Sertoli Cell-Specific Deletion of the Androgen Receptor Compromises Testicular Immune Privilege in Mice 1

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ABSTRACT

In the mammalian testis, meiotic and postmeiotic germ cell antigens are granted immune privilege. Both local immune suppression and specialized intercellular junctions between somatic Sertoli cells have been proposed to contribute to a highly restricted and effective blood-testis barrier (BTB) that helps maintain tolerance to germ cell antigens. Several studies have suggested that androgens play a role in immune suppression, although direct evidence for this is lacking. We previously reported that Sertoli cell-specific ablation of the androgen receptor (*Ar*) decreases expression of *Cldn3*, an androgen-regulated gene and component of Sertoli cell tight junctions, and increases the permeability of the BTB to biotin, a small-molecular-weight tracer. The physiological consequences of Sertoli cell-specific *Ar* (S-*Ar*) ablation on immune privilege are unknown. Here we show that in the testes of S-*Ar* mutant mice, the ultrastructure of Sertoli cell tight junctions is defective and testicular IgG levels are elevated. The interstitium of S-*Ar* mutant testes becomes populated with macrophages, neutrophils, plasma cells, and eosinophils, and serum samples of mutant mice contain antibodies against germ cell antigens. Together, these results suggest that Sertoli cell-specific deletion of the androgen receptor results in loss of testicular immune privilege. Suppressed levels of androgen signaling may be a contributing factor in idiopathic male infertility.

androgens/androgen receptor, blood-testis barrier, immune privilege, Sertoli cells, spermatogenesis

INTRODUCTION

Immune privilege describes prolonged survival of cells expressing self antigens that would otherwise provoke removal by an aggressive immune response. Permissiveness involves several levels of control, including physical barriers that enable antigen sequestration and interactive networks of cellular and soluble factors that down-regulate self-recognition [1]. Tolerance to self-antigens is typically acquired by continuous exposure of the developing immune system to self-antigens and the removal of strongly reactive cells, referred to as negative selection [2, 3].

In the mammalian testis, the blood-testis barrier (BTB) and localized immune suppression promote tolerance to germ cell antigens that emerge with the onset of spermatogenesis at puberty. Within the seminiferous tubules, tight junctions between adjacent somatic Sertoli cells, the major physical component of the BTB, act by separating differentiating meiotic and postmeiotic germ cells from blood and lymphoid elements [4–6]. Outside the seminiferous epithelium, tight junctions between peritubular myoid cells add additional compartmentalization of differentiating germ cells [4]. Finally, anti-inflammatory cytokines, such as macrophage-migration-inhibitory factor from Leydig cells, suppress cytotoxic T lymphocyte and NK cell activities and down-regulate autoimmunity against testis-specific antigens [7–10].

Androgens are required for male sexual differentiation during embryogenesis, testicular descent, masculinization at puberty, and spermatogenesis. Null mutations in the androgen receptor gene cause complete androgen insensitivity and external feminization of XY individuals (*Ar^{Tfm}*) [11]. Androgen receptor function in Sertoli cells is required for progression of spermatogenic cells through meiosis and spermatid differentiation [12–15].

Both clinical and experimental studies suggest a role for androgens in modulating inflammatory responses. Testosterone reduces the expression of several proinflammatory cytokines in culture [16–18], and testosterone therapy has been correlated with suppression of several autoimmune diseases [18, 19]. There is strong evidence for an involvement of androgens in regulating Sertoli cell tight junctions [20]. We have previously reported that *Cldn3*, a gene encoding a Sertoli cell tight junction protein, is regulated by androgens and that CLDN3 levels are reduced in mice with Sertoli cell-specific deletion of the androgen receptor. In addition, compromised permeability of the BTB to small molecules (e.g., biotin) was observed in these *Ar* mutant mice, presumably due to deregulation of *Cldn3*, suggesting that androgens are critical for maintaining a specialized microenvironment for germ cell differentiation [21].

Despite the suggestion that androgens maintain a properly functioning BTB and regulate immune privilege within the testis, no evidence currently exists for how androgens directly promote immune tolerance to testicular antigens in vivo. In the present study, we characterized a mouse model of reduced androgen signaling both ultrastructurally and functionally. Our results demonstrate a critical role of Sertoli cell androgen receptors in maintaining immune privilege for male germ cells.

MATERIALS AND METHODS

Mice

Ar^{tm1.1Reb} mice used in these studies lack Sertoli cell-specific androgen receptors (*Ar*) and are hypomorphic for *Ar* in all other tissues. They were generated by mating floxed *Ar^{tm1Reb}* mice with anti-Mullerian hormone (Amh) cre recombinase (Cre) transgenic mice as described in Holdcraft [13].

Hereafter, the *Ar^{tm1.1Reb}* mice will be referred to as S-*Ar* mutant mice to denote hypomorphic *Ar* mice with conditional disruption of *Ar* in Sertoli cells. The mutant and wild-type (WT) 129S4/SvJaeSor control mice were bred in the research colonies of Dr. R.E. Braun. The *Ar^{Tfm}* (B6-*A^{w-J}*.Cg-*Eda^{Ta-6J}* +/+ *Ar^{Tfm}*/J) mice (stock no. 001809) were purchased from the Jackson Laboratory. The University of Washington's Institutional Animal Care and Use Committee and the Jackson Laboratory Animal Care and Use Committee approved all animal studies. At least two replicates of each experiment were performed on at least three animals of control and mutant genotypes.

Electron Microscopy

Mouse testes from 2-mo WT and S-*Ar* males were cut into 1.5-mm cubes and immersed in half-strength Karnovsky paraformaldehade-glutaraldehyde solution [22] overnight at 4°C. After washing in phosphate buffer containing 2% sucrose, tissue blocks were then postfixed in 1% OsO4 for 1 h at room temperature. After washing, the tissues were dehydrated in a graded series of ethanol and embedded in Epon plastic. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM 1200EXII electron microscope.

Immunofluorescence and Immunocytochemistry

Mouse testes from 2-mo-old WT mice were prepared as described in [23]. To determine if S-*Ar* sera contained germ cell-specific antibodies, deparaffinized sections were incubated in a blocking solution (PBS containing 3% normal goat serum) for 1 h, incubated overnight at 4°C in 1:200 diluted serum of WT or S-*Ar* mutant male mice in a moist chamber, and washed 3× with PBS. The slides were stained with Alexa Fluor 568 goat anti-mouse IgG (H+L; Molecular Probes-Invitrogen) at a dilution of 1:1000 for 30 min at 25°C, washed with PBS, and then mounted in Vectashield mounting medium with DAPI.

To determine the location of mouse IgG in WT and S-*Ar* mutant testis sections, slides were deparaffinized, blocked 1 h in 3% goat serum in PBS + 0.1% Triton X-100 (PBS-T), and incubated 1 h with a 1:500 dilution of goat anti-mouse IgG (H+L chains) conjugated to Alexafluor 568 (Invitrogen; A11031). Slides were washed 3× in PBS-T and mounted with Vectashield Mounting medium with DAPI (Vector Labs; H1200).

To determine the location of CD11B (ITGAM)(+) leukocytes, testis sections from WT and S-*Ar* mutant mice were deparaffinized, blocked 1 h in 3% goat serum in PBS-T, and incubated 1 h with a 1:100 dilution of anti-CD11B-CY3 antibody (Jackson Laboratory). Slides were washed 3× in PBS-T and mounted with Vectashield Mounting medium with DAPI.

For immunohistochemical detection of macrophages, the sections were incubated with 3% normal goat serum in PBS for 1 h and then incubated overnight at 4°C in a moist chamber with a 1:100 dilution of rat anti-F4/80 antibody (eBioscience) in PBS containing 3% normal goat serum. After several washes, the sections were incubated with a 1:200 dilution of biotin-goat-anti-rat IgG (Zymed Laboratories-Invitrogen) for 30 min at 25°C, washed in several changes of PBS, incubated with a 1:100 dilution of horseradish peroxidase (HRP)-streptavidin (Zymed Laboratories) in PBS containing 3% normal goat serum for 20 min at 25°C, and washed in four changes of PBS. The sections were stained with aminoethyl carbazole (Zymed Laboratories), washed in water for 10 min, counterstained with hematoxylin, rinsed with water for 10 min, and mounted with GVA mounting medium (Invitrogen).

Biochemistry

Testis protein preparation and Western blotting were performed as described elsewhere [23]. Binding of a 1:200 diluted mouse anti-β-catenin antibody (BD Biosciences) or a 1:25 000 dilution of mouse anti-β-actin (Sigma; A3854) was used to standardized protein labeling. An HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (Bio-Rad) was used to assess testicular IgG concentration. Protein sizes were measured against a prestained protein ladder (Fermentas Life Sciences).

Autoantigens were detected by incubating blots of WT testicular proteins with a 1:200 dilution of WT, S-*Ar*, and *Ar^{Tfm}* mutant sera overnight at 4°C. Primary antibodies were detected with a 1:3000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Bio-Rad) and the ECL Western blotting detection system (GE Amersham).

To examine age-dependent development of autoantigens, blots of testicular proteins were prepared from WT juvenile males at Postnatal (PN) Days 5, 7, 10, 15, 20, 30, and 40. Blots were incubated with a 1:200 dilution of serum from a single S-*Ar* mutant male and HRP-conjugated goat anti-mouse secondary antibody and ECL. Blots were washed with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and reprobed with a 1:200 dilution of WT serum as control.

Flow Cytometry

Single-cell suspensions from WT and S-*Ar* mutant whole testes, interstitia, and seminiferous tubules were reacted with a panel of antibodies identifying leukocyte subpopulations. Cells from whole testis were obtained by incubating decapsulated testes in an enzyme solution containing 0.1% collagenase IV (Sigma; C1889), 0.2% hyaluronidase (Sigma; H4272), and 0.04% Dnase (Sigma; DN25) with gentle shaking at 37°C for 1 h and then filtering through a nylon mesh to remove clumps. Cells were washed and resuspended in FACs staining buffer (eBioscience) at 2×10^7 cells/ml. Interstitial cells were obtained from decapsulated testes by repeated pipetting of seminiferous tubules into PBS +2% Fetal bovine serum, centrifuging eluted cells at 1350 × g for 6 min, suspending cells in 3 ml FACs buffer, filtering through the strainer cap of 12 × 75 tubes (BD 352235), and adjusting the single-cell suspension concentration to 2×10^7 cells/ml. Cells from seminiferous tubules were obtained by incubating the remaining tubules with 100 μl of 5 mg/ml collagenase for 30 sec. After four quick rinses in PBS, tubules were incubated 10 min with 2 ml of prewarmed 0.25% trypsin and 50 μl of 5 mg/ml DNAse I. At the end of incubation, 40 μl of 5 mg/ml soybean trypsin inhibitor (Sigma; T9003) were added, and cells were mixed by repeated aspiration. Approximately 3 ml FACs buffer were added to cells before filtering them through a 40-μm screen into a sterile 50-ml tube. Cells were rinsed twice in FACs buffer and centrifuged at 1350 × g for 6 min. The final pellet was suspended in 3 ml FACs buffer and filtered through a strainer cap of 12 × 75 tubes to eliminate clumped cells.

To block nonspecific Fc binding, all cell preparations were incubated in a 1:100 dilution of rat anti-mouse CD16/CD32 (FCGR3/FCGR2B; eBioscience; 14-0161) for 5 min at 4°C. Cells from the whole testes were incubated 30 min at 4°C with a 2-antibody mixture containing a 1:40 dilution of FITC-conjugated rat anti-mouse CD3E (eBioscience; 11-0031) and a 1:80 dilution of PE-Cy5-conjugated rat anti-mouse CD11B (eBioscience; 19-0112). Cells from the interstitia and seminiferous tubules were incubated either with a 2-antibody mixture reactive with plasma cells (CD138 [SDC1]-PE, 1:30; BD Biosciences; 347192) and B lymphocytes (CD45R [PTPRC] [B220]-APC; 1:30; eBioscience; 17-0452) or with a mixture of 6-antibodies (all from BioLegend) reactive with major leukocyte subpopulations, including pan-T lymphocytes (CD3E-APC Cy7; 1:40; 100329), $T_{cytotoxic}$ lymphocytes (CD8-PE Cy5; 1:80; 100709), T_{helper} lymphocytes (CD4-Alexa700; 1:40; 100429), monocytes and neutrophils (CD11B-PE Cy7; 1:100; 301321), dendritic cells (CD11C [ITGAX]-APC; 1:120; 301613), and granulocytes (GR1 [LY6G]-Pacific orange; 1:40; Jackson Laboratory). After 30 min of incubation with antibodies, cells were washed and resuspended in 0.2 ml FACs buffer containing 1 μg/ml propidium iodide. For each sample, 10 000 cells from whole testis were analyzed on a BD FACSCanto flow cytometer with FACSDiva software (Becton Dickinson). For interstitial and seminiferous tubule samples, 100 000 cells were analyzed on a BD LSR flow cytometer and interpreted with FlowJo software. An aliquot of each cell suspension was incubated without antibody to set gates and to eliminate autofluorescence. Percent antibody reactivity was calculated on the basis of viable cells excluding propidium iodide. Haploid germ cells were distinguished from diploid cell types based on staining patterns with Hoescht dye.

In contrast to the monocyte-macrophage population residing in the epididymis and seminal vesicles, monocyte-macrophages found in the interstitium, smooth muscle, and epithelial regions of the testis down-regulate expression of CD45 and show little to no immunostaining with specific antibody [24]. Our preliminary experiments suggested that we would lose a significant cell population if we gated on CD45(+) cells. Instead, we analyzed fluorescence intensity by gating on viable diploid cells showing no autofluorescence.

Statistical Analysis

Data are shown as mean ± SEM. Percentage data were arcsine transformed and then analyzed by two-tailed Student *t*-test using JMP-9 software. A value of $P < 0.05$ was considered significant.

RESULTS

Ultrastructural Defects in Sertoli Cell Tight Junctions in Androgen Receptor Deficient Mice

We began by examining the ultrastructure of tight junctions in the seminiferous tubules of WT and S-*Ar* mutant animals. In WT tubules, Sertoli-Sertoli tight junctions contain a series of kissing points (arrows) that bring the lipid bilayers of apposing Sertoli cells into intimate contact (Fig. 1, A and B;
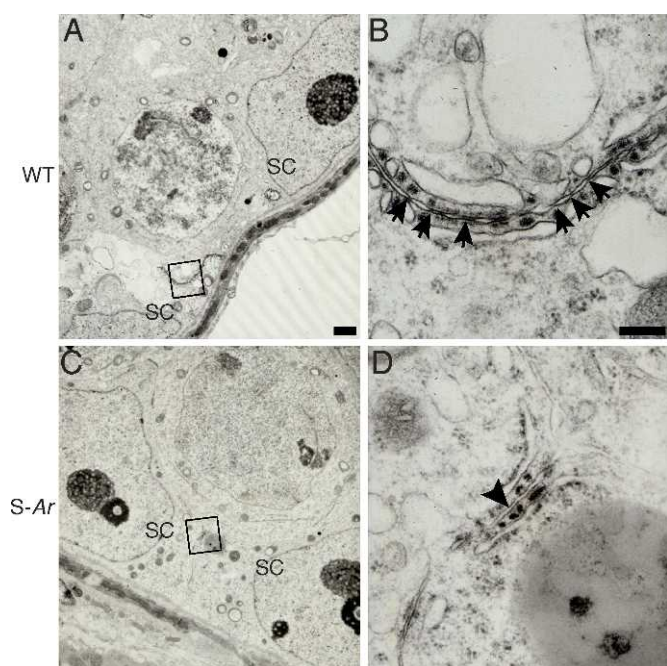
FIG. 1. Altered ultrastructure of tight junctions in S-*Ar* mutant testes. Electron micrographs of WT and S-*Ar* mutant seminiferous epithelium. **A**) WT seminiferous tubule. Boxed region contains a tight junction formed between the two adjacent Sertoli cells. **B**) Higher magnification of the boxed region in **A**. Arrows indicate regions of close apposition of Sertoli cell lipid bilayers (kissing points). **C**) Seminiferous tubule of an S-*Ar* mutant. Boxed region shows the intercellular space of two neighboring Sertoli cells. **D**) High magnification of the boxed region in **C**. Note the absence of kissing points between the membranes of the adjacent Sertoli cells (arrow heads). SC, Sertoli cells. Bar in **A** = 2 μm for **A** and **C**; bar in **B** = 200 nm for **B** and **D**.
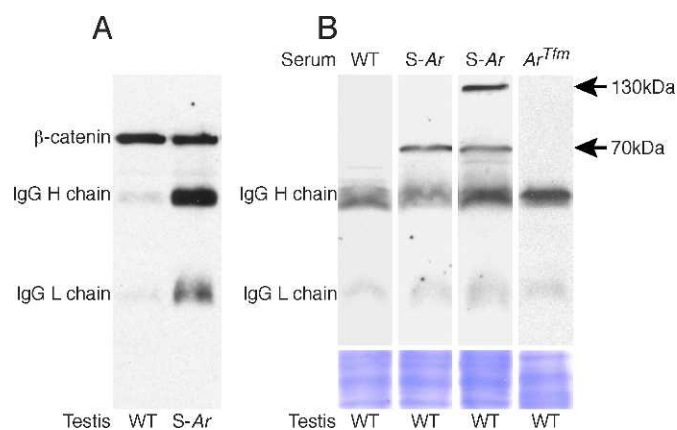

FIG. 2. Loss of androgen signaling in Sertoli cells induces a humoral autoimmune response. **A**) Western blot analysis of extracts prepared from the testes of WT and S-*Ar* mice probed with a mouse anti-β-catenin antibody. β-catenin served as a loading control. Higher concentrations of IgG heavy and light chains were evident in the mutant testicular extract. **B**) Western blot analysis of extracts prepared from the testes of WT mice probed with sera from WT and S-*Ar* and *Ar*^*Tfm* mutant mice. A portion of the gel was stained with Coomassie blue to demonstrate equal loading. Arrows indicate 70- and 130-kDa testicular antigens recognized by antibodies present in serum samples from two different S-*Ar* mutant males.

Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org). In mutant seminiferous tubules, 90% of the Sertoli-Sertoli tight junctions lack obvious kissing points (Fig. 1, C and D; Supplemental Fig. S2). In the remaining 10% of the Sertoli-Sertoli cell interfaces, a few kissing points are present (arrows; Supplemental Fig. S2), although their density is considerably less than WT controls (Fig. 1, A and B; Supplemental Fig. S1). These ultrastructural defects could be directly responsible for the increased permeability of the BTB found in the S-*Ar* mutant testes [21].

Testes of Androgen Receptor Deficient Mice Contain High Levels of IgG

To test the hypothesis that androgens mediate immune privilege to testicular antigens, we examined IgG levels in testicular protein extracts from WT and S-*Ar* mutant mice using Western blot analysis. We found that the level of IgG in the testes of the S-*Ar* mutant males, relative to the level of control proteins (β-catenin and β-actin), was considerably higher in mutant animals (Fig. 2A and Supplemental Fig. S3, respectively).

To determine the location of mouse IgG in the testis, age-matched testis sections were incubated with anti-mouse IgG (H+L chains) conjugated to Alexafluor 568. As shown in Supplemental Figure S4, A and B, none of the WT tubules contained germ cells coated with anti-mouse IgG, whereas approximately 10% of the atrophic tubules contained germ cells coated with mouse immunoglobulin. These observations suggest that germ cells within the tubules of mutant testes were

targeted by germ cell-reactive antibodies. The accumulation of immunoglobulin with germ cell reactivity in S-*Ar* mutant testis suggests the importance of humoral immunity in the spermatogenic phenotype of S-*Ar* mice.

Accumulation of Autoantibodies Against Germ Cell Antigens

A defective BTB in S-*Ar* mutant male mice could compromise testicular immune privilege and result in testis-specific antibodies. To test this possibility, we incubated testicular protein extracts from 2-mo-old WT mice with sera taken from WT, S-*Ar*, and *Ar*^*Tfm* male mice. *Ar*^*Tfm* males contain a whole-body knockout of *Ar* such that spermatogenesis is arrested early in meiosis I [11]. These animals contain early meiotic germ cells but lack postmeiotic germ cells. Western blotting was used to visualize differences in antibody binding patterns (Fig. 2B). As expected, WT serum lacked detectable antibodies against endogenous testicular proteins. However, serum samples collected from two S-*Ar* mutant male mice contained antibodies that recognized testicular antigens of 70 and 130 kDa not observed with control serum samples. Interestingly, antibodies that recognized germ cell proteins of different sizes were detected using sera from two different mutant mice, suggesting animal-to-animal heterogeneity in the autoimmune response. To confirm this observation, sera from an additional four S-*Ar* mutants also showed individualized responses to WT testicular lysate antigens between 24 and 130 kDa (Supplemental Fig. S5).

Spermatogenesis in S-*Ar* mutant mice proceeds through meiosis, but spermatid differentiation is severely altered. Meiotic and postmeiotic cells lie on the immune privileged side of Sertoli cell tight junctions. We wondered if progression through meiosis was necessary for the autoimmune response in androgen signaling-defective mice. To test this, we assayed antibodies in sera from *Ar*^*Tfm* males. As shown in Figure 2B, serum from an *Ar*^*Tfm* male did not contain autoantibodies, suggesting that the antigens responsible for triggering the immune response were expressed predominantly in germ cells at later stages of differentiation.

To determine the developmental timing associated with the appearance of the autoantigens, we performed Western blot analyses of total testis protein extracts prepared from juvenile WT male mice on PN Days 5–40. Probing with serum from a single S-*Ar* mutant animal, we detected the emergence of a 130-kDa autoantigen by PN Day 15 and observed abundant autoantigen levels by PN Day 30, when both round and elongated spermatids are present in the seminiferous tubules (Fig. 3A, upper blot). In contrast, autoantigens were not detected using serum from a WT mouse (Fig. 3A, lower blot).

An additional 5 S-*Ar* mutant and five WT sera were used to probe developmental Western blots (data not shown). Two of the six mutant sera reacted with a protein band at 70 kDa that emerged by Day 15 and showed increasing levels of reactivity out to Day 40. One of six mutant serum samples reacted with a protein doublet at 70–72 kDa emerging at Day 15 and also reacted with elongating spermatids shown in Figure 3B (row III). Three of six WT sera also showed reactivity with a protein band at 70 kDa emerging around Day 15–20. Without further characterization, it is not possible to know if the 70-kDa proteins recognized by S-*Ar* mutant and WT sera share identity. Therefore, the mutant serum sample recognizing round spermatids by IFA (Fig. 3B, row II) was imaged in Figure 3A.

To determine the localization of the autoantigens within the testis, we performed immunofluorescence microscopy on sections of WT testis (Fig. 3B). Using WT serum as primary antibody, no germ cell antigens were detected within the seminiferous tubules (Fig. 3B, I). In contrast, sera from two different S-*Ar* mutant mice detected unique antigens associated with round and elongated spermatids (Fig. 3B, II and III), suggesting that individualized events initiate immune responses to germ cell antigens. Antibodies from a third mutant mouse detected germ cell antigens near the basement membrane of the seminiferous tubules (Fig. 3B, IV). These antibodies were possibly induced by incomplete central immune tolerance or were secondary to the initial inflammation induced by haploid germ cell antigens in the testes of mutant mice.

Detection of Inflammatory Cell Infiltrate

Elevated levels of immunoglobulin in mutant testes suggested a humoral inflammatory response to testicular antigens. To determine if a cellular immune response was also generated against self-antigens, we used flow cytometry to characterize leukocyte subpopulations present in WT and mutant whole testis cell preparations. In comparison to WT, CD11B-positive cells (monocytes/macrophages, neutrophils, eosinophils) were significantly increased in S-*Ar* whole testicular cell preparations (Fig. 4A). A low but significant increase in CD3-positive T lymphocytes was also observed in mutant testes (Fig. 4A; both $P \leq 0.008$; n = 4). Increased percentages of monocytes/macrophages were confirmed in mutant testes by immunohistochemistry using the pan-macrophage marker F4/80 showing that the interstitial spaces of S-*Ar* mutant testes, but not control testes, were infiltrated with F4/80+ macrophages (Fig. 4B).

To determine if other inflammatory cell types were present in mutant testes that could account for localized antibody production, we separated whole testes into interstitial and seminiferous tubule populations as detailed in *Materials and Methods*. Cells from WT and mutant mice were stained with either a 2-antibody mixture reactive with CD138+ plasma cells and B220+ B lymphocytes or a 6-antibody mixture reactive with CD11B CD3, CD4 (T$_{helper}$), CD8 (T$_{cytotoxic}$), and GR1+ granulocytes and analyzed by flow cytometry. Percentages of
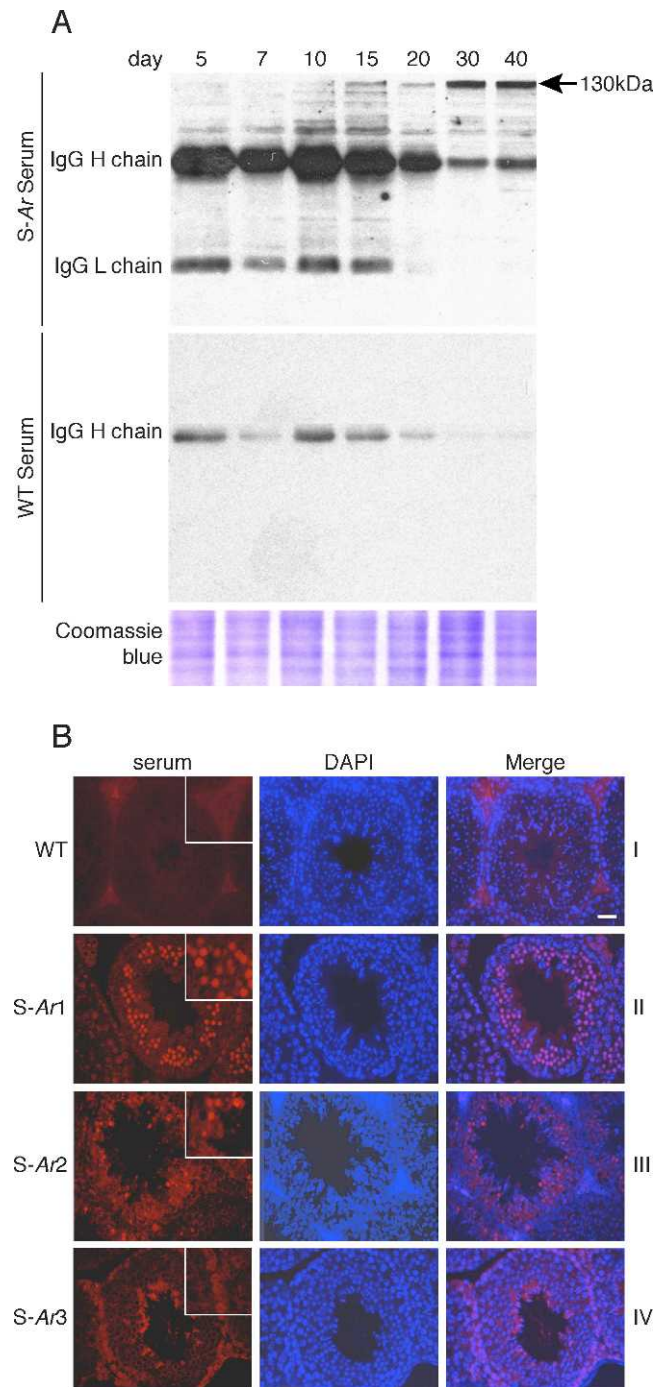
FIG. 3. Autoantigens emerge with development of haploid spermatids. **A**) Western blot analysis of total testis protein prepared from juvenile male mice on PN Days 5–40 and (top) probed with serum from an S-*Ar* mutant male or (bottom) serum from a WT control male. A portion of the gel was stained with Coomassie blue to demonstrate equal loading of samples. **B**) Immunofluorescence detection of self-antigens in WT testis sections. Serum sample from a WT male mouse (**I**). Sera from two different S-*Ar* mutant males (S-*Ar* 1 and S-*Ar* 2, **II–IV**). Serum antibodies from the S-*Ar* 3 mutant male recognize antigens associated with basal germ cells (**IV**). Bar = 20 μm (applies to all panels). Insets show 2× enlargements of highlighted regions.

plasma cells in whole blood, testicular intersitium, and seminiferous tubules were significantly higher in S-*Ar* mutant mice (Fig. 4C; all $P \leq 0.015$; all n = 3), which helps explain circulating and localized accumulation of immunoglobulin in mutant testes. The interstitial cell preparation of S-*Ar* mutants
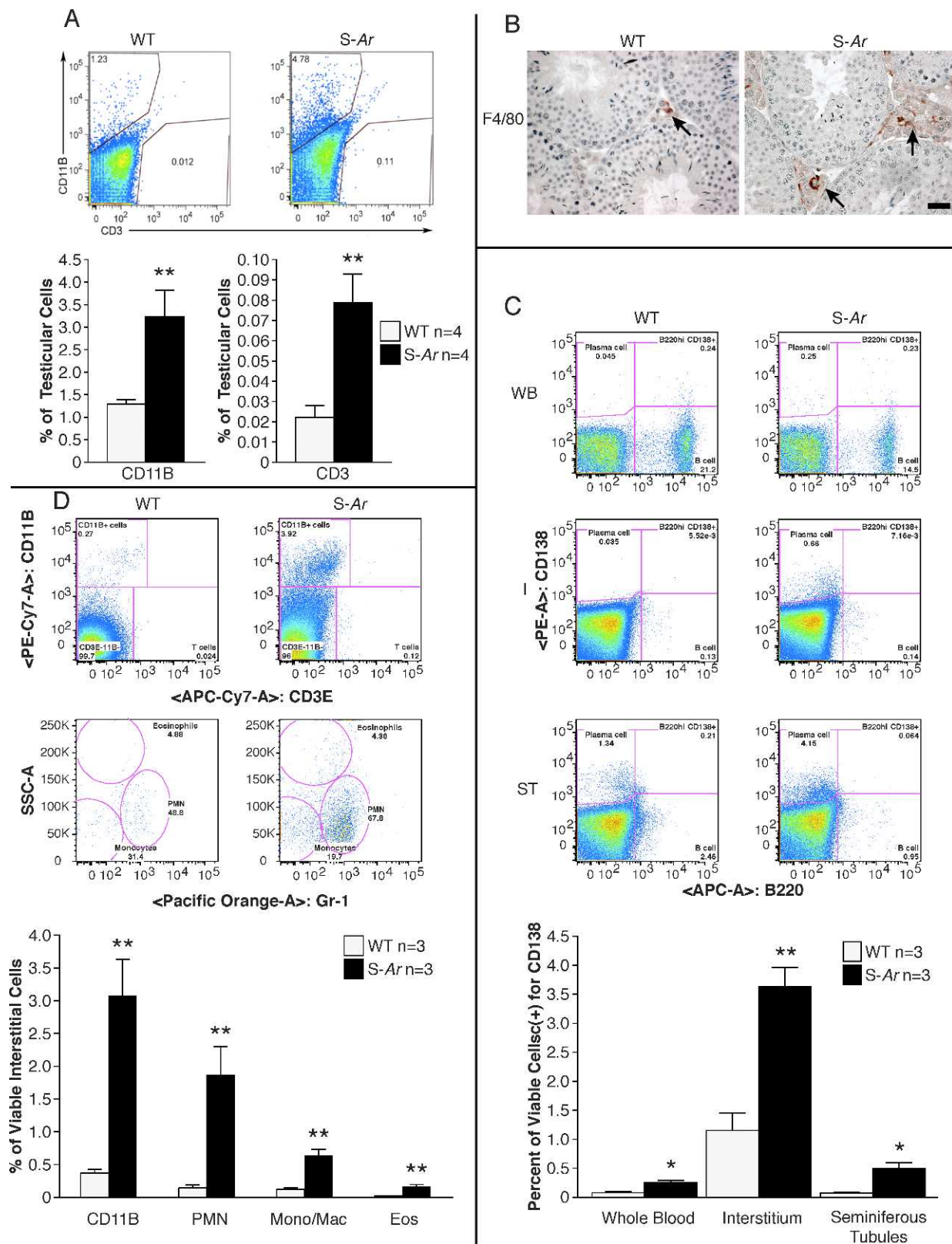
FIG. 4. Inflammatory cells infiltrate the testes of S-*Ar* mutant mice. **A**) Flow cytometric analysis of whole testicular cell suspensions from WT and mutant mice show significantly higher percentages of CD11B-positive macrophages, neutrophils, and eosinophils and CD3-positive T lymphocytes in S-*Ar* mutant mice (both n = 4). An unstained control was used to exclude negative testicular cells and set gates for the CD11B and CD3 populations. **B**) Immunohistochemistry of macrophages stained with F4/80 antibody. Positive cells are stained red in the interstitium of WT and S-*Ar* mutant testes and noted with arrows. Bar = 20 μm (applies to both images). **C**) In comparison to WT, percentages of CD138(+) plasma cells in whole blood, testicular intersitium, and seminiferous tubules were significantly higher in S-*Ar* mutant mice (all n = 3). **D**) In comparison to WT, the interstitia of S-*Ar* mutants contained significantly higher percentages of CD11B(+) cells. It was possible to subdivide the CD11B-positive population into neutrophils, monocytes, and eosinophils based on costaining with anti-GR-1 antibody and by side scatter to detect granularity (all n = 3). *$P < 0.05$; **$P < 0.01$.

also contained significantly higher percentages of CD11(+) neutrophils, monocytes, and eosinophils (Fig. 4D; all $P \leq$ 0.006; n = 3).

Hematoxylin and eosin-stained testis sections from adult WT and S-*Ar* mutant testes showed significant differences in both the cellular composition of the interstitium and the condition of germ cells within the tubules. However, the low percentages of leukocytes made it difficult to identify subtypes and determine location based solely on morphology (Supplemental Fig. S4, E and F). Therefore, to localize CD11B(+) leukocytes, testis sections from WT and S-*Ar* mutant mice were incubated with anti-CD11B-CY3 antibody. Immunofluorescence was detected primarily in the interstitium of both WT and S-Ar mice; however, considerably higher numbers of CD11B(+) cells were present in S-*Ar* mutant interstitium. CD11B(+) cells were not detected in significant numbers in the seminiferous tubules of either WT or S-*Ar* mutant testes (Supplemental Fig. S4, C and D). Together, these findings demonstrate that S-*Ar* mutant mice mount a humoral as well as vigorous cellular immune response against testicular self-antigens.

DISCUSSION

These studies demonstrate the importance of androgen receptor signaling in maintaining proper functioning of the BTB and immune privilege in the testis. Previous studies have shown that the *Cldn3* gene is selectively lost at the blood-brain barrier of mice with experimental autoimmune encephalomyelitis [25] and at the BTB of mice with a Sertoli cell-specific androgen receptor mutation [21]. We do not know if the autoimmune response present in S-*Ar* mutant mice directly contributes to the spermatogenic phenotype or if the spermatogenic defects present in mutant mice are due to the increased permeability of the Sertoli cell tight junctions to small molecules [21]. Cell-specific ablation of *Cldn3* will be necessary to determine if this defect is solely responsible for both the observed increase in the permeability of the Sertoli cell tight junctions to small molecules and the induction of an autoimmune response to germ cell antigens.

Claudin 11 (CLDN11) is a protein constitutively expressed by Sertoli cells, and both CLDN3 and CLDN11 proteins contribute to the integrity of Sertoli cell tight junctions [21, 26]. Knockdown of *Cldn11* in Sertoli cells results in male sterility, but in contrast to S-*Ar* mutants, sterility is not immune mediated, as germ cell antibodies are undetected in the circulation and testes of *Cldn11$^{-/-}$* mice and testes show no evidence of inflammatory cell infiltration [26]. In S-*Ar* mutant mice, spermatogenesis proceeds through meiosis and early spermatid differentiation [13]. In *Cldn11$^{-/-}$* male mice, however, spermatogenesis does not proceed beyond meiosis [27]. Therefore, spermatogenic arrest in S-*Ar* mutant mice may be best explained by the increased permeability of the BTB resulting from disruption of *Cldn3* expression combined with accessibility to germ cell antigens that are no longer sequestered in S-*Ar* mutant mice.

CLDN5, like CLDN3 and 11, contributes to the formation of the BTB. CLDN5 is expressed in Sertoli cells, spermatogonia, and preleptotene spermatocytes and regulated by the transcription factor ets variant 5 (ETV5) [28]. *Etv5$^{-/-}$* mice transplanted with WT spermatogonial stem cells result in testicular inflammation consistent with deficient formation of the BTB [29]. CLDN5 is expressed primarily during stage VIII, the stage at which preleptotene spermatocytes start to migrate across the BTB. CLDN3 expression also peaks in stage VIII. Additional studies are needed to determine if CLDN3 ×

CLDN5 heterotypic interactions are important for maintaining BTB integrity.

Autoimmune processes have previously been shown to play a role in male and female infertility [30–32]. However, despite many important studies on experimental autoimmune orchitis (EAO) in animals [33, 34] and infertile men [35], little is known about the regulation of this process. The S-*Ar* model shares similarities with experimental autoimmune orchitis induced by multiple immunizations with syngeneic testicular germ cells [36, 37], as these mice develop germ cell antibody responses and extensive interstitial infiltration of inflammatory cells and severe hypospermatogenesis. In both models, cellular and humoral autoimmune responses are directed against germ cell antigens. However, S-*Ar* mutant mice provide a valuable model for evaluating the natural progression of male infertility related to disruption of androgen signaling.

In the present study, we probed Western blots with sera from six different S-*Ar* mutant mice and detected unique patterns of antibody reactivity against testicular antigens ranging between 24 and 130 kDa. Autoantigens emerged by PN Day 15 and immunofluorescence staining showed autoantibodies against premeiotic, round, and elongating spermatids. Our findings are similar to those of Qu et al. [38] in which injections of syngeneic testicular germ cells without adjuvants or bacterial components resulted in antibodies recognizing antigens of 15–200 kDa specific to round spermatids. Together, these findings may contribute to ongoing efforts to identify prominent germ cell antigens for use as diagnostic biomarkers or relevant targets for contraceptive vaccines [39, 40].

Approximately 10% of couples worldwide have difficulty conceiving children. About half of infertility is due to the male, and among these cases, 10% are associated with microdeletions of the Y chromosome [41]. The causes of the infertility in the remaining 90% of patients are largely unknown. Extrapolation of our findings to the human condition would predict that at least some of these idiopathic cases may have an autoimmune etiology, readily detected by the presence of antitesticular antibodies. This loss in fertility could be initiated by disrupted integrity of the BTB resulting from androgen insufficiency or genetic defects in androgen signaling in Sertoli cells. Our results may therefore provide a mechanism for idiopathic infertility in men.

ACKNOWLEDGMENTS

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