mRNA level and protein level of PTH1R in both the testis and the cauda of the epididymis were significantly decreased in mouse and human in response to LPS stimulation (Fig. 5 *A*–*E*). ABL-stimulated

Table 1. Effects of ABL on MuV-induced sperm injury in the epididymis

	Control		ABL	
Variable	Sham	MuV	Sham	MuV
SSR (%)	76.67 ± 3.28	57.20 ± 4.95*	88.67 ± 2.33*	78.33 ± 1.45#
VAP (μm/s)	109.30 ± 4.41	82.98 ± 1.07*	121.90 ± 1.11	93.60 ± 3.01#
VSL (μm/s)	66.45 ± 2.24	40.90 ± 1.93*	74.67 ± 1.25*	58.47 ± 6.24#
VCL (μm/s)	246.00 ± 1.19	221.00 ± 2.01*	269.10 ± 4.94*	239.70 ± 1.82#
ALH (μm)	11.93 ± 0.50	7.38 ± 0.74	13.28 ± 1.24	9.55 ± 0.46
BCF (Hz)	33.17 ± 0.95	34.03 ± 1.38	34.43 ± 1.01	34.38 ± 2.02
STR (%)	52.40 ± 1.94	51.00 ± 3.36	62.20 ± 2.71*	59.60 ± 3.82
LIN (%)	24.67 ± 1.67	22.00 ± 0.41	33.00 ± 1.92*	28.80 ± 2.48#

*$P < 0.05$ compared with vehicle-treated samples (control). #$P < 0.05$ compared with MuV-treated samples (control). SSR, sperm survival rate; VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

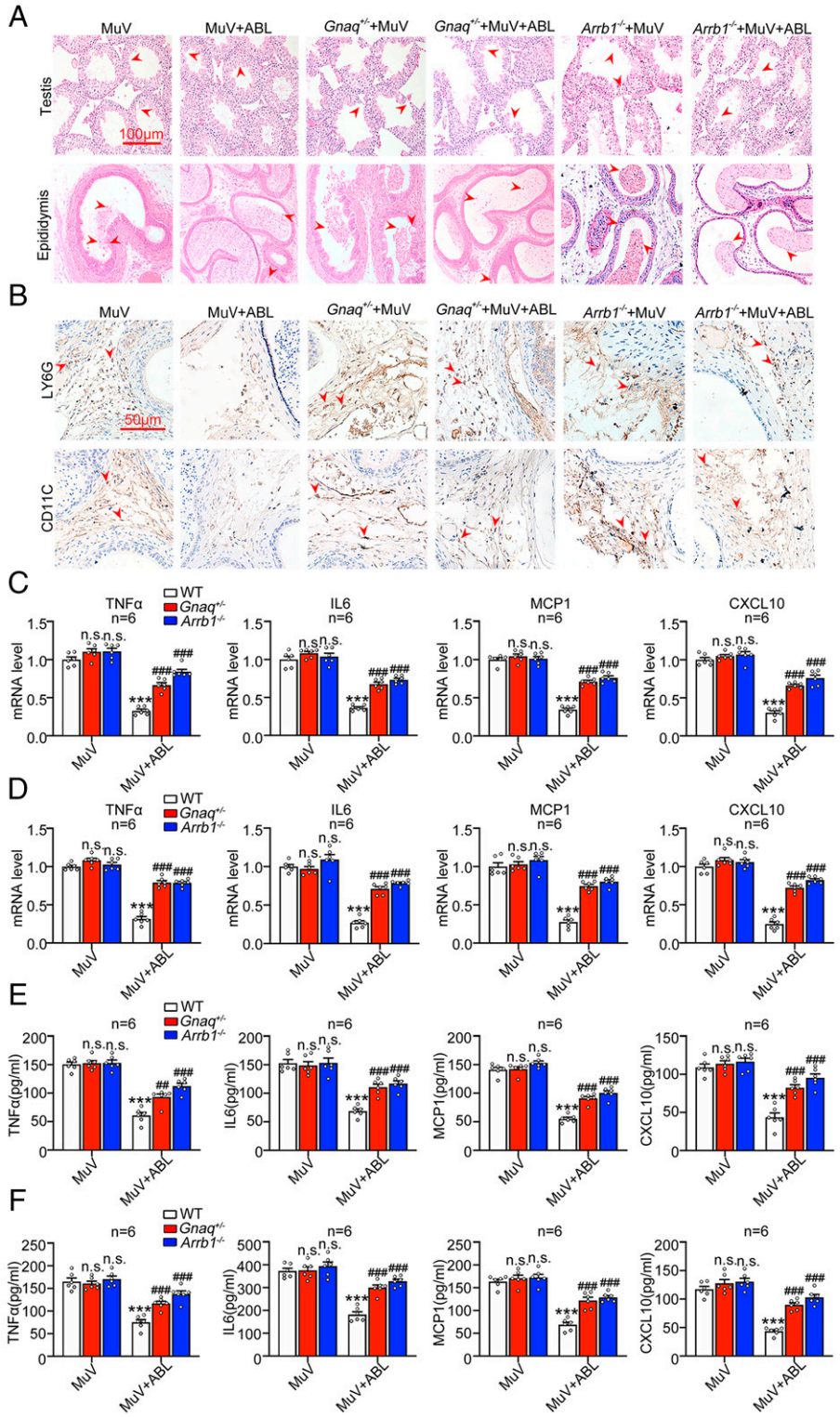

Fig. 4. Gq and β-arrestin-1 signaling were required for ABL-mediated anti-inflammatory effects through PTH1R. (*A*) Hematoxylin and eosin staining of the testis (*Upper*) and the cauda of the epididymis (*Lower*) of mice from different groups. The testis or the cauda of the epididymis of WT, *Gnaq*$^{\pm}$, or *Arrb1*$^{-/-}$ mice was challenged with MuV (1×10^7 PFU) for 24 h. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after MuV treatment. (*B*) Inflammatory infiltration of LY6G$^+$ cells and CD11C$^+$ cells revealed by immunohistochemistry staining in the cauda of epididymis of mice from different groups. (*C*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the testis of mice from different groups ($n = 6$). Data were normalized to the mRNA levels of respective inflammatory factors in WT group upon MuV challenge. (*D*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the cauda of the epididymis of mice from different groups ($n = 6$). Data were normalized to the mRNA levels of respective inflammatory factors in WT group upon MuV challenge. (*E*) TNF-α, IL6, MCP1, and CXCL10 levels in the testis of mice from different groups measured by enzyme-linked immunosorbent assay (ELISA) ($n = 6$). (*F*) TNF-α, IL6, MCP1, and CXCL10 levels in the cauda of the epididymis of mice from different groups measured by ELISA ($n = 6$). Data information: (*C–F*) *n.s.* no significance, ***$P < 0.001$ compared with MuV-treated WT group. ##$P < 0.01$, ###$P < 0.001$ compared with MuV- and ABL-treated WT group.

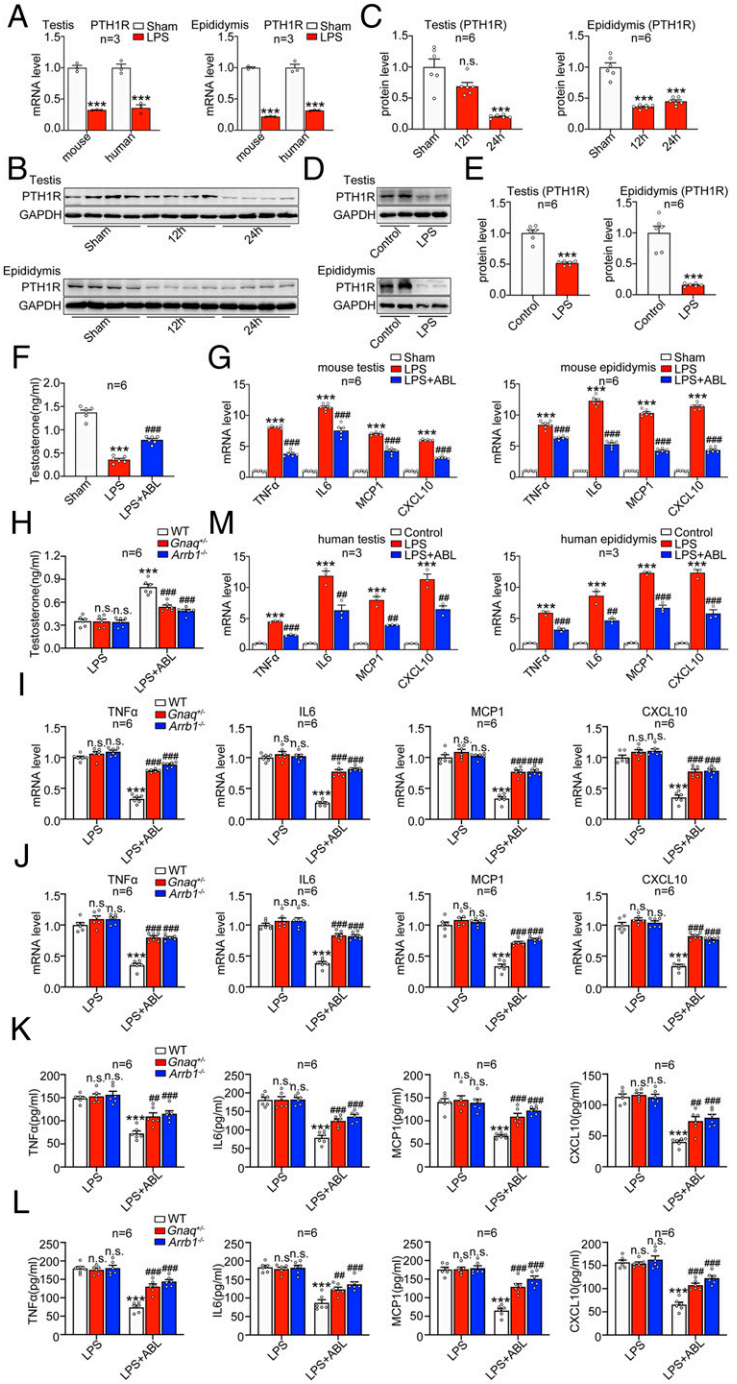

Fig. 5. PTH1R activation by ABL alleviated LPS-induced inflammatory responses in the testis and epididymis. (*A*) Relative mRNA levels of *PTH1R* in the testis (*Left*) and the cauda of the epididymis (*Right*) of mouse 24 h after intraperitoneal injection of LPS (3 mg/kg) or from human sample 12 h after ex vivo infection of LPS (1 μg/mL) ($n = 3$). (*B* and *C*) Representative blots (*B*) and quantification (*C*) of PTH1R protein level in the testis and the cauda of epididymis of mouse treated with LPS for different time ($n = 6$). (*D* and *E*) Representative blots (*D*) and quantification (*E*) of PTH1R protein level in the testis and the cauda of the epididymis of human 12 h after ex vivo infection of LPS ($n = 6$). (*F*) Effect of ABL on the serum testosterone levels in mice 24 h after LPS (3 mg/kg) injection. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after LPS treatment ($n = 6$). (*G*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the testis (*Left*) or the cauda of epididymis (*Right*) of mice from different groups ($n = 6$). (*H*) Effect of ABL on the serum testosterone levels in WT, *Gnaq*$^{+/-}$, or *Arrb1*$^{-/-}$ mice 24 h after LPS (3 mg/kg) injection. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after LPS treatment ($n = 6$). (*I*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the testis of mice from different groups ($n = 6$). Data were normalized to the mRNA levels of respective inflammatory factors in WT group upon LPS challenge. (*J*) Relative mRNA levels of *Tnf-α*, *Il6*, *Mcp1*, and *Cxcl10* in the cauda of the epididymis of mice from different groups ($n = 6$). Data were normalized to the mRNA levels of respective inflammatory factors in WT group upon LPS challenge. (*K*) TNF-α, IL6, MCP1, and CXCL10 levels in the testis of mice from different groups measured by enzyme-linked immunosorbent assay (ELISA) ($n = 6$). (*L*) TNF-α, IL6, MCP1, and CXCL10 levels in the cauda of the epididymis of mice from different groups measured by ELISA ($n = 6$). (*M*) Relative mRNA levels of *TNF-α*, *IL6*, *MCP1*, and *CXCL10* in the testis (*Left*) or the cauda of the epididymis (*Right*) of human samples from different groups ($n = 3$). Data information: (*A*, *C*, *E–G*, and *M*) *n.s.* no significance, ***$P < 0.001$ compared with vehicle-treated samples. ##$P < 0.01$, ###$P < 0.001$ compared with MuV-treated samples. (*H* and *L*) *n.s.*, no significance, ***$P < 0.001$ compared with MuV-treated WT group. ##$P < 0.01$, ###$P < 0.001$ compared with MuV- and ABL-treated WT group.

PTH1R activation significantly alleviated LPS-induced reduction of serum testosterone level and the accumulation of inflammatory factors, including TNF-α, IL6, MCP1, and CXCL10, in the testis and epididymis (Fig. 5 *F–M*). Notably, the beneficial effects of ABL on serum testosterone secretion and inflammatory factor suppression were significantly diminished in the *Gnaq*$^{\pm}$ and *Arrb1*$^{-/-}$ mice, demonstrating the contribution of Gq and β-arrestin-1 pathways to the antagonizing of LPS-induced inflammatory responses (Fig. 5 *H–L*). These results collectively indicated that the activation of PTH1R-Gq and β-arrestin-1 signaling showed anti-inflammatory effects on testis and epididymis pathogenesis in response to both viral and nonviral causes.

Discussion

Epididymitis and orchitis, which are both an inflammation of the epididymis and testis, respectively, are significant etiological factors contributing to male infertility. Antibiotics are commonly prescribed to treat epididymitis and orchitis since urinary tract pathogens, such as *Escherichia coli* and *Neisseria gonorrhoeae*, represent the most frequent causes of the inflammatory responses (4). However, the current therapy is far from perfect due to numerous adverse side effects, and there is also accumulating evidence indicating that epididymitis and orchitis could be caused by viral infections, which is insensitive to regular antibiotic therapies (4). Therefore, alternative therapeutic strategies are needed to be developed to treat epididymitis and orchitis. In the present study, we identified PTH1R, a class B1 GPCR participating in bone turnover and calcium homeostasis, in murine epididymal and testicular systems that regulates the anti-inflammatory responses to maintain the immune homeostasis. The activation of PTH1R by its specific agonist ABL effectively counteracts MuV- or LPS-induced inflammatory responses in the testis as well as in the epididymis. Previous findings have shown that ABL and teriparatide, another PTH analog, could induce chondrogenesis and reduce osteoarthritis (24, 34, 35). Therefore, PTH1R might represent an immunomodulatory target for the therapeutic intervention of inflammatory conditions in the male reproductive system including but not limited to epididymitis and orchitis.

PTH is primarily secreted from the parathyroid gland and regulates PTH1R activity in the bone and kidney through an endocrine manner, whereas PTHrP is synthesized in a wide range of tissues and paracrinely activates PTH1R. We found that both of these two endogenous agonists of PTH1R were expressed in the epididymis and testis. Whereas MuV challenge led to an expression level change of PTH and PTHrP, the knocking down of these two ligands' expression aggravated the MuV-induced inflammatory response. These results raised the possibility of the existence of local PTH/PTHrP-PTH1R signaling that functionally participated in the maintenance of immune homeostasis. The mammalian testis is unique in possessing an immune privileged environment where the antigens are tolerated without evoking detrimental inflammatory responses (36, 37). The maintenance of this immune-privileged state is attributed to multiple mechanisms, such as the blood–testis barrier structure and the local immunosuppressive machinery. Our results suggested that PTH1R activation in Leydig cells directly suppressed MuV- or LPS-induced inflammatory responses and potentially contributed to the testicular immune-privileged status, thus playing active roles in the testicular defense system. These results are mainly carried out using a mouse model and then partially confirmed in human samples. Notably, albeit similar primary sequences and acting through the same receptor, PTH and PTHrP usually regulate distinct physiological functions (20, 21). It would be of interest to further investigate the potentially different functions of PTH1R engaged by these two endogenous agonists in the local immunoregulation of the epididymis and testis.

PTH1R signals primarily through coupling to Gs and Gq proteins, which activate the cAMP-PKA and DAG/Ca^{2+}-PKC signaling pathways, respectively (21). The activated PTH1R also recruits β-arrestins, which not only regulate receptor desensitization and internalization but also redirect the signaling to a G protein–independent pathway by interacting with multiple downstream effector molecules (38–43). Our results from both in vitro and in vivo studies demonstrated a predominant role of Gq- and β-arrestin-1–mediated signaling in the anti-inflammatory effects of ABL through PTH1R in epididymitis and orchitis. Considering that Gq and β-arrestin-1 also regulate fluid reabsorption in efferent ductules of the epididymis as revealed by our previous study (1, 3), these data collectively indicated a critical role of Gq and β-arrestin-1 in the maintenance of the homeostasis of the male reproductive system. Whereas both Gs and Gq signaling contribute to the PTH1R-mediated phosphate reabsorption in the renal proximal tubule, the Gs pathway is primarily responsible for the bone turnover in a subtle manner in that the intermittent activation of PTH1R-Gs leads to bone formation, while continuous activation paradoxically results in bone resorption (44–47). Accordingly, biased agonists of PTH1R selectively activating Gq and β-arrestin-1 signaling might serve as an effective approach to elicit anti-inflammatory effects in the male reproductive system. Our current results suggested that ABL administration significantly alleviated but could not eliminate MuV- or LPS-induced inflammation, possibly because of the dramatical decrease of PTH1R expression. It is worth noting that, although the present study indicated that the PKC activity downstream of ABL activation was regulated by Gq but not by Gs, β-arrestin-1, or β-arrestin-2 in both TM3 cells and epididymal primary epithelial cells, we could not exclude the possibility that PKC might also be activated by other upstream regulators in different cellular contexts.

Another unexpected finding in the present work is the positive effect of ABL on normal sperm functions in terms of both increased survival rate and enhanced motility. The effect of ABL might be attributed to a direct activation of PTH1R on sperm considering the relatively high expression of PTH1R in sperm as revealed by RNA sequencing (22). The regulation of sperm functions and behaviors by manipulating sperm-expressed GPCRs has been previously reported (48). However, it is also possible that ABL-induced activation of PTH1R expressed in the male reproductive tract, such as the epithelium of the epididymis, contributes to the increased functionality of sperms in an interdict manner. Further studies are needed to explore whether ABL treatment could improve the fertilizing capability of sperms.

Finally, despite that most mammalian testes display immune privilege and innate immunity, human testis tends to show a weaker defensing effect compared with murine testis, suggesting species-specific regulatory machinery (49). Our present data using human samples suggested a preliminary therapeutic effect of ABL on the male reproductive system. Moreover, since conventional knockout of *Pth1r* in mice leads to midgestational lethality (50), studies using mouse models with PTH1R specifically ablated in the epididymis and/or testis would be expected to further solidify the present conclusion.

In summary, we found that PTH1R and its endogenous ligands were expressed in the epididymis and testis, which were involved in the regulation of inflammatory responses. Activation of PTH1R by ABL effectively alleviated MuV- or LPS-induced inflammation through a Gq and β-arrestin-1 signaling–dependent mechanism (Fig. 6). Targeting PTH1R has the potential to develop new therapeutics to treat epididymitis, orchitis, or other inflammatory-related diseases in the male reproductive system.

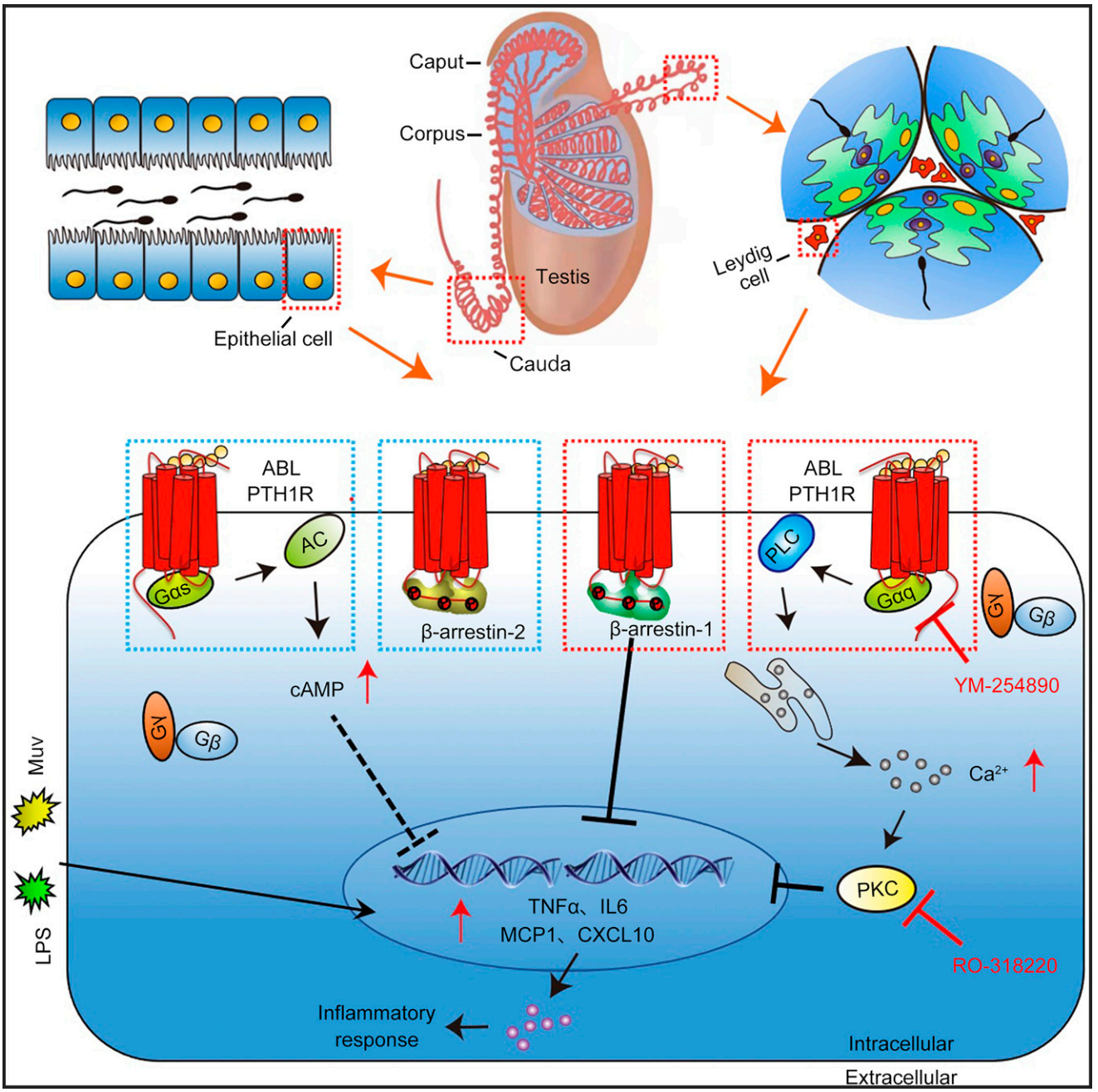

Fig. 6. Schematic representation of PTH1R-mediated anti-inflammatory effects through Gq and β-arrestin-1 pathways. PTH1R is expressed in the Leydig cells of testis and the epithelial cells of the cauda of the epididymis. LPS or MuV infection in the testis and epididymis induces acute inflammatory responses as evidenced by the morphologic injury, increased cell apoptosis, and aberrant production of inflammatory factors such as TNF-α, IL6, MCP1, and CXCL10. Activation of PTH1R by ABL, an FDA-approved drug for postmenopausal osteoporosis, effectively alleviates MuV- or LPS-induced inflammation in the testis and epididymis mainly through combined Gαq-PLC-PKC and β-arrestin-1 signaling–dependent mechanisms. The Gs-cAMP pathway partially contributes to this anti-inflammatory process. AC, adenylyl cyclase; PLC, phospholipase C; PKC, protein kinase C; RO-318220, a specific PKC inhibitor; and YM-254890, a specific Gαq inhibitor.

Materials and Methods

Animals. WT (C57BL/6J) male mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. *Gnaq*$^{\pm}$ mice were acquired from JL Liu at Shanghai Jiao Tong University, Shanghai, China.

Arrb1$^{-/-}$ mice were supplied by R.J. Lefkowitz, Duke University, Durham, NC. Mice were housed at a room temperature of 22 to 24 °C on a 12-h light/12-h dark cycle with ad libitum access to water at the Shandong University Animal Care Facility. All mice care and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University.

Virus, LPS, and ABL Administration. The testis or the cauda of the epididymis of mice were injected with MuV in vivo (1×10^7 PFU) or in vitro (5 MOI), as previously described (51–53). A single dose of ABL (MCE HY-108742A) was intraperitoneally injected in vivo (5 mg/kg) 6 h after MuV injection or in vitro (40 nM) 3 h after MuV injection. A single dose of LPS (Sigma L2630; 3 mg/kg) was intraperitoneally injected into C57BL/6J male mice, which induced inflammation in the testis and epididymis. A single dose of ABL (5 mg/kg) was intraperitoneally injected 6 h after LPS injection. The same volume of 1× phosphate buffer saline was injected as negative control.

Human Tissue Collection. Testicular and epididymal biopsy was obtained from discarded samples after surgery at the Second Affiliated Hospital of Chengdu Medical College Nuclear Industry 416 Hospital and Peking University Third Hospital. The study was approved by the Ethical Review Board of West China Second University Hospital, Sichuan University (No. 2020–31). Informed consent was obtained from each subject in our study.

Data Availability. All data, protocols, and materials are detailed in the manuscript or *SI Appendix*.

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