Phenotypic characterization of testicular immune cells expressing immune checkpoint molecules in wild-type and pituitary adenylate cyclase-activating polypeptide-deficient mice

Matyas Meggyes1,2 | Adrienn Lajko1 | Balazs Daniel Fulop3 | Dora Reglodi3 | Laszlo Szereday1,2

Abstract

Problem: Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide having several regulatory functions in the nervous system and in peripheral organs including those of the reproductive system. PACAP-deficient male mice have several morphological, biochemical, behavioral defects and show disturbed signaling in spermatogenesis affecting fertility in PACAP KO mice. Reproductive functions such as fertility, mating, and maternal behaviors have been widely investigated, but no immune analyses are available regarding the testicular immune-privileged environment in male PACAP-deficient mice.

Method of Study: We performed detailed immunophenotyping of testicular immune cells and investigated the expression of TIM-3 and PD-1 Immune checkpoint molecules of immune cells together with the detection of galectin-9 and perforin. We investigated the percentage of numerous immune cell populations in the testis of wild-type and PACAP-deficient mice.

Results: We demonstrated a significant increase in the frequency of testicular CD8+ T cells together with the decrease in Treg cell number obtained from PACAP KO mice compared with wild-type mice. Investigating Immune checkpoint receptors, only PD-1 showed a significantly decreased expression in CD8+ T cells in PACAP KO mice compared with wild-type suggesting an impaired PD-1/PD-L1 pathway. Regarding TIM-3 expression, we did not find any significant difference between the investigated groups.

Conclusion: We hypothesize that these local changes may result in an immune activation with disturbed testicular immunoregulation in PACAP KO mice; however, determining the exact function requires further investigations. Our data further support the view that besides a systemic immune tolerance, localized active immunosuppression is involved in the regulation of testicular immune privilege.
1 | INTRODUCTION

There are certain “immunologically privileged” sites in our body, where immune responses are naturally suppressed. The anterior chamber of the eye, central nervous system, and pregnant uterus are all considered physiological immune-privileged sites in humans.\(^1\) The mammalian testis also represents an immune-privileged organ\(^2\) maintained through the coordination of systemic immune tolerance, the local physical structure, and active local immunosuppression. The local immune modulatory milieu has been intensively investigated, but the mechanisms underlying immune privilege are still not well understood.\(^2,4\) The blood-testis barrier (BTB) is much more complex than other blood-tissue barriers, and its function relies on the complex interaction between the anatomical, physiological, and immunological barriers.\(^3\) Besides the anatomical BTB, testicular somatic cells, including Sertoli cells, peritubular cells, and Leydig cells together with the local active immunosuppression, play vital roles in maintaining the immune-privileged environment.\(^2,4\) The testis has most types of immune cells found in interstitial spaces, including macrophages, α/β T lymphocytes, natural killer (NK) cells, natural killer T (NK) cells, dendritic cells (DCs), and mast cells.\(^5\)

These cells are important in the maintenance of the special testicular immune environment.

Immune checkpoint molecules, such as TIM-3, PD-1, and CTLA-4, are negative regulators of immune responses occurring on the surface of several immune cells.\(^6\) After ligand (galectin-9, PD-L1/PD-L2, CD80/CD86) binding, these regulators are capable of transducing inhibitory signals leading to decreased immunostimulation.\(^6\)

TIM-3 and PD-1 were shown to be expressed by many types of immune cells, including Th1, Th17, NK and NKT cells, γ/δ T cells, Tregs, and also on antigen-presenting immune cells.\(^6-8\) The ligand of TIM-3 receptor is galectin-9 (Gal-9), a β-galactose binding protein. Both in mice and humans, binding of TIM-3 to its ligand Gal-9 leads to the apoptosis of Th1 and Th17 cells and induces active immunotolerance.\(^6,10\) Known ligands of PD-1 include PD-L1, which can be found on several immune cells (resting T cells, B cells, dendritic cells, macrophages), in various tissues, like placenta, heart, and spleen and by Sertoli and peritubular cells in the testis of mice.\(^6,11\) In contrast to that, PD-L2 expression is limited to dendritic cells and macrophages.\(^12\) The PD-1/PD-L1 pathway is thought to be associated with the promotion of peripheral tolerance and subsequent prevention of immune-mediated tissue damage by favoring Th2 development and expansion.\(^13,14\)

Immune checkpoint regulators are thought to actively participate in the immune defense of infections, prevention of autoimmunity, immune reaction during transplantation, tumor immune evasion, and maternal immune tolerance during pregnancy.\(^6\)

Collaboration between these mechanisms is beneficial to healthy testicular function. However, the disturbance of this physiological status can lead to orchitis leading to male infertility, followed by impaired androgen synthesis and diminished spermatogenesis.\(^15\)

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that belongs to the glucagon/VIP/secretin family.\(^16\) It has major relevance in the nervous system and in several peripheral organs, including reproductive organs. PACAP is one of the peptides having several regulatory functions in reproduction,\(^17,18\) and regulating germ cell development in the ovary and testis.\(^19,20\)

Pituitary adenylate cyclase-activating polypeptide-deficient (KO) mice display various abnormalities in several physiological and pathological processes, including reproductive functions.\(^17,18\) Lack of PACAP in male mice leads to disturbed signaling in spermatogenesis,\(^20\) which could be an important factor responsible for reduced fertility in PACAP KO mice.\(^21\) PACAP is a known modulator of innate and adaptive immune responses.\(^22\)

The aim of our research was to investigate the role of immune checkpoint pathways in the maintenance of immune regulation in testicular immune-privileged environment, especially in PACAP-deficient mice. We investigated the expression pattern of TIM-3, PD-1, and Gal-9 on different immune cell subsets in the testis of wild-type and PACAP KO mice.

2 | MATERIALS AND METHODS

2.1 | Animal model

Generation of PACAP-deficient mice on CD-1 background was described earlier.\(^23\) Two-month-old wild-type (WT) and homozygous knockout (KO) female mice were maintained on a 12-hour light/dark cycle at 20-22°C and 40%-60% humidity and were fed with standard feed pellets and tap water. Animals were handled in accordance with an approved protocol by the animal ethics committee of the University of Pecs (BA02/2000-24/2011, University of Pecs, Hungary). Genotyping was performed using Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Primer sequences used for detection of wild-type and KO DNA signatures of PACAP were identical with those used earlier.\(^23,24\)

2.2 | Cell isolation from the testis

Three-month-old adult male mice (n = 10 WT and n = 11 KO) were killed by cervical dislocation, and both testes were removed. To avoid selective cell death or selective loss of surface proteins, mechanical disaggregation rather than enzymatic digestion was used to process the testicular tissue for isolating testicular immune cells. Later, testicles were homogenized thoroughly with a syringe plunger, and single-cell suspensions were prepared using
a 100-μm nylon cell strainer (BD Biosciences). Subsequently, cells were washed in PBS and the supernatant was aspirated. The pellet was resuspended in PBS and filtered again via 70 μm nylon cell strainer (BD Biosciences). Then, cells were washed in PBS and the supernatant was aspirated and filtered via 40 μm nylon cell strainer (BD Biosciences). Cells were washed again in PBS, and supernatant was aspirated. Separated cells were collected and resuspended in RPMI1640 (Lonza) medium supplemented with penicillin (1 × 105 U/L; Lonza), streptomycin (0.05 g/L; Lonza), and 10% fetal bovine serum (FBS; Gibco).

2.3 | Mononuclear cell surface staining, antibodies, and flow cytometric analysis

Isolated cells were resuspended in PBS and incubated for 30 minutes at room temperature with fluorochrome-labeled monoclonal antibodies for characterization (Figure 1). Antibodies used in the present study are shown in Table 1. Figure 2 shows representative flow cytometric dot plots and histograms for TIM-3, Gal-9, and PD-1. The samples were protected from light. After wash, the cells were resuspended in 300 μl PBS containing 1% paraformaldehyde and stored at 4°C in complete darkness until fluorescence-activated cell sorting (FACS) analysis. Before sample analysis, settings of the flow cytometer were checked using Cytometer Setup and Tracking beads (CS&T beads; BD Biosciences) according to the manufacturer's instructions. Compensation beads were used with single stains of each antibody in order to determine the compensation settings and were applied in FACS Diva V6 software (BD Biosciences) before data collection. Labeled cells were analyzed with FACS Canto II flow cytometer, and 10 000 events were collected in the lymphogate after CD45 staining. FACS Diva V6 software was used for data acquisition and FCS Express IV software (De Novo Software) for data analysis.

2.4 | FoxP3 staining

Following surface labeling, intracellular staining of FoxP3 was performed using the FoxP3 Staining Buffer Set (eBioscience) in accordance with the manufacturer’s protocol. Briefly, isolated cells were permeabilized in 1ml fixation/permeabilization buffer (Concentrate/Diluent 1:4; eBioscience) for 1 hour at 4°C in complete darkness. Then, the samples were washed twice in the buffer and stained with APC-conjugated anti-mouse FoxP3 monoclonal antibody for 1 hour at 4°C in complete darkness. FACS analysis was performed by FACS Canto II flow cytometer with the BD FACS Diva software.
2.5 | Statistical analysis

Statistical analysis was performed using statistical software SPSS version 23 package. Statistical comparisons were made using Student’s t test. Differences were considered significant if the P value was equal to or less than .05.

3 | RESULTS

3.1 | Immunophenotypic analysis of testicular immune cells from wild-type and PACAP KO mice

We investigated the percentage of CD3+ T, CD4+ T, and CD8+ T cells; γ/δ T cells; Treg cells; and NK and NKT cells in the testis of wild-type and PACAP KO mice. We observed a significant increase in the CD3+ and CD8+ T-cell frequency (Figure 3A,C), while the γ/δ T and Treg cells (Figure 3D,E) significantly decreased in the testis of PACAP KO mice compared with the wild-type. The frequency of CD4+ T cells, and NK and NKT cells (Figure 3B, F, and G) showed no difference between the investigated groups.

3.2 | TIM-3 and PD-1 expression by testicular immune cells from wild-type and PACAP KO mice

Flow cytometry was used to analyze the cell surface expression of TIM-3 by CD3+ T, CD4+ T, CD8+ T, γ/δ T, and NK cells in the testis of wild-type and PACAP KO mice.

We found that TIM-3 expression by CD4+ T and γ/δ T cells was significantly decreased in PACAP KO compared with wild-type mice (Figure 4B,D). We could not determine statistical differences in TIM-3 expression by CD3+, CD8+ T, and NK cells between the investigated groups (Figure 4A, C and E).

During our investigation of the PD-1 expression by testicular CD3+ T, CD4+ T, CD8+ T, γ/δ T, and NK cells in male mice, we discovered that CD4+ and CD8+ T cells demonstrated a decrease in PD-1 expression in PACAP KO mice compared with wild-type mice (Figure 5B,C). We could not determine statistical differences in PD-1 expression by CD3+ T, γ/δ T, and NK cells between the investigated groups (Figure 5A, D and E).

3.3 | Cell surface Gal-9 expression by testicular immune cells from wild-type and PACAP KO mice

We measured the surface expression of Gal-9 on different lymphocyte subsets by flow cytometry. None of the investigated testicular immune cell population showed any significant difference between wild-type and PACAP KO mice (Figure 6A-E).

3.4 | Perforin expression by testicular immune cells from wild-type and PACAP KO mice

Flow cytometry was used to analyze the perforin expression by CD3+ T, CD8+ T, and NK cells in the testis of wild-type and PACAP KO mice.

CD3+ and CD8+ T cells from PACAP KO mice showed significantly higher perforin expression compared with wild-type mice (Figure 7A,B). No significant difference in the expression regarding perforin by NK cells was detected between the investigated groups (Figure 7C).

4 | DISCUSSION

The testis is a very complex organ with a unique structure and a large number of cell types. The mammalian testis consists of two distinct compartments: the seminiferous tubules responsible for spermatogenesis and the interstitial spaces between the tubules containing steroidogenesis performing cells. The interstitial spaces represent...
only a very small part of the testis, but they are composed of a large number of immune cells. Lymphocytes are found in the interstitial spaces under physiological conditions. Most testicular lymphocytes are T cells, with CD8+ cells being predominant and CD4+ T cells are rather infrequent. In infertile patients with sperm autoimmunity, the number of lymphocytes is significantly higher, suggesting their role in testicular pathogenesis during inflammatory conditions. The rat testis also contains immunoregulatory T cells, including Tregs, NKT, and γδ T cells. Different experimental models have also shown the important role of macrophages in orchitis development in rats.

Several studies have shown the presence of PACAP and its receptors in human and mouse testis, and it has been shown that PACAP can cross the BTB. PACAP influences hormonal secretion via regulating Leydig and Sertoli cell growth and functions. A recent study described that several factors playing an important role in spermatogenesis are significantly influenced by partial or complete lack of PACAP.

Our present study aimed to investigate the immunological alterations behind the disturbance in testicular functions and decreased fertility rate in PACAP-deficient mice. In order to better understand the alterations regarding testicular immunoregulation in PACAP KO mice, we quantified and phenotypically and functionally characterized immune cell subsets of the testis.

We detected for the first time that the number of CD8+ T cells was significantly increased in the testis of PACAP KO mice compared with wild-type. Regarding TIM-3 expression by CD8+ T cells, we did not find any significant difference between the investigated groups. There is considerable evidence that Gal-9 and PD-L1 are expressed and that they are playing a supporting role at immune-privileged sites. Gal-9 expression was found to be the highest regarding testicular CD4+ T cells in both groups, but none of the investigated immune cells showed a significant difference between PACAP-deficient and wild-type mice. Under physiological situations, peritubular cells, spermatocytes, and spermatids in seminiferous tubules of the testis constitutively express or secrete PD-L1. PD-L1 is inducibly expressed by testicular Sertoli cells. On the other hand, the testis does not constitutively express PD-L2. PD-L1 binding to the PD-1 receptor expressed by activated CD8+ T cells leads to T-cell apoptosis, inhibition of proliferation, and cytokine secretion, which represent immune suppression and might play a role in the maintenance of testicular immune privilege. The decreased PD-1 expression by CD8+ T cells in PACAP-deficient mice suggests an impaired PD-1/PD-L1 pathway where immune cells might shift from immune suppression toward immune activation leading to disturbed testicular immunoregulation.

We previously found no significant alterations in testicular morphology or motility of sperm in homozygous and heterozygous PACAP-deficient mice in spite of the moderately increased number of severely damaged sperms. However, we found robust changes in mRNA and/or protein expression of several factors that play an important role in spermatogenesis. Our results regarding CD8+ T cells and immune checkpoint molecules revealed a local immune activation creating a proinflammatory environment, which could contribute to changes in spermatogenesis and could also affect the PACAP-induced signal transduction pathways.
CD8+ T cells are more sensitive to the PD-1/PD-L1 inhibitory pathway than CD4+ T cells. This could be the reason that our investigations could not reveal a significant increase in the frequency of CD4+ T cells regarding their decreased PD-1 receptor expression. Similarly, in experimental autoimmune orchitis (EAO) models, CD4+ T-cell numbers dramatically increase at the onset of the disease but CD4+ T-cell numbers decrease during disease progression suggesting that CD4+ T cells may be involved in the initiation of the chronic phase of EAO. Further functional assays might elucidate the precise role of testicular CD8+ T cells in PACAP KO mice.

Tregs modulate the function of a variety of immune cells and are critical for maintaining self-tolerance, suppressing autoimmunity and preventing graft rejection, which has been reported in many animal models. Nasr et al showed that antigen-specific Tregs promote transplantation tolerance to pancreatic islet allografts in testis. We quantified other T-cell subsets, like Foxp3+ Treg cells and γδ T cells. Both effector/regulatory subsets decreased in the testis of PACAP KO mice compared with wild-type controls. The decreased number of testicular Tregs can suggest some disturbance in local immunotolerance mechanism since their role in down-regulating the local immune response could be perturbed. Further investigations are needed to fully understand the possible role of decreased testicular Tregs frequency in PACAP-deficient mice since this contradicts with the finding of Jacobo et al who found an increased number of Foxp3+ Tregs in testis of rats with EAO.

Makusa et al demonstrated that the depletion of testicular γδ T cells accelerates inflammatory response in a model of autoimmune orchitis induced by bacterial infection of the testis. In contrast to CD8+ T cells, γδ T cells showed a decreased TIM-3 expression, while PD-1 expression remained unaltered in PACAP KO mice compared with wild-type mice.
**FIGURE 4** TIM-3 expression by testicular immune cells from wild-type and PACAP KO mice. The expression of TIM-3 by CD3+ T (A), CD4+ T (B), CD8+ T (C), γ/δ T (D) cells, and NK (E) cells in the testis of wild-type and PACAP KO mice. Box plot of the median, the 25th and 75th percentiles, range, and individual data values for the expression of TIM-3. The middle line within the box represents the median, the boxes indicate the interquartile ranges, and the whiskers show the most extreme observations. Differences were considered statistically significant for P-values ≤ .05. *P < .05

**FIGURE 5** PD-1 expression by testicular immune cells from wild-type and PACAP KO mice. The expression of PD-1 by CD3+ T (A), CD4+ T (B), CD8+ T (C), γ/δ T (D) cells, and NK (E) cells in the testis of wild-type and PACAP KO mice. Box plot of the median, the 25th and 75th percentiles, range, and individual data values for the expression of PD-1. The middle line within the box represents the median, the boxes indicate the interquartile ranges, and the whiskers show the most extreme observations. Differences were considered statistically significant for P-values ≤ .05. ***P < .01; **P < .03
Among the lymphocyte subpopulations present in the mouse testis, we found predominantly adaptive immune cells (CD4+ and CD8+ T cells) and a low number of innate immune cells (NK, NKT, and γ/δ T cells). These data are consistent with previous studies on testicular lymphocyte populations in mice. Since NK cells play an indispensable role in female reproduction, we investigated the immune checkpoint receptor expression along with the perforin positivity in testicular environment in PACAP-deficient mice. The frequency of NK cells in the testis was the same in both investigated groups. None of the investigated immune checkpoint molecules (PD-1 and TIM-3) expressed by NK cells showed any significant difference between wild-type and PACAP KO mice. Finding no significant difference in the expression regarding perforin by NK cells shows very limited or no role of NK cells is testicular immunological mechanisms.

PACAP KO mice are known for their reduced fertility. This can be due to disturbed mechanisms of this complex process at several levels. For example, PACAP affects hormonal regulation that may affect fertility. At the gonadal levels, PACAP affects both spermatogenesis and folliculogenesis. PACAP affects mating behavior as well as fertilization itself, as it stimulates the penetration of sperm through the zona pellucida and cumulus layers. After fertilization,
reduced implantation was also observed in PACAP-deficient mice.\textsuperscript{39} Based on these findings, PACAP affects reproduction at basically all levels. However, our study can further reveal another possible candidate to explain the abovementioned reduced fertility. Male seminal fluid is implicated in regulating the size and suppressive function of the Treg cell pool during the peri-implantation phase of early pregnancy leading to a state of active tolerance during embryo implantation to prevent the conceptus from maternal immune attack.\textsuperscript{40,41} PACAP deficiency-related changes could modify the immunomodulatory effects of seminal fluid by altering either its cytokine signals or antigen content and also the male leukocyte priming during maternal implantation.

In summary, in our present study, we found that the increased number of testicular CD8\textsuperscript{+} T cells together with the decrease of Treg cell number could be a key player behind the immunological and fertility alteration documented in male PACAP KO mice. The expression of PD-1 was significantly decreased by these immune cells suggesting an impaired PD-1/PD-L1 pathway. We speculate that these local changes may result in an immune activation with disturbed testicular immunoregulation in PACAP KO mice; however, determining the exact functions requires further investigations. Our data further support the view that besides a systemic immune tolerance, localized active immunosuppression is involved in the regulation of testicular immune privilege. Since the prevention and treatment for testicular inflammation-related male infertility are still unresolved, we believe that analyzing immunological changes associated with testicular immune regulation may pave a new direction in the techniques and innovations in the field of obstetrics and gynecology.

ACKNOWLEDGMENTS

This work was supported by grants from National Research, Development and Innovation Office (NKFI K119529, K115874, and K119759; EFOP-3.6.1-16-2016-00004; NAP.2017-1.2.1-NKP-2017-00002, GINOP-2.3.2-15-2016-00050 "PEPSYS," MTA-TKI 14016, EFOP-3.6.2-16-2017-00008 "The role of neuro-inflammation in neurodegeneration: from molecules to clinics," Neuroscience Centre of Pecs, Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary (FIKPII) and PTE ÁOK KA Research Grant (KA-2018-07).

CONFLICT OF INTEREST

None.

ORCID

Laszlo Szereday https://orcid.org/0000-0002-1208-2969

REFERENCES

1. Wang L-L, Li Z-H, Hu X-H, Muyayalo KP, Zhang Y-H, Liao A-H. The roles of the PD-1/PD-L1 pathway at immunologically privileged sites. \textit{Am J Reprod Immunol.} 2017;78(2):e12710.
2. Li N, Wang T, Han D. Structural, cellular and molecular aspects of immune privilege in the testis. \textit{Front Immunol.} 2012;3:152.
3. Mital P, Hinton BT, Dufour JM. The blood-testis and blood-epidymis barriers are more than just their tight junctions. \textit{Biol Reprod.} 2011;84(5):851-858.
4. Wang L-L, Li Z-H, Duan Y-G, Yuan S-Q, Mor G, Liao A-H. Identification of programmed cell death 1 and its ligand in the testicular tissue of mice. \textit{Am J Reprod Immunol.} 2019;81(2):e13079.
5. Zhao S, Zhu W, Xue S, Han D. Testicular defense systems: immune privilege and innate immunity. \textit{Cell Mol Immunol.} 2014;11(5):428-437.
6. Miko E, Meggyes M, Doba K, Barakonyi A, Szereday L. Immune checkpoint molecules in reproductive immunology. \textit{Front Immunol.} 2019;10:846.
7. Zhao J, Lei Z, Liu Y, et al. Human pregnancy up-regulates Tim-3 in innate immune cells for systemic immunity. \textit{J Immunol.} 2009;182(10):6618-6624.
8. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. \textit{Annu Rev Immunol.} 2008;26:677-704.
9. Seki M, Oomizu S, Sakata K-M, et al. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. \textit{Clin Immunol.} 2008;127(1):78-88.
10. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. \textit{Nat Immunol.} 2005;6(12):1245-1252.
11. Dal Secco V, Riccioli A, Padula F, Ziparo E, Filippini A. Mouse sertoli cells display phenotypical and functional traits of antigen-presenting cells in response to interferon gamma. \textit{Biol Reprod.} 2008;78(2):234-242.
12. Ishida M, Iwai Y, Tanaka Y, et al. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. \textit{Immunol Lett.} 2002;84(1):57-62.
13. Habicht A, Dada S, Jurewicz M, et al. A link between PDL1 and T regulatory cells in fetomaternal tolerance. \textit{J Immunol.} 2007;179(8):5211-5219.
14. Chikuma S. Basics of PD-1 in self-tolerance, infection, and cancer immunity. \textit{Int J Clin Oncol.} 2016;21:448-455.
15. Chen Q, Deng T, Han D. Testicular immunoregulation and spermatogenesis. \textit{Semin Cell Dev Biol.} 2016;59:157-165.
16. Rawlings SR, Hezareh M. Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland*. \textit{Endocr Rev.} 1996;17(1):4-29.
17. Lajko A, Meggyes M, Fulop BD, Gede N, Reglodi D, Szereday L. Comparative analysis of decidual and peripheral immune cells and immune-checkpoint molecules during pregnancy in wild-type and PACAP-deficient mice. \textit{Am J Reprod Immunol.} 2018;80(4):e13035.
18. Reglodi D, Tamas A, Koppan M, Szogyi D, Welke L. Role of PACAP in female fertility and reproduction at gonadal level—recent advances. \textit{Front Endocrinol (Lausanne).} 2012;3:155.
19. Cecconi S, Rossi G, Barberi M, Scaldaferrri L, Canipari R. Effect of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on mouse preantral follicle development in vitro. \textit{Endocrinology.} 2004;145(4):2071-2079.
20. Reglodi D, Cseh S, Somoskoi B, et al. Disturbed spermatogenic signaling in pituitary adenylate cyclase activating polypeptide-deficient mice. \textit{Reproduction.} 2018;155(2):129-139.
21. Reglodi D, Kiss P, Szabadfi K, et al. PACAP is an endogenous protective factor-insights from PACAP-deficient mice. \textit{J Mol Neurosci.} 2012;48(3):482-492.
22. Ganea D, Delgado M. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. \textit{Crit Rev Oral Biol Med.} 2002;13(3):229-237.
23. Hashimoto H, Shintani N, Tanaka K, et al. Altered psychomotor behaviors in mice lacking pituitary adenylate
cyclase-activating polypeptide (PACAP). Proc Natl Acad Sci USA. 2001;98(23):13355-13360.
24. Farkas J, Sandor B, Tamas A, et al. Early neurobehavioral development of mice lacking endogenous PACAP. J Mol Neurosci. 2017;61(4):468-478.
25. Jacobo P, Guazzzone VA, Jarazo-Dietrich S, Theas MS, Lustig L. Differential changes in CD4+ and CD8+ effector and regulatory T lymphocyte subsets in the testis of rats undergoing autoimmune orchitis. J Reprod Immunol. 2009;81(1):44-54.
26. Rival C, Theas MS, Suescun MO, et al. Functional and phenotypic characteristics of testicular macrophages in experimental autoimmune orchitis. J Pathol. 2008;215(2):108-117.
27. Brubel R, Kiss P, Vincze A, et al. Effects of pituitary adenylate cyclase activating polypeptide on human sperm motility. J Mol Neurosci. 2012;48(3):623-630.
28. Lacombe A, Lelievre V, Roselli CE, et al. Delayed testicular aging in pituitary adenylate cyclase-activating polypeptide (PACAP) null mice. Proc Natl Acad Sci U S A. 2006;103(10):3793-3798.
29. Mizushima H, Nakamura Y, Matsumoto H, et al. The effect of cardiac arrest on the blood-testis barrier to albumin, tumor necrosis factor-alpha, pituitary adenylate cyclase activating polypeptide, sucrose, and verapamil in the mouse. J Androl. 2001;22(2):255-260.
30. Tani I, Aradate T, Matsuda K, Komiya A, Fuse H. PACAP-mediated sperm-cumulus cell interaction promotes fertilization. Reproduction. 2011;141(2):163-171.
31. Cheng X, Dai H, Wan N, Moore Y, Vankayalapati R, Dai Z. Interaction of programmed death-1 and programmed death-1 ligand-1 contributes to testicular immune privilege. Transplantation. 2009;87(12):1778-1786.
32. Carter L, Fouser L, Jussif J, et al. PD-1:PD-L inhibitory pathway affects both CD4+ and CD8+ T cells and is overcome by IL-2. Eur J Immunol. 2002;32(3):634.
33. Nasr IW, Wang Y, Gao GE, et al. Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells. J Immunol. 2005;174(10):6161-6168.
34. Mukasa A, Hiromatsu K, Matsuzaki G, O’Brien R, Born W, Nomoto K. Bacterial infection of the testis leading to autoaggressive immunity triggers apparently opposed responses of alpha beta and gamma delta T cells. J Immunol. 1995;155(4):2047-2056.
35. Tumubatbar T, Kanasaki H, Oride A, et al. Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) in the regulation of hypothalamic kisspeptin expression. Gen Comp Endocrinol. 2019;270:60-66.
36. Ross RA, Leon S, Madara JC, et al. PACAP neurons in the ventral premammillary nucleus regulate reproductive function in the female mouse. Elife. 2018;7.
37. Gräs S, Hannibal J, Georg B, Fahrenkrug J. Transient peri-oovulatory expression of pituitary adenylate cyclase activating peptide in rat ovarian cells. Endocrinology. 1996;137(11):4779-4785.
38. Shintani N, Mori W, Hashimoto H, et al. Defects in reproductive functions in PACAP-deficient female mice. Regul Pept. 2002;109(1–3):45-48.
39. Isaac ER, Sherwood NM. Pituitary adenylate cyclase-activating polypeptide (PACAP) is important for embryo implantation in mice. Mol Cell Endocrinol. 2008;280(1–2):13-19.
40. Robertson SA, Guerin LR, Moldenhauer LM, Hayball JD. Activating T regulatory cells for tolerance in early pregnancy—the contribution of seminal fluid. J Reprod Immunol. 2009;83(1–2):109-116.
41. O’Leary S, Jasper MJ, Warnes GM, Armstrong DT, Robertson SA. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. Reproduction. 2004;128(2):237-247.

How to cite this article: Meggyes M, Lajko A, Fulop BD, Reglodi D, Szereday L. Phenotypic characterization of testicular immune cells expressing immune checkpoint molecules in wild-type and pituitary adenylate cyclase-activating polypeptide-deficient mice. Am J Reprod Immunol. 2020;83:e13212. https://doi.org/10.1111/aji.13212