

# An update on Immunologic Infertility-special Emphasis on Role of PD-1 (Programmed Death 1) Gene

Kulvinder Kochar Kaur\*, Allahbadia G and Singh M

Dr. Kulvinder Kaur Centre for Human Reproduction, Jalandar, Punjab, India

## Abstract

The association of antisperm antibodies and autoimmunity or presence of gamma delta T cells in semen associated with men with ASA related infertility has been recognized as early as the early 90's with specific correlation with antiphospholipid antibodies but it has been concluded in a review in 2002 by Mclachlan RI 2002 that since IUI/ICSI is the final answer there is no point in bothering about looking or investigating for antisperm antibodies and hence not much interest was given till recently interest got rekindled by the appearance of a report on the presence of programmed death 1(PD-1) gene polymorphisms in men with ASA related infertility as PD-1 is a negative costimulatory molecule also found in sertoli cells and associated with the transplantation potential or helping in the immune privileged site of testis as reported by Dal secco and hence this review is done to highlight the implications of getting insight into the reason for male immune infertility and its difference from female one and correlation with other autoimmune disorders and future therapeutic potentials including in recurrent mishaps as well as for future orthotopic testicular transplantation in men with testis in inguinal region other causes of immune infertility as simple as just subtle chlamydia infection.

**Keywords:** Antisperm antibody; Antiphospholipid antibody; Programmed death-1(PD-1); Costimulatory molecule; Sertoli cells as APC; Orthotopic testicular transplantation; Autoimmune diseases

## Introduction

The blood-testis barrier normally isolates sperms from immune recognition (sperms develop after immunocompetence is established), but if it is disrupted and sperms are exposed to blood, an antigenic response can result. Risk factors for development of antisperm antibodies (ASAs) which occurs in approximately 6% of men presenting with infertility include: i) ductal obstruction ii) previous genital infection [e.g. Chlamydia trachomatis infection] iii) testicular torsion iv) testicular trauma v) reversal of sterilization vi) vasovasostomy and vii) vasoepididymostomy [1]. ASAs can be found in the serum although evidence indicates that they have no clinical significance [2,3]. Antibodies bound to sperm may be clinically relevant since they can interfere with sperm motility or prevent fertilization [4,5]. ASAs may further be detected in blood, semen and follicular fluid besides being in cervico vaginal secretions where they appear to hinder sperm movement, capacitation, fertilization and ultimately inhibiting embryo implantation [6]. While trying to localize sites where ASA binding occurred in men with ASAs related infertility or vasectomy, Bohring et al. [6] found using sperm immunofluorescence especially in impaired acrosomal reactions found sperm binding occurred in acrosomal region, midpiece and tail. Binding occurred in both groups to midpiece alone or in combination with other regions of spermatozoa. Binding to all 3 regions of sperms was shown by very few ASAs containing semen samples. ASAs binding to head influenced acrosomal reaction, while that to tail and/or midpiece was not associated with a significant alteration of viability and motility. Further ASAs were found in upto 28% patients with varicocele. Although some cases showed changes in the ASAs titers after vai cocoelectomy, Knudson et al. [7] did not find that the presence of sperm bound immunoglobulins affected the response to surgical correction, nor did they postulate an immunological mechanism as a major factor in varicocele induced infertility.

Human ASAs bound to specific cognate antigens of mouse spermatozoa like 30% of ASAs recognized apo lactate dehydrogenase (LMC4), 30% voltage dependent ion channel (VDAC2) and ASA of 20% bound to outer dense fiber protein and 20% of samples recognize

glutathione S transferase mu-5 which offers a possibility to study their functional relevance in mouse models and mechanism of autoimmune infertility [8]. Further Dorr et al. [9] found that ASAs are sperm specific as an autoimmune model and hence suitable to use these cognate antigens as possible immune contraceptive agents. Marked sperm clumping or agglutination like isolated asthenospermia can signal the presence of ASAs, but neither is observed with much frequency. Some also regard unexplained infertility as an indicator of ASAs. The 2 most widely used tests for determination of ASAs involve the use of beads or latex particles with attached antibodies (both) (raised) against human immunoglobulins that bind to the surface of sperm [10]. Unfortunately the threshold for a positive test having clinical importance is not well defined. Moreover the levels of antibodies can fluctuate even without treatment [11]. Pregnancy rates are reportedly lower for men with demonstrable ASAs than for those without antibodies and amongst those with ASAs, pregnancy rates are lower when more than 50% of sperms are antibody bound [12]. ASAs have been found with a poor post coital test result but routine PCT is no longer performed because results have no proven value. Because intrauterine insemination (IUI) was amongst the most popular and effective treatment for ASAs [13], as it was for presumed cervical factor infertility IUI has become a core element of most treatments for unexplained infertility other than IVF. The result of ASA testing, like those of PCT, rarely offers any information that affects decisions or outcomes. Hence these days ASA testing is seldom performed as when IUI fails ICSI is supposed to circumvent any adverse effects of ASAs [13] hence we usually don't even try to find an answer for ASAs.

Although Human seminal plasma and cervical mucus both contain

\*Corresponding author: Kulvinder Kochar Kaur, Dr. Kulvinder Kaur Centre for Human Reproduction, Jalandar, Punjab, India, E-mail: [kulvinder.dr@gmail.com](mailto:kulvinder.dr@gmail.com)

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an immunoglobulin binding factor (IgBF) which interacts with IgG as monomers under reducing conditions, Antibody production against allogeneic sperms maybe prevented in the female reproductive tract by them. Since IgG is secreted as a homodimer it does not bind IgG, and *in-vivo* systems activation is needed to do so. GSH is known to reduce the inactive native dimer to the active monomer. Protein disulphide isomerases (PDA), alters the configuration of dimers to inactive monomers. PDA produced by activated T cells cleaves the dimers in the presence of GSH to active fragments. Mori et al. hypothesized that since these enzymes are produced upon stimulation by the immune system, immunocompetent cells interact with the allogeneic sperms leading to the local production of these enzymes that will activate IgBF [14].

Koide et al. [15] in a review to establish a cause and effect relationship identified seven target sperm antigens to be associated with infertility and described their coding genes. The ASAs examined were from infertile women or were monoclonal antibodies (mAb) raised against human sperm proteins. All the ASAs studied possessed potentially sperm agglutinating and/or immobilizing activation. The seven target antigens isolated from human and mammalian sperms are YWKII, BE 20, rSMP-B, BS-63 (nucleosporin related), BS-17(calpastatin), HED-2 (zyxin) and 75-kDa. Each antigen is a distinct and separate entity produced by different cells of the reproductive tract (e.g. germ cells, epididymal epithelial cells, and sertoli cells). No single predominant target component has been found to interact with ASAs It is thus proposed by Koide et al. [15] that immunologic infertility is the consequence of the combined actions of multiple ASAs in immobilizing and/or agglutinating spermatozoa blocking sperm egg interaction ,preventing implantation, and or arresting embryo development. Further they characterized an 80 kDa human sperm antigen responsible for immune infertility as well as suitable candidate for immune contraception [16].

Further Wang et al. [17] cloned a 339 bp cDNA fragment of mouse testis form of nuclear autoantigenic sperm protein (t NASP) and developed a recombinant rmt NASP and a synthetic human tNASP (a histone binding protein) and found that significant antifertility effect in animals immunized with rmt NASP antigen or the synthesized peptide which was reversible. This further provides evidence that active immunization with rmt NASP antigen may induce a strong antibody response which causes an inhibitory effect on fertility.

Recently antibodies against ACTL7a, a spermatozoan specific protein has been demonstrated to be the cause of immunologic infertility in males following vasectomy as proved by Fu et al. [18] by mass spectrometry, direct immunostaining, sperm agglutination tests and standard infertility assays, where Anti ACTL7a antibodies may cause infertility in mice because *in vitro* treatment of mouse spermatozoa with ACTL7a antibody containing serum markedly reduced the fertilizing potential of the spermatozoa. In addition active immunization of mice with ACTL7a resulted in significant reduction in fertility. The expression of ACTL7a is upregulated via PKA pathway and undergoes this during the early period of capacitation in mouse spermatozoa. Fu et al. [18] further demonstrated how ACTL7a is an essential component of mouse capacitation and dynamic changes occur in the expression and localization of ACTL7a which maybe a primary biochemical event in the induction of capacitation in mouse spermatozoa [19].

## Role of T Cells

In a study by Munoz et al. [20] studying 30 semen samples by immunoperoxidase techniques and monoclonal antibodies to  $\beta$  chain and  $\delta$  chain of human T cell receptors (TCR) i) in 7 men with ASAs in

both ejaculated sperms and serum mean concentration of  $\gamma\delta$  and  $\alpha\beta$  cells were 3560 and 3230 cells/ml semen, respectively. ii) in 7 men with ASA in serum only concentration of  $\gamma\delta$  and  $\alpha\beta$  T cells were 860 and 1280 cells/ml, while in iii) 16 men with no evidence of autoimmunity to sperms there was a mean of 350  $\gamma\delta$  T cells and 610  $\alpha\beta$  T cells. In contrast the concentration of  $\gamma\delta$  and  $\alpha\beta$  T cells in peripheral blood from same men were unrelated to ASA status. The mean ratio of  $\alpha\beta$ : $\gamma\delta$  T cells in peripheral blood of all subjects was 12. The ratio of  $\alpha\beta$ : $\gamma\delta$  T cells in semen were 0.9 for men with sperm bound and serum ASAs, 1.5 for men with ASA in serum only and 1.7 for men lacking these autoantibodies. Thus  $\gamma\delta$  T cells in human semen comprise a greatly increased proportion of total T cell population as compared to the circulation. Further gamma delta T cells are further elevated in semen from men with evidence of localized autoimmunity to their own sperm. Hence these results suggested that  $\gamma\delta$  T cells may be functioning in immune surveillance in the non-sterile proximal portions of the male genital tract and that replication of T cells bearing the  $\gamma\delta$  TCR accompanies an autoimmune response to sperm [20].

Subsequently studying the relation of asymptomatic chlamydia trachomatis genital tract infection in 48 male partners of couples with unexplained infertility Munoz and Witkin [21] found IgA antibodies to C. trachomatis in seminal fluid from 14 (29.2%) of men. Only 4 of these were positive for circulating antichlamydial IgA, suggesting that the stimulus for antibody production was within the genital tract. In contrast 4 men were positive for antichlamydial IgG in their semen; they all were also seropositive for antichlamydial IgG. Tlymphocytes bearing the alpha beta and gamma delta antigen receptors, which were present in every semen sample. However men having seminal chlamydial IgA had significantly increased gamma delta T cell concentrations (median-3100cells/ml) as compared to men lacking this antibody (median 1400 cells/ml) although concentrations of alpha beta T cells were comparable in both groups. Genital tract sperm autoimmunity was shown by antibodies bound to motile ejaculated spermatozoa which were shown in 13 (27.1%) men. Antibodies presence was associated with elevated concentrations of both gamma delta (median 4200 v 700 cells/ml) and alpha beta (median 5000 vs. 850 cells/ml) T cells ( $p=0.0002$  &  $p=0.00001$  respectively). Men with ASA only in their serum had seminal T cell concentrations comparable with men who tested negative for ASAs. Antichlamydial IgA was found in 4/10 men with IgA bound to their spermatozoa and in none of men with only spermatozoon bound IgG [21].

Munoz and Witkin [21] showed that ASAs are associated with increased numbers of both  $\gamma\delta$  and cytotoxic T cells in seminal fluid of males with unexplained infertility especially with chlamydia trachomatis infections. Bertotto et al. [22] carried out direct immunofluorescence staining following two colour cytoflorometric analysis on mononuclear cells (MCs) suspensions from ejaculates of 10 healthy fertile volunteers to study the  $\gamma\delta$  T cell subset distribution in normal human serum. Autologous peripheral blood MC were also simultaneously analysed and results were used for statistical comparison. They found that proportion of normal human semen lymphocytes bearing the  $\gamma\delta$  T cells receptors for antigens was greatly increased as compared with autologous circulating counterparts. The rise was mainly due to overexpansion of cells expressing V $\delta$  1 gene encoded determinants on their surface. This was in contrast with their normal blood picture where most  $\gamma\delta$  T cells express V $\delta$  2 conformational epitope. Hence, Bertotto et al. [22] concluded that there is a numerical as well as phenotypical difference in semen  $\gamma\delta$  T lymphocyte which provides evidence of a migratory lymphocyte subset balance in anatomically and physiologically distinct areas of body. They further suggested that their functional role in terms of both helper and suppressor cytotoxic activities in the non-

sterile proximal portion of the male genital tract needs to be explored. Presence of antigenic specific T cells in semen fluid implicates them in the development of local inflammation and auto antibodies.

## Role of Programmed Death 1

The peripheral tolerance pathways normally protect against any autoreactive responses. Programmed death-1(PD1) is a 55 kDA transmembrane protein along with being a member of CD28 family and inhibitor of cellular activation of T and B cells is also known to actively participate in maintaining peripheral tolerance via activation of immunoreceptor tyrosine-based inhibitory motif (ITIM) pathway [23]. Recently B7/CD28 superfamily has expanded to include the costimulatory and inhibitory receptors, including inducible costimulator (ICOS) and PD1 which are inducibly expressed on the surface of T cells and provide unique secondary signals that shape the immune response [24,25]. PD1 has 2 ligands PD-ligand 1 (PD-L1) which is broadly expressed on haematopoietic and parenchymal cells including pancreatic cell while PD-L2 is restricted to macrophages and dendritic cells [26]. Mice deficient in PD1 developed autoimmune diseases despite different genetic back grounds [27]. Nishimura et al. [28] reported that allele A of a SNP PD1.3 in intron 4 is associated with SLE in Europeans and Mexicans with a relative risk of 2.6 and 3.5 respectively.

Recently, Zamani et al. [29] investigated for PD1 gene polymorphism in patients with antisperm antibody related infertility in Iranian men. They performed genotyping by PCR and restriction enzyme digestion (PCR-restriction fragment length polymorphisms (RFLP) for PD 1.3 polymorphism in 145 cases (61 of ASA related infertility and 84 controls). They found a lower frequency of GG and GA and higher frequency of AA genotype in patients vs. controls (29.6%, 32.7%, and 37% in patients vs. 41.6%, 38.2%, and 20.2% in controls ( $p=0.042$ ,  $p=0.035$ ,  $p=0.0001$ ) respectively. Also a significantly higher frequency of an allele in patients (55.8%,  $p=0.0012$ ) as compared to controls (39.1%) was observed. Hence they concluded that a correlation exists between PD-1 gene polymorphism and susceptibility to ASA related infertility In their group investigated.

In a previous study el-Roiey et al. [30] had investigated the autoimmune profile to search for antinuclear antibodies, autoantibodies in (IgG, igM, and IgA isotypes) to 7 phospholipids (cardiolipin, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidylinositol and phosphatidic acid) and to 4 histone subfractions (H1, H2A, H3, H4) and to 4 polynucleotides (ssDNA, dsDNA, poly(I) and poly (dT) total immunoglobulin levels and ASAs titres in sera from 25 women and 27 men positive for ASAs. The sera were also evaluated for the presence of a common anti DNA epitope and an anticardiolipin idio type. IgG levels were elevated in both groups while only women with ASA showed elevated total IgM. 24% of women and 11% of men with ASAs demonstrated antinuclear antibodies $>1:40$ . The most striking autoabnormalities were found amongst anti phospholipid antibodies. Women demonstrated higher auto antibody production as compared to males. A significant correlation was found between ASA and Anticardiolipin antibody (ACA) and IgA antiphosphatidylinositol in women and between ASAs and IgA Phosphatidyl serine antibodies in men. The presence of ACA and anti DNA Antibody idiotypes was significantly more frequent in women than in men. Identification of 24 of 25 women and 26 of 27 men with ASAs was correctly predicted with the use of a discriminant analysis and variable selected using a mathematical model. Hence they concluded that women and men respond differently to sperm antigens. The apparent cross-reactivity

suggests that a polyclonal B cell activation similar to that seen in autoimmune diseases occurs in patients of ASAs related infertility.

How polyclonal B and T cells over activation occur in autoimmune conditions has remained difficult to understand and remains an on-going challenge. PD-1 like CTLA4 can be induced in different immune cell types like CD4<sup>+</sup>T cell, CD8<sup>+</sup> T cell NKT Cell, B cell as well as monocyte [26]. Normally interactions between PD1 and PDL1 inhibit positive selection during the double negative to CD4<sup>+</sup>CD8<sup>+</sup>(DP) maturational stage [31]. These DP thymocytes undergo DNA rearrangements in the TCR $\alpha$  chain locus and are subsequently subjected to positive and negative selections [32-34]. Only positively selected thymocytes mature into CD4<sup>+</sup> or CD8<sup>+</sup> single positive (SP) cells. A maturation process has been further defined within the DN compartment, by the expression of IL-2receptor $\alpha$  chain (CD25) and the phagocytic glycoprotein 1(CD44). CD44<sup>+</sup> CD25<sup>-</sup> DN cells mature in the order of CD44<sup>+</sup> CD25<sup>+</sup>, CD44<sup>-</sup> CD25<sup>+</sup> and CD44<sup>-</sup> CD25<sup>-</sup> cells [35,36]. Earlier Nishimura et al. [31] had demonstrated that although PD1 was expressed only in 3-5% of total thymocytes, 34% of the CD4<sup>+</sup> CD8<sup>-</sup> double negative (DN) fraction are PD-1<sup>+</sup> cells with two distinct expression levels (low and high). PD-1 high thymocytes belonged to TCR  $\gamma\delta$  lineage cells. In the DN compartment of the TCR  $\alpha\beta$  lineage, PD-1 expression started at the low level from the CD44<sup>+</sup> CD25<sup>+</sup> stage and the majority of the thymocytes expressed PD-1 at the CD44<sup>-</sup> CD25<sup>-</sup> stage in which thymocytes express TCR  $\beta$  chains. The fraction of the PD-1 low cells in the CD4<sup>+</sup> CD8<sup>+</sup> double positive (DP) compartment was very small (<5%) but increased by stimulation with the anti-CD3 antibody, although the total number of DP cells were drastically reduced. Hence they concluded that PD-1 expression is specifically induced at the stages preceding clonal selection [37]. PD1 is not only important for negative selection [38] but also regulates self-reactive T cells which escapes negative selection to periphery [39]. Mouse models of autoimmune disease show that PD:PDL1 interaction besides being important in the primary phase of T cell activation and expansion, also modulate T cell effector function upon antigen reencounter [40].

Previous studies showing PD1 gene polymorphisms correlate with autoimmune diseases like ankylosing spondylitis [41], rheumatoid arthritis [42], SLE [43]. PD1.3 SNP has been associated with type 1 diabetes [44], multiple sclerosis [45], ankylosing spondylitis [46]. Further there is a suggestion that increased number of  $\alpha\beta$  and  $\gamma\delta$  T cell lineage with ASA suggests that local autoimmune Tcell mediated immune reaction towards spermatozooids [19,47] may be responsible for elimination of sperms from reproductive tract and possibly upregulation of PD1 upon activation of T cells could potentially reverse this with the recent demonstration of PD1 ligand *in vitro* by Interferon  $\gamma$  treatment in mouse testis sertoli cells *in vitro* [48]. Dal Secco [48] showed that in response to interferon gamma, mouse sertoli cells, strongly upregulated the negative costimulatory molecule -PD-1 (B7-H1/CD274) although remaining devoid of positive costimulatory molecules. PD-1/B-7-H1 blockade on sertoli cell surface was associated with marked proliferation of CD8<sup>+</sup> Tcells co-cultured with sertoli cells. Since Interferon gamma stimulated sertoli cells were found to express along with PD1, MHC class II, they hypothesized that possibly sertoli cells could function as nonprofessional tolerogenic APC by inducing enrichment in Treg population in a mixedT lymphocyte population. Co-culturing T cells with sertoli cells did induce CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg and a decrease in CD4<sup>+</sup>CD25<sup>-</sup> Tcells which suggest sertoli cell mediated T reg conversion; while this process was PD1 independent. Hence they concluded sertoli cells can potentially downregulate the immune responses locally either by directly inhibiting CD8<sup>+</sup> T cells proliferation through PD-1 on one hand or by increasing Treg cells which might suppress other bystander T cells.



Recently, Nasr et al. [49] in trying to understand the mechanism of immunological privilege for testis for transplantation tolerance found that by transplantation of islets in testis, an immunologically privileged site, much less memory CD8<sup>+</sup> T cells got generated but induced more Ag-specific CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in a conventional site (beneath renal capsule), although these allografts got rejected within 42 days (although greater survival than renal subcapsular islet allografts). Blocking CD40/CD40L costimulation induced tolerance in testicular allografts but not in renal ones. This tolerance to intratesticular islet allografts was broken by either the transfer of memory CD8<sup>+</sup> T cells or deletion of CD25<sup>+</sup> T cells. Thus Nasr et al. [49] concluded that transplantation tolerance requires both costimulatory blockade, along with a favourable balance between memory and regulatory T cells.

Dendritic Cells (DC's) are the key antigen presenting cells and they play an important role by not only by priming naïve T cells but are also important for maintenance of peripheral T cell tolerance. Cognate interactions between Foxp3(+) Treg and steady DC's are crucial for the tolerogenic potential of DC's. Muth et al. [50] using DIETER mice showed that following induction of antigen presentation selectively on DC's without altering their maturation show here that breakdown of CD8(+) T cell tolerance which ensues after depletion of CD4(+) T cells is driven by a positive feedback loop in which autoreactive CD8(+) T cells activate DC's via CD40. Hence they concluded this data identified that ligation of CD40 on DC's as a stimulus which prevents autoreactive T cell priming when regulatory T cell suppression fails and suggested that a feedback from autoreactive T cells to DCs may contribute to the well documented involvement of CD40 in many autoimmune diseases.

## Conclusions

Further studies are required to corroborate studies by Zamani et al. [29] and simultaneous study of antiphospholipid antibodies could go a long way in helping solve the puzzle of ASAs rather than just sticking to ICSI or IUI for cervical factor infertility or unexplained infertility as has been our current approach. A successful orthotopic testicular transplantation in mice [51] further goes to show how understanding this immune privilege of testis may help in future even in testicular transplantation in men with undescended testis or following cryopreservation after cancer surgery or any other immune cause of infertility, besides finding simpler answers for men who get immune infertility just with a subtle infection of chlamydia trachomatis which goes even unnoticed as only in 4 semen samples Munoz et al. could demonstrate IgA antibodies in semen following chlamydia infection although IgG was positive and gamma delta cells had appeared.

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