UNIVERSITÀ DEGLI STUDI DI MILANO

PHD COURSE IN CLINICAL RESEARCH

IMMUNOLOGICAL CHARACTERIZATION OF SEVERE INFERTILE MEN REVEALED ALTERATIONS OF THE LYMPHOID AND MYELOID COMPARTMENTS

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INDEX

I. INTRODUCTION 5
1. Male infertility 5
1.1 Diagnostic work-up in male infertile patients 5
1.1.1 Medical history and Physical examination 5
1.1.2 Semen analysis 6
1.1.3 Hormone levels 9
1.1.4 Imaging 9
1.1.5 Genetic screening 10
1.2 Causes of male infertility 10
1.2.1 Physical problems 11
1.2.2 Hormonal defects 12
1.2.3 Environment and lifestyle 13
1.2.4 Genetic and Epigenetic factors 13
1.3 The role of cancer in male infertility 14
1.3.1 Testis cancer 14
1.3.2 Prostate cancer 16
1.3.3 Other cancers 17
1.4 Comorbidities and male infertility 17
1.4.1 Cardiovascular disease 22
1.4.2 Insulin resistance and diabetes 24
1.4.3 Metabolic syndrome 25
1.4.4 Autoimmune diseases 26
1.4.5 Mortality 27
1.5 The role of age and aging 28
1.6 Insights and possible treatments in male infertility 29
2. The Testis: an immune privilege 31
2.1 Resident immune cells in the testis 31
2.1.1 Macrophages 31
2.1.2 Dendritic cells 32
2.1.3 Lymphocytes 33
2.1.4 Others immune cells 33
2.2 Tolerogenic molecules associated with infertility: HLA-G and IL-10 35
2.2.1 HLA-G 36
2.2.2 Interleukin-10 37
2.3 IL-10 related regulatory cells: DC-10 and Tr1 cells 38
2.3.1 DC-10 38

2.3.2 T regulatory Type 1 (Tr1) cells 40
2.3.3 Pro-inflammatory and senescent signature in infertile men……..42
II. AIM AND DESIGN OF THE STUDY 44
III. MATERIALS AND METHODS 45
1. Study population 45
2. Clinical and anamnestic variables 46
3. Experimental Methods 47
4. Statistical analysis 55
IV. RESULTS 56
1. Seminal, clinical, and hormonal, and characteristics of the whole cohort 56
2. Immunologic profile 57
2.1 Leucocyte composition in seminal fluid 58
2.2 Dendritic cell evaluation in the seminal fluid of infertile men 59
2.3 Cytokines and chemokines in seminal plasma 60
2.4 Leucocyte subsets in whole blood 61
2.5 Regulatory T cells and DC-10 in peripheral blood 62
2.6 T cells characterization in peripheral blood 64
2.7 Functional characterization of T cells 67
2.8 Chemokines and cytokines in peripheral blood……..69
V. DISCUSSION 71
BIBLIOGRAPHY 77

I. INTRODUCTION

1. Male infertility

Couple's infertility is defined as not conceiving a pregnancy after at least 12 months of unprotected intercourses regardless of whether or not a pregnancy ultimately occurs (World Health Organization (WHO) Manual for the Standardized Investigation and Diagnosis of the Infertile Couple, 2000).

Primary infertility refers to couples that have never had a child and cannot achieve pregnancy after at least twelve consecutive months having sex without using birth control methods. Secondary infertility refers to infertile couples who have been able to achieve pregnancy at least once before.

In Western countries infertility touches about 15% of couples of reproductive age. Large-population-based studies have found that approximately 50% of all cases of infertility happen because of female factors, 20 to 30% due to male factors, and 20 to 30% because of common causes of both genders (Babakhanzadeh et al., 2020).

1.1 Diagnostic work-up in male infertile patients

Current Guidelines mandate a focused diagnostic work-up of both partners of infertile couples. For males, this should include a medical and reproductive history, a physical examination, hormonal investigation and semen analysis, with adherence to World Health Organization (WHO) reference values

1.1.1 Medical history and Physical examination

The medical history must contain the reproductive history, a comprehensive surgical and medical history, including allergies and treatments, and excluding any potential known gonadotoxins. Moreover, medical history should contain family history, including testis cancer; a survey of past infections, for example respiratory infections and sexually transmitted diseases; a comprehensive analysis of (any) comorbid conditions, such as diabetes mellitus, obesity, metabolic syndrome (MetS), and hypertension (Salonia et al., EUA guidelines on Sexual and Reproductive health, 2020).

Typical findings from the history of a patient with infertility include:

- cryptorchidism (uni- or bilateral);
- testicular torsion and trauma;
- genitourinary infections;
- exposure to environmental toxins;
- gonadotoxic medications (anabolic drugs, chemotherapeutic agents, etc.);
- exposure to radiation or cytotoxic agents.

A complete physical examination is required over the diagnostic assessment of every infertile male. The physical exam must include the palpation of the testes and the measurement of their consistency, size and texture. In clinical practice, testis volume is measured by Prader's orchidometer, which consists of a string of twelve numbered wooden or plastic beads of increasing size from about 1 to 25 milliliters (Salonia et al., EUA guidelines on Sexual and Reproductive health, 2020).

The mean Prader's orchidometer-derived testis volume reported in the European general population is 20.0±5.0 mL; conversely, for in infertile patients is 18.0±5.0 mL (Jensen et al., 2004a).

In addition, physical examination should include the determination of presence of vas deferens, varicocele and the fullness of epididymis. Likewise, should be analyzed any palpable abnormalities of the testis and other physical alterations, for example epispadias, hypospadias, fibrotic nodules, phimosis and short frenulum. Lastly, the assessment of secondary sexual characteristics, including hair distribution and gynecomastia, that is an endocrine disorder in which a noncancerous increase in the size of male breast tissue occurs should be evaluated (Salonia et al., EUA guidelines on Sexual and Reproductive health, 2020)

1.1.2 Semen analysis

Semen analysis is the cornerstone of the laboratory evaluation of the infertile man and helps to describe the severity of male factors. Before semen collection all men must abstain from sexual activity for 2 to 3 days (WHO Laboratory Manual for the Examination and Processing oh Human Semen, 2010).

A complete andrological examination is required if semen analysis suggests alterations compared to reference values (Table 1).

Semen and ejaculate analysis have been standardized by the WHO (last update, WHO Laboratory Manual for the Examination and Processing oh Human Semen (5th edn) - WHO Laboratory Manual for the Examination and Processing oh Human Semen, 2010). A complete analysis according to these widely recognized standardization is necessary, since treatment decisions are based on sperm parameters.

Table 1: Lower reference limits for semen (Salonia et al., EUA guidelines on Sexual and Reproductive health, 2020).

Parameter	**Lower reference limit (range)**
Semen volume (mL)	1.5 (1.4-1.7)
Total sperm number (10^6/ejaculate)	39 (33-46)
Sperm concentration (10^6/mL)	15 (12-16)
Total motility (PR + NP)	40 (38-42)
Progressive motility (PR, %)	32 (31-34)
Vitality (live spermatozoa, %)	58 (55-63)
Sperm morphology (normal forms, %)	4 (3.0-4.0)
Other consensus threshold values	
pH	> 7.2
Peroxidase-positive leukocytes (10^6/mL)	< 1.0
Optional investigations	
MAR test (motile spermatozoa with bound particles, %)	< 50
Immunobead test (motile spermatozoa with bound beads, %)	< 50
Seminal zinc (µmol/ejaculate)	≥ 2.4
Seminal fructose (µmol/ejaculate)	≥ 13
Seminal neutral glucosidase (mU/ejaculate)	≤ 20

CIs = confidence intervals; MAR = mixed antiglobulin reaction NP = non-progressive; PR = progressive.

It is important to distinguish among the following conditions (Salonia et al, EAU guidelines on Sexual and Reproductive health, 2020):

- oligozoospermia: < 15 million spermatozoa/mL;
- asthenozoospermia: < 32% progressive motile spermatozoa;

- teratozoospermia: < 4% normal forms.

Frequently, all these three anomalies appear at the same time and this condition is defined as oligo-astheno-teratozoospermia (OAT) syndrome. In severe cases of oligozoospermia (i.e., spermatozoa < 1 million/mL), as in azoospermia, a higher incidence of genetic abnormalities and obstruction of the male genital tract are present (Grimes et al., 2007).

1.1.3 Hormone levels

Endocrine disorders, particularly hormonal abnormalities of the HPG are unusual in men with normal semen parameters.

The first hormonal analysis must comprise measurement of total testosterone and, at least, FSH levels. If circulating total testosterone is low (<3 ng/mL), additional evaluations are usually required and must include a second early morning measurement of total testosterone and the assessment of lutein hormone (LH) and prolactin (PRL) (Practice Committee of the American Society for Reproductive Medicine, 2015).

Both LH and FSH are involved throughout spermatogenesis produced by gonadotropic cells in the anterior pituitary gland that act synergistically to stimulate testosterone production by Leydig cells and the activation of Sertoli cells, respectively.

While serum gonadotropin (i.e., FSH and LH) concentrations vary due to their pulsatile secretion, a single evaluation typically is enough to define the clinical endocrine status. The relationship among serum levels of testosterone, PRL, FSH and LH could help to understand the causes of abnormal total testosterone levels (Practice Committee of the American Society for Reproductive Medicine, 2015).

While many men with impaired spermatogenesis present normal serum FSH level, a significantly elevated serum FSH concentration suggests an abnormality of the spermatogenesis. Moreover, in men with FSH levels in the upper normal range, spermatogenesis may be altered as well. Finally, evaluation of the thyroid-stimulating hormone (TSH) levels could be required in men who need an even more thorough endocrine evaluation (Practice Committee of the American Society for Reproductive Medicine, 2015).

Serum inhibin B levels are also considered as a marker of spermatogenesis. Indeed, inhibin B concentration is usually remarkably decreased in infertile men. Furthermore, inhibin B levels are associated with sperm parameters better than FSH levels. However, the cost of measuring inhibin B is remarkably higher, thus at present FSH continues to be the favorite test for screening purposes (Kumanov et al., 2006).

1.1.4 Imaging

Ultrasound (US) evaluation is currently commonly applied over the evaluation of male reproductive issues (Lotti et al., 2015)

TRUS. Transrectal US (TRUS) has also recently become important over the diagnostic work-up of infertile men. Indeed, it allows the assessment of congenital bilateral absence of the vas deferens (CBAVD), either the presence or the absence of seminal vesicles and/or the epididymis. TRUS plays a crucial role in evaluating abnormalities associated with ejaculatory duct obstruction (e.g., ejaculatory duct cysts, seminal vesicles dilatation or hypoplasia/atrophy); moreover, retrograde ejaculation may be excluded as a differential diagnosis. Furthermore, TRUS can detect a decreased prostate volume, sometime suggestive of hypogonadism, or conversely a prostate enlargement, which can be associated to metabolic abnormalities or aging (Lotti et al., 2015).

Scrotal US. Scrotal US could identify signs and markers of testicular dysgenesis, frequently associated to an altered form of spermatogenesis, an increased risk of malignancy or of testicular lesions suggestive for malignancy. Moreover, hyperemia is frequently detected in the case of epididymo-orchitis or, once again, in certain types of malignant diseases (e.g., lymphoma and leukemia); while a lower testis vascularization may be suggestive for testicular torsion (Isidori et al., 2008).

The clinical management of varicocele is primarily based on physical examination; however, CDUS is helpful also in evaluating a pathologic venous reflux (Liguori et al., 2004).

The dilatation of head and/or tail of the epididymis may be suggestive of male genital tract inflammation or obstruction and both conditions are associated, along with echo-texture anomalies, with the alteration of sperm parameters (Lee et al., 2008).

1.1.5 Genetic screening

The most frequent genetic abnormalities found in infertile men are structural and numeric chromosomal aberrations that damage testicular function, and Y-chromosome microdeletions that are related to alterations in spermatogenesis. Moreover, men with CBAVD often present an abnormality on the cystic fibrosis transmembrane conductance regulator (CFTR) gene.

According to recent EAU Guidelines it is suggested to perform karyotype examination in all men with sperm concentration <10 x10^6/ml, to request CFTR mutation test and and Y-microdeletions in infertile men with <5 x10^6/ml.

1.2 Causes of male infertility

Male fertility can be compromised due to several factors, examples of which are listed in Table 2.

Broadly speaking, the most relevant factors associated with male infertility are immunological factors, endocrine disturbances, congenital or acquired urogenital abnormalities, urogenital tract infections, malignancies, genetic or epigenetic abnormalities, and factors associated with an increased scrotal temperature, such as varicocele. As detailed, in as many as 30-40% of cases no male factors associated to infertility are found: this condition is called “idiopathic male infertility”.

Table 2. Summary of the main male infertility causes and associated factors
(Salonia et al., EUA guidelines on Sexual and Reproductive health, 2020).

Diagnosis	Unselected patients	Azoospermic patients
All	100%	11.2%
Infertility of known (possible) cause	42.6%	42.6%
Maldescended testes	8.4	17.2
Varicocele	14.8	10.9
Sperm autoantibodies	3.9	-
Testicular tumour	1.2	2.8
Others	5.0	1.2
Idiopathic infertility	30.0%	13.3
Hypogonadism	10.1%	16.4%
Klinefelter’s syndrome (47, XXY)	*2.6*	13.7

XX male	*0.1*	0.6
Primary hypogonadism of unknown cause	*2.3*	0.8
Secondary (hypogonadotropic) hypogonadism	*1.6*	1.9
Kallmann syndrome	*0.3*	0.5
Idiopathic hypogonadotropic hypogonadism	*0.4*	0.4
Residual after pituitary surgery	*< 0.1*	0.3
Late-onset hypogonadism	*2.2*	-
Constitutional delay of puberty	*1.4*	-
Others	0.8	0.8
General/systemic disease	2.2%	0.5%
Cryopreservation due to malignant disease	7.8%	12.5%
Testicular tumour	5.0	4.3
Lymphoma	1.5	4.6
Leukaemia	0.7	2.2
Sarcoma	0.6	0.9
Disturbance of erection/ejaculation	2.4%	-
Obstruction	2.2	10.3
Vasectomy	0.9	5.3
Cystic fibrosis (CBAVD)	0.5	3.0
Others	0.8	1.9

CBAVD = Congenital Bilateral Absence of the Vas Deferens

1.2.1 Physical problems

Varicocele, a pathologic enlargement of testicular vessels affecting up to 15% of men in the general population, is one of the most frequent male infertility causes, thus being associated with infertility in up to 25% of cases.

Testicular torsion within the testicle sac determines testicular damage because of pressure on the sperm vessels and consequently compromise testicular circulation (Sengupta et al., 2017).

Acute and chronic genital tract infections can determine infertility in men, especially sexually transmitted diseases (STD), such as Chlamydia and Gonorrhea, due to their obstruction in the epididymis and mumps that causes testicular atrophy and infertility in

men. Indeed, a recent study (Boeri et al., 2020) points out the importance of an accurate investigation of semen infection in the everyday clinical practice diagnostic workup of infertile men, having found that one out of five men presenting for a couple's primary infertility had asymptomatic semen infections, which were significantly associated with impaired sperm concentration.

1.2.2 Hormonal defects

The male reproductive hormone axis is known as the HPG axis and it contains three major components: the hypothalamus, the pituitary gland and the testes. HPG axis operates regularly to supply the correct concentration of hormones needed for male sexual development and functioning. Any alterations over this system can ultimately determine infertility (Corradi et al., 2016).

When the hypothalamus is unable to supply gonadotropic releasing hormone (GnRH), this condition results in a deficiency of testosterone production and the interruption of sperm production. On the other hand, GnRH absence causes a set of disorders known as hypogonadotropic hypogonadism.

Similarly, the incapacity of the pituitary gland to produce correct amounts of FSH and luteinizing hormone (LH) may lead to a failure in stimulating the testes, along with a depressed production of sperm and testosterone. Long-term hormonal therapy is required for men with pituitary deficiency, which may determine a number of complications, such as heart disease, bone defects and diabetes mellitus (Pienkowski et al., 2016).

Increased levels of FSH and LH along with low testosterone levels depict the condition of primary hypogonadism (otherwise called hypergonadotrophic hypogonadism), leading to abnormalities in spermatogenesis (Wdowiak et al., 2014).

Lastly, higher concentrations of prolactin may be associated with reduce libido and altered sperm production; indeed, hyperprolactinemia leads to male infertility in about 10% of men with oligospermia. Usually the use of dopamine agonists, such as cabergoline, is the correct treatment for this condition (Dabbous and Atkin, 2017).

1.2.3 Environment and lifestyle

Exposure to dangerous substances in the workplace, including radiation, insecticides, solvents, silicones, adhesives and similar substances – can lead to reduced sperm production and infertility (Liu et al., 2017).

Regarding smoking and alcohol consumption, there is no unequivocal evidence of a damaging role on fertility outcomes and sperm parameters (Boeri et al., 2019). Moreover, a decrease in sperm concentration and sperm quality may be related to alcohol consumption, cigarette smoking and poor nutrition, particularly saturated fat intake.

The recurrent use of illicit drugs, otherwise called substances of abuse, such as cannabinoids and cocaine have been associated to a lower sperm concentration and decreased urinary testosterone in men (Onyije, 2012).

1.2.4 Genetic and Epigenetic factors

Both genetic and epigenetic factors play an important role in male infertility. About 2% of men with oligospermia and 14% of men with azoospermia present chromosomal anomalies; this percentage is increased compared to general population, where this rate is about 0.6% (Pylyp et al., 2013).

The most frequently genetic cause of azoospermia is Klinefelter syndrome which accounts for about 14% of male infertility cases. This syndrome is a sex chromosomal aneuploidy characterized by a karyotype of 47XXY, and is responsible for about two thirds of all chromosomal abnormalities described in infertile men, and is mostly associated with non-obstructive azoospermia (Corona et al., 2017)

Microdelections on the Y-chromosome are termed AZFa, AZFb and AZFc deletions (Vogt et al., 1996). Clinically relevant deletions remove partially, or in most cases completely, one or more of the AZF regions, and are the most frequent molecular genetic cause of severe oligozoospermia and azoospermia (Krausz et al., 2017).

Moreover, certain gene mutations with pathological syndromes, e.g., CBAVD, are caused by a mutation in the CFTR and leads to impaired spermatogenesis and obstructive azoospermia in 80 to 90% of cases (Jarzabek et al., 2004).

1.3 The role of cancer in male infertility

It has been shown that infertile men present an increased risk to develop several malignancies, with the most thoroughly investigated association between infertility and testicular cancer. In infertile men the risk of developing testicular malignancies is double compared to the fertile population; similar findings have been also reported for high-grade prostate cancer.

Moreover, a number of studies have also shown that male infertility can be consider a biomarker for cancer risk in first- and second-degree relatives. The risk of testicular cancer is 52% higher in first-degree relatives of infertile men than in relatives of fertile control men. Moreover, male infertility has been related to a two- to threefold elevation in risk of childhood cancer in the siblings of infertile men (Hanson et al., 2018).

The relationship between cancer and infertility underlies, beyond the semen analysis, the importance of clinical evaluation and long-term follow-up in infertile men. Particularly, a detailed urologic evaluation, including scrotal ultrasound, may be indicated to screen infertile men for testicular cancer (Hanson et al., 2016).

1.3.1 Testis cancer

One of the most well described association between malignancy and male infertility is that with testicular cancer. In some ways, this is not unexpected, since testicular tumors and spermatogenesis are both some of the highest-throughput processes in the human body in the benign and malignant states, respectively.

Hanson et al. performed a large retrospective cohort study from the Utah Population Database including 20.433 men undergoing semen analysis. The authors showed an elevated risk of testicular cancer in patients with oligozoospermia based on sperm concentration (hazard ratio (HR 11.9) and sperm count (HR 10.3). Moreover, men in the lowest quartile of morphology, viability, motility or total motile count were also described to have an increased risk of testicular cancer (Hanson et al., 2016).

The detection of scrotal masses in the infertile male is not unusual, although most of these findings are benign and can be safely followed with surveillance.

Scrotal US is widely used in everyday clinical practice with oligozoospermia or azoospermia, as infertility has been found to be an additional risk factor for testicular cancer (Bieniek et al., 2018)

One of the first study on this topic, was performed by Pierik and colleagues which showed that ultrasound appears as a superior means to detect testicular abnormalities compared to physical examination. The results showed that 67% of ultrasound findings were not detectable on palpation, and only one out of seven testicular tumors were discovered by physical exam alone (Pierik et al.,1999).

The association between testicular cancer and male infertility is multifactorial, with a combination of hormonal, genetic, in utero, and environmental factors contributing to the development of cancer in the infertile population.

Testicular abnormalities are also significant risk factors for the development of malignancies. For instance, cryptorchidism is associated with an increased risk of testicular cancer ranging from four to nine and is strongly associated with azoospermia and infertility in adults. Although improvements in fertility outcomes for patients who have undergone surgical correction of cryptorchidism as infants, infertility in adulthood remains a concern. Indeed, almost 30% of men with a history of unilateral cryptorchidism and about 80% of adult men with a history of bilateral cryptorchidism show abnormal sperm parameters (Cortes et al., 2003).

Between 20% and 30% of male infertility is thought to be caused by genetic defects, and some of these genetic changes may also play a role in the development of testicular cancer. The possibility of an underlying genetic component to the link between male infertility and testicular cancer is highlighted by epidemiologic studies suggesting that the brothers of men with testicular cancer may also have decreased fertility and be at an elevated risk of developing testicular cancer (Hanson et al., 2017).

Finally, environmental factors, such as smoking, may predispose men to both infertility and testicular cancer. Smoking has been proposed in certain studies as a risk factor for testicular cancer, and men who smoke have been shown to have higher rates of erectile dysfunction, elevations in chromosomally abnormal sperm, and decreased sperm concentration, motility, and morphology (Eisenberg et al., 2016).

1.3.2 Prostate cancer

A possible association between prostate cancer and infertility is less well established compared to testicular cancer, with conflicting data in literature. Walsh et al., conducted a retrospective cohort study, analyzing 22.562 Californian men who had undergone fertility testing. The author discovered that men with infertility did not present an overall higher risk of prostate cancer, although a subcategories analysis showed that infertile patients presented a greater risk of developing high-grade prostate cancer in comparison to age-matched control men (Walsh et al., 2010).

On the other hand, Hanson et al. performed a 2016 retrospective cohort study, including 20.433 men who underwent semen analysis, but the authors fail to find a relationship between greater prostate cancer risk and infertility (Hanson et al, 2016). Moreover, a Swedish case-control study of 445 prostate cancer men, has observed lower probabilities of developing prostate cancer in infertile men (Ruhayel et al., 2010).

Remarkably, Walsh et al., studying the association between infertility and prostate cancer, suggested a significant chronologic separation between a diagnosis of prostate cancer and the diagnosis of infertility. In fact, a diagnosis of infertility in a men's fourth decade of life seems to bestow a higher risk of prostate cancer in their sixth decade (Walsh et al., 2011).

Along with testicular cancer, the hypotheses concerning genetic influences, hormonal functions and environmental modulators have been suggested to clarify the association between prostate cancer and male infertility, as well.

For example, with impaired gonadal function, the prostate may present a higher risk of cancer due to aberrant signals received during key phases of its development. Moreover, problems with DNA mismatch repair or alterations of CAG sequences in genes that encode the androgen receptor may have consequences for both prostate cancer and male infertility (Walsh, 2011).

However, identify the mechanisms associating prostate cancer with male infertility and validate a causal relationship between them remains a challenge.

1.3.3 Other cancers

The relationships between other types of cancer and male infertility are less known in comparison to testicular and prostate cancer. To this regard, Eisenberg et al, using a large United States insurance claims dataset, performed a study including 76.083 infertile men. The authors showed that infertile men present higher risk of thyroid cancer, bladder cancer, melanoma, leukemia, Hodgkin lymphoma and non-Hodgkin lymphoma. In the same study, infertile men presented a 49% increased overall cancer risk in comparison to the control group (Eisenberg et al., 2015).

Moreover, Hanson et al. performed a study including 20.433 men which underwent semen analysis. The authors found a double risk of developing melanoma in men within the highest quartile of total motile sperm count. On the other hand, they showed a higher risk of malignancies related to decrease sperm motility; although, they did not describe an elevated risk of cancers in relation to alterations in viability, total motile counts or morphology (Hanson et al., 2016).

Aside from testicular and prostate cancer, described relationships between other types of cancer and male infertility are relatively scarce and further studies are needed to validate these relationships and identify possible causal pathways.

1.4 Comorbidities and male infertility

Numerous studies have attempted to explain the correlation between male infertility and health status (Table 3).

Table 3. Relevant studies assessing the association between overall health and mortality and male infertility (Capogrosso et al., 2018).

Study	Study design	Patients	Main outcome	Main findings
Salonia *et al.*	Case-control	344 infertile men *vs.* 293 controls	Infertility and comorbidities	Infertile men had significantly higher CCI score (0.33 *vs.* 0.14)
Eisenberg *et al.*	Retrospective cohort	9387 infertile men	Semen quality and comorbidities	Higher CCI scores were significantly associated with worse semen parameters Men with hypertension, peripheral vascular and cerebrovascular disease, and non-ischemic heart disease had higher rates of semen abnormalities
Ventimiglia *et al.*	Retrospective cohort	2100 infertile men	Semen quality and comorbidities	Sperm concentration was inversely correlated with CCI score (OR=0.06; CI: 0.01-0.00)
Eisenberg *et al.*	Prospective cohort	501 couples attempting to conceive	Semen quality and comorbidities	Men with hypertension were had lower morphology scores compared to normotensive men (17% *vs.* 21%)
Eisenberg *et al.*	Prospective cohort	13,027 infertile men	Risk of developing comorbidities	Men with infertility had higher risk of developing subsequent comorbidities (any type)
Latif *et al.*	Prospective cohort	4712 infertile men	Semen quality and comorbidities	Higher risk of hospitalization among patients with sperm concentration <15 $\times 10^6$/mL (HR=1.5; CI: 1.4-1.6)

Salonia et al. presented a case-control study involving 344 European men with a diagnosis of male factor infertility and 293 age-comparable fertile men. They applied the Charlson Comorbidity Index (CCI) to accurately measure the burden of patient's comorbidities; the CCI is view as the most rational and valid index to evaluate the hospital-based comorbidity (Table 4) (Charlson et al., 1987).

Each comorbidity category has an associated weight (from 1 to 6), based on the adjusted risk of mortality or resource use, and the sum of all the weights results in a single comorbidity score for a patient. A score of zero indicates that no comorbidities were found. The higher the score, the more likely the predicted outcome will result in mortality or higher resource use.

The study revealed that infertile men showed considerably higher CCI scores in comparison to control groups (0.33 vs. 0.14; $P<0.01$). This relationship was preserved at multiparameter analysis after accounting for patients' educational status, BMI and age.

Table 4. Charlson comorbidities index score (Yang et al., 2016)

Comorbidity	Score
Prior myocardial infarction	1
Congestive heart failure	1
Peripheral vascular disease	1
Cerebrovascular disease	1
Dementia	1
Chronic pulmonary disease	1
Rheumatologic disease	1
Peptic ulcer disease	1
Mild liver disease	1
Diabetes	1
Cerebrovascular (hemiplegia) event	2
Moderate-to-severe renal disease	2
Diabetes with chronic complications	2
Cancer without metastases	2
Leukemia	2
Lymphoma	2
Moderate or severe liver disease	3
Metastatic solid tumor	6
Acquired immuno-deficiency syndrome (AIDS)	6

Remarkably, among comorbidities the authors described a higher percentage of diabetes mellitus (DM), pulmonary diseases, cardiovascular diseases (CVDs), liver disease and connective tissue disorders (Salonia et al., 2009).

These results were supported by a study including a cohort of 9,387 men with accessible semen analysis; greater CCI scores were related to reduced semen volume, lower concentration, decreased motility, worse morphology scores and lower total sperm count at uniparameter analysis. Among the examined comorbidities, nonmalignant diseases involving the skin and the endocrine and genitourinary systems were associated with greater rates of semen abnormalities (Eisenberg et al., 2015).

Later, longitudinal studies reinforce the relationship between male infertility and comorbidities. Indeed, data from the Marketscan claim dataset revealed that among 13.027 men diagnosed with male infertility, the possibility of developing successive comorbidities was considerably higher in relation to 23.860 controls. More specifically, the probability of DM, CVDs, and also pathological behaviors, for example the abuse of alcohol, was considerably higher in the infertile group (Eisenberg et al., 2016).

Ventimiglia et al. presented additional data concerning the association between CCI scores and semen parameters. In their cross-sectional study, with single-institution data of 2100 men requesting aid for couple infertility, sperm concentration was found being inversely and linearly associated with higher CCI scores. Comparably, a greater rate of patients with non-obstructive azoospermia (NOA) and sperm concentration $<15 \times 10^6$/mL had a CCI score ≥1. Moreover, after accounting for length of infertility, BMI and age, they presented a multiparameter analysis predicting a poorer health status (CCI≥1), showing a remarkable connection with sperm concentration (Ventimiglia et al., 2015).

Recently, Ventimiglia et al. proposed two different mechanisms to describe the relationship between comorbidities and infertility: the presence of a common mechanism promoting both infertility and subcategories associated to pathological conditions and comorbidities, directly conflict with male reproductive function (Figure 1) (Ventimiglia et al., 2016b).

Regarding the first model, substantial evidence indicates that men with reproductive health problems may have deficiency of certain regulatory genes, involved not only to abnormal spermatogenesis but also to a greater probability of cancer and aberrant mechanisms of controlling cell division. In this line, a lot of DNA repair genes earlier discovered in cancer syndromes have been observed to regulate crucial processes in gamete production (Matzuk et al., 2008).

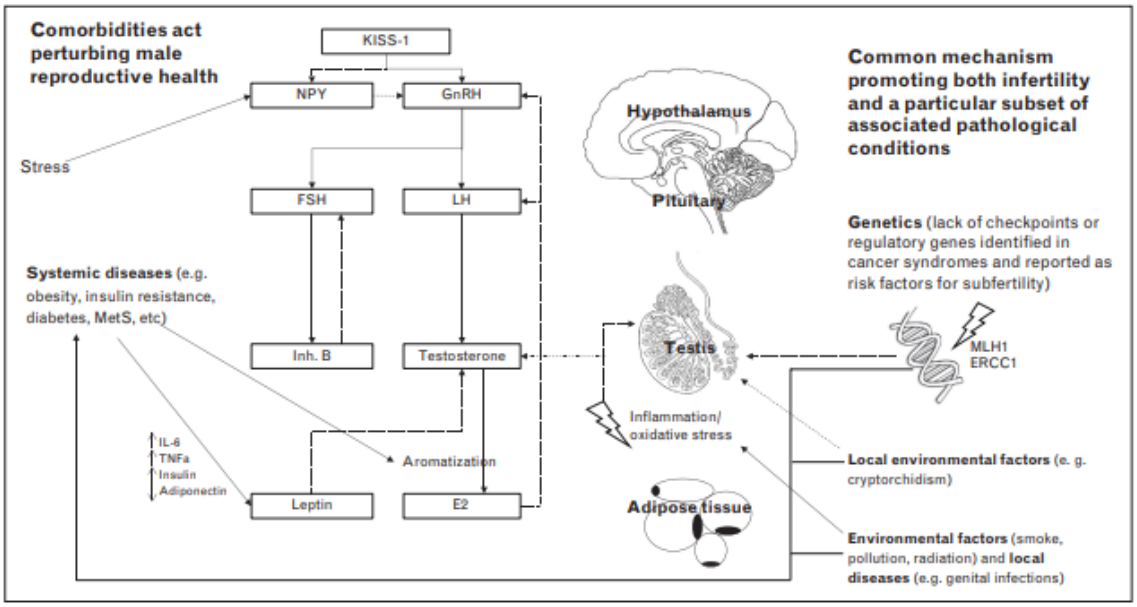


Figure 1. Two possible models describing the connection between male infertility and comorbidities. The presence of a common mechanism promoting both infertility and a subcategories of associated pathological conditions and comorbidities; directly interfere with male reproductive function. E2, estradiol; ERCC1, DNA repair gene ERCC1; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; IL-6, interleukin 6; Inh. B, inhibin B; LH, luteinizing hormone; MetS, metabolic syndrome; MLH1, Lynch syndrome gene MLH1; NPY, neuropeptide Y; TNF-α, tumor necrosis factor alpha Solid arrows indicate stimulation; dashed arrows represent inhibition (Ventimiglia et al., 2016b).

For instance, a single nucleotide polymorphism in the Lynch syndrome gene MLH1, involved in mismatch repair pathway, was defined as a risk factor in men with oligozoospermia and azoospermia. Furthermore, this single nucleotide polymorphism was associated, in men with normal sperm concentration as well, with a higher risk of DNA fragmentation (Ji et al., 2012).

Concerning to this issue, the problem of cryptorchidism is evident; undescended testis is one of the most important risk factors for testicular cancer and is also associated with impaired fertility. Postnatal germ-cell development after the first year is deteriorated in the undescended testis with an increasing risk of infertility with ageing. Despite of the genetic background alleged to partially connect cryptorchidism to both infertility and testicular cancer, data demonstrated a link between an increased proportion of cancer/infertility and delayed orchiopexy; that suggests a key role for 'local environmental factors' as well (Huff et al., 2001).

On the other hand, it is also possible that comorbidities per se may have a negative effect on male reproductive function. Indeed, several of the conditions observed, which are well-known to have a negative impact on reproductive function, are common in the male general population. La Vignera et al. showed that up to 39% of the adult population in the US has criteria indicative of metabolic syndrome (MetS); similar percentages were noted in the Italian population. Comparably, high proportions have been noted for dysmetabolic conditions, such as diabetes, obesity and also hypertension (La Vignera et al., 2012).

While hormonal homeostatic changes, for example higher rates of hypogonadism, carried on by MetS and obesity, have been extensively described, the consequences on semen parameters are still uncertain. Comparable results are described for diabetes; indeed, though not univocal, altered markers of spermatogenesis and semen parameters have been described in diabetic men. Although the greater of these potential detrimental factors have been studied separately, recent data suggest the idea of a shared pathophysiology pathway where they work together to alter overall reproductive health (Aggerholm et al., 2008).

1.4.1 Cardiovascular disease

The link between male infertility and CVDs has been proposed by many studies (Table 5).

Table 5. Relevant studies on the association between cardiovascular diseases and male infertility (Capogrosso et al., 2018).

Study	Study design	Patients	Main outcome	Main findings
Lawlor *et al.*	Retrospective cohort	4252 men from a population database	Offspring and CVDs	Men with no or 1 child show higher risk of CVDs than those with more children
Eisenberg *et al.*	Prospective cohort	137,903 men without prior CVDs	Offspring and risk of developing CVDs	Childless men had higher risk of death from CVDs (HR=1.17; CI: 1.03-1.32)
Punab *et al.*	Case-control	1737 infertile men *vs.* 325 controls	Primary causes of infertility	CVDs were significantly more prevalent among infertile men
Eisenberg *et al.*	Prospective cohort	501 couples attempting to conceive	Semen quality and comorbidities	Men with hypertension were had lower morphology scores compared to normotensive men (17% *vs.* 21%)
Eisenberg *et al.*	Retrospective cohort	9387 infertile men	Semen quality and comorbidities	Men with hypertension, peripheral vascular and cerebrovascular disease, and non-ischemic heart disease had higher rates of semen abnormalities
Eisenberg *et al.*	Case-control	76,083 infertile men *vs.* 112,655 vasectomized men *vs.* 760,830 controls	Incidence of comorbidities	Infertile men had higher risk of ischemic heart disease (HR=1.48; CI: 1.19-1.84)
Latif *et al.*	Prospective cohort	4712 infertile men	Semen quality and comorbidities	Higher risk of CVDs for patients with sperm concentration <15 $\times 10^6$/mL (HR=1.4; CI: 1.2-1.6)

CVDs: cardiovascular diseases; HR: hazard ratio; MI: male infertility.

The analysis of association between prevalence of CVDs with offspring number in an European population revealed that men with ≤1 child had a higher risk of CVDs as compared with those with more children (Lawlor et al., 2003).

A study comparing the CVDs risk and fatherhood, using data from the National Institutes of Health–American Association of Retired Persons Diet and Health Study, showed that childless men have higher risk of death from CVDs during the study period (an average of 10.2 years) in relation to fathers (Eisenberg et al., 2011).

From this point of view, childlessness could be considered as an incomplete surrogate for infertility, because childless men may not inevitably be infertile. Wang et al. show that men with varicoceles have a greater incidence of heart disease; while varicoceles may give a contribute to male infertility, the presence of a varicocele does not automatically determine infertility (Wang et al., 2018).

Eisenberg et al. described that peripheral vascular diseases, non-ischemic heart diseases, hypertension and cerebrovascular diseases, were related to a considerably greater percentage of any type of semen abnormality (Eisenberg et al., 2015).

In a recent study, analysis of 23.860 men tested for infertility, 79.099 vasectomized men and 13.027 men with a diagnosis of male infertility, after correcting for many confusing factors, such as obesity, health care utilization and smoking, showing that infertile men presented considerably greater risk of presenting ischemic heart disease compared to controls (HR=1.48; 95% CI: 1.19-1.84) and vasectomized men (1.20; 95% CI: 1.09-1.32) (Eisenberg et al., 2015).

In a more recent study, using insurance claim data, an attempt to verify the incidence of chronic medical conditions in infertile men was made. The study confirmed that men diagnosed with male-factor infertility had a higher possibility of developing ischemic heart disease compared to a first control group (men who had merely undergone fertility testing) and a second control group (men who had undergone vasectomy and were thought to be likely fertile) (Eisenberg et al., 2016).

1.4.2 Insulin resistance and diabetes

Insulin resistance and DM may affect male fertility. Agbaje et al. matched semen samples of healthy controls with insulin-dependent diabetic patients and showed a considerably decreased semen volume and increase sperm DNA fragmentation proportions among diabetic patients compared to healthy controls (Agbaje et al., 2007).

A study including 500 male partners, in whom 1.2% was discovered with either type 1 or type 2 DM, revealed that morphology and progressive sperm motility were considerably decreased in men with DM compared to controls. The authors found a higher rate of infertility among patients with diabetes, reporting a 35% of either primary or secondary male infertility compared to the control group (Bener et al., 2009).

Similarly, La Vignera et al. matched 32 patients with type 1 DM with 20 healthy controls; lower sperm concentration and progressive motility were found among young men with type 1 DM (La Vignera et al., 2009).

A meta-analysis including 12 observational studies which analyze the consequences of DM on seminal parameters of patients screened for fertility showed a 14.3% lower rate of motile cells in DM patients compared to healthy controls. Nevertheless, no difference was discovered in sperm morphology and total sperm count (Pergialiotis et al., 2016).

Finally, Glazer et al. conducted a large prospective trial including 39.516 patients from the Danish national IVF register, with the aim of evaluating the risk of further developing DM. Overall, 1.6% of men developed DM throughout follow-up; patients with oligospermia had a considerably increased risk (Hazard Ratio (HR)=1.44; 95% CI: 1.01-2.06) compared to fertile men, along with men with azoospermia (HR: 2.10; 95% CI: 1.25-3.56) and aspermia (HR=3.20; 95% CI: 1.00-10.31); these results powerfully propose a mutual pathogenic background between male infertility and diabetes (Glazer et al., 2017a).

Regarding lipids alterations, preclinical in vivo studies provided evidences that a low semen quality is connected to an abnormal lipid profile (Yamamoto et al., 1999) (Shalaby et al., 2004).

These results have been confirmed by clinical findings; in the LIFE study the non-fasting blood samples of included patients was used for quantification of serum lipids. Results

showed that a lower semen volume were associated with total cholesterol levels; while a higher phospholipid levels and free cholesterol were related to sperm shape abnormalities (Eisenberg et al., 2015a).

An independent analysis on the same dataset to evaluate the consequences of serum lipid profile on time-to-pregnancy revealed that both female and male serum free cholesterol concentrations were associated with higher time to pregnancy (Schisterman et al., 2014).

In contrast, a recent cross-sectional study including 7601 undergoing sperm analysis showed that increased total cholesterol was positively related to sperm motility (Liu et al., 2017).

1.4.3 Metabolic syndrome

Metabolic syndrome, characterized by the presence of three or more of the following risk factors: elevated fasting glucose, elevated triglycerides, low high-density lipoprotein (HDL) cholesterol, elevated blood pressure and abdominal obesity, has been associated to male infertility. Moreover, patients with MetS are at higher risk of having a decreased rate of normal semen morphology at multivariable analysis, after accounting for hormonal profile and age (Lotti et al., 2013) (Ventimiglia et al., 2016a).

In a small case-control study, men with MetS presented poorer sperm parameters in terms of vitality, concentration, total motility and total count (Leisegang et al., 2014). In contrast, in a cohort of 1337 primary infertile men, no relationship between semen parameters and MetS was observed, while decreased levels of inhibin-B, SHBG and total testosterone were related to MetS (Ventimiglia et al., 2016a).

Particularly, men with MetS had 11% lower calculated free testosterone, 19% decreased total testosterone, and 18% lower SHBG than controls (Laaksonen et al., 2003).

Makhside et al. pointed to observational studies which reported that decreased levels of SHBG and testosterone are considerably associated with MetS and its associated components, such as BMI, waist-height ratio and waist circumference (Makhside et al., 2005).

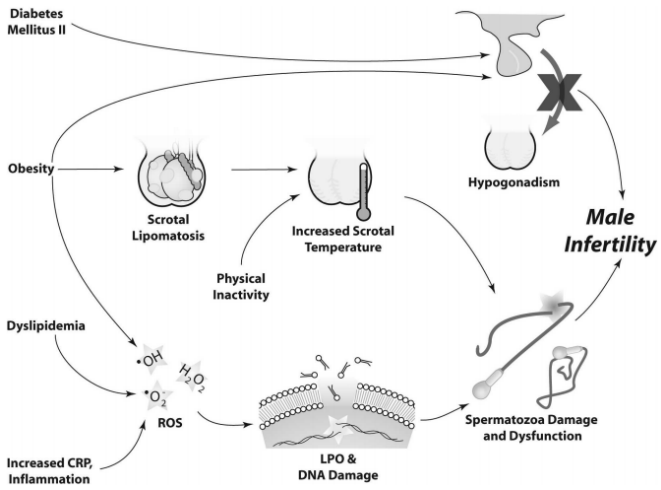
Remarkably, a significant positive association between MetS and the inflammatory marker CRP, which is indicated as a pathogenic correlate of MetS was also reported (Malik et al, 2005).

Similarly, total testosterone was negatively associated with BMI, insulin resistance and insulin level in male patients with MetS, thus indicating that insulin resistance is a probable underlying aberration in MetS (Robeva et al., 2006).

Currently, there is evidence to propose a MetS-male infertility paradigm (Figure 2).

Obesity/overweight may lead to increased scrotal temperatures, hypogonadism, altered spermatogenesis, decreased sperm concentration and motility, and higher sperm DNA damage. Dyslipidemia with higher oxidative stress in the testicular microenvironment and/or excurrent ductal system could reduce fertility. In the same way, type 2 DM/insulin resistance may participate to produce this scenario. Additional studies are needed to fully elucidate the pathophysiologic link between the components of MetS and male infertility (Kasturi et al., 2008).

Figure 2. MetS-male infertility paradigm. CRP indicates C-reactive protein; ROS, reactive oxygen species; OH, hydroxyl free radical; $O2^{\cdot}$, superoxide anion; H_2O_2, hydrogen peroxide; and LPO, lipid peroxidation (Kasturi et al., 2008).



1.4.4 Autoimmune diseases

Autoimmune diseases of the human testis and epididymis have been analyzed by several authors. For example, Kisand and Peterson studied that patients suffering from autoimmune polyendocrinopathy syndrome 1, because of inactivating mutations of the AIRE gene in 30% of cases, develop sperm autoantibodies and testicular failure connected to multi-organ autoimmune disease (Kisand and Peterson, 2011).

Additionally, several systemic autoimmune diseases, for example lupus erythematosus and various systemic vasculitis including Behcet's disease, could affect the epididymis and blood vessels of the testis. As a consequence, it determines damaging local inflammatory diseases (Silva et al., 2014).

Recently, a nationwide Danish cohort study including more than 24.000 infertile men showed that they had greater risks of both incident and prevalent of multiple sclerosis in comparison to a control-group of fertile men (Glazer et al., 2017b).

An epidemiologic study using insurance claim data to study and validate a possible association between autoimmune disease and male infertility, found that the cohort of infertile men presented a greater risk of developing multiple sclerosis, autoimmune thyroiditis, Graves disease, psoriasis and incident rheumatoid arthritis in relation to a group of vasectomized, assumed fertile, men and/or a cohort of age-matched control men (Brubaker et al., 2018).

Although the underlying mechanism of the relationship between autoimmunity and infertility is still unclear, there is some hypothesis that androgens may play a protective role against autoimmunity, which could be altered in the setting of hypogonadism (Ortona et al., 2016). Furthermore, it has been shown that infertile men have decreased baseline testosterone levels compared to fertile men (Andersson et al., 2004).

An additional possible mechanism affecting both autoimmunity and infertility is the development and presence of cross-reacting antibodies. This relationship has been discovered from studies in gonadotropin targets showing that the presence of autoantibodies to gonadotropic cells or gonadotropins themselves may impair the testicular spermatogenesis and lead the spermatogenic cells to apoptosis (Cocco et al., 2014).

1.4.5 Mortality

Due to the relationship between chronic disease and male infertility, several studies have tried to establish a possible association between mortality and infertility. At first, Gross et al. performed a research including a historic German cohort of 600 men over the span of 35 years; although the authors did not find any association between mortality and semen quality, subgroup analysis indicated a probable relationship among oldest participants of the cohort (Groos et al., 2006). However, the study was limited to subjects who lived in post–World War II Germany; thus, the generalizability of the results remains uncertain.

By then, two large-scale cohort researches have separately indicated that compromised semen quality is associated with higher risk of mortality. Jensen et al. performing a study in a cohort of 43.000 Danish men who presented semen analyses conducted in the context of infertility, discovered that a higher sperm concentration, up to a threshold of $40x10^6$/mL, is linked to lower mortality. Moreover, the authors described that mortality rate reduces in a dose-response way as semen volume, sperm motility and morphology increases (Jensen et al., 2009).

A further American cohort study of 11.935 men presenting for infertility established that patients with altered semen parameters, particularly lower sperm motility, semen volume, total sperm count and sperm concentration, had considerably increased mortality levels in comparison to men with regular semen parameters. Furthermore, while the total rate of mortality in this study have being <1%, men with two or more altered semen parameters presented a 2.3-fold higher risk of death (Eisenberg et al., 2014).

1.5 The role of age and aging

Together with cancers and comorbidities, the age of the man per se is a factor of male fertility. Age acts as a continuum behind this scenario, leading to a progressive decline in both general and reproductive health. Indeed, on the one hand, male reproductive health declines as time goes by; moreover, comorbidity load certainly increases with age (Kidd et al., 2001).

More in detail, within the 34–40 years of age it is possible to find the first age-related consequences on seminal parameters. The likelihood of intercourse resulting in pregnancy diminishes continuously in men older than 34 years of age, regardless of the woman's age (Stone et al., 2013).

Interestingly, this age range appears to be progressively more frequently chosen by European men to father a child (Salonia et al., 2012).

Regarding semen parameters, Stone et al. conducted a study in which sperm concentration, the rate of sperm with normal motility and morphology, and ejaculate volume were discovered to reduce respectively after 40, 43, and 45 years, with total sperm count decreasing even previously (Stone et al., 2013).

Growing paternal age more than 35–40 years was discovered to be associated with late child's birth in a population of 8.515 fertile UK couples (Ford et al., 2000); a higher possibility of spontaneous abortion (Slama et al., 2005); a lower rate of success at both IVF (Klonoff-Cohen et al., 2004) and intrauterine insemination (Mathieu et al., 1995).

1.6 Insights and possible treatments in male infertility

Primary prevention is widely considered best practice to obtain a healthy status.

Recent studies delineated that at their presentation infertile men frequently present with significant burden of comorbidities; this consideration suggests that curing infertile men is not centered on the simple reproductive question but must be concentrated on their general health condition (Eisenberg et al., 2015) (Ventimiglia et al., 2015).

Similarly, Corona et al. studying erectile dysfunction, suggested the idea of the impotent men as a blessed man, because he has the possibility to undertake medical examination, bettering not just his sexual life but also his overall health status (Corona et al., 2008).

Indeed, infertility may be considered as a possibility of getting to the medical attention a young adult cohort of men, with undiscovered existing comorbidities, thus consequently promoting their treatment. On the other hand, evaluating comorbidities in infertile men could offer helpful insights in the management and treatment of infertility.

To this point, a randomized control trial discovered how a 4-month weight-loss schedule, with behavioral adjustment and a considerably low-energy diet, determine higher levels

of total testosterone, high-density lipoprotein cholesterol and serum SHBG; moreover, this program led to lower concentrations of insulin and leptin (Kaukua et al., 2003).

Similarly, Morgante et al. performed a study that demonstrated how oligo-terato-asthenozoospermic men with MetS treated with metformin for 6 consecutive months had obtained improvements in metabolic and hormonal parameters, and in all semen parameters as well (Morgante et al., 2011).

2. The Testis: an immune privilege site

The testis is considered an immune-privileged organ since it is able to tolerate autoantigens from germ cells without evoking detrimental inflammatory immune responses. This microenvironment contributes to protect spermatogenesis. The testis contains most types of immune cells and these cells regulate testicular functions under physiological conditions (Table 6).

Table 6. Immune cells in the normal testis of adult mouse and human (Fijak et al., 2018).

Immune cells	Common markers	Testis	
		Mouse	Human
Macrophages M1 classically activated M2 alternatively activated	CD68, F4/80 (mouse), CD11b CD86, MHC class II CD163, CD206	+++	+++
Dendritic cells	CD11c, CD209, MHC class II, CD80, CD86	+	(+)
T cells	CD3, CD4, CD8, Foxp3	+	+
B cells	CD19, CD 20, B220 (CD45R)	+	(+)
Natural killer cells	CD56, CD161 (NK1.1)	+	(+)
Mast cells	Tryptase Fc epsilon RI alpha, CD117 (c-kit), CD23, CD203c	(+)	+

Circulating immune cells infiltrate into the testis under inflammatory conditions and are involved in tissue pathogenesis and host defense. Notably, many immune cells infiltrating the testis in physiological conditions adopt anti-inflammatory phenotype to favor anti-inflammatory and tolerogenic status. The most represented immune cells in the testis are myeloid cells, including macrophages and dendritic cells (DC) (Fijak et al., 2018).

2.1 Resident immune cells in the testis

2.1.1 Macrophages

Testicular macrophages represent approximately 20% of all interstitial cells and 80% of total leukocytes in the rat testis under physiological conditions. Resident testicular macrophages constitute the frontline of testicular innate defense against microbial infections from circulating blood (Hedger, 2002).

However, testicular macrophages are defecting in producing pro-inflammatory cytokines compared to macrophages from other tissues, and present immunosuppressive phenotypes. The immunosuppressive phenotype of macrophages is indicated, amongst others, by the production of the anti-inflammatory cytokine IL-10 and show M2 surface markers (Wang et al., 2017).

Historically, Frungieri et al. demonstrated that CD68+ and CD163+ macrophages are present in human testes and express pro-inflammatory cytokines IL-1 (α and β isoforms) and TNF-α. Testicular macrophages are located in the lumen of the seminiferous tubules and are significantly increased in the case of hypo-spermatogenesis or complete absence of testicular germ cells, suggesting that macrophages and/or their secretory products may be involved in the cause of male infertility (Frungieri et al., 2002).

2.1.2 Dendritic cells

DC are the most potent professional antigen-presenting cells (APC) that play a pivotal role in activation of naïve T cells and regulations of immune response and immunological tolerance in numerous organs, thus including the male genital tract; here they are located only in the testicular interstitial space. Testicular DC are immature, since they express low levels of major histocompatibility complex class II antigens (MHC II), co-stimulatory molecules, such as CD80 and CD86, and chemokines acting via the C–C chemokine receptor type 7 (CCR7) (Rival et al., 2007).

Interestingly, 95-100% of DC from normal testis express MHC II and co-stimulatory molecules at similar concentrations to those noticed in DC from inflamed rats. Notwithstanding, the negative IL-12p35 mRNA expression and the reduced CCR7 mRNA concentration revealed that DC from normal testis are immature (Rival et al., 2007).

Guazzone et al. confirmed that DC isolated in normal rats from testicular draining lymph nodes and testis were unable to stimulate T cell proliferation, reinforcing the idea that testicular DC are tolerogenic in physiological conditions (Guazzone et al., 2009).

Conversely, during experimental autoimmune orchitis (EAO), an increased frequency of mature DC has been identified in the testis of rats, suggesting that testicular DC can be activated and involved in autoimmune response. Thus far, the roles of immature DC in

the testis under the pathophysiological conditions remain to be clarified (Guazzone et al., 2011).

2.1.3 Lymphocytes

Despite its immune privileged status, the testis is not isolated from the immune system. As in many other tissues, circulating immune cells, including T lymphocytes, have relatively free access to the testis and the testis has lymphatic draining lymph nodes (Hedger et al., 2003).

T Lymphocytes are indeed the second largest population of leukocytes in the testis and represent ∼15% of total testicular leukocytes in rats. In normal rat and human testis several immunoregulatory T cell subpopulations, including suppressor CD8+ cells, natural killer (NK) cells and CD4+ Foxp3+ regulatory T cells (Tregs) have been reported (Klein et al., 2016).

Testicular Tregs favor the testicular immune privilege by inhibiting the activation antigen-specific T cell responses of effector T cells. Remarkably, under physiological conditions T lymphocytes are present at low levels in human testis (Nasr et al., 2005); on the other hand, their levels significantly increase in the testis of rats that suffer from autoimmune orchitis, supporting the idea that T cells are implicated in the pathogenesis of the disease itself (Jacobo et al., 2009).

2.1.4 Others immune cells

Mast cells are also found in the interstitial and peritubular areas of the testis. Mast cells stimulate tissue fibrosis by inducing collagen production and fibroblast proliferation. Interestingly, a higher number of mast cells and more fibrosis are often observed in the testicular biopsies of infertile men (Apa et al., 2002).

The major testicular cells, including, Sertoli, Leydig, and germ cells, have innate immune activities. (Chen et al., 2016). Particularly, Sertoli cells (SCs) express immunoregulatory factors that participate to create a tolerogenic environment in the testis. As an example, SCs produce Galectin-1, which downregulates pro-inflammatory cytokine secretion and stimulates the induction of $CD4^+$ Tregs (Gao et al., 2016), and apoptosis inhibitors, such as SerpinA3N and protease inhibitor-9, which inhibit NK and T cell-mediated death (Sipione et al., 2006).

It has been shown that SCs stimulated with IFN-γ, up-regulate MHC II expression and B7-H1, a ligand for negative regulatory receptor that allows induction of $CD4^+$ Tregs (Dal secco et al., 2008).

Furthermore, activin A produced by SCs under inflammatory conditions caused a loss of blood testis barrier (BTB) function and led SCs to become functionally immature. As a consequence, this process may impair male germ cell development and lead to a compromised spermatogenesis (Nicholls et al., 2012).

Overall, several mechanisms are active in the testis to prevent autoimmunity (Figure 3):

a) the presence of an immunological BTB formed by two contiguous SCs near the basal lamina (BL) of the seminiferous epithelium (SE) and highly specialized inter-Sertoli tight, gap and adherent junctions. The BTB limits the access of immune cells to the luminal compartment and sequesters the autoantigens of the late stage of germ cells within the luminal compartment;
b) the secretion of immunosuppressive factors, mainly by tissue testis cells. DC in the testis with immature phenotype suppress the activation of T lymphocytes (T). Macrophages (Mφ) by producing IL-10 concur in inhibiting T cells. SCs secret transforming growth factor β (TGF-β) and activin A, which inhibit the production of pro-inflammatory cytokines by DC and Mφ. Testosterone synthesized by Leydig cells (LC) regulates SCs activity, and LC by secreting the growth arrest-specific gene 6 (Gas6) inhibit DC, Mφ and SCs. The tolerogenic testis microenvironment is also sustained by spermatogonia (Sg) that release soluble programmed death ligand-1 (sPDL-1), which inhibits T cells and promotes T cell apoptosis;
c) a limited number of T cells;
d) the presence of regulatory T cells (Tregs), known to be modulators of immune response acting through local and systemic mechanisms. The delicate equilibrium between immune suppression and inflammation is modulated by cytokines (Fijak et al., 2010) (Zhao et al., 2014).

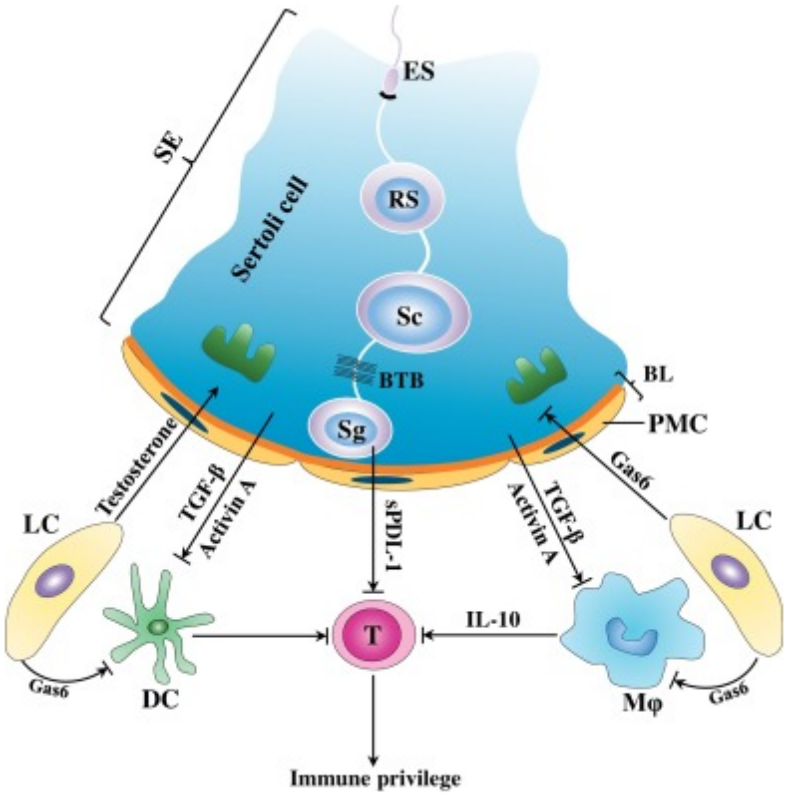


Figure 3 Schematic of mechanism's underlying testicular immune privilege. BTB indicates blood-testis barrier; BL, basal lamina; SE, seminiferous epithelium; DC, dendritic cells; T, T lymphocytes; Mφ, macrophages, TGF-β, transforming growth factor β; LC, Leydig cells; Sg, spermatogonia; ES, elongated spermatid; PMC, peritubular myoid cell; RS, round spermatid; Sc, spermatocyte; ↑ induction; ⊼ inhibition (Chen et al., 2016).

2.2 Tolerogenic molecules associated with infertility: HLA-G and IL-10

The evaluation of the role of immunoregulatory factors in male infertility is complex. Three decades ago, HLA-G, a non-classical human leukocyte antigen (HLA) molecule was identified (Ellis, 1990). HLA-G endowed with immune-regulatory functions, is primarily expressed on extravillous trophoblasts lining the placenta and on the naturally occurring tolerogenic DC, named DC-10, which are enriched in the human first trimester decidua (Amodio et al., 2013). Decidual DC-10 are involved in HLA-G-mediated tolerance at the maternal–fetal interface. Only recently, studies to examine whether HLA-G could have paternal origin and serve important functions during fertilization and implantation have been performed (see below) (Hiivd, 2015).

The examination of detailed immunologic pathways that may control the immune response and support tolerance in both fertile and infertile men has been poorly investigated. Notably, these studies could be also important for explaining the poorer overall health condition of male infertile individuals, in addition could be of crucial importance to further define the immunogenic profile in the male infertile status.

2.2.1 HLA-G

HLA-G is a non-classical HLA class I molecule that regulates immune response and promotes tolerance. In healthy conditions, a basal level of HLA-G gene transcription is detected in most tissues and cells; although translation into HLA-G protein is limited to trophoblasts at the fetal-maternal interface (Carosella et al., 2003), and in adults, to cornea, mesenchymal stem cells (MSCs), pancreatic beta cells, thymic epithelial, nail matrix and precursors of endothelial and erythroid cells. HLA-G can be also newly-expressed in pathological conditions, such as allogeneic transplantation, viral infections, malignant transformation, inflammatory and autoimmune diseases (Carosella, 2011). HLA-G function is favorable during pregnancy or after transplantation, since it protects from rejection, or detrimental when expressed by tumors or during chronic infections, inducing immune escape mechanisms.

Myeloid antigen-presenting cells (APC) are also modulated by HLA-G, through the expression of both ILT2 and ILT4. HLA-G impairs the differentiation and myeloid APC functions, thus resulting in impaired T lymphocyte activation and improper NK cytotoxic activity (Gros et al., 2008). Notably, HLA-G does not inhibit the functions of myeloid APC but stimulates them to differentiate into tolerogenic cells. Horuzsko et al. showed that in a murine model HLA-G treatment of monocyte-derived DC lead to the induction of cells with tolerogenic properties and able to induce T cell anergy (Horuzsko et al., 2001).

Finally, HLA-G participates in the induction of tolerogenic cells including HLA-G-expressing Tregs (LeMaoult et al., 2004), $CD4^{low}$ and $CD8^{low}$ suppressor T cells (Naji et al., 2007b), T regulatory type 1 (Tr1) cells and DC-10 (Gregori et al., 2010) (see below).

Only recently, the expression of HLA-G in the male reproductive system has been investigated. Soluble HLA-G is present at different levels in seminal plasma (Larsen et

al., 2011). Moreover, HLA-G is expressed in normal testis and epididymal tissues, but it is still unclear where in the male reproductive system, the soluble HLA-G detected in seminal plasma originates from. It has been postulated that the presence of soluble HLA-G in seminal fluid may play an immunoregulatory roles during fertilization and implantation (Dahl et al., 2012). In the testis, HLA-G might have a role as an immunosuppressive factor, and thereby avoiding recognition of 'self' sperm cells, which can be perceived as autoantigens by the immune system. On the other hand, paternal sHLA-G in the seminal plasma, may be associated with the induction of tolerance in the mother to paternal antigens. This induction may be important for the success of the pregnancy.

Costa et al. (2016) enrolled couples undergoing assisted reproduction treatment and couples who conceived naturally. In that study the haplotype HLA-G/01:01:01b/HLA-G/01:01:01 showeda significant higher frequency in control groups and protection against infertility. Moreover, the HLA-G UTR-4 haplotype (possessing -964G,-725G, 14 bp del) was associated with a shorter time to achieving pregnancy in an infertility treatment setting when both female and male partners were carriers. More recently, it was observed that fertile men differ in the profile of HLA-G polymorphism from men participating in IVF. Among all HLA-G haplotypes, the most unfavorable for male fertility is the G-C-ins haplotype, which determines the secretion of the lowest concentration of the soluble HLA-G molecule. This haplotype may reduce sperm parameters.

Thus far, no studies have been performed to determine soluble HLA-G levels in seminal plasma from men with fertility problems.

2.2.2 Interleukin-10

Interleukin-10 (IL-10) is an immunomodulatory cytokine that performs a central function in regulating inflammation, down-regulating immune responses, stimulating tolerance and in preventing chronic inflammatory pathologies.

IL-10 has been shown to be produced by practically all leukocytes, including monocytes, macrophages, neutrophils, eosinophils, mast cells, B and all T cell subsets (Saraiva et al., 2009).

IL-10, through the interaction with IL-10 receptor (IL-10R), controls the expression of numerous genes with the subsequent upregulation of immune-modulatory molecules, downregulation of pro-inflammatory mediators and inhibition of antigen presentation. Overall, IL-10 stimulates regulatory cell differentiation, inhibits, directly and indirectly cytokine production and effector T cell proliferation, and regulates APC (Gregori et al., 2012).

Moreover, insufficiency or aberrant expression of IL-10 or IL-10R favorites inflammatory responses to microbial challenge and lead to the development of inflammatory bowel disease (Engelhardt et al., 2014) and several autoimmune diseases (Groux et al., 2003).

2.3 IL-10 related regulatory cells: DC-10 and Tr1 cells

2.3.1 DC-10

DC-10 are a subcategory of human tolerogenic DC characterized by the ability to spontaneously secrete IL-10. DC-10 can be differentiated in vitro by culturing peripheral blood monocytes for 7 days in the presence of GM-CSF, IL-4, and IL-10 (Gregori et al., 2010).

DC-10 express at high-level HLA-G and other tolerogenic molecules including ILT2, ILT3, and ILT4. DC-10 promote the differentiation of Tr1 cells in vitro (see below) and are present in vivo in peripheral blood of healthy donors where they represent 0.3% of the mononuclear cells, but they were also found in the spleen (3.1% of total cells). Recently, it has been demonstrated that circulating DC-10 can be identified in vivo by the co-expression of CD14, CD16, CD141, and CD163 (Figure 4).

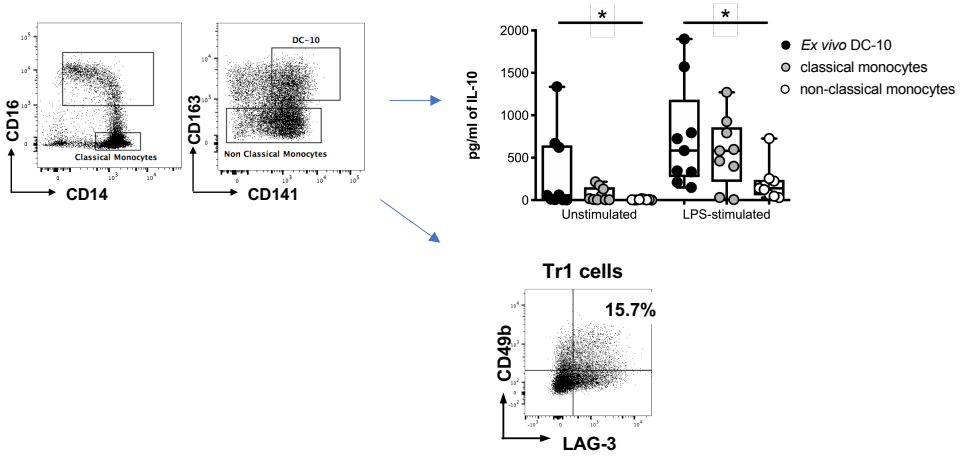


Figure 4. Circulating DC-10 can be isolated from peripheral blood according to the expression of CD14, CD16, CD141, CD163. Ex vivo DC-10 ($CD14^+CD16^+CD141^+CD163^+$), classical ($CD14^+CD16^-$) and non-classical ($CD14^+CD16^+CD163^-$) monocytes were FACS-isolated from peripheral blood of healthy donors and left unstimulated or stimulated with LPS for 48 hours (n=9). Upper panel: concentration levels of IL-10 in culture supernatants were evaluated by multiplex microbead-based cytokine array. Each dot represents a single donor, lines indicate median, while whiskers are minimum and maximum levels. *P≤.05, **P≤.01 (Wilcoxon matched pairs test, two-tailed). Lower panel: naïve $CD4^+$ T cells were cultured with allogeneic ex vivo DC-10 ($CD14^+CD16^+CD141^+CD163^+$) FACS-isolated from peripheral blood of healthy donors (ratio 20:1) for 10 days. After culture, percentages of Tr1 cells within cells stimulated with DC-10 were evaluated by CD49b and LAG-3 expression on $CD45RA^-CD4^+$ T cells. One representative donor is presented (Comi et al., 2018).

DC-10 produce IL-10, which inhibits T cell proliferation and cytokine production, promotes T-cell anergy and induces the up-regulation of ILT-4 and HLA-G not only on the adjacent DCs but also on T cells. The ILT4/HLA-G interaction promotes the differentiation of IL-10-producing Tr1 cells with suppressive activity and enhances IL-10 production, establishing a tolerogenic loop (Gregori et al., 2010) (Figure 5).

Interestingly, DC-10 have been identified in the decidua in the first trimester of pregnancy (Amodio et al, 2013). The decidua is an immunologically privileged tissue that gives a rise to the essential environment for pregnancy maintenance. Gregori and colleagues (Gregori et al, 2015) demonstrated that DC-10 frequency is low in the decidua of women

with early pregnancy miscarriage. In cancer patients it has been reported an altered DC-10 frequency.

Particularly, a significantly higher frequency of DC-10 was observed, in peripheral blood of patients affected by gastric cancer (Xu et al, 2016) and acute myeloid leukemia (Locafaro et al, 2014), compared with the healthy donors. Relying on these findings, it was postulated that the presence of DC-10 contributes to the inhibition of the immune system promoting tumor immune-escape.

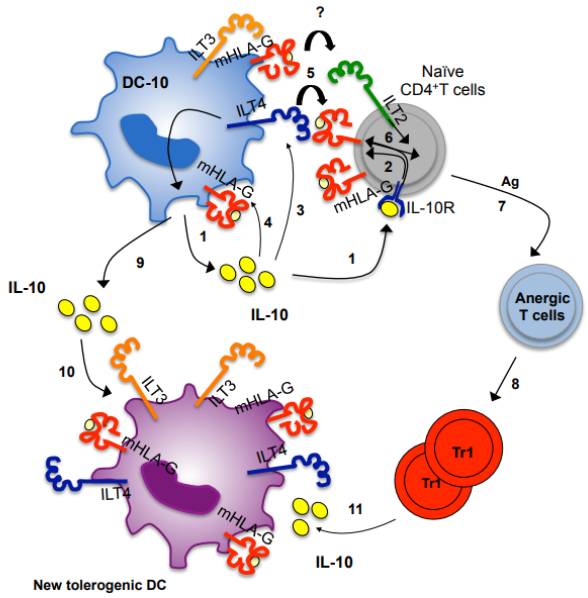


Figure 5. Tr1 cells induction via the IL-10-dependent ILT4/HLA-G pathway. DC-10 secrete high levels of IL-10 (1). During T-cell priming, IL-10 produced by DC-10 inhibits T cell proliferation and promotes the up-regulation of HLA-G on allogeneic $CD4^+$ T cells (2). IL-10-derived by DC-10 also up-regulates ILT4, ILT3 (3), and HLAG (4) on DC-10. HLA-G expressed on T cells interacts with ILT4 on DC-10 (5) and provides negative signals to T cells (6) that induce T-cell anergy (7) and Tr1 cell differentiation (8). Concomitantly, interaction between HLA-G on T cells and ILT4 on DC-10 (5) enhances IL-10 secretion by DC-10 (9). DC-10-derived IL-10 promotes de novo differentiation of tolerogenic DC by inducing ILT3, ILT4, and HLA-G expression (10). In addition, Tr1 cells secrete IL-10, which contributes to amplify this tolerogenic circuit (11) (Gregori et al, 2010).

2.3.2 T regulatory Type 1 (Tr1) cells

T-regs play a crucial role in the preservation of immune homeostasis and in the development and conservation of peripheral tolerance. Treg populations are composed by

the forkhead box P3 (FOXP3)-expressing, $CD4^+CD25^+$ Tregs ($FOXP3^+$ Tregs) and the $CD4^+$ IL-10-producing Tr1 cells (Roncarolo et al, 2006).

Tr1 cells and $FOXP3^+$ Tregs are different sub-populations of Tregs that perform a non-redundant role in preserving tolerance.

Tr1 cells are characterized by a unique cytokine production profile: they produce high levels of IL-10, Transforming Growth Factor-β (TGF-β), and IL-5, low levels of interferon-γ (IFN-γ) and IL-2, and absence of IL-4 after stimulation (Groux et al.,1997) (Roncarolo et al., 2006) (Roncarolo et al, 2018).

Tr1 cells are induced by chronic stimulation of $CD4^+$ T cells in the presence of IL-10. Indeed, once stimulated through their specific TCR, Tr1 cells can regulate immune responses via the secretion of TGF-β and IL-10, which directly suppress the proliferation of effector T cell and IL-2 and TNF-α production by $CD4^+$ T cells (Roncarolo et al., 2006).

Tr1 cells also downregulate T cell responses through granzyme B (GzmB) and perforin secretion, which induce selective killing of APCs via both cognate and non-cognate mechanism (Magnani et al., 2011). Cytolysis of the APCs results in inhibition of both antigen-specific CD4+ and CD8+ T cells and bystander T cells (Tree et al., 2010) (Figure 6).

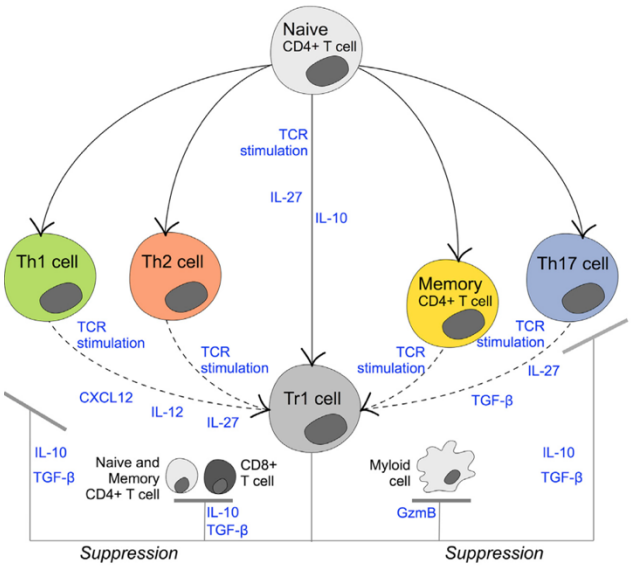


Figure 6. Tr1 Cell Development, Origin, and Function Naive CD4+ T cells can develop into Th1, Th2, Th17, and Tr1 cells. Some Th1, Th2, memory CD4+ T and Th17 cells can acquire a Tr1 cell phenotype and function when activated through their TCRs in the presence of specific cytokines and chemokines as depicted. Tr1 cells exert their anti-inflammatory and regulatory function by secreting IL-10, TGF-b, and GzmB. Th1, Th2, and Th17 cells, CD4+ memory and naive cells, CD8+ T cells, and myeloid cells are suppressed by Tr1 cells (Tree et al., 2010).

The importance of Tr1 cells in controlling immune response has been further proved by several studies that claim their involvement in many T cell-mediated diseases and in the regulation of tolerance in vivo. The first suggestion that human Tr1 cells are involved in maintaining peripheral tolerance in vivo came from studies in severe combined immunodeficiency (SCID) patients that developed long-term tolerance after HLA-mismatched allogeneic stem cells transplantation without the need of immunosuppression. Till then, Tr1 cells have been repetitively demonstrated to play a critical role in promoting and maintaining tolerance in pre-clinical and clinical models of autoimmune diseases, in chronic inflammation, after allogeneic transplantation and in cancer (Roncarolo et al., 2018).

2.3.3 The pro-inflammatory and senescent signature of immune cells in infertile men

A detailed characterization of the inflammatory status of resident immune cells in testicular tissue has recently been explored (Alfano et al. 2021). In testicular tissue of NOA men with Sertoli Cells Only Syndrome (SCOS) a pro-inflammatory phenotype was seen in resident immune cells. In macrophages, an upregulated pathway related to MHC class II antigen presentation, interferon-gamma (INF), and NF-kB signalling was found. Moreover, CD8 + CD69 + TCL expressed granzyme K and M, the proinflammatory chemokines CCL4/MIP1β and CCL5/RANTES and cytokine IL-32, supporting the conclusion that these somatic tissue-resident cytotoxic T cells might be generated by a previous inflammatory or autoimmune insult and contribute to chronic local inflammation. Moreover, a senescent phenotype was observed in iNOA somatic cells of infertile men but not in those from the control group. To support the hypothesis of senescence, a strong accumulation of p16 in Leydig cells (a known indicators of cellular senescence-like cell cycle-related proteins) was found in infertile vs. fertile men. As a whole, Alfano et al. (2021), showed that the upregulated proteins of the innate immunity and cytokine signals, a pro-inflammatory environment, p16 staining, low levels of circulating testosterone, along with the downregulated pathways of RNA processing and amino acids metabolism, reported for iNOA men, are consistent with altered biological pathways orchestrating the process of ageing. To this regard, the association between a pro-inflammatory/exhausted phenotype of immune cells and ageing has recently been explored. Marquez et al. (2020) characterized peripheral blood mononuclear cells from

172 healthy adults with flow cytometry and revealed a shared epigenomic signature of aging including declining naïve T cell and increasing monocyte and cytotoxic cell functions. In particular, they quantified the frequencies of CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes in PBMC from men and women. Authors reported that CD8+ and CD4+ T cells significantly decreased with ageing in men. A similar pattern was observed for B cells but not for CD14+ monocytes. Importantly, cells from older men were characterized by a pro-inflammatory signature compared to those from younger individuals, with higher levels of IL18 and IL6. Overall, these data suggest that the ageing signature is characterized by declining adaptive responses and increased systemic inflammation.

The previously reported findings in infertile men documented a shift from the immune-privileged status to a proinflammatory testicular environment, eventually associated with iNOA. From a translational point of view, the pro-inflammatory status of testicular cells and the senescent phenotype characterized by decreased T cells number and higher level of inflammatory cytokines observed in infertile men could be associated with the infertility status per se, an exhausted pattern of circulating immune cells and it could predispose to future risk of developing malignancies and chronic diseases.

However, a detailed characterization of the local (e.g. testicular) and systemic immune status of infertile and fertile men has never been investigated.

II. AIM AND DESIGN OF THE STUDY

Severe infertile men develop comorbidities earlier than their fertile counterpart and a number of these comorbidities could be associated with defects of the immune system. Moreover, data from testicular biopsies showed the presence of activated macrophages and T cells in severe infertile men. Based on these premises, the hypothesis of the study was that the immunological asset of infertile individuals could be altered by means of either downregulation of the tolerogenic, or upregulation of the pro-inflammatory arm of immune system. These hypotheses have been explored at tissue-specific (seminal samples) and at systemic (peripheral blood) levels.

For the first time, we investigated the immunological profile of both infertile men and fertile individuals, as for WHO classification criteria. Since a chronic pro-inflammatory environment has been associated with autoimmune diseases, neoplasms and chronic conditions, all of which are highly prevalent in infertile men, we thought that this study could help to explain the biological rationale behind the lower overall health status of infertile men in comparison to fertile controls. Given the existing lack of research regarding the role of the immunological status over men's fertility potential, our study was the first to address a clinically relevant question.

This project has been designed as a case-control study including men seeking medical help for primary couple's infertility (cases) and fertile (controls) both enrolled at Ospedale Maggiore Policlinico and IRCCS Ospedale San Raffaele, Milan.

Our main hypothesis that infertile men have alterations/defect of the immune system has been pursued with the following specific aims:

AIM 1. To assess lymphoid and myeloid cell distribution in seminal fluid and peripheral blood of fertile and infertile men.

AIM 2. To evaluate the presence of regulatory cells in seminal fluid and peripheral blood.

AIM 3. To evaluate the presence of potential biomarkers of an inflammatory/exhausted immune status of infertile men.

III. MATERIALS AND METHODS

1. Study population

We enrolled 38 infertile men with a diagnosis of oligo-astheno-teratozoospermia (OAT), 13 infertile iNOA men and 40 age-matched fertile controls that provided comprehensive medical data, peripheral blood (PB) and seminal fluid (SF) samples. Infertile men have been enrolled at the "Andrology and Reproductive health office" at the Policlinico of Milan and at the Urological Research Institute (URI)/Division of Experimental Oncology at IRCCS San Raffaele Hospital. Age-matched fertile men have been recruited via their partners who had been new and expectant mothers in the department of Obstetrics and Gynaecology at both institutions. PB and SF samples have been collected upon informed consent in accordance with the Helsinki Declaration and with local ethical committee approvals. All samples have been collected between December 2019 and June 2022.

To limit potential biases associated with causal heterogeneity of male factor infertility (MFI), we included in the study only infertile men matching the following inclusion criteria: age 18-50 years; white-Europeans; belonging to primary infertile couples, associated with pure MFI; and having complete clinical data available at enrollment.

Age-matched fertile men have been included based on the following inclusion criteria: age 18-50 years; white-Europeans; having fathered at least one child with a time to pregnancy within 12 months, according to WHO classification criteria; having complete clinical data available at enrollment. Both fertile and infertile men with a known history of hematologic disease, that could alter their immune profile, over the last 6 months, have been excluded from the study.

2. Clinical and anamnestic variables

For all participants included, the following information have been collected: (i) Clinical variables: date of birth, race, ethnicity, complete medical history, with comorbidities scored with the Charlson Comorbidity Index, measured Body Mass Index, partner's age, partner's health status, presence of varicocele, history of undescended testis/testes,

testicular volume; (ii) hormonal parameters: total testosterone, FSH, LH, AMH, prolactin, TSH; (iii) genetic profile: karyotype, according to EAU guidelines on male infertility; (iv) semen parameters according to WHO 2010 standard references; and, (v) partner's age and health status,.

3. Experimental Methods

Experimental design

We defined the leukocyte composition and the presence of different subsets of myeloid cells in the peripheral blood and seminal fluid by multiparameter flow cytometry. The levels of several cytokines and chemokines were measured in serum and seminal plasma by multi-beads array, and T cell responsiveness was performed on peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation (Figure 7). To identify leucocyte subpopulations and evaluate presence and frequency of regulatory cells and of effector/memory, exhausted T and myeloid cells in peripheral blood and in seminal fluid we used the panels shown in Table 7 and the antibodies (Abs) listed in Table 8 and Table 9.

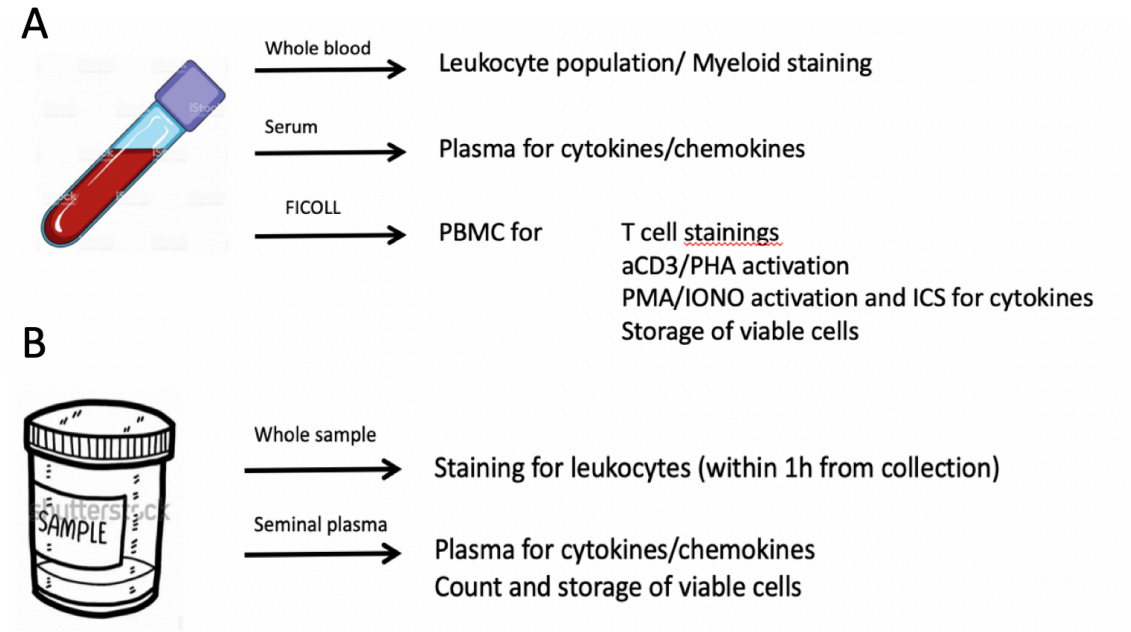


Figure 7. Experimental design. Leukocyte composition and the presence of different subsets of myeloid cells was assessed on total blood (A) and seminal fluid (B) by multiparameter flow cytometry. After density gradient centrifugation, PBMCs were collected, and the frequency of T

cell subsets and proliferative capacity of cells were analyzed (A). The levels of several cytokines and chemokines were measured in serum and seminal plasma by multi-beads array (A and B).

Flow Cytometry staining on whole peripheral blood.

The frequencies of major leucocyte populations, of DC-10 and of other monocyte/myeloid populations were assessed on EDTA peripheral blood within 6 h after blood sampling.

In brief, 50 µl of antibody (Ab) mix in brilliant stain buffer (BD biosciences, San Jose, CA) were directly added to 150 µl of whole blood and incubated for 15 min at room temperature in the dark. The samples were then incubated for additional 13 min at room temperature in the dark with lysis buffer (EDTA 1mM, KHCO3 10 mM, NH4Cl 150 mM) to perform red blood cell lysis, and washed twice with PBS (Sigma, CA, USA). Finally, samples were stained for 10 minutes at room temperature (RT) with Live/Dead PromoFluor 840 maleimide reactive dyes (Invitrogen, CA, USA) at a concentration of 0.25 µg/µl, washed and re-suspended in PBS (Sigma, CA, USA) with 2% FBS (Lonza, Italy). Cells were identified using a multiparametric approach based on the combination of monoclonal Abs listed in Table 7, 8, and 11.

Table 7. Multicolor flow cytometry panel to identify leucocyte subpopulations

MIX	FITC	PerCp5.5	PE-CY7	PE	APC	APC-H7	BV421	BV510	BV605	BV650	BUV395	BUV737	PF840
DC-10	HLA-DR	CD163		HLA-G	ILT4	CD14	CD141	CD16		CD11c	CD45	CD83	L/D
MyDC	CD303	CD86		CD1c	CD141	CD14		HLA-DR		CD11c	CD45	CD83	L/D
Emocromo	CD15	CD3	CD56	CD19	CD14	CD8	CD4	CD16		CD11c	CD45		L/D
Activation/Treg	FOXP3	CD127	CD45RA	LAG-3	CD49b	CD8	CD25	HLA-DR	CD4		CD45	CD3	L/D
Exhaust	EOMES	CD3	TIM3	PD1		CD8	CD45RA	KLRG1	CD4	tBET	CD45	CD62L	L/D

We first defined the overall leukocytes composition using multiparametric flow cytometric analysis. Specifically, using CD45, CD3, CD4, CD8, CD14, CD15, CD16, and CD56 markers and the gating strategy shown in Figure 8, we evaluated the frequency

of $CD4^+$, $CD8^+$, B ($CD19^+CD3^-$), NK ($CD56^+CD3^-$), NKT ($CD56^+CD3^+$) cells, neutrophils ($CD15^+CD16^+$), eosinophils ($CD15^+CD16^-$), and monocytes ($CD14^+$).

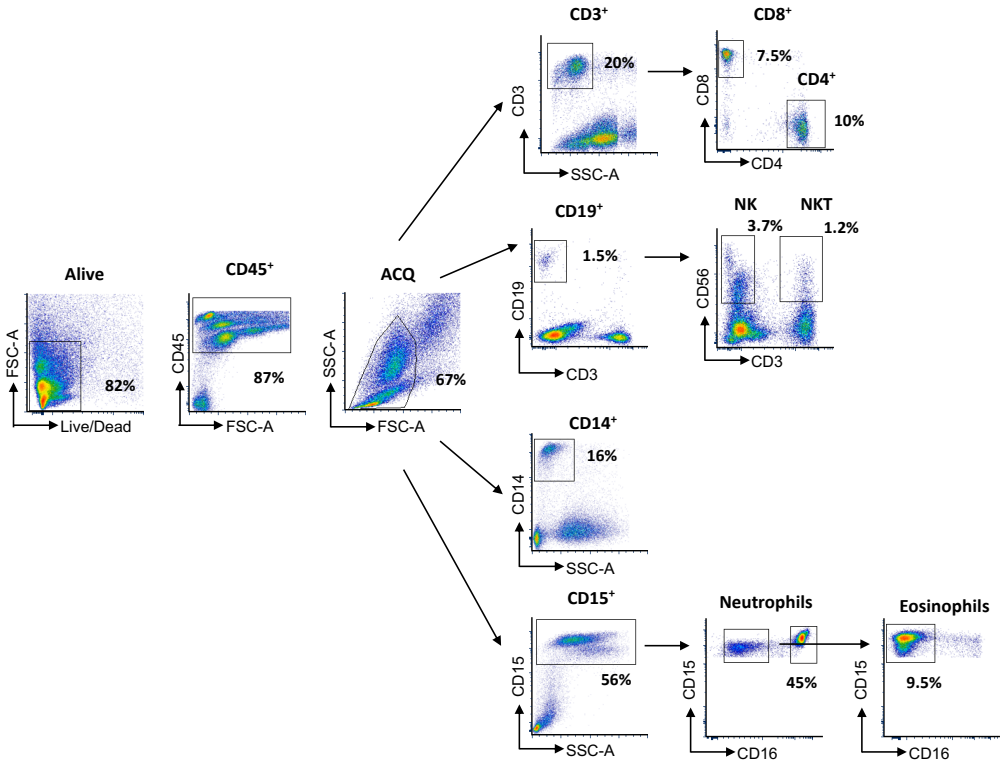


Figure 8. Gating strategy to identify leucocyte subsets in whole blood. Major leucocyte subsets were identified in the peripheral whole blood as alive and $CD45^+$ cells, and according to FSC/SSC, physical parameters and the indicated markers. Percentages indicate the frequency of the different populations within the physical parameters. Dot plots from one representative donor are shown.

In parallel, we identified tolerogenic DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$) and immunogenic cDC1 ($CD14^-CD11c^+CD141^+$), and cDC2 ($CD11c^+CD1c^+$) subsets by using the gating strategy reported in Figure 9.

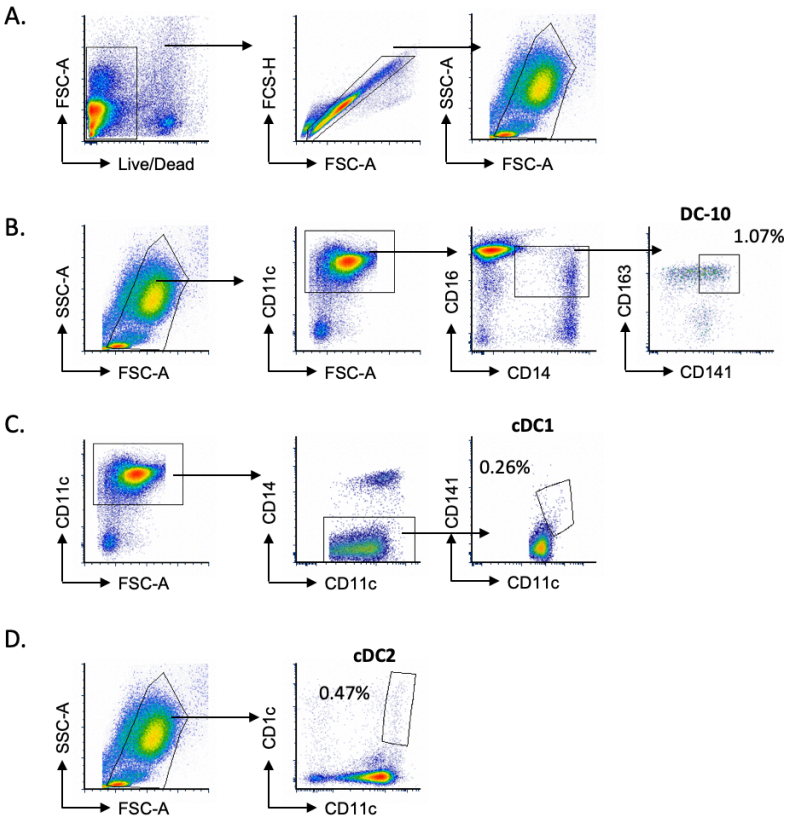


Figure 9. Gating strategy to identify DC subsets by flow cytometry in blood samples. (A) DC subsets were identified in whole blood according to FSCarea/SSCarea physical parameters after exclusion of dead cells and doublets; **(B)** DC-10 were defined according to CD11c, CD14, CD16, CD141 and CD163 co-expression; **(C)** cDC1 were identified according to the lack of CD14 expression and CD11c and CD141 co-expression; **(D)** cDC2 were identified according to CD11c and CD1c co-expression. Dot plots from one representative donor are shown.

Peripheral blood mononuclear cells (PBMCs) isolation.

PBMCs were isolated via density gradient centrifugation over Lymphoprep (Figure 10) (Cedarlane, Canada). After centrifugation, PBMCs were collected and remaining red blood cells were lysed by incubation with ammonium chloride solution, and platelets removed by three low speed centrifugations. Cells were then re-suspended in PBS (Sigma, CA, USA) and counted.

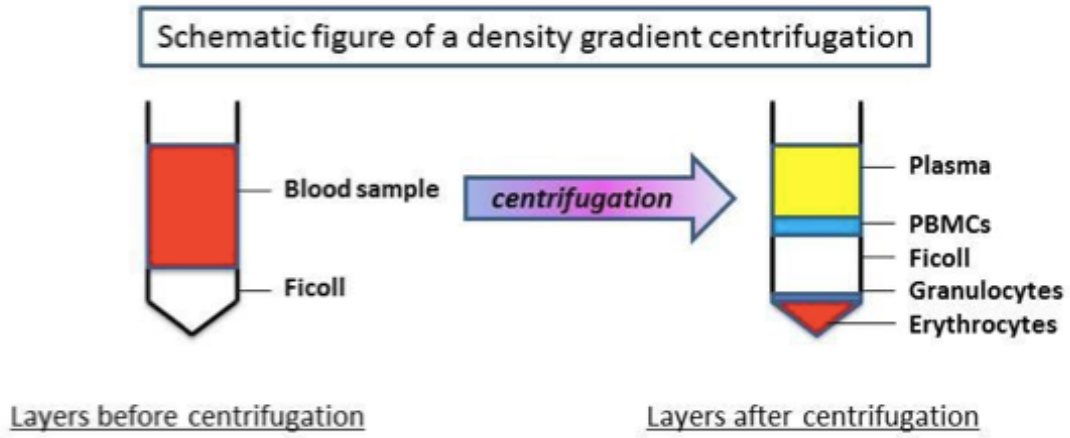


Figure 10. Schematic figure of a density gradient centrifugation (from PBMC isolation and cryopreservation, version 2015 03 05, Centre Physiopathologie de ToulousePurpan)

Flow Cytometry staining for T cells.

T cell subsets have been identified by multicolor flow cytometry on PBMCs. Briefly, 0.5 M of PBMCs were stained for 10 minutes at room temperature (RT) with Live/Dead PromoFluor 840 maleimide reactive dyes (Invitrogen, CA, USA) at a concentration of 0.25 µg/µl, washed and re-suspended in 50 µl of antibody (Ab) mix in brilliant stain buffer (BD biosciences, San Jose, CA) and incubated for 15 min at room temperature in the dark. Finally, cells were washed and re-suspended in PBS (Sigma, CA, USA) with 2% FBS (Lonza, Italy).

Effector/memory T cell populations and regulatory T cells were identified using a multiparametric approach based on the combination of monoclonal Abs listed in Table 7 and 9. Briefly, effector/memory T cell populations were identified using the combination of CD45RA, CD45RO, and CD62L monoclonal Abs (Table 9). The expression of activation markers (i.e. CD25, HLA-DR, LAG-3) and of costimulatory/inhibitory molecules (i.e. PD-1, KLRG-1, TIM3, HLA-G) were analyzed in parallel.

Tr1 cells were identified using the combination of CD3, CD4, CD45RA, CD49b and LAG3. For identification of $FOXP3^{+}$ Tregs, PBMCs were washed, re-suspended in 1X e-bioscience FOXP3 Fix/Perm* solution (Biolegend, San Diego, CA), and incubated at room temperature in the dark for 20 minutes. Cells were than washed with 1X e-bioscience FOXP3 Perm buffer (Biolegend, San Diego, CA) and incubated with 50 µl of

intracellular staining mix in 1X ebioscience FOXP3 Perm buffer at room temperature in the dark for 25-30 minutes. Lastly, cells were washed once with 1X ebioscience FOXP3 Perm buffer, once with 200µl of PBS-2%FBS, and then re-suspended in PBS (Sigma, CA, USA) with 2% FBS (Lonza, Italy).

Table 8. List of monoclonal antibodies used to identify myeloid cell populations

Marker	Fluorophore	Clone	Producer
CD1c	PE Y585		
CD3	Percp-Cy5.5	HIT3a	BioLegend, CA,USA
CD4	Pacific Blue	SK3	BioLegend, CA,USA
CD8	APC-H7	SK1	Biosciences, NJ, USA
CD11c	BV650	B-ly6	BD Horizon, NJ, USA
CD14	APC	MϕP9 BD	Biosciences, NJ, USA
CD15	FITC	MMA	Biosciences, NJ, USA
CD16	BV510	3G8	BD Horizon, NJ, USA
CD19	PE	4G7	Biosciences, NJ, USA
CD45	BUV395	HI30	BD Horizon, NJ, USA
CD56	Pe-Cy7	NCAM	BioLegend, CA,USA
CD83	BUV740		
CD86	Percp-Cy5.5	HIT3a	BioLegend, CA,USA
CD141	Pacific Blue	SK3	BioLegend, CA,USA
CD141	APCR660		
CD163	Percp-Cy5.5	HIT3a	BioLegend, CA,USA
CD303	FITC	MMA	Biosciences, NJ, USA
HLA-DR	BV510	G46-6	BD Horizon, NJ, USA
HLA-DR	FITC	MMA	Biosciences, NJ, USA
CTLA-4	PE/585		
ILT-4	APC		

Table 9. List of monoclonal antibodies used to identify T cell populations

Marker	Fluorophore	Clone	Producer
CD3	Percp-Cy5.5	HIT3a	BioLegend, CA,USA
CD4	BV605	RPA-T4	BD Horizon, NJ, USA
CD8	APC-H7	SK1	Biosciences, NJ, USA
CD25	BV421	M-A251	BD Horizon, NJ, USA
CD45	BUV395	HI30	BD Horizon, NJ, USA
CD45RA	Pacific Blue	HI100	BioLegend, CA,USA

CD49b	APC	REA188	Miltenyi Biotech, Germany
CD62L	BUV737	DREG-56	Biosciences, NJ, USA
CD127	Pe-Cy7	eBioRDR5	Biosciences, NJ, USA
CTLA4	APC	L3D10	BioLegend, CA,USA
EOMES	FITC	WD1928	Biosciences, NJ, USA
FcR Blocking Agent			Miltenyi Biotech, Germany
FOXP3	Alexa Fluor488	259D	BioLegend, CA,USA
HLA-DR	BV510	G46-6	BD Horizon, NJ, USA
KLRG1	BV510	2F1/KLRG1	BioLegend, CA,USA
LAG-3	PE	REA351	Miltenyi Biotech, Germany
PD1	PE	MIH4	Biosciences, NJ, USA
T-bet	BV650	O4-46	Biosciences, NJ, USA
TIM3	PE-Cy7	F38-2E2	BioLegend, CA,USA

Functional tests on PBMCs

T-cell cytokine detection after short term stimulation

0.2M of PBMCs were left unstimulated or stimulated for 6 hours with 50 ng/ml of Phorbol-12-myristate-13-acetate (PMA) and 1 ug/ml of Ionomycin (both from Sigma-Aldrich, St. Louis, MO, USA). 10 ug/ml of Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) were added for the last 5 hours of culture to block granules release. Subsequently, cells were stained with with Live/Dead PromoFluor 840 maleimide reactive dyes (Invitrogen, CA, USA), anti-CD3, and anti-CD4, and fixed with a PBS plus 4% PFA. The intracellular cytokine staining (ICS) was performed permeabilizing with Saponin 0.5% (Sigma-Aldrich, St. Louis, MO, USA) and staining with FITC-conjugated anti-IFNγ (BD Bioscience) and PerCp5.5-conjugated anti-IL-2 (BD Bioscience) antibodies.

T cell proliferation and activation status after long term stimulation.

3M PBMCs were stained with 10µM of Cell Proliferation Dye eFluor® 450 (Invitrogen, CA, USA), according to manufacturer's instructions, and then plated at a concentration of 1M/ml in a final volume of 200 µl of completed X-VIVO in 96-flat well culture plates. Cells were activated or not with soluble CD3 Monoclonal Antibody (OKT3) at a final concentration of 0.05 µg/ml or with PHA (as a non-TCR-mediated stimulation). After 4 days culture supernatants were collected and cells were harvested, washed with PBS, and

stained for 10 minutes with Live/Dead PromoFluor 840 maleimide reactive dyes (Invitrogen, CA, USA).

Cells were then washed with PBS 2% FBS and then re-suspended in 50 µL of antibody mixture that included the following mAbs: anti-CD3, anti-CD4, anti-HLA-DR, anti-CD25, and anti-LAG-3.

T cell proliferation was evaluated through their Cell Proliferation Dye eFluor® 450 content because in each round of division that a T cell does there is a dilution of Cell Proliferation Dye signal and so proliferating cells show a lower eFluor® 450 staining than non-proliferating once. The percentage of proliferated cells was evaluated as the percentage of cells experiencing at least one round of division.

The level of IL-10, INF-γ and GM-CSF were evaluated by ELISA in culture supernatant. The standard curves were created diluting rhIL-10, rhINF-γ and rhGM-CSM in Blocking Buffer (PBS with 2% of bovine serum albumin) at the concentration defined by the manufacturer and the limits of detection were: IL-10: 15 pg/mL, INF- γ: 30 pg/mL and GM-CSF: 30 pg/mL

Peripheral and seminal plasma isolation.

Two rounds of centrifugation at room temperature at 1500 rpm for 15 minutes and at 1500 rpm for 10 minutes were performed to obtain plasma from EDTA peripheral blood.

Two rounds of centrifugation at room temperature at 2000 rpm for 15 minutes and at 1500 rpm for 10 minutes were performed to obtain plasma from seminal fluid.

All the samples were processed within 2 hours after collection and stored immediately at -80 °C until use.

Flow Cytometry staining on seminal fluid.

The frequencies of major leucocyte populations and of tolerogenic DC-10 and immunogenic cDC1 and cDC2 were assessed in SF within 2 h after sample collection. In brief, 50 µl of antibody (Ab) mix in brilliant stain buffer (BD biosciences, San Jose, CA) were directly added to 150 µl of sample and incubated for 15 min at room temperature in

the dark, and washed twice with PBS (Sigma, CA, USA). Then, samples were stained for 10 minutes at room temperature (RT) with Live/Dead PromoFluor 840 maleimide reactive dyes (Invitrogen, CA, USA) at a concentration of 0.25 µg/µl, washed and re-suspended in PBS (Sigma, CA, USA) with 2% FBS (Lonza, Italy). Cells were identified using a multiparametric approach as shown in Table 7 and based on the combination of monoclonal Abs listed in Table 8 and 11 and following the gating previously showed.

Table 11. List of monoclonal antibodies used in seminal fluid samples

	Fluorophore	**Clone**	**Producer**
CD3	Percp-Cy5.5	HIT3a	BioLegend, CA,USA
CD4	Pacific Blue	SK3	BioLegend, CA,USA
CD8	APC-H7	SK1	Biosciences, NJ, USA
CD11c	BV650	B-ly6	BD Horizon, NJ, USA
CD14	APC	MϕP9 BD	Biosciences, NJ, USA
CD15	FITC	MMA	Biosciences, NJ, USA
CD16	BV510	3G8	BD Horizon, NJ, USA
CD19	PE	4G7	Biosciences, NJ, USA
CD45	BUV395	HI30	BD Horizon, NJ, USA
CD56	Pe-Cy7	NCAM	BioLegend, CA,USA

Flow Cytometry Analysis.

Samples were acquired within 12 h after the staining using a FACS Cytoflex LX flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed with FCS express 6 (De Novo Software, Glendale, CA). Quadrant markers were set accordingly to the relative fluorescence minus one (FMO) staining.

Detection of cytokines and chemokines.

The presence of cytokines and chemokines in plasma from peripheral blood and seminal fluid was evaluated by Bio-Plex Multiplex immunoassays using Luminex magnetic beads (BIO-RAD, CA, USA) according to manufacturer's instructions. The limits of detection of the different analytes are indicated in Table 12.

Table 12 List of analytes and their limit of detections

Analyte	Detection limit (pg/ml)	Analyte	Detection limit (pg/ml)
IL-10	0.000810791	Basic FGF	0.003659973
IL-12 (p70)	0.001871765	Eotaxin	9.08203E-05
MCP-1	0.000584045	IFN-γ	0.000599365
MIP-1β	0.000360474	IL-1β	0.000272644
RANTES	0.001365906	IL-1Rα	0.010764343
TNF-α	0.003352356	IL-8	0.000809265

4. Statistical analysis

All results are presented as mean values ± Standard Error of Mean (SEM) or median and range, where appropriate. Continuous data of both groups was compared using the non-parametric Mann-Whitney U test while categorical variables were compared with the Fisher exact test. Where reached, statistically significant p values have been reported in the relative figures. Differences were regarded as significant at $P \leq 0.05$.

Results were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA) and SPSS v.26.

IV. Results

To characterize the immunological profile of infertile men, we collected peripheral blood and seminal fluids from infertile and fertile men enrolled based on criteria specified in Materials and Methods section.

Herein, we will describe the characteristics of this population and the analyses we performed on blood and seminal samples.

1. Seminal, clinical, and hormonal, and characteristics of the whole cohort

Overall, we enrolled in the study 51 infertile and 40 fertile (FER) men. Semen analyses were performed in all participants according to WHO reference criteria. Based on results from semen analyses (Table 13), the infertile subjects were further classified based on the diagnosis of oligo-astheno-teratozoospermia (OAT, n=38) and of idiopathic-Non-Obstructive Azoospermia (iNOA, n=13). Overall, no significant differences were observed in terms of sperm volume (p=0.8), pH (p=0.7) and number of leukocytes in semen among the three cohorts.

Table 13. Semen analyses of the enrolled men (median, min-max)

Semen parameters	FER (40)	OAT (38)	iNOA (13)	Lower limit
Concentration, 10^6/ml (Av and range)	47.9 (2-200)	4.4 (0.2-14)****	N.A.	15
Progressive motility (Av and range)	38.4 (15-64)	11.2 (0.0-28)****	N.A.	32
Normal Morphology (Av and range)	2.9 (1-10)	1.2 (0.0-3.0)****	N.A.	4

Groups were comparable in terms of age and BMI. As expected, testicular volume was smaller in infertile than fertile men (Boeri L, et. al 2020). Furthermore, waist circumference was longer in OAT and iNOA men compared to fertile controls. A higher rate of current smoking status was observed in infertile compared to fertile men. The rate of health significant comorbidities was low, with only 4 and 1 men showing a CCI >1 in the OAT and iNOA group, respectively. None of the fertile men enrolled displayed comorbidities (Table 14).

Table 14. Clinical characteristics of the enrolled men (median, IQR)

	FERTILE	OAT	iNOA
Patients, n	**40**	**38**	**13**
Age, years	36 (32-42)	37 (34-42)	36 (32-40)
BMI, Kg/m²	25.7 (23.4-28.0)	25.5 (24.0-27.3)	26.3 (22.7-30.1)
Waist circumference, cm	89 (85-94)	97 (92-100)*	100 (92-106)*
Average testicular volume, Prader	20 (15-21)	16 (13-20)	12 (6-15) *
Active smoker, n (%)	17 (43)	6 (16.2)*	3 (23)*
Presence of varicocele, n (%)	5 (13)	16 (51)*	4 (36)*
Comorbidities, n (%)			
Charlson comorbidity index >= 1	0	4	1

*p≤0.05 vs fertile

Partners of all infertile men were in overall good health status.

All participants underwent a complete set of laboratory exams, with values within normal ranges in all individuals. Likewise, a complete hormonal assessment was also performed in each group (Table 15).

Table 15: Hormonal levels in the entire cohort

	FERTILE	OAT	iNOA
Total testosterone, ng/mL	4.1 (3.5-5.1)	4.9 (3.6-5.6)	5.3 (2.9-6.3)
FSH, mUI/mL	3.3 (2.6-5.8)*	5.3 (3.9-7.1)*	21.9 (6.3-36.0)
LH, mUI/mL	4.8 (3.7-5.8)*	4.6 (4.0-5.8)*	7.4 (5.9-11.4)
Prolactin, ng/mL	14.3 (11-26)	11.1 (8.0-13.1)	9.1 (6-17.2)
Estrogens, pg/mL	20.3 (14.4-26.2)	21.1 (16.2-27.5)	22.7 (18.0-24.0)
TSH, mUI/mL	2.1 (1.7-2.8)	1.8 (1.4-2.4)	1.3 (0.9-2.1)
PSA, ng/mL	0.6 (0.5-1.0)	0.5 (0.4-0.7)	0.6 (0.6-0.8)

*p≤0.05 vs iNOA

As expected, iNOA men showed higher FSH and LH values compared to OAT and fertile men (all $p<0.01$). Conversely, total testosterone was similar among groups.

Karyotype and molecular studies of chromosome Y and CFTR gene were normal in all subjects.

2. Immunologic profile

2.1 Leucocyte composition in seminal fluid

The presence of immune cells and leukocyte composition in seminal fluid was evaluated by multiparameter flow cytometry.

Overall, the percentage of hematopoietic $CD45^+$ cells was higher in both OAT [mean (SD), 8.7% (11.7)] and iNOA [21.2% (16.2)] men compared to fertile men [0.6% (1.1)] (Figure 15A), probably as a consequence of the low presence/lack of spermatozoa in the seminal fluid from OAT and iNOA. Next, we investigated the overall leukocyte composition using CD45, CD3, CD4, CD8, CD14, CD15, CD16, and CD56 markers and the gating strategy previously described in Materials and Methods section. Myeloid cells represented the major leukocyte subsets in the seminal fluid. In details, OAT [45.1% (21.1)] and iNOA [58.9% (18.6)] men had higher neutrophil cells ($CD15^+CD16^+$) compared to fertile controls [32.6% (20.8)] ($p \leq 0.05$ and $p \leq 0.01$, respectively). Similarly, OAT participants [24.7% (20.9)] showed more eosinophils ($CD15^+CD16^-$) than fertile ones [15.5% (15.9)] ($p \leq 0.04$) (Figure 15B). No major differences in the frequency of CD14, CD3, and NK cells among the different cohorts were observed.

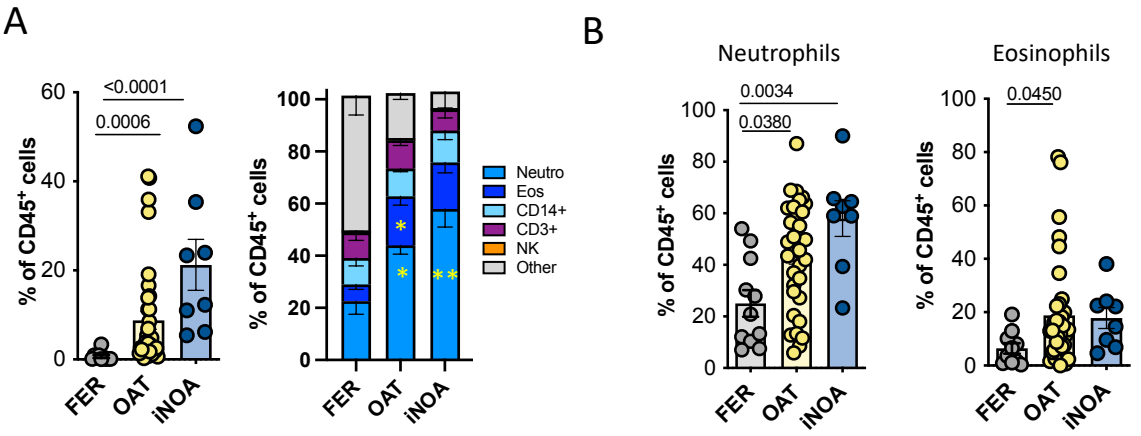


Figure 15. Leucocyte composition in seminal fluid of infertile and fertile men. Major leucocyte subsets were identified in the seminal fluid as alive and $CD45^+$ cells, and according to specific markers as indicated in Figure 8. **(A)** Percentages of $CD45^+$ cells (left panel) and of the indicated cell populations [right panel, (mean±SEM)] in the different cohorts of donors are shown. **(B)** Percentage of neutrophils and eosinophils in FER, OAT, and iNOA men. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in

association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Significant P values are reported.

2.2 Dendritic cell evaluation in the seminal fluid of infertile men

Next, we further dissected the myeloid compartment of seminal fluid by analyzing in each group of donors the presence and phenotype of tolerogenic DC-10 and the proportion of immunogenic (e.g. cDC1 and cDC2) DC subsets based on the markers and the gating strategy reported in Figure 9.

The overall rate of DC-10 was similar between fertile, OAT, and iNOA men (Figure 16A). Moreover, we evaluated the percentage of DC-10 expressing the tolerogenic molecule HLA-G and the activation marker CD83 as indicators of DC-10 tolerogenic potential (Amodio, 2015; Amodio, 2021). Results did not reveal any differences neither in DC-10 frequency, nor in DC-10 phenotype among the three groups of subjects (Figure 16A). On the contrary, a significant higher rate of pro-inflammatory cDC2 was observed in OAT participants compared to fertile controls [5.8 (5.5) vs. 2.8 (3.2), p=0.03)] (Figure 16B). On the same line, DC-10/cDC2 ratio was lower in OAT and iNOA men than fertile ones (all $p<0.01$) (Figure 16B). A similar trend was observed for cDC1 but without statistical significance. These results revealed a shift toward pro-inflammatory DC in peripheral blood of infertile men.

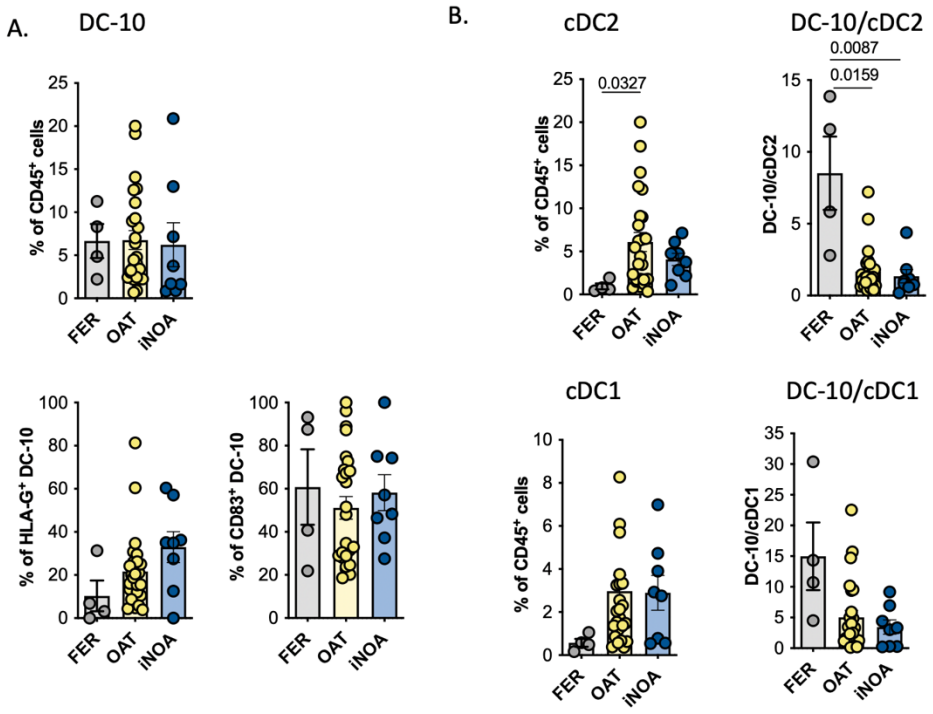


Figure 16. Proportion of DC-10 in seminal fluid of infertile and fertile men. Percentage of DC-10, cDC2, and cDC1 in OAT, fertile and iNOA men. **(A)** The frequency and phenotype of DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$) and **(B)** the proportion of cDC2 ($CD11c^+CD1c^+$), and of cCD1 ($CD11c^+CD14^-CD141^+$) have been evaluated in the indicated cohorts of donors. **(C)** The ratio between the percentage of DC-10 and of cDC2 (upper panel) and between the percentage of DC-10 and of cDC1 (lower panel) was calculated for each donor. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

2.3 Cytokines and chemokines in seminal plasma

Levels of several cytokines in seminal plasma of infertile and fertile men were next investigated using multi beads array. Of the cytokines analyzed, we observed an overall upregulation of myeloid-derived pro-inflammatory cytokines and of IL-10 in the seminal fluid of infertile compared to fertile men (Figure 17A). On the contrary, chemokines involved in leukocyte recruitment, such as MCP-1, MIP1b, RANTES, Eotaxin, and IL-8, were detected at lower or comparable concentration in samples from infertile men compared to fertile ones (Figure 17B).

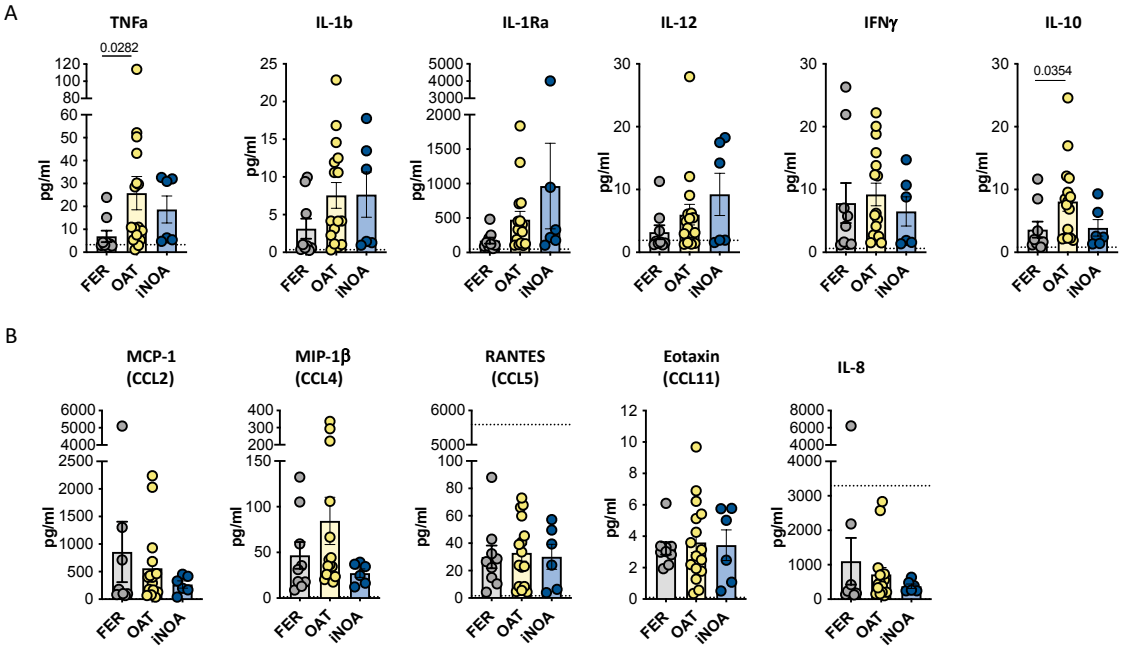


Figure 17. Seminal plasma levels of cytokines and chemokines in infertile and fertile men. The levels of the indicated (**A)** cytokines and **(B)** chemokines were measured in the seminal plasma of FER (n=9), OAT (n=16), and iNOA (n=6) men by multibeads array. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

2.4 Leucocyte subsets in whole blood

Similar to data from seminal plasma, the analysis of leucocyte subsets in the peripheral blood revealed a higher frequency of neutrophils in infertile men compared to fertile controls reaching statistical significance for OAT [44.4% (22.2) vs. 34.5% (17.8), p=0.01]. Conversely, in OAT compared to fertile men, we observed a lower proportion of $CD3^+$ [21.6% (2.1) vs. 33.9% (2.4), p<0.001] and $CD19^+$ [3.6% (2.3) vs. 4.4% (2.5), p=0.04] lymphocytes (Figure 18A). Notably, the reduction of the $CD3^+$ fraction was due to a decrease in both $CD4^+$ [11.4% (6.4) vs. 17.0% (7.5), p<0.01] and $CD8^+$ T cells [8.1% (5.7) vs. 12.1% (6.5), p<0.01] (Figure 18B). No major differences in terms of eosinophils, $CD14^+$ monocytes, natural killer (NK), and NKT cells frequency between infertile and fertile men were observed.

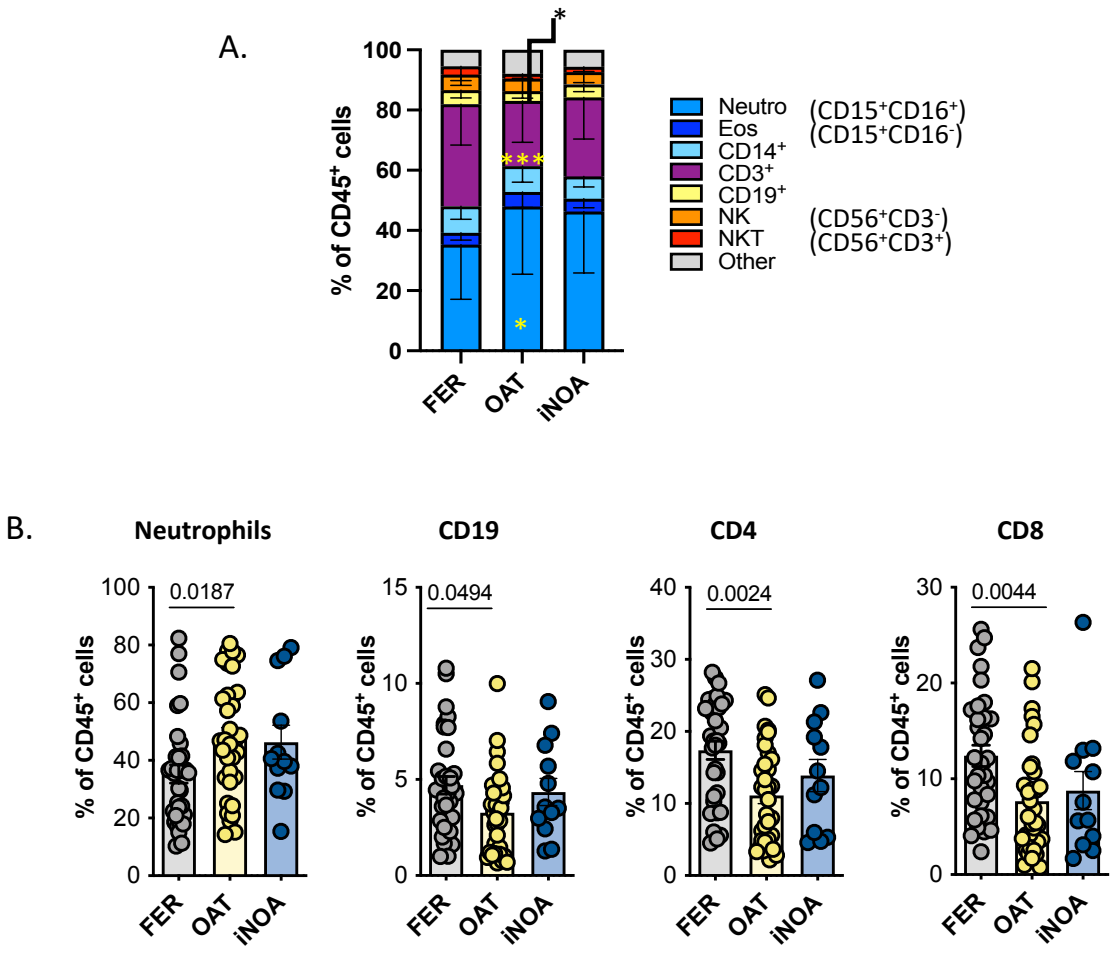


Figure 18. Leucocyte populations in peripheral blood of infertile and fertile men. The proportion of the major leukocyte populations in the peripheral blood of subjects enrolled in the study has been defined by multicolor flow cytometry in FER (n=33), OAT (n=34), and iNOA (n=12) men. **(A)** Bars represent the percentage of the indicated cell subset (mean±SEM). **(B)** Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

2.5 Regulatory T cells and DC-10 in peripheral blood

We next investigated whether the different proportion of leucocyte in the peripheral blood of infertile men was linked to alteration of the frequency of lymphoid and myeloid tolerogenic compartment by analyzing the proportion of $CD4^+$ regulatory T cells (Tregs) and of DC-10. The analysis of Tregs, focused on the detection of both Tr1 cells and $FOXP3^+$ Tregs, did not reveal any difference between infertile and fertile men (Figure 19A).

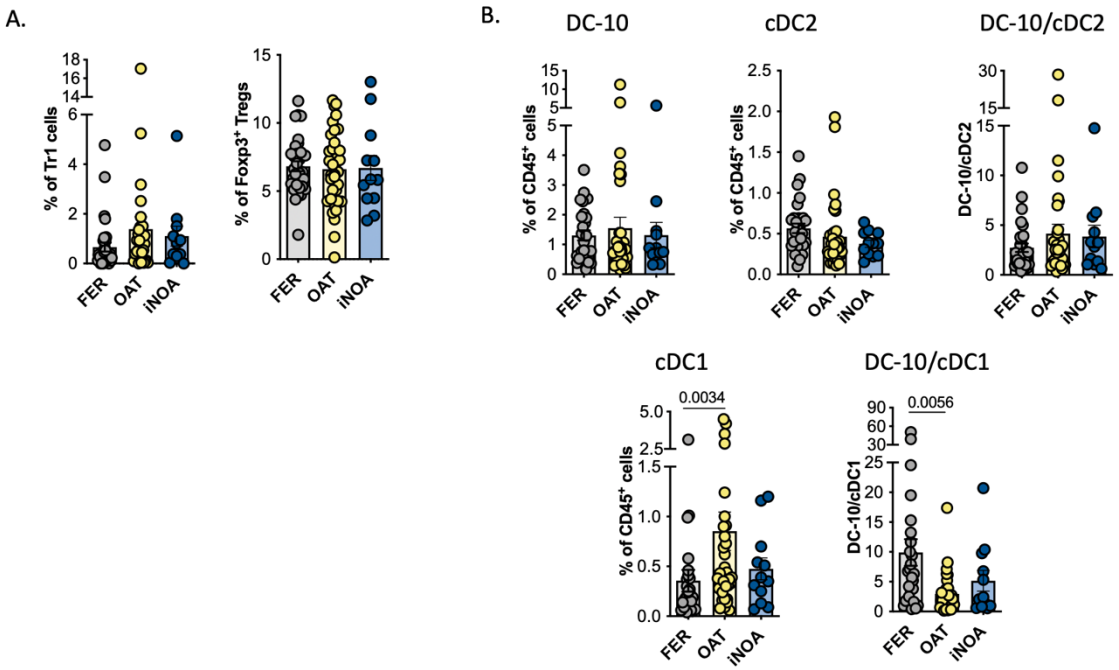


Figure 19. Analysis of the regulatory compartment in the peripheral blood of infertile and fertile men. The frequency of Tr1, Foxp3 Tregs, DC-10, cDC1, and cDC2 in peripheral blood of FER (n=29), OAT (n=35), and iNOA (n=12) men has been defined by multicolor flow cytometry based on the markers and the gating strategy reported in Figure 9. **(A)** Proportion of Type 1 regulatory T cells (Tr1, $CD4^+CD45RA^-CD49b^+LAG\text{-}3^+$) and $Foxp3^+$ regulatory T cells ($CD4^+CD25^+CD127^-Foxp3^+$) in the whole cohort. **(B)** The frequency and phenotype of DC-10 ($CD11c^+CD14^+CD16^+CD141^+CD163^+$) and the proportion of cCD1 ($CD11c^+CD14^-CD141^+$) and of cDC2 ($CD11c^+CD1c^+$) have been evaluated in the indicated cohorts of donors. **(C)** The ratio between the percentage of DC-10 and of cDC1 (upper panel) and between the percentage of DC-10 and of cDC2 (lower panel) was calculated for each donor. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

Similarly, the frequency of DC-10 was comparable among fertile, OAT, and iNOA men (Figure 19B). Of note, the analysis of the pro-inflammatory DC subset revealed that the rate of cDC1 was higher in OAT men as compared to fertile controls (p=0.003) while the proportion of cDC2 was comparable between infertile and fertile men (Figure 19B). In line with this result the DC-10/cDC1 ratio was lower in OAT than fertile men (p=0.005, Figure 19C), indicating a shift toward immunogenic DC in the peripheral blood of infertile men.

2.6 T cells characterization in peripheral blood

Activation status

To better characterize T cells in the peripheral blood, we firstly evaluated $CD4^+$ and $CD8^+$ T cell activation status by assessing the levels of expression of CD25, LAG-3 and HLA-DR activation markers in both populations (Figure 20).

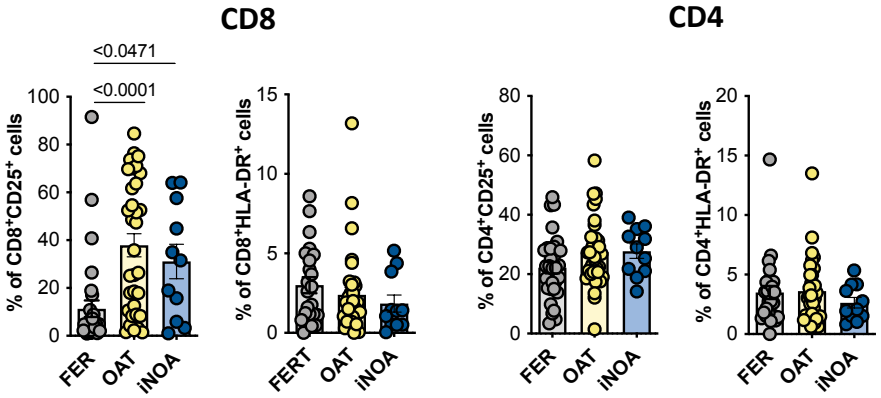


Figure 20. Frequency of activated T cells in peripheral blood of infertile and fertile men. The proportion of $CD8^+$ and $CD4^+$ T cells expressing activation markers in the peripheral blood of FER (n=31), OAT (n=33), and iNOA (n=11) subjects enrolled in the study has been defined by multicolor flow cytometry. The percentage of CD25- and of HLA-DR-expressing CD8 (left panels) and CD4 (right panels) has been evaluated in each cohort. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

We observed an overall higher frequency of circulating $CD8^+CD25^+$ T cells in infertile [36.2% (27.7) for OAT and 33.2% (27.3) for iNOA] compared to fertile men [15.9% (23.7)] (all $p<0.04$) (Figure 20). Conversely, no difference in activation status of CD4 T cells was noted among groups.

Exhaustion status

T cell exhaustion is a state of T cell dysfunction that arises during chronic T cell stimulation *in vivo*. It is defined by the sustained expression of inhibitory receptors including PD1, TIM-3 and KLRG-1, and the expression of specific transcription factors such as Eomes and T-bet (Wherry, 2011).

Exhaustion prevents optimal control of infection and tumors. Based on the hypothesis that in infertile men T cells might be less responsive compared to those in fertile men, we analyzed the presence and frequencies of $CD4^+$ and $CD8^+$ T cells expressing one or more exhaustion-associated markers.

In $CD4^+$ T cells, we observed a higher frequency of cells expressing KLRG1 in infertile men [7.3% (6.8) for OAT and 8.0% (9.0) for iNOA] compared to fertile men [5.0% (6.1)] (all $p \leq 0.02$) (Figure 21A). Similarly, PD1 and TIM3 were more expressed by CD4 of OAT and iNOA men vs. fertile participants (all $p \leq 0.01$). Moreover, we showed higher proportion of $CD4^+EOMES^+$ T cells in infertile men [12.3% (11.1) for OAT and 10.5% (12.2) for iNOA] compared to fertile men [6.7% (8.9)] (all $p<0.04$) (Figure 21A). Since the definition of exhausted phenotype for T cells relies on the co-expression of more than one exhausted-associated markers, we also performed this type of analysis. Results showed that both OAT and iNOA patients had a significantly higher frequency of $CD4^+$ T cells co-expressing KLRG-1 and PD-1 [3.2% (0.8) for OAT and 2.6% (0.7) for iNOA] compared to fertile participants [0.7% (0.2) (all $p<0.01$)] (Figure 21B). Similar results, even not statistically significant, were obtained from the analysis of the co-expression of KLRG-1, PD1, and TIM-3 (Figure 21B).

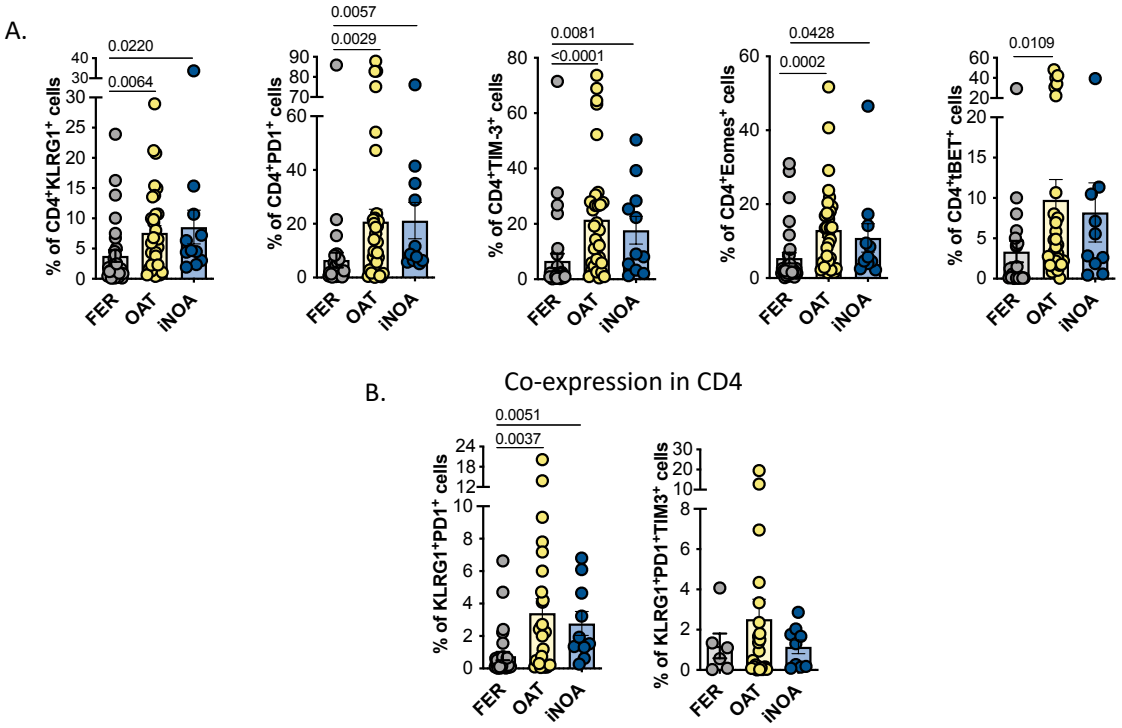


Figure 21. Frequency of exhausted CD4$^+$ T cells in peripheral blood of infertile and fertile men. The proportion of CD4$^+$ T cells expressing exhaustion markers in the peripheral blood of FER (n=31), OAT (n=33), and iNOA (n=11) subjects enrolled in the study has been defined by multicolor flow cytometry. **(A)** The frequency of CD4$^+$KLRG1$^+$, CD4$^+$PD-1$^+$, CD4$^+$TIM-3$^+$, CD4$^+$Eomes$^+$, and CD4$^+$Tbet$^+$, and **(B)** the frequency of CD4$^+$KLRG1$^+$PD1$^+$ and of CD4$^+$KLRG1$^+$PD1$^+$TIM3$^+$ co-expressing cells have been evaluated in the indicated cohort of donors. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

Interestingly, we obtained similar results from the analysis on CD8$^+$ T cells. Indeed, in CD8$^+$ T cells, we observed a higher frequency of T cells expressing PD1 and TIM3 from OAT and iNOA men vs. fertile participants (all p≤0.02). Moreover, we showed higher proportion of CD8$^+$EOMES$^+$ T cells in OAT men compared to fertile man [39.1% (20.1) vs. 25.8% (18.1), p<0.01] (Figure 22A). Notably, even though the percentage of CD8$^+$KLRG-1$^+$ cells was not different between infertile and fertile men, the percentage of CD8$^+$ T cells co-expressing KLRG-1 and PD-1 was higher in OAT and iNOA [3.9% (1.0) for OAT and 3.4% (0.9) for iNOA] compared to fertile participants [0.5% (0.2) (all p<0.001)] (Figure 22B).

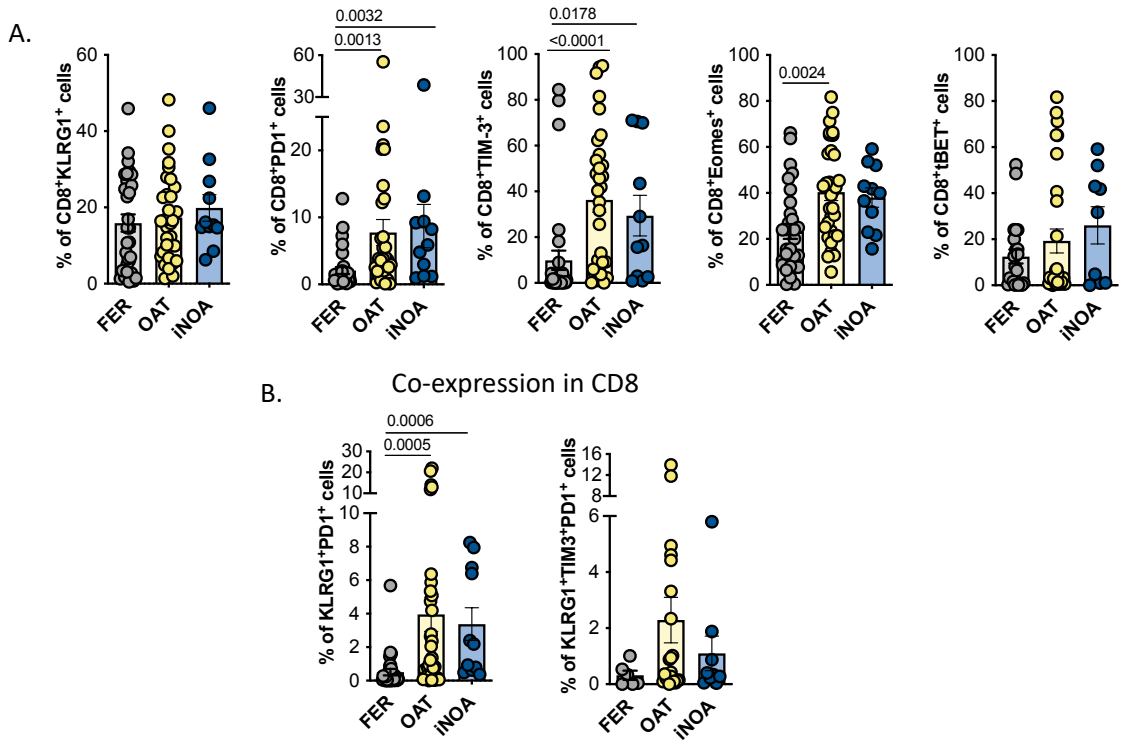


Figure 22. Frequency of exhausted CD8$^+$ T cells in peripheral blood of infertile and fertile men. The proportion of CD8+ T cells expressing exhaustion markers in the peripheral blood of FER (n=31), OAT (n=33), and iNOA (n=11) subjects has been defined by multicolor flow cytometry. **(A)** The frequency of CD8$^+$KLRG1$^+$, CD8$^+$PD-1$^+$, CD8$^+$TIM-3$^+$, CD8$^+$Eomes$^+$, CD8$^+$Tbet$^+$, and **(B)** the frequency of CD8$^+$KLRG1$^+$PD1$^+$ and of CD8$^+$KLRG1$^+$PD1$^+$TIM3$^+$ co-expressing cells have been evaluated in the indicated cohort of donors. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

2.7 Functional characterization of T cells

We next investigated the ability of CD4$^+$ and CD8$^+$ T cells isolated from peripheral blood of infertile and fertile men to proliferate in response to TCR-dependent or independent stimulation. To this end, T cells were labeled with a proliferation dye and stimulated with anti-CD3 mAb or with phytohemagglutinin (PHA) for 4 days. Both CD4$^+$ and CD8$^+$ T cells from infertile and fertile men proliferated at similar levels in response to tested stimuli (Figure 23).

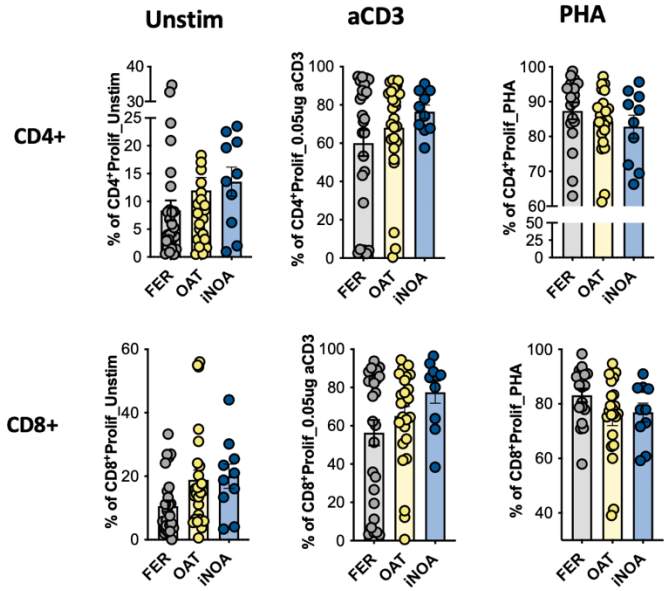


Figure 23. Proliferative response of T cells. Freshly isolated PBMCs from FER (n=27), OAT (n=26), and iNOA (n=10) subjects were stained with a proliferation dye (E-Fluor) and left unstimulated or activated with anti-CD3 or PHA for 4 days. The percentage of proliferated cells was evaluated by multicolor flow cytometry as percentage of cells that diluted the dye. The upper

panel represent the percentage of unstimulated and stimulated $CD4^+$ cells. The lower panel represent the percentage of unstimulated and stimulated $CD8^+$ cells. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

In parallel to the proliferative capacity, we evaluated the levels of IFN-γ, IL-10, and GM-CSF secreted in culture supernatants by PBMCs in response to the abovementioned stimuli. No differences in the ability of PBMCs to secrete IFN-γ, IL-10, and GM-CSF were detected when cells were stimulated with anti-CD3 mAbs. However, the stimulation with PHA revealed an overall lower ability of PBMCs from infertile men compared to fertile to secrete IL-10 and GM-CSF reaching statistical significance for iNOA ($p<0.05$ for all the cytokines tested) (Figure 24).

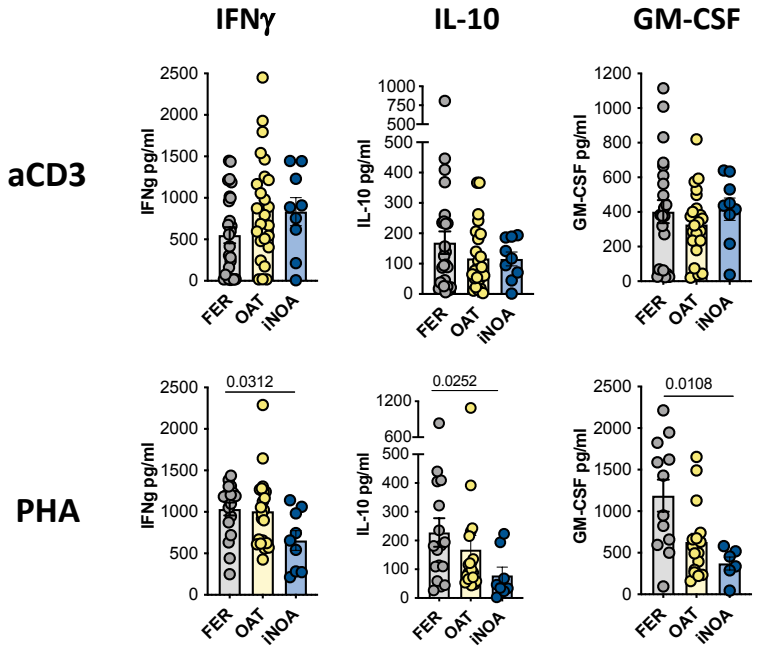


Figure 24. Cytokines expression at proliferative response. PBMCs from FER (n=24), OAT (n=17), and iNOA (n=6) subjects were stimulated with anti-CD3 mAbs (upper panels) or PHA (lower panels). The levels of INF-γ, IL-10, and GM-CSF were evaluated by ELISA in culture supernatant after 4 days of stimulation. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

Since 4 days of stimulation did not allow the evaluation of IL-2, which is rapidly up taken by proliferating cells, we performed a short-term stimulation of PBMCs from fertile, OAT and iNOA subjects with PMA and ionomycin. Results showed that, similarly to what observed for IFN-γ, IL-10, and GM-CSF, both $CD4^+$ and $CD8^+$ T cells from infertile men produced less IL-2 compared to cells from fertile men (Figure 25).

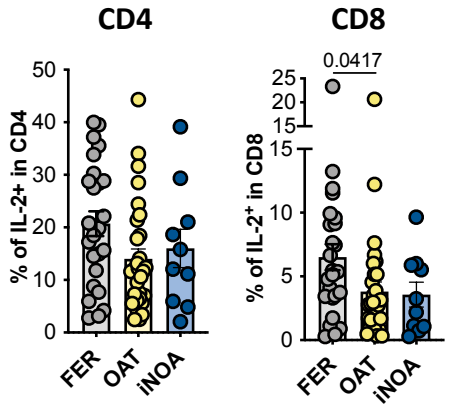


Figure 25. IL-2 production from $CD4^+$ and $CD8^+$ T cells activated with PMA and ionomycin. The proportion of $CD4^+$ and $CD8^+$ T cells producing IL-2 in response to PMA and IONO stimulation has been defined by multicolor flow cytometry. The frequency of $CD4^+$ (left panel) and of $CD8^+$ (right panel) cells expressing IL-2 has been evaluated in FER (n=25), OAT (n=30), and iNOA (n=10) subjects. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data. Statistically significant P values are reported.

2.8 Chemokines and cytokines in peripheral blood

Finally, the levels of cytokines and chemokines present in plasma of infertile and fertile men were investigated using the same multi beads array panel applied for seminal fluid. Results showed no major differences between infertile and fertile men for all the cytokines analyzed (e.g. TNFα, IL-12, and IFNγ,) (Figure 26A). Among the chemokines analyzed, MIP-1β, involved in promoting NK and monocytes recruitment during inflammation, appeared to be higher in OAT compared to fertile men, but without statistical significance. Similarly, the levels of MCP-1 and of RANTES, involved in T cell and eosinophil recruitment, seemed to be higher at least in a fraction of OAT subjects compared to fertile men (Figure 26B). No differences were detected among the groups in

the amounts of Eotaxin. The levels of IL-1β, IL-8, IL-10, and IL-1Ra, were undetectable in all the samples analyzed.

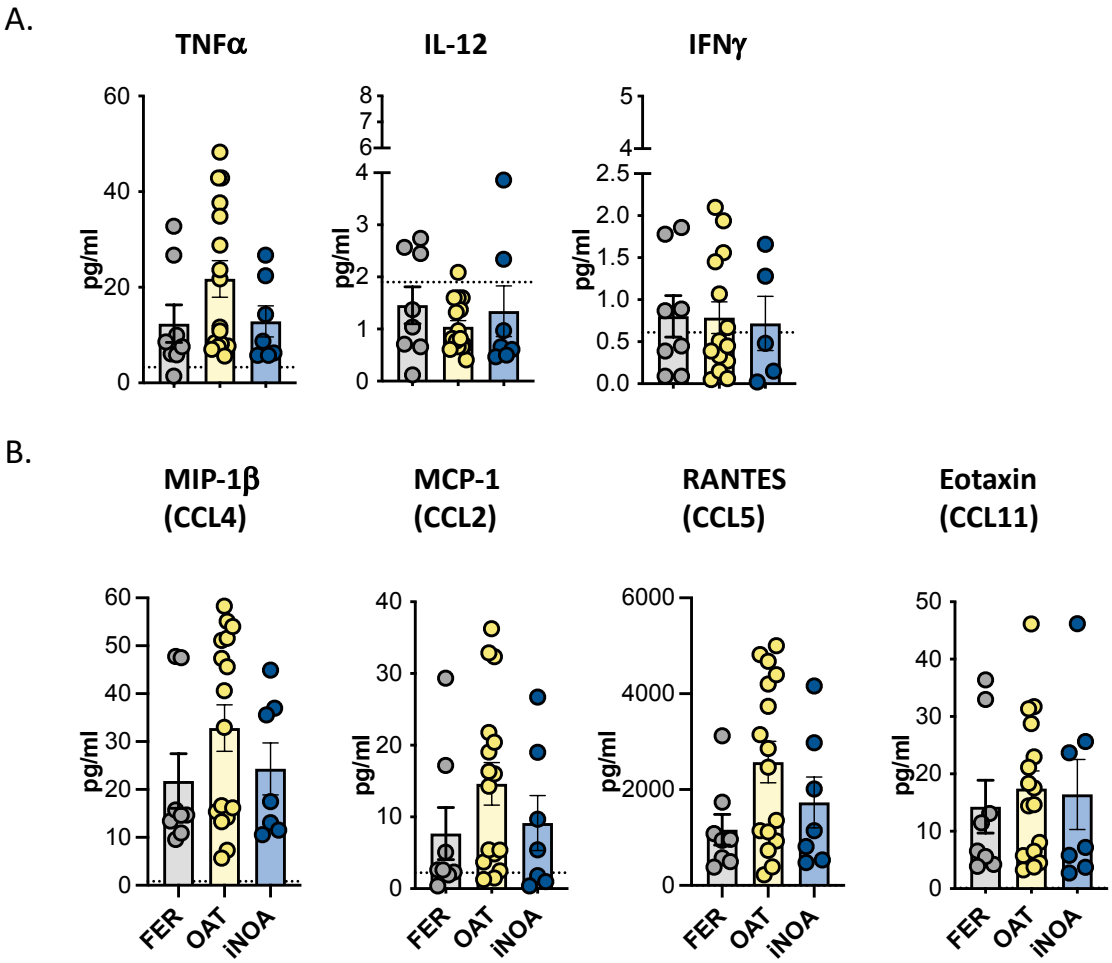


Figure 26. Serum levels of cytokines and chemokines in infertile and fertile men. The levels of the indicated **(A)** cytokines and **(B)** chemokines were measured in the seminal plasma of FER (n=8), OAT (n=17), and iNOA (n=7) men by multibeads array. Each dot represents a single donor; scatter plots indicate mean mean±SEM. Kruskal-Wallis test in association with Dunn's multiple comparison test were used to determine the statistical significance of the data.

V. DISCUSSION

Over the last decade several studies have shown that infertile men are “less healthy” individuals compared to age-matched fertile counterparts. Epidemiological studies have revealed that infertile men are at higher risk of cancer, chronic non-malignant comorbidities, endocrinological and autoimmune disorders and show an overall higher mortality rate compared to fertile men. However, the pathological mechanism underling the association between male infertility and health status is still a matter of debate.

The testis has always been considered an immune privileged site, with myeloid cells (macrophages and DC) representing the most prevalent resident immunological type. However, recent studies have shown that in severe infertile men, there is a shift toward a pro-inflammatory environment and a senescent phenotype of immune cells in the testis. Moreover, signs of systemic inflammation (e.g. upregulation of proinflammatory chemokines CCL4/MIP1β and CCL5/RANTES) have been identified also in the peripheral blood of NOA men (Alfano et al. 2021). Moreover, an impaired testicular biological signature associated with senescence was observed in NOA men. A similar immunological characterization of senescence was investigated by Marquez et al (2020). Authors reported that, in men, $CD8^+$ and $CD4^+$ T cells significantly decreased with ageing. Of note, cells from older men displayed a pro-inflammatory signature compared to those from younger individuals, suggesting that the ageing signature was characterized by declining adaptive responses and increased systemic inflammation.

From a translational point of view, the pro-inflammatory status of testicular cells and the senescent phenotype characterized by decreased T cells number and by higher level of inflammatory cytokines observed in infertile men could be associated with the infertility status per se, and it could also predispose to future risk of developing malignancies and chronic diseases.

However, an in-depth characterization of the local (e.g. testicular) and systemic immune status of infertile males and healthy controls has never been performed.

In this context, we designed a set of experiment to analyse phenotypically and functionally the leukocyte populations, and to investigate the presence of a pro-

inflammatory microenvironment in the peripheral blood and seminal fluid of infertile men.

At a local (testicular) level, we confirmed historical data showing that myeloid cells represent the major leukocyte subsets in the seminal fluid. Of clinical importance, our results indicate that infertile men, compared to fertile controls, have an overall higher local pro-inflammatory signature. This was derived from a higher frequency of neutrophils and from a higher proportion of immunogenic DC. Moreover, we observed an overall upregulation of myeloid-derived pro-inflammatory cytokines and of IL-10 in the seminal fluid of infertile compared to fertile men.

Previous Authors have investigated the association between leukocytes, oxidative stress and semen parameters in infertile men. Taken and colleagues discovered a negative correlation of leukocytes 8-OHdG/106dG with sperm count and sperm motility (Taken et al., 2016). In a recent meta-analysis, it has been reported that several markers of oxidative stress are abnormal in seminal plasma of infertile men, thus indicating that these parameters might be associated with infertility (Huang et al., 2018). Our results indicated that this inflammation is related to the high presence of neutrophils and of immunogenic DC and lead us to speculate that these leucocytes might either sustain the production of ROS, or directly contribute to infertility by affecting spermatozoa. Moreover, our study not only corroborated the presence of a local pro-inflammatory signature in iNOA men (Alfano et al. 2021), but also revealed that this signature is shared by infertile participants with OAT. Finally, we reported for the first time the presence of tolerogenic DC-10 in seminal fluid of both infertile and fertile men. Previous study already described a role of DC-10 in favoring successful pregnancy form the female side (Amodio and Mugione et al., 2013; Liu et al., 2019). Our findings pave the way to additional studies to better define whether DC-10 in seminal fluid play a role also from the male side by directly favoring oocyte fertilization.

To our knowledge, a detailed characterization of immune cells from peripheral blood of infertile men has not been performed yet. Interestingly, results on the overall leucocyte composition paralleled what observed in seminal fluid. Indeed, our analysis revealed a high frequency of neutrophils and of immunogenic DC also at systemic level (e.g. in the peripheral blood) of infertile men.

Moreover, the increase of neutrophils seemed to be paralleled by a lower proportion of $CD3^+$, both $CD4^+$ and $CD8^+$ T cells, and $CD19^+$ lymphocytes in infertile men compared to fertile controls. The analysis of the regulatory T cell compartment, including both IL-10-producing Tr1 and $FOXP3^+$ $CD4^+$ T cells, revealed no alteration of these subsets in infertile men, excluding the possibility that the reduction of $CD4^+$ T cells was associated to a contraction of the regulatory compartment. The reduction of $CD3^+$ lymphocyte, especially in males, has been described as a physiological contraction related to ageing (Marquez et al., 2020). Interestingly, despite the age of our cohort of infertile men correspond to the young cohort described by Marquez et al., the percentages of $CD4^+$ and $CD8^+$ T cells that we measured in our infertile patients, are closer to the range previously associated with older subjects (e.g. over 65 years of age).

A more detailed characterization of T cells including the evaluation of activatory and inhibitory surface markers, revealed that $CD8^+$ T cells from OAT and iNOA men were highly activated compared to those from fertile counterparts. Moreover, we observed a higher frequency of $CD4^+$ and $CD8^+$ T cells expressing at least one inhibitory molecule, such as PD1, KLRG1 and TIM-3, compared to fertile men. PD1, KLRG1 and TIM-3 are markers of exhaustion, and their expression appears as a consequence of repeated antigenic stimulation and hyper-activation of the immune system. In infertile subjects we described the high proportion of immunogenic DC but also a pro-inflammatory signature due to the presence of high cytokines and chemokines that can contribute to the development of the exhausted T cells. Further sustaining the presence of exhausted T cells in infertile subjects, are the functional data showing an impaired ability of these cells to secrete IFNγ, GM-CSF and IL-2.

Overall, the reduced levels of $CD4^+$ and $CD8^+$ T cells, along with the higher pro-inflammatory and exhausted signatures seem to depict a senescent phenotype for infertile men. Immunological senescence could be one of the underlaying mechanism behind the infertility status per se and the higher risk of developing comorbidities later in life typical of infertile men.

It has been repetitively demonstrated that exhausted T cells may contribute to tumor growth and the incidence of tumors in infertile men has been reported to be higher than

fertile subjects. More in detail, the most thoroughly investigated relationship is between infertility and urological cancer. Indeed, men in the lowest quartile of spermatozoa morphology, viability, motility or total motile count have an increased risk of testicular cancer (Hanson et al., 2016). Furthermore, Walsh and colleagues described that infertile men had an increased risk of developing testicular cancer by nearly threefold as compared with fertile men (Walsh et al., 2009). Beside urogenital malignancies, other types of tumor have been also correlated with male infertility. Eisenberg and colleagues showed that patients with male infertility presented a 49% higher risk for being subsequently diagnosed with any cancer compared to fertile men (Eisenberg et al., 2015). A possible association between male infertility and the risk of prostate cancer has also been described. Recently, Boeri et al. (Boeri et al. 2020) reported that infertile men have higher PSA values than fertile age-matched controls. Therefore, a role for prostate cancer development/progression by exhausted T cells with senescent phenotype could also be speculated.

All these evidences indicated that the pro-inflammatory signature present in infertile men is mainly related to a hyper-activation of the immunogenic arm of the immune system rather than to alterations in the regulatory compartments such as Tregs and DC-10. Indeed, we did not observe impairment neither in the frequency nor in the phenotype of DC-10 in the peripheral blood of infertile men.

These data are in line with the presence of an overall pro-inflammatory and exhausted signature in the local and peripheral tissue of infertile men. Since infertile men are more likely to develop autoimmune and chronic metabolic conditions earlier in life than fertile controls, we could speculate that this immunogenic impaired signature could be one of the reasons associated with impaired health of infertile men.

The pro-inflammatory environment was supported by cytokine and chemokines production. We reported a higher level of MIP-1β, which is implied not only to activate neutrophils, basophils and eosinophils, but also to promote T cell activation and induction of pro-inflammatory cytokines, such as IL-1β, IL-6 e TNF-α. Similarly, the levels of RANTES, involved in T cell and eosinophil recruitment, were higher in infertile as compared with fertile men. Overall, these findings suggest that higher levels of pro-inflammatory chemokines might be associated with an activation of adaptive immunity

and T cells; this high level of inflammation would be paralleled to the increased number of cDC1 and cDC2 cells, thus increased inflammatory signature and cellular senescence.

Finally, we showed that PBMCs from iNOA patients had lower ability to secrete IL-10 in response to stimuli. IL-10 is an immunomodulatory cytokine playing a central role in regulating inflammation, down-regulating immune responses, stimulating tolerance and in preventing chronic inflammatory pathologies (Gregori et al., 2012). Moreover, insufficiency or aberrant expression of IL-10 favors inflammatory responses to microbial challenge and lead to the development of inflammatory bowel disease (Engelhardt et al., 2014) and several autoimmune diseases (Groux et al., 2003). Since autoimmune disease and chronic inflammation are more prevalent in infertile than in fertile men, a possible role for IL-10 imbalance can be suggested.

Our results are novel since we performed, for the first time, a detailed tissue-specific and systemic immunological characterization of infertile men. Our findings can be summarized as follows:

Infertile men showed local:

- High frequency of neutrophils
- No alteration in the proportion of DC-10
- High frequency of immunogenic DC
- High pro-inflammatory mediators.

Infertile men showed systemically:

- High frequency of neutrophils
- Low frequency of T cells
- High proportion of exhausted T cells
- No alteration in the proportion of DC-10 and Tregs
- High frequency of immunogenic DC
- High inflammatory cytokines and chemokines.

Therefore, for the first time, we reported that male infertility is associated to a deregulation of the immune system without defect of the regulatory compartment (DC-

10 and Tr1 cells) and with a pro-inflammatory signature that leads to the expansion of exhaustion T cell compartment.

Our study is not devoid of limitations. First, the relatively small cohort of patients in each group may limit the generalizability of the results and the reliability of the association between study variables. Moreover, a detailed correlation between our findings on peripheral blood and seminal fluid and variables from the medical history could not be effectively assessed; therefore, it would be relevant to further analyze whether our findings are related or not to any specific characteristics observed in infertile men (e.g., the presence of varicocele, overweight, comorbidities, hormonal parameters).

Given these premises, this study, although preliminary, provides novel evidence that the immunological status of infertile men both at systemic and local levels is different from that of fertile controls. If further corroborated by studies including larger cohorts of patients and longer follow up, we could speculate that the immunological profile of infertile men, characterized by a pro-inflammatory and senescent signature, can help the knowledge on the pathobiology behind the lower overall health status observed in infertile compared to fertile men. Moreover, results might be also important to define the pathophysiological mechanism associated with idiopathic infertility.

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