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Immune mediators associated to male infertility in a mouse model of DNA immunization with the sperm protease proacrosin

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ABSTRACT

The immune response has relevant physiological functions both in the male and female reproductive system, and must be tightly controlled to achieve a successful pregnancy. Several immune factors have been related to infertility, among them humoral and cellular immune responses triggered by sperm antigens. The present study was aimed at evaluating the immune profile induced by DNA immunization against the sperm protease proacrosin in CF1 male mice and its effect upon fertility.

Immunized animals exhibited higher anti-proacrosin antibodies levels than controls (indirect ELISA), both in serum ($p < 0.01$) and in seminal vesicle fluid (SVF; $p < 0.05$). IgG2a levels were higher than IgG1 in serum ($p < 0.01$) and similar in SVF. IL-10 and TGF-β1 mRNA levels were lower in testis ($p < 0.05$), whereas TNF-α and IFN-γ transcript levels were increased in SV tissue ($p < 0.05$). Immunized mice showed a trend toward higher IFN-γ concentration in serum and SVF than controls. Male fertility rate was diminished in immunized mice ($p < 0.01$) and inversely correlated with serum and SVF anti-proacrosin IgG levels ($p < 0.001$). Immunized animals also had fewer pups born than controls ($p < 0.01$).

To our knowledge, this is the first report on DNA immunization done in CF1 mice. Injection of proacrosin DNA induces an immune response in the male reproductive tract characterized by high levels of specific antibodies and cytokine changes. These factors may alter the crucial balance of the genital tract microenvironment required for adequate fertilization and pregnancy.

1. Introduction

Numerous reports have associated the immune response to spermatozoa with fertility impairment, and several animal models have been designed to study this condition. AntiSperm Antibodies (ASA) play an important role in interfering with normal sperm functions, since they may affect sperm performance by blocking sperm transport and capacitation, Acrosome Reaction (AR), sperm binding and penetration of the ZP, sperm binding and fusion to the oolemma, early embryo development and implantation (Chamley and Clarke, 2007; Vazquez-Levin et al., 2014). In particular, agglutinating ASA can reduce sperm forward progressive motility and may affect sperm penetration through cervical mucus (Kremer and Jager, 1992). In addition to antibody production, immunity to sperm leads to the expression of other immune factors, among them cytokines, known to play multiple roles in male and female reproduction, by participating in intercellular communication and mediating inflammatory responses (Ingman and Jones, 2008; Fraczek and Kurpisz, 2015; Schumacher and Zenclussen, 2015). There are reports describing alterations in fertility due to inflammation or infection caused by an imbalance in cytokine production (Hedger and Meinhardt, 2003; Ochsenkuhn et al., 2006). In the testis, cytokines have two relevant functions in physiological conditions, as they mediate immune-endocrine interactions during inflammation and work as growth and differentiation factors in the regulation of cell–cell interactions (Hales et al., 1999). Regarding pro-inflammatory cytokines, TNF-α is produced by germ cells and plays a role in the maintenance of the Blood-Testis-Barrier (BTB) (Li

Abbreviations: AR, Acrosome Reaction; ASA, AntiSperm Antibodies; BTB, Blood Testis Barrier; INF-γ, interferon gamma; IL-4, interleukin 4; IL-10, interleukin 10; TGF-β, transforming growth factor beta; OD, optical density; h-proacrosin, human proacrosin; Rec-40, recombinant h-proacrosin; TNF-α, tumor necrosis factor alpha; SVF, seminal vesicle fluid; ZP, zona pellucida; RE, relative expression.

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et al., 2006), whereas INF-γ production has been linked to anti-viral protection (Hedger and Meinhardt, 2003). On the other hand, regulatory cytokines such as IL-4, IL-10 and TGF-β1, -β2 and -β3 are constitutively expressed in the rodent testis and could have a role in immunosuppression as well as other functions in testis physiology (Yotsukura et al., 1997; Robertson et al., 2002; Winnall et al., 2011).

Cytokines are also present in the seminal plasma, and their immunomodulatory activities are essential to protect spermatozoa from the female immune system and to prepare the uterus for pregnancy (Robertson et al., 2011, 2013). Although controversy still exists about their origin, the seminal vesicles seem to be the most important contributors of components with immunomodulatory properties, such as cytokines (Robertson et al., 1996) and prostaglandins (Skibinski et al., 1992). In particular, TGF-β1 is essential for induction of immune tolerance against sperm antigens (Robertson et al., 2002); moreover TGF-β1 and -β2 appear to mediate the pro-inflammatory cascade elicited in the female tract at insemination (Ingman and Robertson, 2002). In addition, an increased concentration of pro-inflammatory cytokines in seminal plasma, such as IFN-γ and TNF-α, has been associated with poor semen quality and male infertility as well as with recurrent pregnancy loss (Daher et al., 2004; Chaouat et al., 2007). Despite its relevance, data is still scarce about the induction of immune mediators in male genital tract after an immunization.

Acrosin (EC 3.4.21.10) is a trypsin-like protease and localized to the sperm acrosome as an inactive zymogen (proacrosin; 53 kDa) that is converted to the active enzyme and released during the AR (Zahn et al., 2002). Several functions have been attributed to the proacrosin/acrosin system, mainly binding to glycoproteins of the *zona pellucida* (ZP) and participation in the release of acrosomal content (Vazquez-Levin et al., 2005) in mammals. Specific antibodies towards human (h-)proacrosin were shown to impair some of its functions in *in vitro* assays (Veaute et al., 2010) and antiacrosin antibodies were found in sera of women consulting for infertility (Veaute et al., 2009a). Moreover, the proacrosin/acrosin system has been found immunogenic after DNA immunization with the coding sequence of h-proacrosin of BALB/c female and male mice, an inbred strain with a low fertility rate. Mouse anti-h-proacrosin antibodies have been shown to bind to the mouse proenzyme and to inhibit *in vitro* fertilization, early embryonic development, sperm-ZP binding, and Ca^{+2}-ionophore-induced AR; moreover, immunized animals had diminished their fertility (Veaute et al., 2009b; Garcia et al., 2012).

Based on this background information, the present study was aimed at evaluating the ability of DNA immunization with proacrosin to modulate male fertility in the outbred strain CF1, known to have a high fertility rate (Katz-Jaffe et al., 2013). In addition to the assessment of presence and levels of antibodies towards proacrosin/acrosin in serum and SVF of immunized and control animals, the expression of a subset of pro-inflammatory and regulatory cytokines in the male gonad and seminal vesicles as well as its presence in serum and SVF was evaluated.

2. Materials and methods

2.1. Materials

A plasmid containing the h-proacrosin coding sequence was kindly donated by Prof. Dr. Wolfgang Engel (Institut für Humangenetik, Göttingen, Germany) to MVL, as previously described (Furlong et al., 2000) and subcloned in the pSF2-CMV eukaryotic expression vector for DNA immunization kindly provided to MVL by Dr. Stephen Johnston (Texas University, Southwestern Medical Center, Dallas, TX, USA).This construction was named pSF2-Acro (Veaute et al., 2009b). Plasmid DNA was obtained from bacterial cultures and purified by anion exchange chromatography using a commercial kit (Jetstar, Genomed, Löhne, Germany) following the manufacturer's instructions. The concentration of purified DNA was determined by optical density (OD) reading at 260 nm, and its quality was assessed by agarose gel electrophoresis and by the ratio of OD at 260 nm and at 280 nm (Sambrook and Russell, 2001).

Recombinant h-proacrosin was cloned and expressed using the pET22b expression vector as previously reported, and recombinant proacrosin (Rec-40: 42–44 kDa, aminoacids 1-402) was purified by preparative SDS-PAGE as previously described (Furlong et al., 2000).

2.1.1. Animal immunization protocol

For the immunization protocol, six to eight weeks old CF1 male mice were used. Animals were bred in a colony established at the IBYME animal facility. All experimental procedures were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals (Ilar, 2010). Adult male mice were inoculated in the quadriceps muscle with 50 μg of pSF2-Acro (n = 12) or pSF2 (n = 8) (plasmid without insert, control) in 50 μL of sterile phosphate-buffered saline (PBS). Injections were repeated four times, every three weeks. Blood samples were taken by tail vein puncture one week before each inoculation. Three weeks after the last dose (week 12), animals were bled by cardiac puncture and sacrificed, one serum aliquot was stored at −80 °C for cytokine determination, and another was kept at −20 °C for antibody analysis.

2.1.2. Assessment of immune response

The humoral immune response to proacrosin was evaluated by a previously optimized indirect ELISA (ELISA-Acro (Garcia et al., 2012)). Dilution of sera, as well as dilution of secondary antibodies, time of incubation, blocking agent and amount of antigen, was previously optimized in order to have an adequate positive to negative discrimination. Briefly, 96 well-polystyrene plates (Costar, Corning, NY, USA) were coated with 0.8 μg/well of recombinant h-proacrosin (Rec-40). After blocking non-specific binding sites with PBS supplemented with 3% skimmed milk, 1:200 dilutions of sera in PBS-1%-skimmed milk or pure seminal vesicle fluid (SVF) were tested. Anti-proacrosin IgG, IgG1, and IgG2a were assessed by incubation with peroxidase conjugated anti-mouse IgG (Jackson, Baltimore, MD, USA), anti-mouse IgG1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) or anti-mouse IgG2a (Abcam Inc. Cambridge, MA, USA), respectively, followed by incubation with hydrogen peroxide and tetramethylbenzidine (Zymed, San Francisco, CA, USA). In order to find the dilution of secondary antibodies giving a comparable OD between both subtypes of IgG, the ELISA was optimized by immobilizing different amounts of monoclonal antibodies from each subtype. OD was measured using a microplate reader (Emax Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). For serum samples, results are expressed as the ratio between the sample OD and the preimmune sera OD (OD/OD preimmune). For SVFs, results are expressed as the ratio between the sample OD value and the average OD value of samples from pSF2 inoculated mice (OD/OD control).

2.1.3. Assessment of male mice fertility

Immunized male mice were mated with non-treated 14 to16 week-old female mice with 1 male per 3 females in a cage, starting at week 9 of the immunization protocol. Fertility of female mice was proven by their mating with untreated males prior to the initiation of the immunization protocol; only female mice that had offspring

were included in the study. Animals were monitored daily for the presence of vaginal plugs or spermatozoa in the vaginal secretion that was considered day 0.5 of pregnancy. At term, female mice were monitored and numbers of born pups were recorded.

2.1.4. Evaluation of sperm parameters and testis histology

Caudae epididymides were excised, diced in small pieces and placed into 0.5 mL Whittingham's culture medium at 37 °C. Spermatozoa were allowed to swim out into the medium for 10 min and the concentration was estimated using a hemocytometer. Sperm motility was assessed by counting progressively motile cells and expressed as percentage of the total sperm count. Testes were fixed in Bouin's solution and embedded in paraffin. Testes transversal sections (5 μm–thick) were stained with hematoxylin–eosin and analyzed.

2.1.5. SVF extraction

Seminal vesicles were excised and cleaned avoiding exposure to coagulating glands secretion. One vesicle was perfused with 0.5 mL of sterile PBS; the collected fluid was centrifuged at 6000*g* for 5 min, and the supernatant was kept at −80 °C until use for cytokine determination. An aliquot was stored at −20 °C for antibody analysis. The other seminal vesicle was used for RNA extraction.

2.1.6. RNA extraction and RT-PCR

Total RNA was purified from testis and seminal vesicles using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and quantified by OD reading at 260 nm, as previously described (Sambrook and Russell, 2001), using a NanoDrop Lite spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One μg of total RNA of each sample was retrotranscribed to cDNA with MMLV reverse transcriptase. IL-4, IL-10, IFN-γ, TNF-α, TGF-β1, TGF-β2, and TGF-β3 mRNA relative expression levels were determined by quantitative Real Time PCR with specific primers designed using Primer3 software (http://primer3plus.com/) (Table 1). Each PCR was performed in triplicate using 5X SYBR green PCR master mix HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Estonia) and 0.5 μM of each primer.

PCR reactions were performed on an Applied Biosystems Step One thermocycler (Life Technologies, Carlsbad, CA, USA) using a basic program, as follows: denaturation (15 min at 95 °C), followed by 40 cycles of denaturation (20 s at 95 °C), annealing (30 s at the adequate temperature for each primer pair) and extension (30 s at 72 °C), and a final extension of 10 min at 72 °C. The specificity of each reaction was routinely verified by melting curve analysis; random samples were additionally tested by gel electrophoresis. Amplification efficiency was calculated for all PCR reactions based on the slope of a standard curve.

Relative gene expression was calculated by the ΔΔCt method including the efficiency of amplification and using the REST-2009 software, which uses a statistical model based on Pair Wise Fixed Real location and Randomization Test (Relative Expression Software Tool, Qiagen, Hilden, Germany, http://www.REST.de.com) (Pfaffl et al., 2002). Cyclophilin B was used as endogenous control for normalization of the relative mRNA levels.

Table 1
Primers used for cytokine mRNA quantification by real time PCR.

Gene	Gene ID	Primers	Product size
IL-10	NM_010548.2	F: TACAGCCGGGAAGACAATAA R:CACTCTTCACCTGCTCCAC	369 bp
IL-4	NM_021283.1	F:CCCCAGCTAGTTGTCATC R:CGAAAAGCCCGAAAGAGT	306 bp
TGF-β1	NM_011577	F:ATGCTAAAGAGGTCACCCGC R:GTATCAGTGGGGGTCAGCAG	273 bp
TGF-β2	NM_009367.3	F: CTGCCTTCGCCCTCTTTACA R:GATCCTGGGACACACAGCAA	273 bp
TGF-β3	NM_009368.3	F: GGACTTCGGCCACATCAAGA R: ATAGGGGACGTGGGTCATCA	112 bp
TNF-α	NM_013693	F:TAGCCAGGAGGGAGAACAGA R:CACTTGGTGGTTTGCTACGA	436 bp
INF-γ	NM_008337.3	F:GCGTCATTGAATCACACCTG R: CGCAATCACAGTCTTGGCTA	448 bp
Cyclophilin B	NM_011149.2	F: GCACAGGAGGAAAGAGCATC R: AAAATCAGGCCTGTGGAATG	460 bp

F: forward, R: reverse.

2.1.7. IL-4 and IFN-γ quantification by ELISA

Cytokine levels in sera and SVF were measured using a commercially available capture ELISA kit (OptEIA™, BD Biosciences, San Diego, CA, USA) following the manufacturer's instructions.

2.1.8. Statistical analysis

Results are reported as mean ± Standard Deviation of the Mean (SDM), which was calculated with the denominator N-1, for each group, unless specified. Statistical differences between groups were analyzed by two tailed Fisher's Exact test and Mann-Whitney test. Spearman's correlation test was applied to assess the relationship between antibody levels and litter size. P values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Assessment of humoral immune response

CF1 male mice were immunized with a plasmid containing the cDNA encoding human proacrosin (pSF2-Acro, n = 12) and control mice received the empty plasmid (pSF2, n = 8). Specific antibodies, assessed by indirect ELISA with recombinant h-proacrosin, were detected in pSF2-Acro inoculated mice after the second inoculation (week 8). At the end of the trial (week 12), levels of specific antibodies were 16.6 ± 7.4 times over the preimmune value in the pSF2-Acro group and they were higher than those of pSF2 inoculated mice (1.7 ± 0.8; $p < 0.01$) (Fig. 1A). The immunization protocol induced production of both IgG1 and IgG2a immunoglobulin subclasses. Serum levels of anti-proacrosin IgG2a immunoglobulins were higher than IgG1 (OD/OD preimmune: 8.2 ± 5.39 *versus* 3.6 ± 1.4, respectively; $p < 0.01$) at the end of the immunization scheme.

Anti-proacrosin antibodies were also detected in SVF, being more abundant in immunized than control animals (1.89 ± 0.88 *versus* 1.0 ± 0.1, respectively, expressed as OD/OD control; $p < 0.05$). Unlike serum antibodies, anti-proacrosin IgG1 and IgG2a reached similar levels in SVF of pSF2-Acro immunized mice (OD/OD control = 2.60 ± 1.91 *versus* 2.01 ± 1.72, respectively; $p > 0.05$) (Fig. 1B).

3.2. Evaluation of cytokines in tissues and fluids

Giving their prominent role as immune response modulators, TNF-α, INF-γ, IL-4, IL-10, TGF-b1, TGF-b2, and TGF-b3 mRNA expression levels were evaluated in the testes and seminal vesicles, as well as INF-γ and IL-4 protein levels in sera and SVF at

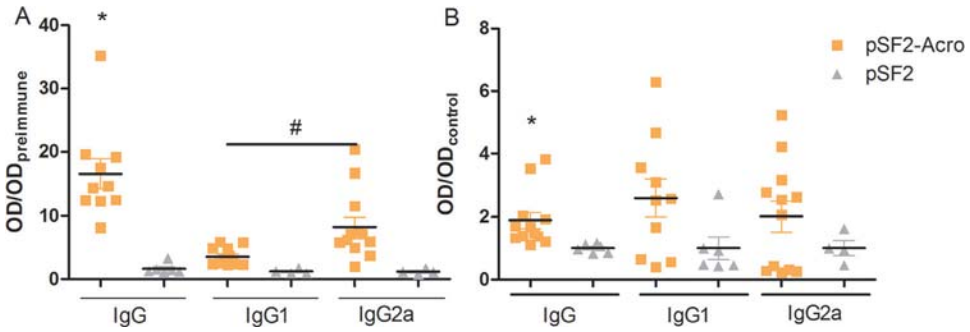

Fig. 1. Humoral immune response of mice immunized with pSF2-Acro or inoculated with pSF2 (controls) at week 12, assessed by indirect ELISA. (A) Antibody levels in animals sera expressed as OD/OD preimmune (mean ± SDM). Anti-proacrosin IgG levels were increased in immunized mice compared to controls (16.62 ± 2.34 *vs* 1.65 ± 0.33; *$p < 0.01$). Specific IgG2a was higher than IgG1 (8.24 ± 1.56 *vs* 3.57 ± 1.41; #$p < 0.05$). (B) Antibody levels in SVF expressed as OD/OD control (mean ± S.E.M). Anti-proacrosin IgG levels were significantly increased in immunized compared to control mice (1.89 ± 0.26 *vs* 1.00 ± 0.06; *$p < 0.05$). Levels of IgG2a and IgG1 subclasses were similar (2.6 ± 0.60 and 2.01 ± 0.49, respectively).

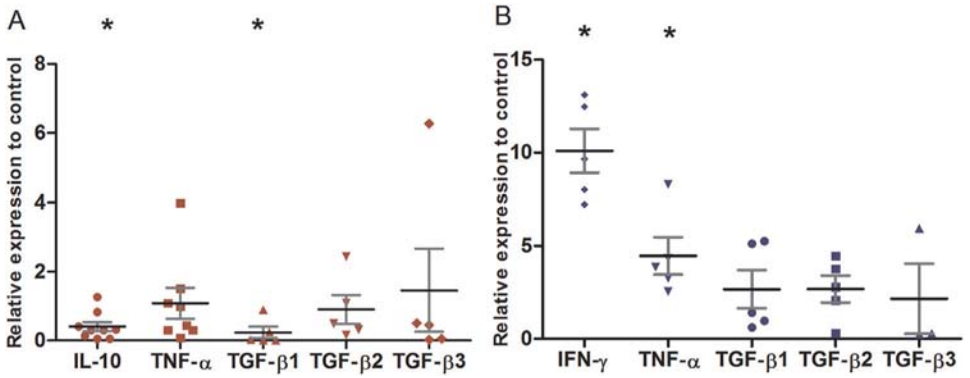

Fig. 2. Effect of DNA immunization against proacrosin on cytokine mRNA expression in testis (A) and in seminal vesicle (B). Results are expressed as the mean relative expression of each mRNA from pSF2-Acro treated animals to control animals, calculated by the ΔΔCt method with efficiency correction. Transcript levels were normalized using cyclophilin as housekeeping gene. Immunization with pSF2-Acro plasmid was associated to a significant decrease of IL-10 (Relative expression (RE): 0.40 ± 0.13) and TGF-β1 (RE: 0.23 ± 0.017) mRNA in testis, and a significant increase in TNF-α (RE: 4.47 ± 1.00) and IFN-γ (RE: 10.09 ± 1.17) mRNA levels in seminal vesicles (*$p < 0.05$).

the end of the immunization protocol. In the testis, TNF-α, TGF-β2, and TGF-β3 mRNA were detected, but their levels were not altered by DNA immunization with Acrosin (Relative Expression (RE) to controls RE: TNF-α = 1.08 ± 0.13; RE: TGF-β2 = 0.90 ± 0.98; RE: TGF-β3 = 1.46 ± 2.70; $p > 0.05$). In contrast, the expression of testicular IL-10 and TGF-b1 diminished in immunized mice (RE: IL-10 = 0.39 ± 0.16; RE: TGF-b1 = 0.23 ± 0.58; $p < 0.05$) (Fig. 2A). Neither IFN-g nor IL-4 mRNAs were detected in testes of animals before or after DNA immunization (data not shown; mouse spleen included as positive control assay).

In seminal vesicle tissue, In seminal vesicle tissue, IFN-γ and TNF-α mRNA levels were higher in immunized mice than in controls (RE: IFN-γ=10.2 ± 2.0 and RE: TNF-α = 3.9 ± 2.0; $p < 0.05$). TGF-β mRNA levels of the three isoforms slightly increased in immunized animals compared to control but differences did not reach significance (RE: TGF-β1 = 2.64 ± 0.31; RE: TGF-β2 = 2.38 ± 0.79; RE: TGF-β3 = 2.16 ± 3.28; $p > 0.05$) (Fig. 2B). Finally, a non-significant increase in IFN-γ was observed in both fluids recovered from pSF2-Acro injected mice compared to controls (Fig. 3A), whereas no differences were observed in IL-4 protein levels between immunized and control animals (Fig. 3B).

3.3. Fertility assessment

To assess the influence of the immune response upon animal fertility, male mice were mated with fertility proven females in a 1:3 ratio at week 9 of the immunization protocol. Females were separated when either a vaginal plug or the presence of spermatozoa in the vaginal secretion was detected. A mouse was considered fertile when it had at least one pup.

In the immunized mice group, the fertility rate was significantly lower than in the control, finding only 5 fertile out of 12 mice evaluated (41.7%). Contrasting, all control mice were fertile (8/8, 100%; $p < 0.05$). Regarding the litter size, mice from the control group had more pups born than immunized animals (8.08 ± 4.59 *versus* 3.73 ± 4.45 pups/male mice; $p < 0.01$) (Fig. 4A). This decrease in fertility was inversely correlated with serum and SVF anti-proacrosin IgG levels ($r = -0.71$, $p < 0.001$ and $r = -0.54$, $p < 0.05$, respectively) (Fig. 4B and C).

3.4. Evaluation of testis histology and cauda epididymal sperm parameters

At week 12 of the immunization protocol, male mice were sacrificed and the testes and spermatozoa from the *cauda* epididymis were recovered and analyzed. Histological examination of testis sections stained with hematoxylin-eosin showed normal appearance of seminiferous tubules and interstitial tissue. In particular, no lympho-mononuclear cell infiltrates were observed in the interstitium of any of the animals studied (Fig. 5). Moreover, a similar sperm concentration and motility was estimated in animals immunized with proacrosin and controls (14.4 ± 3.9 × 10^6 sperm/mL *versus* 11.8 ± 2.5 × 10^6 sperm/mL, respectively; % motile sperm 77 ± 2% *versus* 67 ± 13%, respectively).

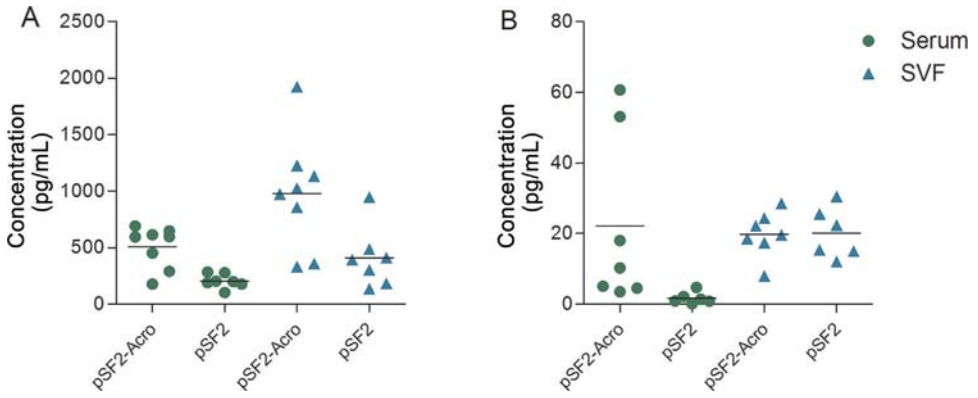

Fig. 3. Serum and SVF levels of IFN-g(A) and IL-4 (B) quantified by capture ELISA assay. IFN-γ and IL-4 levels showed non-significant differences between immunized mice (pSF2-Acro) and controls (pSF2) in both serum and SVF. IFN-γ Serum: 506 ± 185 pg/mL versus 203 ± 62.2 pg/mL, IFN-γ SVF: 977 ± 507 pg/mL versus 407 ± 268 pg/mL; p>0.05. IL-4 Serum: 22.86 ± 26.59 pg/mL vs 3.39 ± 4.27 pg/mL, IL-4 SVF: 20.20 ± 6.6 pg/mL versus 23.36 ± 6.49 pg/mL; p > 0.05.

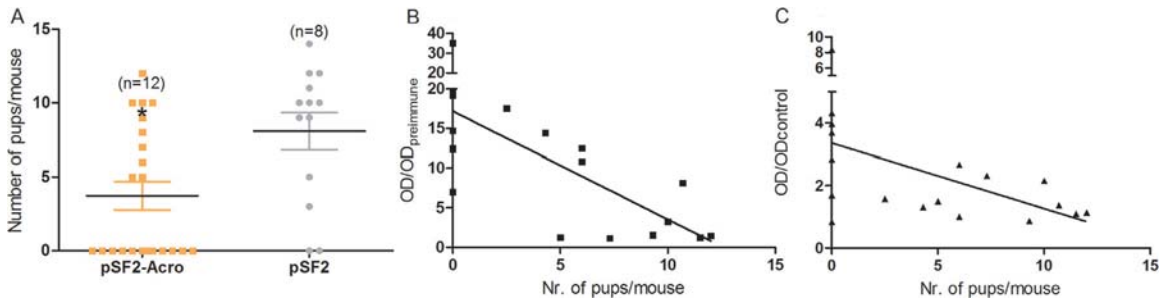

Fig. 4. Immunized male mice were mated with female mice of proven fertility in a 1:3 ratio, until evidence of mating was obtained. The number of pups born per animal was registered. A) Number (mean ± SDM) of born pups per male mouse: female mice mated with immunized male gave significantly fewer pups than those mated with control mice (3.7 ± 0.9 *vs* 8.1 ± 1.3; *$p < 0.01$). B and C) Relationship between serum (B) or SVF (C) antibody levels and litter size from pSF2-Acro immunized male mice. A significant negative correlation between antibody levels and litter size was observed for both fluids ($r = -0.71$, $p < 0.001$ and $r = -0.54$, $p < 0.05$, respectively, Spearman correlation test).

4. Discussion

The present report describes a set of investigations done on CF1 male mice subjected to DNA immunization with a eukaryotic expression vector carrying the nucleotide sequence of h-proacrosin. Studies involved assessment of the animals immune response, by evaluating the humoral response (specific antibodies towards the sperm protein) in sera and SVF, as well as determining mRNA expression levels of regulatory and pro-inflammatory cytokines in the male gonad and the seminal vesicles, and assessing the presence of these factors in sera and SVF in animals injected with a plasmid carrying the sequence of h-proacrosin and in control animals injected with the empty plasmid. Animals immunized towards proacrosin produced high levels of antibodies towards the sperm protease. In addition, they expressed regulatory and pro-inflammatory cytokines in the male gonad and seminal vesicles. The immune response to DNA immunization was associated to a significant lower fertility.

In a previous study, the induction of high antibody levels towards h-proacrosin in BALB/c female and male mice was achieved using the same immunization scheme used in this investigation. Mouse and h-proacrosin have a high degree of protein identity; antibodies induced by h-proacrosin were reported to recognize mouse-proacrosin and impair fertilization-related events and female and male fertility in BALB/c mice (Veaute et al., 2009b; Garcia et al., 2012). To our knowledge, the present work is the first reporting DNA immunization of CF1 male mice. This outbred strain is typically used in biology of reproduction research, mainly owing to its excellent response to ovarian hyperstimulation (Hogan et al., 1994) as well as in other areas of biomedical research (Cebral et al., 2011; Miranda et al., 2012). The time-course response to immunization was similar between both strains, and antibody levels attained at the end of the immunization trial in CF1 were comparable with those observed in our previous work done with BALB/c mice (Veaute et al., 2009b; Garcia et al., 2012).

Anti-proacrosin immunity was associated with male infertility. DNA immunization of CF1 mice led to a significant reduced fertility (41.7% *vs* 100%; $p < 0.05$) despite that outbred strains are reported to have a good reproductive performance (Silver, 1995). Levels of anti-proacrosin antibodies were related to the degree of infertility, as reflected by the reduced number of born pups in animals carrying antibodies and the inverse correlation between antibodies levels in SVF and fertility blockade. Anti-proacrosin antibodies were found in serum and in SVF. Both systemic and mucosal immune systems contribute to the presence of antibodies in semen. IgG is the dominant isotype and a significant part of this immunoglobulin is derived from plasma (Moldoveanu et al., 2005). Nevertheless, giving that IgA isotype has been described in semen mainly produced by the prostate and the urethra (Anderson and Pudney, 2015), anti-proacrosin antibody levels in ejaculate semen may be higher than those reported here, and their effect on female reproductive system deserves further studies. Regarding distribution of IgG sublcasses in semen, a previous study reported similar levels in serum (Shakib, 1986), but data are scarce about the subclasses content after an immunization, even less with DNA immunization. Intramuscular DNA immunizations usually lead to Th1-biased immune responses with predominant serum IgG2a production (Cohen et al., 1998) and this is in agreement with the profile induced by the immunization method described in this report. However, in SVF both subclasses were found balanced, suggesting that semen antibodies may have an additional origin other than serum; alternatively, relative levels of IgG subclasses may be influenced by seminal components. How-

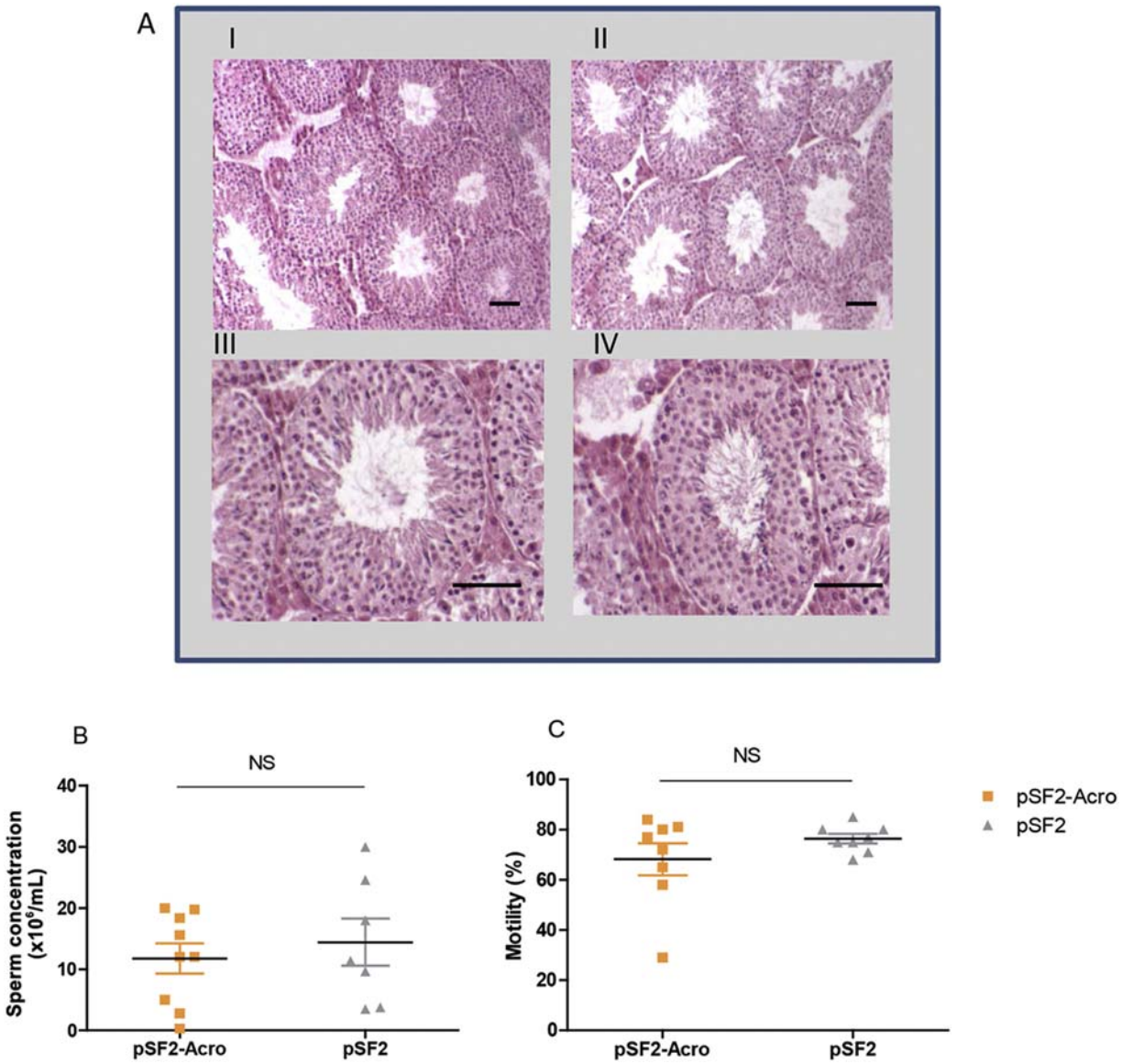

Fig. 5. Effects of immunization on testis histology (A) and sperm parameters (B,C). A) Representative microphotographs of the testis from a control (I and III) and an immunized mouse (II and IV) showing normal spermatogenesis and absence of lymphocytic infiltrates. FFPE and hematoxylin-eosin stained tissue. I and II: 400×., III and IV: 1000×, Bar represents 50 μm. B) Sperm concentration of the epididymal content (pSF2-Acro: 11.77 ± 2.50, pSF2: 14.43 ± 3.85 (spermatozoa $\times 10^6$/mL)). C) Progressive motility of epidydimal spermatozoa (pSF2-Acro: 68.25 ± 6.40, pSF2: 76.38 ± 1.90). Results are expressed as mean ± SDM. NS: No significant differences ($p > 0.05$).

ever, other IgG subclasses are produced in mice and may contribute to the total pool of IgG induced after DNA immunization.

In addition to the antibodies, other immune factors, among them cytokines, are part of a complex network of cell-to-cell communication entities that are involved in fertility regulation, both in a positive or a negative fashion. A balance between pro- and anti-inflammatory molecules is assumed to support successful implantation. Cytokines in seminal plasma, mainly as TGF-β1 and -β2, are involved in the generation of Treg cells specific for paternal antigens (Robertson et al., 2011, 2013; Schumacher and Zenclussen, 2015). As a consequence, an altered cytokine balance with elevated concentrations of several cytokines, including IL-1, IL-2, IL-6, and TNF-α in seminal plasma has been associated to poor sperm performance (Politch et al., 2007). Furthermore, an excess of inflammatory cytokines, including TNF-α, INF-γ and IL-2, was associated with negative effects on implantation (Robertson et al., 2011).

Regarding cytokine production in the male gonad, immunized animals showed similar levels of TNF-α than controls, and INF-γ was under the detection limit of the technique. These findings, together with the lack of leukocyte infiltrates, suggest the absence of an important inflammatory reaction in testis, at least at the time point of the evaluation. With regard to the regulatory cytokines, testis immune privilege is given by the BTB as well as by a set of cells and molecules that contribute to generate a tolerogenic environment. IL-10 is mainly produced by testicular resident macrophages. It was previously reported that IL-10 administration in different autoinmmune conditions, such as experimental autoimmune orchitis, suppresses the development of the disease (Bettelli et al., 2003; Watanabe et al., 2005). On the other hand, TGF-β is produced by Sertoli and Leydig cells, as well as by M2 macrophages. This cytokine plays a vital role in testis development and spermatogenesis, and in the maintenance of the immune privilege (Lui et al., 2001; Dobashi et al., 2002; Itman et al., 2006). In our experimental model, immunized mice showed a decrease in IL-10 and TGF-β1

mRNA levels compared to control animals. These changes could be related to a perturbation of the immunosuppressive microenvironment of the testis and with concomitant changes in gamete development.

In the present work, IFN-γ and IL-4 were detected in SVF, and higher levels of IFN-γ in immunized animals were observed. In line with these findings, higher IFN-γ transcript levels were also found in the seminal vesicles of immunized animals, suggesting this tissue as a relevant site of production of IFN-γ after immunization. TNF-α transcript levels were also found increased in seminal vesicles of proacrosin-immunized mice. IFN-γ and TNF-α were previously found to impair *in vitro* sperm motility (Eisermann et al., 1989; Estrada et al., 1997); other studies reported alterations of both the spontaneous and induced-AR (Dimitrov and Petrovska, 1996; Carrasquel et al., 2014), and cytokine receptors on the sperm surface were associated to some of these effects (Brackett et al., 2007). Specifically regarding IFN-γ, specific receptors for this cytokine have been immunodetected in human sperm (Naz et al., 2000) and this cytokine was found to affect sperm membrane permeability to calcium ions influx when added at high concentrations (Carrasquel et al., 2014). In agreement with these observations, increased IFN-γ concentrations in seminal plasma of infertile subjects have been reported (Paradisi et al., 1996). These findings and the increased INF-γ is in line with the Th1-biased profile identified in serum, and may explain, at least in part, the negative effects of immunization upon fertility in spermatozoa with sperm that do not present major abnormalities in sperm concentration, motility and morphology.

In summary, DNA immunization of male mice against proacrosin was associated with an impairment of fertility. The inhibitory effect could be associated to the blockade of the proacrosin/acrosin system functions caused by antibodies toward the acrosomal protein, but also to other immune mediators induced by the immunization which may exert their effects directly on the sperm or alter the crucial balance of genital tract microenvironment needed for fertilization and normal pregnancy progress.

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