nature portfolio

Peer Review File

Chronic inflammation drives epididymal tertiary lymphoid structure formation and autoimmune fertility disorders in mice

Corresponding Author: Dr Maria Battistone

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Review report
Key results
This manuscript studied autoimmune orchitis and epididymitis in a Treg depletion model. The authors demonstrate the subsequent development of anti-sperm autoantibodies, identify targets for these antibodies, and show the isotypes and deposition in testis and different region of the epididymis. They demonstrate for the first time the development of tertiary lymphoid structures (TLS) in special areas of the epididymis and characterize the cellular composition of these. They also demonstrate similar autoantibodies in seminal plasma linking a common mechanism of infertility in mice and humans. This manuscript focuses on the role of B cells and autoantibodies and is based on a previous published model where they describe the T cells. This paper extent the previous work by the addition of analyses at week 8 after Treg depletion together with analyses at week 2. The data is novel and provide valuable insight into mucosal immunology and the role of TLS.
The amount of data provided is extensive and the use of methods is valid. The results are presented comprehensively. However, the discussion does not give the result any justice.

I have some questions and suggestions for improvement of this extensive work:
Results:
The number of animals used is missing.
Suppl Fig 4 show distinct patterns of the different IgG isotypes. The authors have not addressed this. Could the different patterns reflect different antigenic specificity between the isotypes? The proteomics data may indicate the formation of immune complexes. This could also affect the autoantibody binding pattern observed in the testis. Could the binding of autoantibodies in the basal lamina of the seminiferous tubules influence the spermatogenesis?
The sperm antigens detected could have been presented better with more details on their expression levels. Line 169-195 might fit better in the discussion?
The distinct cell subtypes shown in the t-SNE and FlowSOM analysis is not completely described in the figures. The colors and cells should be defined in the figure text.
The authors claim that spermatogenesis was not chronically affected, but they observed reduced distal sperm counts. The picture in Suppl. Fig. 13E is clearly showing a morphological change of the semi in the seminiferous tubules of the Treg depleted animals. Did the authors count the different spermatocytes?
The definition of TLS includes T and B cell areas with germinal centers (GC) and High endothelial venules (HEVs). Figure 6G is not convincing evidence for TLS as very few CD3 cells are detected. The Videos also lack evidence for T cells in these structures. However, TLS have been shown to differ from organs to organs. The authors should try to discuss their finding on TLS in relation to TLS in other organs and diseases.
The authors did not demonstrate HEVs within the TLS although the CD31 staining looks promising. To verify functional TLS and HEVs, PNAD staining could be included.
Figure 8F is a very nice figure illustrating the important findings. However, as pointed out above, the staining pattern in Supplementary Figure 4 indicate binding of autoantibodies on the epithelial cells or basement membrane in the testis. The figure should include an illustration of autoantibody binding in the testis in addition to the binding to the sperm cells.
The order of the supplementary figures is confusing. Consider reorganizing the order of Supplementary Figure 12-14 as they appear in the text.

The Supplementary Tables lack an intuitive explanation of the different columns.

Discussion:
It is difficult to follow the authors reasoning as the use of references makes it difficult to assess if the statements are part of the results or referring to existing literature. The authors need to revise the discussion. Especially for the discussion in section line 408-420 where the steady state immune cell signature (Pleuger et al 2022, ref 42) could be discusses in relation to this manuscript results on TLS development. Pleuger et al demonstrate distinct clusters of immune cells in both Corpus and Cauda areas, among them the γδ T cells known to be involved in TLS formation.
Line 456-459 lacks references.
Line 423-424 need references.

Reviewer #2

(Remarks to the Author)
The paper by Elizagaray et al. investigates the impact of Treg depletion on the adaptive immune response in the epididymis. Using a diphtheria toxin model in Foxp3-DTR mice, the authors deplete Tregs and assess the outcomes 2 and 8 weeks after depletion. They examine the effects on autoantibody formation against testicular and epididymal antigens, alterations in epididymal epithelial parameters, changes in leukocyte populations, and the development of tertiary lymphoid structures (TLS), among other factors.

The model results in a temporary depletion of Tregs, with their numbers fully recovering by 8 weeks post-ablation. The manuscript is well-written, presents a wealth of data, and addresses a research area that remains only marginally understood. One of the paper's key strengths is that it advances our understanding of Tregs in the epididymis and their role in an organ that exhibits a highly localized response to inflammatory signals. Additionally, the study enhances our knowledge of the adaptive immune response, particularly the role of B cells in the epididymis—a topic that is still underexplored, despite the known association between antisperm antibodies and fertility issues in a small cohort of men.

The paper stands out for its comprehensive analysis of T- and B-cell dynamics and provides an in-depth characterization of antibody subtypes formed against testicular and epididymal antigens. This thorough investigation represents a valuable contribution to the field.
While the manuscript provides valuable insights, there are several aspects that warrant further attention and clarification as they dampen the overall enthusiasm for the study.

1. Immunofluorescence controls: The study relies heavily on immunofluorescence, yet only the omission of the primary antibody is used as a control. How can the authors be certain that their primary antibodies bind specifically, especially since no positive controls are included to validate the specificity of the staining?

2. Treg population in the epididymis (Figure 1): Figure 1 suggests that the normal epididymis harbors very few Tregs, likely in the low double-digit range. This point should be more clearly emphasized in the manuscript to avoid any misinterpretation of the data.

3. Y-axis scaling in Figures 1A iii, iv, and 1B ii: For better comparison of the data, it would be helpful if Figures 1A iii and iv, and 1B ii used the same scale on the y-axis.
4. Clarification of Figure 1C: In Figure 1C, it is unclear which part of the epididymis is shown. The left image primarily focuses on the interstitial space, while the right image shows mostly epithelium and lumen, with little interstitial tissue.

5. AQP9 Staining in Figure 1E: Does the AQP9 staining in the interstitial space (IS) compare the same regions in both WT and Foxp3-DTR mice? From the images, it appears that the IS in the Foxp3-DTR mice has a wider lumen compared to the lumen generally shown for IS in other images and the corresponding picture for WT mice.

6. Figure 2F (right panel): Figure 2F (right panel) compares IgG1 and IgG2b in the Foxp3-DTR epididymis, while the three panels on the left compare the same antibody subtype in both WT and Foxp3-DTR. Consistency in the comparison groups should be maintained for clarity.

7. IgG2c localization in Figure 2G and Suppl. Figure 9: The authors claim the presence of IgG2c in the lumen of the epididymis, where it agglutinates sperm two weeks after Treg depletion. However, Suppl. Figure 9 clearly shows that IgG2c, like all other Ig subtypes investigated, is only present in the interstitial space, not in the lumen. The same issue is seen in Figure 3A, where IgG2c staining is shown on the tail of sperm 2 weeks after Treg depletion, contradicting the data in Suppl. Figure 9.

8. In line 696 of Figure 3, the M1 concept of macrophage polarization is mentioned. This concept has been largely superseded by more nuanced classifications and should be updated to reflect current understanding.

9. Human semen sample investigations: The use of human semen samples raises several concerns:
(i) The patient cohort is categorized exclusively as normozoospermic and asthenozoospermic, but no information is provided on leukocytospermia or microbial assessment of the ejaculate. These factors are important for interpreting Ig measurements in seminal plasma.
(ii) It is unclear why asthenozoospermic men (with reduced sperm motility) were chosen, especially when sperm motility in

Treg-depleted mice remains normal (Figure 8C, D). In contrast, the number of sperm is decreased in Treg-depleted mice (Figure 8C), which would reflective of oligozoospermic men.

10. Figure 4G - gating of germinal center B cells: The rationale for the gating of germinal center B cells, particularly in the proximal epididymis of 2-week Treg-depleted mice, is unclear. Is this gating supported by isotype controls? According to the gating used, germinal center B cells are claimed to be increased in the proximal epididymis, but not in the distal epididymis, where authors claim to observe tertiary lymphoid structures (TLS) (Figure 6).

11. A minor typographical error should be corrected: "Follicular" in Figure 5G.

12. TLS characteristics in Figures 5 and 6: In the introduction (lines 79-80), the authors define TLS as secondary lymphoid organs featuring distinct T and B cell zones, follicular dendritic cell networks, high endothelial venules (HEV), and specialized immunofibroblasts. However, Figures 5 and 6, as well as Suppl. Figure 17A and F, show TLS lacking most features—particularly the absence of closely organized T and B cell zones and HEV. Additionally, the anti-smooth muscle actin (SMA) staining labels smooth muscle cells surrounding the tubules, not fibroblasts, which calls into question the identification of the structures as TLS.

13. Clarification of germinal center B cell gating (Figure 6D): The gating of germinal center B cells in Figure 6D is unclear, similar to Figure 5G. The "nose" protruding from the B cell cloud in the bottom right corner may be more appropriate for gating. What do the respective isotype controls reveal? In this regard, there is no mention of these controls in the materials and methods, and relying on unstained cells and FMO controls is not adequate.

14. AID and Ki67 staining (Figure 6K): The use of AID staining to demonstrate immunoglobulin class switch recombination and somatic hypermutation, along with Ki67 as a proliferation marker, is intended to indicate possible germinal center formation. However, both markers are found outside of the B cell clusters in TLS (Figure 6K, L; Suppl. Fig. 17B, C), which raises questions about the interpretation of the staining.

15. CD31+ endothelial cells (Figure 6M): Figure 6M shows CD31+ endothelial cells but does not demonstrate HEV, which are typically a hallmark of TLS.
16. Tissue preservation in Suppl. Figures 2 and 13E: The preservation of testicular and epididymal tissue in Suppl. Figures 2 and 13E appears suboptimal compared to the other morphological images.

17. Fertility implications (abstract, short summary): In the abstract and conclusion, the authors suggest that their findings may lead to therapies for infertility and contraception. However, as Treg-depleted mice remain fertile, this statement appears overstated.

Reviewer #3

(Remarks to the Author)
The investigators have previously demonstrated that Treg depletion in mice can produce autoimmune infertility (PNAS 2023, ref. 11 in the manuscript). In the present study, they use the same mouse model to explore in greater depth the events following Treg depletion - reporting epididymal epithelial changes, lymphocytic infiltration, the formation of tertiary lymphoid structures in the epididymis, and the production of autoantibodies against testicular and epididymal antigens, ultimately resulting in reduced male fertility. Additionally, they test male individuals with fertility challenges for the presence of anti-sperm autoantibodies. This study provides valuable new information regarding the role of Treg-mediated peripheral tolerance in male infertility, with potential relevance for human reproductive health.

I have several questions, particularly regarding the work to characterize autoantibodies.

1. Autoantibody testing by ELISA and using tissue homogenates as antigen sources was conducted at a high serum concentration of 1:25. Bona fide autoantibodies are typically detectable at much lower serum dilutions, often several orders of magnitude lower. The observed differences in IgG autoantibody levels between Treg-depleted and control mice look convincing. However, to further validate these findings, it would be beneficial for the authors to perform a dilution series, at least in a few mice, to determine their autoantibody titers. Using a lower serum concentration could reduce the background in autoantibody assay and enable a better separation of Treg-depleted and control mice.

2. The investigators should be commended for their efforts to characterize the molecular targets of autoantibodies. However, additional information would be helpful to understand the credibility of the reported antigens. The authors used immunoprecipitation with sperm as the antigen source, followed by mass spectrometry, to identify the molecular targets of autoantibodies in Treg-depleted mice, resulting in a long list of putative autoantigens. I cannot find information how many mice were included in this experiment - could the authors clarify this?

3. The manuscript states that immunoprecipitation confirmed the presence of ASAs following Treg depletion. Could the authors clarify in what way this was demonstrated?

4. Seventy-seven proteins were identified as putative autoantibody targets, but it is not clear how this selection was made. Were these 77 proteins those showing the strongest increase in Treg-depleted mice compared to WT mice? Additional explanation of the selection criteria would be valuable. Furthermore, while several of these 77 proteins are reported to have

known functions in sperm maturation and fertility, this alone does not support their validity as autoantibody targets. Even without immunoprecipitation, mass spectrometry of sperm samples would inherently identify proteins involved in sperm function. Thus, additional validation is needed. To strengthen the credibility of the reported autoantibody targets, the investigators could leverage their existing data by performing a reverse analysis. Specifically, they could examine the proteins most strongly increased in healthy control mice compared to Treg-depleted mice. If the top-ranked proteins in Treg-depleted mice include a higher number of sperm-specific proteins, while those in healthy controls are relatively higher number of ubiquitously expressed proteins, this would provide support for the validity of the findings.

Ideally, the authors should experimentally validate the identified putative autoantibody targets to confirm one or more of them. This could be achieved using recombinant proteins in an ELISA-based assay for autoantibody detection.

5. Investigations of gonadal tissue from Treg-depleted mice revealed IgG deposition in the epididymal interstitium. How does this finding relate to the presence of antisperm autoantibodies?

6. The authors should also be commended for their efforts to assess the relevance of their findings in mice to human male infertility. They report that males with fertility challenges exhibit antisperm autoantibodies (ASA); however, the difference in autoantibody levels between affected individuals and controls appears quite subtle. Given this, it is difficult to determine the significance of the findings. Would it be possible to optimize the assay to achieve clearer and more definitive results? Additionally, it would be important to relate these findings to existing literature. As I understand it, ASA are already well-established in male infertility, making this aspect of the study less novel. Furthermore, the connection between the human data and the mouse model remains weak, as the only overlap that can be shown is the presence of sperm ASA. Given these limitations, the authors may want to reconsider including these data in the manuscript.

7. IPEX syndrome may offer insights into the human relevance of the findings. Individuals with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused by loss-of-function mutations in the FOXP3 gene, lack functional regulatory T cells and develop severe multiorgan autoimmunity. In these patients, the gut, pancreatic islet cells, and skin are the primary targets of autoimmunity. Is there any data on male fertility in individuals with IPEX that could support the relevance of the reported findings in humans? Exploratory autoantibody testing in IPEX patients has identified autoantibody targets specifically expressed in enterocytes and pancreatic islet cells - tissues affected by the disease - but has not revealed autoantibodies targeting gonadal proteins (PMID 31027649). It would be relevant to discuss potential parallels between IPEX and the findings in this study in the Discussion section.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors has answered all my questions and concerns, and the manuscript has been revised accordingly.I highly recommend to publish this important research on development of TLS after Treg depletion in the epididymis.

Reviewer #2

(Remarks to the Author)
The authors have submitted a revised version of their manuscript and have addressed the reviewers' comments in a detailed, point-by-point response. This reviewer will focus specifically on the revisions related to the concerns made previously by this reviewer.

Overall, the manuscript has been improved, and many of the concerns have been addressed satisfactorily. However, several critical issues remain unresolved, as outlined below.

Figure 1:
A comparison between Figures 1A and 1C reveals a discrepancy in Treg cell detection. The flow cytometry data suggest a very low number of Tregs in the entire epididymis (in the two-digit range), whereas the Foxp3-EGFP+ staining shows 6–7 positive cells in just a small section of the epididymis indicating a much higher prevalence. Furthermore, similarly intense staining is observed in the apical region of the epithelium and within the lumen. This raises concerns about the specificity of the immunofluorescence staining.

Figure S23 (related to comment #17 in the point-by-point response):
Despite the inclusion of quantification, the image still demonstrates poor tissue preservation. It remains unclear how the authors are able to assess spermatogonia or sperm, as these cells are barely distinguishable as individual entities in the provided images.

Human samples:
The authors now include more semen data from their patient cohort, which is appreciated. However, in the context of autoantibody formation, it would be important to indicate whether any of the patients had a history of male genital tract infection/inflammation, as this may significantly impact the interpretation of the findings.

Figure 7 (related to comments #12, #14, and #15):
Figure 7 aims to illustrate the formation of tertiary lymphoid structures (TLS), a key aspect referenced in the manuscript title. However, the evidence provided remains unconvincing in the revised version, for the following reasons:
1. While an organized B cell cluster is visible, there is no equivalent T cell cluster. T cells appear scattered in relatively low numbers around the B cells and do not form a structured organization—an observation acknowledged by the authors themselves in their rebuttal.
2. The new AID staining image (Figure 7E) does not convincingly support TLS formation. Notably, the prominent AID staining lies outside the CD19+ B cell cluster, and staining within the cluster is at best minimal.

3. In Figure 7G, PNAD staining is used to demonstrate the presence of high endothelial venules (HEVs), an essential TLS marker. However, the staining does not reflect blood vessels and instead appears unspecific—filamentous in pattern, lacking the morphology of blood vessels, and likely also located within the epididymal epithelium (top right corner), where no HEVs can be expected.

4. The Ki67 staining shown in Figure 7F is predominantly outside the B cell cluster, with only faint and punctate staining within the cluster. This aspect has not been clarified by the revision.

5. Additionally, Figure 7F shows a B cell cluster that is tightly surrounded by epididymal epithelium. It is unclear where a putative T cell cluster could be accommodated in this context.

Minor point:
There is inconsistency in the use of mouse and human gene/protein nomenclature, particularly regarding the capitalization of letters. Standardizing this throughout the manuscript would improve clarity and alignment with field-specific conventions.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed all of my questions by providing new data and making appropriate revisions to the manuscript.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have now staisfactorily addressed the concerns of this reviewer by adding new images and providing further explanation.

Response to Reviewers' comments

Reviewer #1 (Remarks to the Author)

Review report

Key results

This manuscript studied autoimmune orchitis and epididymitis in a Treg depletion model. The authors demonstrate the subsequent development of anti-sperm autoantibodies, identify targets for these antibodies, and show the isotypes and deposition in testis and different region of the epididymis. They demonstrate for the first time the development of tertiary lymphoid structures (TLS) in special areas of the epididymis and characterize the cellular composition of these. They also demonstrate similar autoantibodies in seminal plasma linking a common mechanism of infertility in mice and humans. This manuscript focuses on the role of B cells and autoantibodies and is based on a previous published model where they describe the T cells. This paper extent the previous work by the addition of analyses at week 8 after Treg depletion together with analyses at week 2. The data is novel and provide valuable insight into mucosal immunology and the role of TLS.
The amount of data provided is extensive and the use of methods is valid. The results are presented comprehensively. However, the discussion does not give the result any justice.

I have some questions and suggestions for improvement of this extensive work:
Results:

- The number of animals used is missing.
We apologize for the missing information. The number of animals used for each experiment was added to all figure captions.

- Suppl Fig 4 show distinct patterns of the different IgG isotypes. The authors have not addressed this. Could the different patterns reflect different antigenic specificity between the isotypes? The proteomics data may indicate the formation of immune complexes. This could also affect the autoantibody binding pattern observed in the testis. Could the binding of autoantibodies in the basal lamina of the seminiferous tubules influence the spermatogenesis?
We thank the reviewer for this comment. The antibody deposition on the testis 2 weeks post-Treg depletion shows different patterns, possibly due to distinctive specificities among the isotypes. This observation is included in the revised version, lines 140-141 (redlined version).

The immunoprecipitations weren't performed with testicular antigens. Fig. 3E shows sperm proteins (identified by our approach of immunoprecipitation and proteomics) that were only detected by the Foxp3 samples and showed more than a 2-fold increase compared to the WT control samples. Our proteomic data shows C1qa, C1qb, C1qc, C1ra, C1s1, C1s2, C3, C4b, and Serping1 presence in the Foxp3-DTR serum when precipitated against sperm antigens. Only C1qb was found in distal epididymal fluid when immunoprecipitated with epididymal sperm antigens. Several immunoglobulins were detected in the serum, proximal, and distal epididymal fluids of Foxp3-DTR mice when precipitated with sperm antigens. Since sperm antigenic properties vary from their formation in the testis to their transit to the epididymis (Skerrett-Byrne et al., 2022. Cell Reports), future investigations must address whether complement proteins and immune complexes are present in the testicular tissue after Treg depletion.
Sertoli cells play a crucial role in maintaining the integrity of the blood-testicular barrier (BTB) through various mechanical and immunological mechanisms (Dufour et al, 2023, 2024. Int J Mol Sci). It has been reported that anti-collagen antibodies disrupt the tight junctions of the BTB (Siu et al, 2008. Adv Exp Med Biol). Additionally, anti-Sertoli cell antibodies have been linked to human male infertility (Wall et al., 1974. Clinical Endocrinology), and antibodies targeting the basement membrane of the seminiferous tubules are associated with autoimmune orchitis in infertile men (Silva et al., 2012. Clin. Rev. Allerg. Immunol; Qu et al., 2019. Rep. Med. Biol.). Importantly, in our study, the presence of autoantibodies near the basal membrane did not significantly influence spermatogenesis 24 h (Suppl. Fig. 2) nor 8 weeks after Treg depletion (new Suppl. Fig. 23). Importantly, spermatogenesis was not affected 2 weeks after Treg depletion (Barrachina et al.,

2023 PNAS). Based on this comment, we have included these studies in the discussion of the revised version (Lines: 519-522, redlined version).

-The sperm antigens detected could have been presented better with more details on their expression levels. Line 169-195 might fit better in the discussion?
We apologize for the lack of clarity regarding the proteomics results. To enhance readability, we have added headlines and subheadings to Supplementary Tables I and II. Additionally, we have created a new supplementary table (III) listing the proteins recognized by serum (tab 1) and proximal (tab 2), and distal (tab 3) fluid ASAs, exclusively present in the Foxp3 samples, along with a fold increase greater than 2 compared to the control samples. These lists include sperm antigens and complement proteins (also included in the Venn diagram of Fig. 3) and immunoglobulins (in red in Table III). Following the reviewer's suggestions, lines 169-195 were moved to the discussion section. Please see lines 538-553 of the redlined manuscript.

-The distinct cell subtypes shown in the t-SNE and FlowSOM analysis is not completely described in the figures. The colors and cells should be defined in the figure text.
We apologize for the missing information. The colors and population numbers key for the t-SNE and FlowSOM analyses have been added to the captions. Please see the Legends of Figs. 4-6, as well as Suppl. Figs. 11-13 and 17.

-The authors claim that spermatogenesis was not chronically affected, but they observed reduced distal sperm counts. The picture in Suppl. Fig. 13E is clearly showing a morphological change of the semi in the seminiferous tubules of the Treg depleted animals. Did the authors count the different spermatocytes?
We thank the reviewer for this comment. We quantified the different cells (spermatogonia, spermatocyte/spermatid, and spermatozoa) in the 8-week DT-injected WT and Foxp3-DTR testis. We do not observe significant changes between groups (new Suppl. Fig. 23). We also replaced the images as suggested by this reviewer. We proposed that the decrease in distal sperm counts might be due to phagocytosis of sperm in the epididymis. Please see lines 369-370 and 513-515 in the redlined version.

- The definition of TLS includes T and B cell areas with germinal centers (GC) and High endothelial venules (HEVs). Figure 6G is not convincing evidence for TLS as very few CD3 cells are detected. The Videos also lack evidence for T cells in these structures. However, TLS have been shown to differ from organs to organs. The authors should try to discuss their finding on TLS in relation to TLS in other organs and diseases.
We would like to clarify that Fig. 6G (in the previous version) didn't show CD3 staining. Fig 6J and H (now Fig. 7B, D), Movies III-VII, and new Suppl. Fig. 18B and C) show CD3+ cells surrounding/nearby clusters of B cells. In the distal epididymis, 8 weeks after Treg depletion, we detected distinct stages of epididymal TLS development: an initial stage marked by dense lymphocyte aggregates, an intermediate stage with defined T and B cell areas, and a mature stage accompanied by Germinal Center (GC) B cells (AID-positive and class-switched IgD), follicular DCs, HEVs, and fibroblasts. However, the T zone of the epididymal TLSs may not exhibit the same well-organized architecture typically observed in fully matured TLSs in other organs (Zhao et al., 2024. Nat Rev). As noted by this reviewer and discussed in our previous version of the manuscript (lines 461-478 in the redlined version), TLSs exhibit significant heterogeneity across different organs and pathological conditions. Further studies will be performed to study the different stages and distinct characteristics of epididymal TLSs.

To address the comment regarding immunofibroblasts, we conducted immunostaining for podoplanin. Our results revealed cells positive for this marker surrounding the TLSs (new Fig. 7H,

Suppl. Fig. 18I, and new Movies IX-X), indicating the presence of immunofibroblasts. Furthermore, we also added new images for CD31 and PNAD markers (new Fig. 7G and Suppl. Fig. 18F-G). Cells positive for these markers were also present surrounding epididymal TLSs, suggesting the involvement of high venule endothelial cells in the organization of these structures during epididymitis. Together, these observations indicate the presence of mature TLSs in the epididymis.
We have revised Figures 6 (now Figures 6 and 7) and Suppl. Fig. 18 by adding new images, arrows, and white squares to better highlight our findings.

-The authors did not demonstrate HEVs within the TLS, although the CD31 staining looks promising. To verify functional TLS and HEVs, PNAD staining could be included.
Based on the comment provided, we performed immunostaining for PNAD, a marker of HEVs. Our results revealed that HEVs were near TLSs under the experimental conditions. Please refer to the new Figure 7G and Suppl. Fig 18G.

- Figure 8F is a very nice figure illustrating the important findings. However, as pointed out above, the staining pattern in Supplementary Figure 4 indicate binding of autoantibodies on the epithelial cells or basement membrane in the testis. The figure should include an illustration of autoantibody binding in the testis in addition to the binding to the sperm cells.
We thank the reviewer for this comment. In the revised version of Figure 9F, we included the conclusion of the finding of autoantibody isotype levels against testicular antigens. Please also find the summary of anti-sperm and autoantibodies in epi/testis/serum in Suppl. Fig. 21D.

- The order of the supplementary figures is confusing. Consider reorganizing the order of Supplementary Figure 12-14 as they appear in the text. The Supplementary Tables lack an intuitive explanation of the different columns.
We apologize for the confusion. We modified the order of the Suppl. Figs.10-18 as well as the text. We also included Headlines and sub-headlines in rows 1 and 2 of the Suppl. Tables.

Discussion:

-It is difficult to follow the authors reasoning as the use of references makes it difficult to assess if the statements are part of the results or referring to existing literature. The authors need to revise the discussion. Especially for the discussion in section line 408-420 where the steady state immune cell signature (Pleuger et al 2022, ref 42) could be discusses in relation to this manuscript results on TLS development. Pleuger et al demonstrate distinct clusters of immune cells in both Corpus and Cauda areas, among them the γδ T cells known to be involved in TLS formation.
Based on this comment, we rewrote the discussion to improve it in the revised version of the manuscript. We also discussed the role of γδ T cells and TLS formation (lines: 452-454 in the redlined version).

Line 456-459 lacks references.
Line 423-424 need references.
We apologize for the missing information. We added those references. Lines 504-507 (references 10, 60, and 61) and Lines 470-471, references 10, 11 and 34 (redlined version).

Reviewer #2 (Remarks to the Author)
The paper by Elizagaray et al. investigates the impact of Treg depletion on the adaptive immune response in the epididymis. Using a diphtheria toxin model in Foxp3-DTR mice, the authors deplete Tregs and assess the outcomes 2 and 8 weeks after depletion. They examine the effects on autoantibody formation against testicular and epididymal antigens, alterations in

epididymal epithelial parameters, changes in leukocyte populations, and the development of tertiary lymphoid structures (TLS), among other factors.

The model results in a temporary depletion of Tregs, with their numbers fully recovering by 8 weeks post-ablation. The manuscript is well-written, presents a wealth of data, and addresses a research area that remains only marginally understood. One of the paper's key strengths is that it advances our understanding of Tregs in the epididymis and their role in an organ that exhibits a highly localized response to inflammatory signals. Additionally, the study enhances our knowledge of the adaptive immune response, particularly the role of B cells in the epididymis—a topic that is still underexplored, despite the known association between antisperm antibodies and fertility issues in a small cohort of men.

The paper stands out for its comprehensive analysis of T- and B-cell dynamics and provides an in-depth characterization of antibody subtypes formed against testicular and epididymal antigens. This thorough investigation represents a valuable contribution to the field.
While the manuscript provides valuable insights, there are several aspects that warrant further attention and clarification as they dampen the overall enthusiasm for the study.

1. Immunofluorescence controls: The study relies heavily on immunofluorescence, yet only the omission of the primary antibody is used as a control. How can the authors be certain that their primary antibodies bind specifically, especially since no positive controls are included to validate the specificity of the staining?
In response to this comment, we have incorporated positive (lymph nodes) controls for the T- and B-cell and TLS markers in the revised manuscript (Suppl. Fig. 24, line 681 in the redlined version). The epithelial markers (B1 subunit of the V-ATPase and AQP9), macrophage (F4/80), and proliferation (Ki67) markers have been fully validated in prior studies conducted by our group (Battistone et al., 2020, MHR; 2020 JCI; Barrachina et al., 2022. CMLS; 2023. PNAS). Additionally, we would like to emphasize that our immunofluorescence staining experiments included DT-injected WT and naïve mice as controls. The absence of staining in these controls confirms the specificity of the antibody binding and rules out nonspecific interactions. We would like to clarify that all of our immunofluorescence findings are corroborated by complementary analyses, including flow cytometry, ELISA, proteomics, and other methodologies.

2. Treg population in the epididymis (Figure 1): Figure 1 suggests that the normal epididymis harbors very few Tregs, likely in the low double-digit range. This point should be more clearly emphasized in the manuscript to avoid any misinterpretation of the data.
Thank you very much for the comment. The current study and the previous one from our group (Barrachina et al., 2023 PNAS) showed that despite Tregs comprising only a small fraction of the epididymal immune cell population, they are crucial in preventing autoimmune damage and maintaining proper fertility function.
Tregs are well-known for their potent immune-suppressive properties. Similar quantities of Tregs have been observed in other non-lymphoid tissues under physiological conditions, including adipose tissue (Chaoran Li et al., 2018, *Cell*; Spallanzani et al., 2019, *Science Immunology*; Wang et al., 2024, *Immunity*) and muscle (Burzyn D et al., 2013, *Cell*). We included a statement in the revised version as suggested by this reviewer (lines 410-414 in the redlined version).

3. Y-axis scaling in Figures 1A iii, iv, and 1B ii: For better comparison of the data, it would be helpful if Figures 1A iii and iv, and 1B ii used the same scale on the y-axis.
Thank you very much for the suggestion. The mentioned graph axes were edited in Figure 1.

4. Clarification of Figure 1C: In Figure 1C, it is unclear which part of the epididymis is shown. The

left image primarily focuses on the interstitial space, while the right image shows mostly epithelium and lumen, with little interstitial tissue.
Thank you very much for the comment. We replaced the picture for the epididymis of the 1-day DT-injected Foxp3-DTR in Fig. 1C. In addition, “cauda” was added next to the images.

5. AQP9 Staining in Figure 1E: Does the AQP9 staining in the interstitial space (IS) compare the same regions in both WT and Foxp3-DTR mice? From the images, it appears that the IS in the Foxp3-DTR mice has a wider lumen compared to the lumen generally shown for IS in other images and the corresponding picture for WT mice.
It is important to note that AQP9 staining is primarily localized to the apical region of the epididymal principal cells, with no interstitial staining observed 24 h after DT treatment. The size of the epididymal lumen can vary depending on the cross-section, as the epididymis is a single, convoluted tubule. However, it is also possible that the apparent increase in lumen size is due to epithelial shedding. The images presented were taken from the initial segments (IS), where we observed apical blebs in clear cells and luminal-reaching projections of mononuclear phagocytes, both of which are characteristic features of the IS.

6. Figure 2F (right panel): Figure 2F (right panel) compares IgG1 and IgG2b in the Foxp3-DTR epididymis, while the three panels on the left compare the same antibody subtype in both WT and Foxp3-DTR. Consistency in the comparison groups should be maintained for clarity.
We apologize for any lack of clarity in the previous version of this figure; we have made the necessary modifications in the revised manuscript to present our results in a better way. Figure 2F-H illustrates 'proto' cellular aggregations observed at 2 weeks post-treatment, which may potentially contribute to the formation of TLS at a later stage.

7. IgG2c localization in Figure 2G and Suppl. Figure 9: The authors claim the presence of IgG2c in the lumen of the epididymis, where it agglutinates sperm two weeks after Treg depletion. However, Suppl. Figure 9 clearly shows that IgG2c, like all other Ig subtypes investigated, is only present in the interstitial space, not in the lumen. The same issue is seen in Figure 3A, where IgG2c staining is shown on the tail of sperm 2 weeks after Treg depletion, contradicting the data in Suppl. Figure 9.
IgG2c autoantibodies were detected in both the interstitium (Fig. 2E,H, Suppl. Fig. 9) and the lumen (Fig. 2I) by immunofluorescence (IF). We observed higher levels of autoantibodies in the interstitium compared to the lumen by IF, likely due to washout effects in this technique. Detecting antibodies binding to sperm in the lumen of epididymal sections is more challenging, so we used higher laser intensity for imaging the epididymal lumen. We further confirmed the presence of IgG2c in the lumen through proteomics using antibodies from the epididymal fluid (gene name Ighg2c in Tables II-III (in red)) and detecting this isotype on sperm through sperm suspension staining (Fig. 3A; line 146 in the redlined manuscript). We have added a sentence in the revised manuscript to clarify the luminal staining (Legend Fig. 2).

8. In line 696 of Figure 3, the M1 concept of macrophage polarization is mentioned. This concept has been largely superseded by more nuanced classifications and should be updated to reflect current understanding.
We agree with this comment. We modified the manuscript (line 154 in the redlined manuscript) and Figure Legend 3 accordingly.

9. Human semen sample investigations: The use of human semen samples raises several concerns (i) The patient cohort is categorized exclusively as normozoospermic and asthenozoospermic, but no information is provided on leukocytospermia or microbial assessment of the ejaculate. These factors are important for interpreting Ig measurements in seminal plasma.

We appreciate the reviewer's comment. Supplementary Table III (now Tables IV and V) includes the descriptive parameters, including leukocytospermia (% round cells, RCs) of the normozoospermic and asthenozoospermic semen samples used in this study. To examine the association between ASAs and potential immune-mediated male infertility, patients were categorized based on the concentration of RCs in their ejaculates, without considering the sperm motility parameter. A threshold of 1 million RCs per milliliter (1 M/mL), as defined by the WHO 2021 reference limits for leukocytospermia, was used for classification. Samples with RC levels ≥1 M/mL were classified into the 'RC' group, while those with RC concentrations <1 M/mL were placed in the 'no RC' group. We observed higher levels of IgA in seminal plasma in patients with RCs. The details of this classification have been added to the Materials and Methods section in the revised version (lines 806-811 in the redlined manuscript).

(ii) It is unclear why asthenozoospermic men (with reduced sperm motility) were chosen, especially when sperm motility in Treg-depleted mice remains normal (Figure 8C, D). In contrast, the number of sperm is decreased in Treg-depleted mice (Figure 8C), which would reflective of oligozoospermic men.

We apologize for the missing information in our manuscript. As initially reported in Barrachina et al. (2023, PNAS), 2 weeks following Treg depletion, an impairment in sperm motility was observed. The human cohort was based on this early finding. Additionally, it has been reported that autoimmune infertility could contribute to a decrease in sperm motility (Fijak et al., 2018, Human Reproduction), further guiding our choice of participants. Based on this comment, we added a statement in the revised version (lines 201-203 (redlined version)).

Our current ongoing collaboration involves a larger and more diverse cohort of patients diagnosed with various fertility disorders, allowing for a more comprehensive analysis. Importantly, the classification of "no RC" versus "RC" was made independently of sperm motility, which has been a crucial factor in our study. We believe that ASA levels could provide valuable insight into the underlying mechanisms of infertility associated with autoimmune conditions.

10. Figure 4G - gating of germinal center B cells: The rationale for the gating of germinal center B cells, particularly in the proximal epididymis of 2-week Treg-depleted mice, is unclear. Is this gating supported by isotype controls? According to the gating used, germinal center B cells are claimed to be increased in the proximal epididymis, but not in the distal epididymis, where authors claim to observe tertiary lymphoid structures (TLS) (Figure 6).

We apologize for the missing information. Two weeks after Treg depletion, we observed cell accumulation in the proximal region of the interstitium (Figs. 2F, H, and 4A), as detected by confocal microscopy, and an increase in B cell abundance by flow cytometry (Fig. 4C-D). Based on these findings, we further investigated the immune phenotype of B cells at that time point. Notably, we found an increase in GC B cells in the proximal epididymis (Fig. 4G-H); however, we did not observe the formation of TLSs in that region 2 weeks after Treg depletion. At 8 weeks post-treatment, the scenario is different, showing accumulation of B cells and TLSs in the distal epididymis, whereas in the proximal, the infiltration of B cells is resolved (Fig. 6). These findings suggest that region-specific cell signaling pathways may be required for the full development of TLSs. For example, it is important to note that the distal epididymal environment contains higher amounts of sperm antigens, which could play a role in the promotion, initiation and/or maintenance of TLSs. This difference in antigenic load between regions may contribute to the absence of TLSs in the proximal epididymis despite an increased presence of GC B cells at early time points after treatment. Please see lines 455-458 in the redlined version. Further studies will be needed to explore the molecular and cellular signals that influence TLS formation in the different regions.

In response to this comment, we have revised the gating strategy to more accurately define GC.

It is important to highlight that the results didn't change. Please refer to Figures 4G and 6D and Suppl. Figures 11I-K,12D, and 17F.

There is an ongoing debate regarding the utility of isotype controls in flow cytometry analysis. While they have traditionally been regarded as helpful for confirming the proper blocking of cells under experimental conditions, the prevailing consensus is that they should not be used to determine positivity in fully stained samples (Holden T. Maecker and Joseph Trotter, Cytometry Part A 69A:1037–1042, 2006; Ruud Hulspas et al., Cytometry Part B (Clinical Cytometry) 76B:355–364, 2009). Even when the isotype control antibody is matched to the test antibody, significant limitations exist. Each antibody conjugate exhibits varying levels of background staining, influenced by factors such as specificity, concentration, aggregation, and the fluorophore-to-antibody ratio, among others. Given the unpredictable nature of finding an isotype control that accurately reflects the background staining of a specific test antibody, isotype controls are increasingly being phased out in favor of alternative methods. Instead of isotype control, we perform negative and FMO (fluorescence minus one) controls to define our gating strategies.

11. A minor typographical error should be corrected: "Follicular" in Figure 5G.
Thank you for pointing this out. Figure 5G was updated.

12. TLS characteristics in Figures 5 and 6: In the introduction (lines 79-80), the authors define TLS as secondary lymphoid organs featuring distinct T and B cell zones, follicular dendritic cell networks, high endothelial venules (HEV), and specialized immunofibroblasts. However, Figures 5 and 6, as well as Suppl. Figure 17A and F, show TLS lacking most features—particularly the absence of closely organized T and B cell zones and HEV. Additionally, the anti-smooth muscle actin (SMA) staining labels smooth muscle cells surrounding the tubules, not fibroblasts, which calls into question the identification of the structures as TLS.
We thank the reviewer for this comment. In the distal epididymis, 8 weeks after Treg depletion, we detected distinct stages of epididymal TLS development: an initial stage marked by dense lymphocyte aggregates (Fig. 7D, Suppl. Fig.18A), an intermediate stage with defined T and B cell areas (Fig. 7D and Suppl. Fig. 18B, C), and a mature stage accompanied by Germinal Center (GC) B cells (AID-positive and class-switched IgD), follicular DCs, HEVs and fibroblasts (Fig. 7A-B, E-H, Suppl. Fig. 18A, D-I). However, the T zone of the epididymal TLSs may not exhibit the same well-organized architecture typically observed in fully matured TLSs in other organs. As noted by reviewer 1, TLSs exhibit heterogeneity across different organs and pathological conditions (Zhao et al., 2024 Signal Transduct Target Ther). Further studies will be performed to study the different stages and distinct characteristics of epididymal TLSs.
To address the comment regarding immunofibroblasts and HEVs, we conducted immunostaining for podoplanin and PNAD (Fig. 7G-H, Suppl. Fig. 18G, I). Our results revealed cells positive for these markers surrounding the TLSs, indicating the presence of immunofibroblasts and HEVs, respectively. These observations suggest the presence of mature structures. We have revised Figures 6, 7, and Suppl. Fig. 18 by adding new images, arrows, and squares to better highlight our key findings.

13. Clarification of germinal center B cell gating (Figure 6D): The gating of germinal center B cells in Figure 6D is unclear, similar to Figure 5G. The "nose" protruding from the B cell cloud in the bottom right corner may be more appropriate for gating. What do the respective isotype controls reveal? In this regard, there is no mention of these controls in the materials and methods, and relying on unstained cells and FMO controls is not adequate.
Based on this comment, we have modified the gating strategy used to define GC B cells in Figures 4G and 6D and Suppl. Figures 11I-K,12D, and 17F. It is important to highlight that the results

didn't change. Please refer to the previous response regarding isotype controls for further clarification.

14. AID and Ki67 staining (Figure 6K): The use of AID staining to demonstrate immunoglobulin class switch recombination and somatic hypermutation, along with Ki67 as a proliferation marker, is intended to indicate possible germinal center formation. However, both markers are found outside of the B cell clusters in TLS (Figure 6K, L; Suppl. Fig. 17B, C), which raises questions about the interpretation of the staining.

We thank the reviewer for this comment. In response to this comment, we added arrows and new images for AID in the new Fig. 7E and Suppl. Fig. 18D. Furthermore, we have clarified the localization of AID/Ki67 in non-B cells in the revised manuscript (Lines: 310-314). We also included positive controls in Suppl. Fig. 24.

We detected AID (Activation-Induced Cytidine Deaminase)-positive B cells within the TLSs (Fig. 7E and Suppl. Fig. 18D, arrows). Under normal physiological conditions, AID is predominantly expressed in mature/activated B lymphocytes. However, chronic inflammation can create a microenvironment that induces aberrant AID expression, not only in B cells but also in non-B-cell populations (Mechtcheriakova et al., 2012. Cancer Immunol Immunother.). Therefore, the AID-positive staining observed in non-B cells (outside the TLSs) could be attributed to the inflammatory state of the epididymis after Treg depletion.

Ki67 was also detected in B cells on the TLSs (Fig. 7F and Suppl. Figure 18E, indicated by arrows). Additionally, other Ki67-positive cells, particularly epithelial cells, were observed. This could be attributed to tissue healing and repair processes occurring in the epididymis 8 weeks after Treg depletion. Indeed, we detected several soluble mediators involved in this process (Fig. 7I).

The formation of GC in the distal epididymis, observed 8 weeks following Treg ablation, was further supported by flow cytometry data, which revealed an increase in resident GL7+ B cells in that region (Fig. 6D).

15. CD31+ endothelial cells (Figure 6M): Figure 6M shows CD31+ endothelial cells but does not demonstrate HEV, which are typically a hallmark of TLS.

Thank you for this comment. We performed immunostaining for PNAD to address this comment. We observed HEVs surrounding the TLSs, please see new Fig. 7G and Suppl. Fig. 18G. Line 315 in redlined version.

16. Tissue preservation in Suppl. Figures 2 and 13E: The preservation of testicular and epididymal tissue in Suppl. Figures 2 and 13E appears suboptimal compared to the other morphological images.

Based on this comment, we added new images of the testis (new Suppl. Figs. 2 and 23) and epididymis (Suppl. Fig. 2). In addition, we included the quantification of spermatogonia, spermatocytes/spermatids, and sperm in the testis (new Suppl. Fig. 23).

17. Fertility implications (abstract, short summary): In the abstract and conclusion, the authors suggest that their findings may lead to therapies for infertility and contraception. However, as Treg-depleted mice remain fertile, this statement appears overstated.

We thank the reviewer for this insightful comment. We modified the abstract and short summary as suggested by this reviewer (Lines: 51, 55, and 569 in the redlined version).

Reviewer #3 (Remarks to the Author):

The investigators have previously demonstrated that Treg depletion in mice can produce autoimmune infertility (PNAS 2023, ref. 11 in the manuscript). In the present study, they use the

same mouse model to explore in greater depth the events following Treg depletion - reporting epididymal epithelial changes, lymphocytic infiltration, the formation of tertiary lymphoid structures in the epididymis, and the production of autoantibodies against testicular and epididymal antigens, ultimately resulting in reduced male fertility. Additionally, they test male individuals with fertility challenges for the presence of anti-sperm autoantibodies. This study provides valuable new information regarding the role of Treg-mediated peripheral tolerance in male infertility, with potential relevance for human reproductive health.

I have several questions, particularly regarding the work to characterize autoantibodies.

1. *Autoantibody testing by ELISA and using tissue homogenates as antigen sources was conducted at a high serum concentration of 1:25. Bona fide autoantibodies are typically detectable at much lower serum dilutions, often several orders of magnitude lower. The observed differences in IgG autoantibody levels between Treg-depleted and control mice look convincing. However, to further validate these findings, it would be beneficial for the authors to perform a dilution series, at least in a few mice, to determine their autoantibody titers. Using a lower serum concentration could reduce the background in autoantibody assay and enable a better separation of Treg-depleted and control mice.*
   Thank you very much to the reviewer for the thoughtful comments. The 1:25 dilution is a standard, arbitrary dilution used for serums in our lab. To further validate this, we performed an ELISA titration of mouse serums collected 2- and 8-week post-DT treatment, assessing their reactivity against sperm antigens (included in the first submission). The results are shown in the new Figure 8Ei-ii (first submission in Fig. 7E i-ii). Notably, OD measurements were detectable even at dilutions as high as ~1:4000 for Foxp3 serum collected 2 weeks post-treatment and ~1:1000 for Foxp3 serum collected 8 weeks post-treatment, demonstrating the specific activity of the autoantibodies (ASAs) in the serum.
   Additionally, we conducted an ELISA to quantify the levels of autoantibodies against epididymal antigens, as presented in the updated Suppl. Fig. 21E. The results demonstrate that the serum could be diluted up to 1:250, both 2 and 8 weeks following Treg depletion. These new data have been incorporated into the revised manuscript, lines 363-364 (redlined version).

   2. The investigators should be commended for their efforts to characterize the molecular targets of autoantibodies. However, additional information would be helpful to understand the credibility of the reported antigens. The authors used immunoprecipitation with sperm as the antigen source, followed by mass spectrometry, to identify the molecular targets of autoantibodies in Treg-depleted mice, resulting in a long list of putative autoantigens. I cannot find information how many mice were included in this experiment - could the authors clarify this?
   Thank you very much to the reviewer for this comment, and apologize for the missing information. We pooled at least 3 samples (3 serums or 6 epididymides) per condition (serum, proximal, and distal epididymal fluids from WT and Foxp3-DTR) and performed two independent experiments. This information was added in the Method section (lines 780-782 (redlined version)).

   3. The manuscript states that immunoprecipitation confirmed the presence of ASAs following Treg depletion. Could the authors clarify in what way this was demonstrated?
   We apologize for the lack of clarity regarding the immunoprecipitation and proteomics data. The immunoprecipitation was conducted by incubating sperm proteins with the different samples, including serum, proximal epididymal fluid, and distal epididymal fluid, from DT-treated WT and Foxp3-DTR mice (Fig. 3D). The proteomics analysis identified several immunoglobulins that were bound to sperm proteins, particularly in the samples from Foxp3-DTR mice (Suppl. Table III in red). This confirms the presence of ASAs in the Treg-depleted samples. Please refer to the

complete list of identified proteins in Suppl. Tables I and II, as well as the new proteomics Suppl. Table III (red).

4. Seventy-seven proteins were identified as putative autoantibody targets, but it is not clear how this selection was made. Were these 77 proteins those showing the strongest increase in Treg-depleted mice compared to WT mice? Additional explanation of the selection criteria would be valuable. Furthermore, while several of these 77 proteins are reported to have known functions in sperm maturation and fertility, this alone does not support their validity as autoantibody targets. Even without immunoprecipitation, mass spectrometry of sperm samples would inherently identify proteins involved in sperm function. Thus, additional validation is needed. To strengthen the credibility of the reported autoantibody targets, the investigators could leverage their existing data by performing a reverse analysis. Specifically, they could examine the proteins most strongly increased in healthy control mice compared to Treg-depleted mice. If the top-ranked proteins in Treg-depleted mice include a higher number of sperm-specific proteins, while those in healthy controls are relatively higher number of ubiquitously expressed proteins, this would provide support for the validity of the findings. Ideally, the authors should experimentally validate the identified putative autoantibody targets to confirm one or more of them. This could be achieved using recombinant proteins in an ELISA-based assay for autoantibody detection.
We apologize for the earlier lack of clarity in reporting these findings. Based on this comment, we added a new table (Suppl. Table III) and rewrote the explanation of these results (Lines 157-172 in redlined version).
To clarify, a total of 87 proteins were commonly identified after immunoprecipitation using serum and epididymal fluids from both proximal and distal regions after Treg depletion, an increase from the 77 proteins noted in our initial submission (Fig. 3E). In Treg-ablated mice, we detected 134 immunoglobulin (Ig)-related proteins using serum, and 91 and 102 Ig-related proteins using the proximal and distal epididymal fluids from Treg-depleted mice, respectively (Suppl. Table III, red). These data support the presence of IgG1 and IgG2c ASAs in the epididymal lumen, as previously observed via immunofluorescence.
Additionally, we identified 8 complement proteins with serum, whereas only 1 was detected using the distal epididymal fluid following Treg depletion (Fig. 3E and Suppl. Table III).
The proteins shown in Fig. 3E (220) include those with a ≥2-fold increase after immunoprecipitation using Foxp3-DTR samples compared to WT, as well as those uniquely detected using the Foxp3-DTR samples. We have excluded Ig-related proteins from this figure, as they are not sperm-antigens; these are now listed separately in Suppl. Table III (highlighted in red).
In response to the reviewer's suggestion, we re-analyzed the data and confirmed that proteins such as ZP3R and PZP, known to be exclusively expressed in sperm, were present only in the Treg-depleted samples. We agree that experimentally validating the identified putative autoantibody targets is important. To address this, we plan to pursue this in future work by utilizing recombinant proteins in ELISA-based assays to confirm the presence of autoantibodies against these targets. Unfortunately, we were unable to find commercially available recombinant proteins for this validation. As a result, we have selected two targets for further investigation: ICOSLG, an immune modulator, and the ZP3R, a well-known sperm protein. We plan to produce these proteins in our lab and establish the ELISA.

5. Investigations of gonadal tissue from Treg-depleted mice revealed IgG deposition in the epididymal interstitium. How does this finding relate to the presence of antisperm autoantibodies?
In our study, we detected anti-sperm antibodies in serum and epididymal fluids from Treg-depleted mice, suggesting that these antibodies may originate from either systemic or local compartments. These humoral responses were confirmed using multiple techniques, including ELISA, proteomics, flow cytometry, and microscopy.

We hypothesize that Treg ablation causes injury to the epididymis, exposing sperm and tissue antigens to the immune system. This triggers systemic B cell activation in the bone marrow and secondary lymphoid organs (e.g., spleen and lymph nodes), leading to the production of anti-sperm (and auto-) antibodies. The anti-sperm antibodies may accumulate in the tissue due to concentration gradients between systemic and local compartments. Concomitantly, the presence of plasma B cells and TLSs within the epididymal tissue suggests the possibility of local anti-sperm (and auto-) antibody production.

6. The authors should also be commended for their efforts to assess the relevance of their findings in mice to human male infertility. They report that males with fertility challenges exhibit antisperm autoantibodies (ASA); however, the difference in autoantibody levels between affected individuals and controls appears quite subtle. Given this, it is difficult to determine the significance of the findings. Would it be possible to optimize the assay to achieve clearer and more definitive results? Additionally, it would be important to relate these findings to existing literature. As I understand it, ASA are already well-established in male infertility, making this aspect of the study less novel. Furthermore, the connection between the human data and the mouse model remains weak, as the only overlap that can be shown is the presence of sperm ASA. Given these limitations, the authors may want to reconsider including these data in the manuscript.

We appreciate the Reviewer's thoughtful feedback. Given the clinical context of male infertility, where over 40% of cases remain unexplained or idiopathic, we believe that our findings are meaningful. Even small changes in ASA levels in the seminal plasma can potentially impact fertility outcomes. We are actively working to expand our study to include a larger, more diverse cohort, which will help better delineate the differences in ASA levels between individuals with fertility challenges and healthy controls. The role of ASAs in male infertility is not yet well-established (Gupta et al., 2022, The World Journal of Men's Health, Mukherjee, A.G. & Gopalakrishnan et al., 2024. *Reprod Sci*). Currently, many clinics do not routinely perform assays to detect ASAs nor classify patients into immunological subgroups. Our study aims to provide new insights into the mechanisms of ASA production and its relationship with immune system dysregulation, particularly Treg depletion. We believe this approach offers a valuable perspective on ASA production and its potential tissue-specific effects in the context of male infertility. While our mouse model provides important mechanistic insights, we agree that additional human samples are necessary to confirm the broader applicability of these results. As we mentioned before, we are currently expanding our studies. The current findings represent an important preliminary step in understanding the relationship between immune responses and male infertility. Thus, they should be included in our manuscript.

7. IPEX syndrome may offer insights into the human relevance of the findings. Individuals with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused by loss-of-function mutations in the FOXP3 gene, lack functional regulatory T cells and develop severe multiorgan autoimmunity. In these patients, the gut, pancreatic islet cells, and skin are the primary targets of autoimmunity. Is there any data on male fertility in individuals with IPEX that could support the relevance of the reported findings in humans? Exploratory autoantibody testing in IPEX patients has identified autoantibody targets specifically expressed in enterocytes and pancreatic islet cells - tissues affected by the disease - but has not revealed autoantibodies targeting gonadal proteins (PMID 31027649). It would be relevant to discuss potential parallels between IPEX and the findings in this study in the Discussion section.

Thank you for your insightful comment. We have now expanded the Discussion section to address the relevance of IPEX syndrome to our findings. As the reviewer noted, IPEX syndrome, caused by mutations in the FOXP3 gene, results in a loss of functional Tregs and leads to severe

multiorgan autoimmunity. Regarding the query about male fertility in IPEX syndrome, it is important to highlight that this condition has not been directly linked to autoantibodies targeting gonadal proteins. Moreover, the precise effects of IPEX syndrome on male fertility are not yet fully understood due to the shortened life expectancy and significantly compromised quality of life in these patients, who often do not reach reproductive age or, if they do, lack the physical condition, opportunity, or expectation to pursue reproduction. As a result, fertility-related outcomes in this population remain largely undocumented and understudied (van der Vliet & Nieuwenhuis, 2017 Clin Dev Immunol; Bacchetta, & Roncarolo, 2024 Allergy Clin Immunol). Furthermore, mutations in the AIRE gene, which is also critical for Treg function, cause orchitis and infertility in autoimmune polyendocrine syndrome type 1 (APS-1) (Kekalainen. et al., 2007. J Immunol). In AIRE-deficient mice, autoimmune responses lead to autoantibodies against testicular tissue, epididymal leukocyte infiltration, and reduced fertility despite normal testicular function (Ahn et al., 2025 bioRxiv; Warren et al., 2021 Am J Pathol). Moreover, autoimmune diseases such as lupus, rheumatoid arthritis, Type 1 diabetes, and Behcet's disease—conditions that affect reproductive vasculature—have been shown to increase the risk of infertility through localized inflammation (Chereshnev et al. 2021 Pathophysiology; Chan & Schlegel J Androl 2002; Silva et al., 2014, Autoimmun). While the direct implications for male fertility in IPEX syndrome remain unclear, these parallels with other autoimmune syndromes, where inflammation impacts reproductive tissues, suggest that similar mechanisms might underlie potential fertility issues in IPEX patients. Therefore, it is plausible that patients with IPEX syndrome may also experience fertility issues. We believe this comparison provides a valuable context for understanding the potential human relevance of our findings and have now included this discussion in the revised manuscript. Lines 554-562 in the redlined version.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors has answered all my questions and concerns, and the manuscript has been revised accordingly.I highly recommend to publish this important research on development of TLS after Treg depletion in the epididymis.

We thank the reviewer for the comment.

Reviewer #2 (Remarks to the Author):

The authors have submitted a revised version of their manuscript and have addressed the reviewers' comments in a detailed, point-by-point response. This reviewer will focus specifically on the revisions related to the concerns made previously by this reviewer.

Overall, the manuscript has been improved, and many of the concerns have been addressed satisfactorily. However, several critical issues remain unresolved, as outlined below.

Figure 1:
A comparison between Figures 1A and 1C reveals a discrepancy in Treg cell detection. The flow cytometry data suggest a very low number of Tregs in the entire epididymis (in the two-digit range), whereas the Foxp3-EGFP+ staining shows 6–7 positive cells in just a small section

of the epididymis indicating a much higher prevalence. Furthermore, similarly intense staining is observed in the apical region of the epithelium and within the lumen. This raises concerns about the specificity of the immunofluorescence staining.

We appreciate the reviewer's comment and the opportunity to clarify. Figure 1C is not an immunofluorescence staining, but rather a fluorescent microscopy image from a Foxp3-DTR-EGFP transgenic mouse, in which EGFP is co-expressed with a membrane-bound receptor. This results in a relatively dim fluorescence signal. The image is included solely as a representative example to illustrate the presence and localization of Foxp3-DTR-EGFP^{+} cells in the epididymal interstitium of naïve and DT-treated mice.

Importantly, this image was not used for quantification. As such, it is not appropriate to compare it directly with the flow cytometry data, which reflects Treg numbers per gram of tissue. Based on this comment, we added a statement to the manuscript: "*It is important to note that in a naïve epididymis, Tregs are relatively sparse in the interstitial compartment*". Lines 106-107 redlined version.

Please see below low-magnification fluorescence microscopy images from the epididymis of FOXP3-EGFP mice (another mouse model), where Tregs express soluble EGFP (green) under the control of the FOXP3 promoter. With this construction, the EGFP signal is clearer and brighter. These images support the findings shown in Figure 1C by confirming the presence of Tregs within the interstitium and highlighting their low abundance. Since Treg localization using this mouse model has been previously reported (Barrachina et al., 2023 PNAS), and to avoid potential misinterpretation, we have decided not to include these images in the current manuscript.

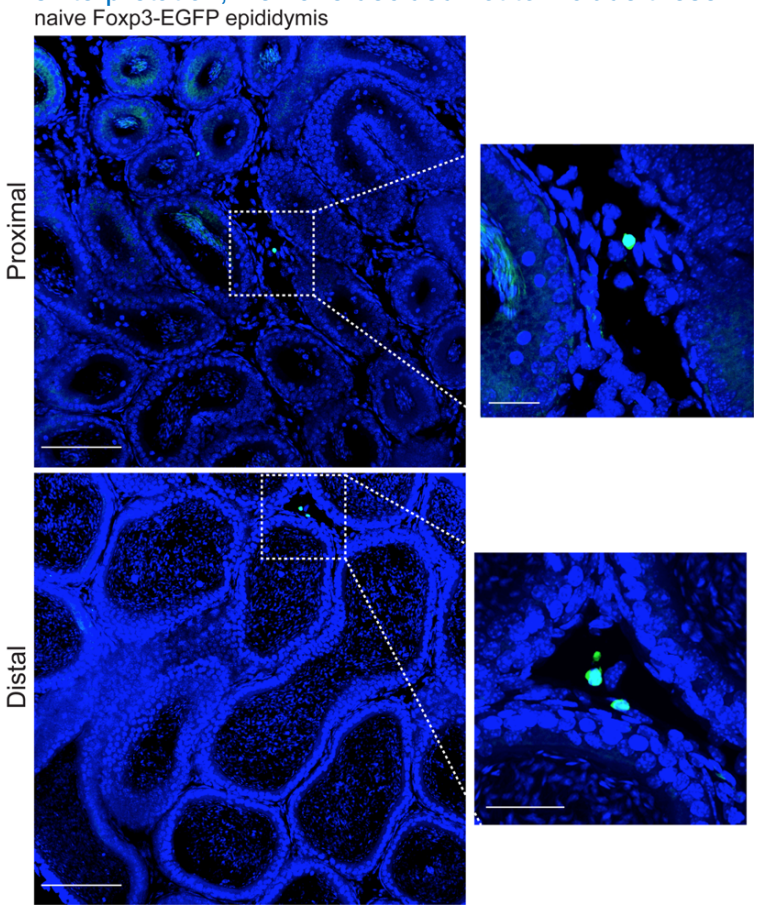

Figure S23 (related to comment #17 in the point-by-point response): Despite the inclusion of quantification, the image still demonstrates poor tissue preservation. It remains unclear how the authors are able to assess spermatogonia or sperm, as these cells are barely distinguishable as individual entities in the provided images.

We apologize for the lack of resolution in the original supplementary figures. To meet file size constraints for uploading, we had to optimize the PDF, which unfortunately affected image quality. To assess spermatogenesis, we used the NDP.View2 software, which allows high-resolution zooming to single-cell detail. This enables us to accurately distinguish and identify the different cell types within the seminiferous tubules, consistent with established methodologies used in previous publications (Meistrich, M.L. & Hess, R.A. Assessment of spermatogenesis through staging of seminiferous tubules. Methods Mol Biol 927, 299-307 (2013)). The quantification of spermatogenesis is based on the classification of germ cells according to their cellular and nuclear morphology, as well as their specific location within the seminiferous tubules. This includes distinguishing spermatogonia, spermatocytes, spermatids, and spermatozoa. The arrangement and proportion of these cells at different stages of the seminiferous epithelium cycle are also evaluated to assess the efficiency and completeness of spermatogenesis.

In response to the reviewer's comments, we have now included high-magnification representative images in the revised Supp. Fig. 24. These images illustrate how we identify and quantify the various cell populations, providing greater transparency and clarity regarding our methodology. We added this information in the Method section, lines 644-650 redlined version.

Human samples:

The authors now include more semen data from their patient cohort, which is appreciated. However, in the context of autoantibody formation, it would be important to indicate whether any of the patients had a history of male genital tract infection/inflammation, as this may significantly impact the interpretation of the findings.

We appreciate the reviewer's thoughtful comment and fully agree that information regarding the history of male genital tract infections would enhance the interpretation of Ig measurements in seminal plasma. We would like to clarify that the semen samples used in our study were obtained from patients attending IVF clinics, where the primary clinical objective was fertility treatment. Patients were classified into normozoospermic and asthenozoospermic groups based on standard semen parameters. Importantly, leukocytospermia was assessed in all samples by quantifying round cells (RCs), following WHO 2021 guidelines. A threshold of 1 million RCs per milliliter (1×10^6/mL) was used to define leukocytospermia, in line with the current reference standards. However, no additional clinical data about the patient's history of epididymal or urogenital infections were collected, nor were microbial cultures performed. These evaluations are not part of the standard diagnostic workup in most fertility clinics and were therefore not included in our protocol. We acknowledge this as a limitation of our study and have included a corresponding statement in the revised manuscript (Lines 775-778, redlined version). "*A limitation of our human study is the lack of additional clinical data regarding participants' histories of epididymal or other urogenital infections. Furthermore, microbial cultures were not performed to assess the presence of subclinical infections*".

Figure 7 (related to comments #12, #14, and #15): Figure 7 aims to illustrate the formation of tertiary lymphoid structures (TLS), a key aspect referenced in the manuscript title. However, the evidence provided remains unconvincing in the revised version, for the following reasons:

1. While an organized B cell cluster is visible, there is no equivalent T cell cluster. T cells appear scattered in relatively low numbers around the B cells and do not form a structured organization—an observation acknowledged by the authors themselves in their rebuttal.

As noted in our previous responses, TLSs exhibit diverse architectures depending on the organ and context in which they develop (Zhao, L. et al. Tertiary lymphoid structures in diseases: immune mechanisms and therapeutic advances. Signal Transduct Target Ther 9, 225 (2024). Sato, Y., Silina, K., van den Broek, M., Hirahara, K. & Yanagita, M. The roles of tertiary lymphoid structures in chronic diseases. Nat Rev Nephrol 19, 525-537 (2023).Sato, Y., Silina, K., van den Broek, M., Hirahara, K. & Yanagita, M. The roles of tertiary lymphoid structures in chronic diseases. Nat Rev Nephrol 19, 525-537 (2023)). Furthermore, TLSs can exist in various stages of maturation, ranging from early lymphoid aggregates to fully organized structures with distinct B and T cell zones and active germinal centers. In our samples, TLSs were observed at different stages of development. We acknowledge that the T cells shown in Figure 7 and Suppl. Figs. 18/19 do not form a densely packed, well-demarcated cluster (mentioned already in the previous revised version, lines 439-441 in the new redlined version). However, their peripheral distribution around the B cell zone, though relatively sparse, is consistent with features of TLS formation, as described in previous studies.

To address this, we have revised the manuscript to clarify that the lymphoid structures observed are likely not fully mature TLSs. This distinction is now explicitly stated in both the Results and Discussion sections (Lines 276, 281-282, 359, and 430-450, redlined version).

2. The new AID staining image (Figure 7E) does not convincingly support TLS formation. Notably, the prominent AID staining lies outside the CD19+ B cell cluster, and staining within the cluster is at best minimal.

We want to clarify that Figure 7E shows clusters of $CD19^+$ B cells, with a few of these cells expressing AID. In the revised version of the manuscript, we have updated the text to reflect this more precisely by replacing "some" with "few" (Line 277). As previously noted, chronic inflammation can create a microenvironment that induces aberrant AID expression in non–B cell populations (Mechtcheriakova, D., Svoboda, M., Meshcheryakova, A. & Jensen-Jarolim, E. Activation-induced cytidine deaminase (AID) linking immunity, chronic inflammation, and cancer. Cancer Immunol Immunother 61, 1591-1598 (2012)). Therefore, the AID-positive staining observed in non–B cells outside the TLSs may reflect the inflammatory state of the epididymis following Treg depletion.

Although AID expression indicates active germinal center (GC) activity, TLSs can be present with or without ongoing GC reactions depending on factors such as tissue type, disease stage, and the specific time point of analysis. (J Gunderson A, Rajamanickam V, Bui C, Bernard B, Pucilowska J, Ballesteros-Merino C, Schmidt M, McCarty K, Philips M, Piening B, Dubay C, Medler T, Newell P, Hansen P, Tran E, Tang E, Bifulco C, Crittenden M, Gough M, Young KH. Germinal center reactions in tertiary lymphoid structures associate with neoantigen burden, humoral immunity and long-term survivorship in pancreatic cancer. Oncoimmunology. 2021 Mar 17;10(1):1900635; Alfranca, A. (2023). Tertiary lymphoid structures and B lymphocytes: A promising therapeutic strategy to fight cancer. Frontiers in Immunology, 14, 1231315; Salomonsson, S., Jonsson, M.V., Skarstein, K., Brokstad, K.A., Hjelmström, P., Wahren-Herlenius, M. and Jonsson, R. (2003), Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. Arthritis & Rheumatism, 48: 3187-3201; Werner, F., Wagner, C., Simon, M., Glatz, K., Mertz, K. D., Läubli, H., Griss, J., & Wagner, S. N. (2021). A Standardized Analysis of Tertiary Lymphoid Structures in Human Melanoma: Disease Progression- and Tumor Site-Associated Changes With Germinal Center Alteration. Frontiers in Immunology, 12, 675146. https://doi.org/10.3389/fimmu.2021.675146; Weinstein AM, Storkus WJ. Biosynthesis and Functional Significance of Peripheral Node Addressin in Cancer-Associated TLO. Front Immunol. 2016 Aug 9;7:301), Consequently, the limited detection of AID within $CD19^+$ clusters does not exclude the presence of TLSs. Instead, it suggests that the TLSs observed in our sample may not have been undergoing active GC reactions at the time of the tissue collection. This aligns with

the well-established dynamic nature of TLSs and secondary lymphoid organs, where GC formation is transient and highly context-dependent (Victora, G. D., & Mesin, L. (2014). CLONAL AND CELLULAR DYNAMICS IN GERMINAL CENTERS. Current Opinion in Immunology, 90.; De Silva, N. S., & Klein, U. (2015). Dynamics of B cells in germinal centres. Nature Reviews Immunology, 15(3), 137-148). We have incorporated this explanation into the revised manuscript (lines 430-450, redlined version).

Moreover, to further characterize the presence of GCs within the TLSs, we performed immunostaining for BCL6, a transcription factor critical for GC formation and function. We observed that in a subset of epididymal TLSs, B cells exhibited BCL6 expression (Fig. 7F, Suppl. Fig. 18E, Lines 278-279 redlined version), providing additional evidence of GC-like activity and suggesting local B cell activation and differentiation within these ectopic lymphatic structures. BCL6 is also essential for the differentiation of T cells into follicular T cells, which provide help for GC formation, affinity maturation, and the development of most high-affinity antibodies and memory B cells (Lines 284 redlined version). We also added a positive control for BCL6 in the Suppl. Fig. 25.

To avoid overinterpretation, we have tempered our language in the revised text and now refer to these findings as supportive of local B cell activation rather than definitive evidence of GC-like activity. Lines 281-282, 359, and 439-442 in the redlined version.

3. In Figure 7G, PNAD staining is used to demonstrate the presence of high endothelial venules (HEVs), an essential TLS marker. However, the staining does not reflect blood vessels and instead appears unspecific—filamentous in pattern, lacking the morphology of blood vessels, and likely also located within the epididymal epithelium (top right corner), where no HEVs can be expected.

Thank you for this valuable observation. In response, we have revised the figure to include new images presented in Fig. 7G (now Fig. 7H) that show clear colocalization of PNAD and CD31 (highlighted by arrows) in areas adjacent to B cell clusters ($CD20^+$), consistent with TLSs. The previous images are now in the new Suppl. Fig. 19. These new images help confirm the presence of high endothelial venules (HEVs) in the epididymis 8 weeks after Treg depletion. Additionally, we would like to clarify that PNAD can be expressed by epithelial cells, such as those in the endometrium (*T. Lai, I. Shih, N. Vlahos, C. Ho, E. Wallach, Y. Zhao, Differential expression of L-selectin ligand in the endometrium during the menstrual cycle, Fertil. Steril. 83 (2005) 1297–1302, and L. Margarit, D. Gonzalez, P.D. Lewis, L. Hopkins, C. Davies, R.S. Conlan, L. Joels, J.O. White, L-Selectin ligands in human endometrium: comparison of fertile and infertile subjects, Hum. Reprod. 24 (2009) 2767–2777,*). Therefore, it is possible that the signal observed in the epithelium, as highlighted by the reviewers, is specific rather than artifactual. Nonetheless, to avoid any potential misinterpretation, we replaced the original image with clearer examples that more accurately represent vascular PNAD expression associated with epididymal TLSs (Fig. 7H).

4. The Ki67 staining shown in Figure 7F is predominantly outside the B cell cluster, with only faint and punctate staining within the cluster. This aspect has not been clarified by the revision.

We apologize for the lack of clarity. Based on this reviewer's comments, we wrote that we detected few TLS cells positive for Ki-67 (line 277 redlined version). In the previous revised version (in the new version, lines 284-287), we had mentioned that “we observed other Ki67-positive cells, particularly among the epithelial population. This likely reflects ongoing tissue repair and regenerative processes in the epididymis, which might occur 8 weeks after Treg depletion.” Based on this comment, we added new images of Ki67 and CD20 in the new Fig. 7G and Suppl. Fig. 18F, confirming that few $CD20^+$ cells are $Ki67^+$ in the epididymis.

5. Additionally, Figure 7F shows a B cell cluster that is tightly surrounded by epididymal epithelium. It is unclear where a putative T cell cluster could be accommodated in this context.

We appreciate the reviewer's observation. We would like to clarify that throughout the manuscript; we presented multiple examples of TLSs located at different sites within the distal epididymal interstitium. As discussed in our responses to points 1 and 2, TLSs are known to be highly heterogeneous and dynamic, both in terms of their cellular composition and spatial organization. This is especially relevant in tissues like the epididymis, where region-specific structural and functional clues may influence TLS morphology. The observation in (former) Figure 7F (now Suppl. Fig. 18F, upper panel) may represent a developing or spatially constrained TLS, where cellular compartmentalization is still emerging or is shaped by the unique tissue architecture. While the proximity of the B cell cluster to the surrounding epithelium may suggest limited space for a segregated T cell zone, it does not preclude its presence, especially given that T cells may be more diffusely distributed in early-stage TLSs or positioned in adjacent regions not captured in the plane of the section. Importantly, our study provides the first detailed characterization of TLSs in the epididymis. It is therefore plausible that these structures possess tissue-specific features distinct from TLSs observed in other organs, further emphasizing the heterogeneity of TLSs across different anatomical sites and inflammatory settings. We now discuss this in greater detail in the revised manuscript (lines 430-450 in the redlined version), noting that the variability in TLS architecture observed may reflect both the dynamic nature of these structures and the influence of local microenvironmental factors.

Minor point:

There is inconsistency in the use of mouse and human gene/protein nomenclature, particularly regarding the capitalization of letters. Standardizing this throughout the manuscript would improve clarity and alignment with field-specific conventions.
Thank you very much. We modified the gene/protein nomenclatures accordingly. Lines 513, 515 in the redlined version.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed all of my questions by providing new data and making appropriate revisions to the manuscript.

We thank the reviewer for the comments.