Immune molecules in the female reproductive path and sperm: relevance to mate choice

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Abstract

Mate choice has been postulated to be MHC-dependent, ensuring the maintenance of polymorphism for species survival. At the molecular level, MHC polymorphism is represented by class-I (MHCI), class-II (MHCII) antigens and their T cell receptors (TCRs). In order to evaluate the presence such immune molecules during male/female interaction, vaginal fluid, vaginal cells, urine, sperm, seminal fluid, cumulus cells, tubal fluid and epithelium were isolated from BALB/c mice and examined for the presence of membrane or soluble MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$, using immunofluorescence and ELISA techniques, respectively. These molecules were expressed on sperm and seminal fluid in a sperm quality-dependent manner and in vagina, fallopian tube, cumulus cells and urine in an estrus cycle-dependent manner. Vaginal cells showed increased expression of all molecules tested during estrus, while vaginal fluid showed an increase of TCR $\gamma\delta$ and decrease of MHCI and MHCII levels, during estrus. Urine showed only increased concentrations of TCR $\alpha\beta$ during estrus. Cumulus cells expressed MHCI, MHCII, TCR $\gamma\delta$ but not TCR $\alpha\beta$, while sperm mainly expressed TCR $\alpha\beta$ and TCR $\gamma\delta$. All molecules were detected in tubal fluids mostly during estrus, while they were almost undetectable during pregnancy. The vaginal environment was shown to affect sperm motility according to the estrus-cycle, whereas sperm motility was affected by antibodies against these molecules. In conclusion, the presence of complementary immune molecules in the male/female interactive environment, except for revealing novel markers for unexplained infertility, provides for the first time evidence for immune-mediated recognition of the two counterparts, enlightening thus a molecular basis for mate choice.

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Introduction

The maintenance of MHC polymorphism in species is a fundamental natural process ensuring survival and evolution (Bernatchez & Landry 2003, Sommer 2005, Piertney & Oliver 2006, Alcaide *et al.* 2010, Spurgin & Richardson 2010). MHC genes and products dictate individual responsiveness to an antigenic stimulus including bacterial, viral or any parasite load (Madsen & Ujvari 2006, Schwensow *et al.* 2007), environmental adaptations (Evans *et al.* 2010), maternal-fetal interactions (Thomas *et al.* 1985, Hedrick & Thomson 1988) and reproductive success (Kalbe *et al.* 2009), while MHC polymorphism is being considered as a major component in mate choice (Penn & Potts 1999, Penn 2002, Eizaguirre *et al.* 2009) or cryptic female choice (as proposed by Eberhard 1996) providing the optimal MHC repertoire to the offspring (Milinski *et al.* 2005, Eizaguirre *et al.* 2009). The advantage of dissimilar MHC molecules contributing to the repertoire of the offspring is almost certainly a wider toolkit for pathogen

detection. An additional and not mutually exclusive benefit could be in form of better fetto-embryonic compatibility, allowing the maternal immune system to better identify and optimally contain the fetal cells.

The most relevant mechanisms in reproduction, concerning species evolution are natural selection, sexual selection and genetic drift (Lewontin 1970, Masel 2011). Natural selection is based on phenotypic variation, differential fitness and the heritability of fitness (Lewontin 1970). According to such mechanism, the most advantageous traits will be propagated to the next generation thus producing a competition between the organisms for survival and reproduction. According to the genetic drift mechanism, random changes of allelic frequencies from one generation to the other dictate evolution, either by excluding drifted alleles from the population or entirely replacing homolog alleles (Masel 2011), mainly due to demographic factors (small populations associated with population bottlenecks or founder events).

According to the genetic drift mechanism individuals with functional deviating MHC are selectively advantageous (Piertney & Oliver 2006). The pressure to maintain high allelic diversity at the MHC loci is in accordance with increased defense to a larger number of pathogenic antigens. Indeed, MHC marker analysis in endangered species has been correlated with extinction risk (Ujvari & Belov 2011). Such MHC diversity can be maintained at high levels through sexual selection, including MHC-related selective fertilization (Wedekind *et al.* 1996, 2004, Rulicke *et al.* 1998), MHC-dependent selective abortion (Alberts & Ober 1993) or disassortative matings based on MHC genotype (Penn & Potts 1998). Thus, MHC-related mate choice would facilitate inbreeding avoidance, enriching genome-wide variation in offspring and maximizing immunological abilities, while maintaining high levels of polymorphism, generating heterozygote excess and linkage disequilibrium characteristics (Hedrick 1992, Apanius *et al.* 1997). In various species, mate choice has been correlated with odortypes, which are considered as soluble forms of MHC molecules in body's excretions, although not yet understood how they become volatile (Wedekind & Penn 2000).

However, it is worth wondering whether mate choice through odortype selection is the only mechanism in evolution ensuring the increase of MHC polymorphism in species. Considering that sperm travel through the vagina to the uterus to enter the fallopian tube, where interaction with the ciliated cells and the oviduct fluid facilitates traversing cumulus cells and reaching zona pellucida for ovum penetration (Abe 1996, Steinhauer *et al.* 2004), it seems logical for the female reproductive tract to display additional check point mechanisms for the selection of the proper spermatozoa for fertilization, pregnancy and healthy offspring development, ensuring the increase of MHC polymorphism under the spectrum of the evolutionary necessity for species survival.

Immune polymorphic molecules include MHC class I (MHCI) and class II (MHCII) molecules as well as their receptors namely, T cell receptor $\alpha\beta$ (TCR $\alpha\beta$) and $\gamma\delta$ (TCR $\gamma\delta$). MHCI and MHCII molecules are membrane-anchored or soluble glycosylated heterodimers bound with an immune antigenic epitope at their active site. The classical, highly polymorphic MHCI molecules are encoded by *B*, *C*, *A* genes in the *HLA* locus in humans and *K*, *D* genes in the *H-2* locus in mice, which during the molecular maturation process in the endoplasmic reticulum are being loaded with peptides and recognized either by TCR $\alpha\beta$ of CD8-positive T cells or TCR $\gamma\delta$. Similarly, classical MHCII molecules are encoded by *DP*, *DQ*, *DR* genes in the *HLA* locus in humans and *I-A*, *I-E* genes in the *H-2* locus in mice, which during the molecular maturation process in the endosomal compartment are being loaded with peptides and recognized by TCR $\alpha\beta$ of CD4-positive T cells. It is

worth noticing that soluble MHCII molecules exert an inhibitory effect on CD4-positive T helper cells, where upon binding to TCR $\alpha\beta$ stimulate inhibitory pathways to the cells (Bakela *et al.* 2015).

Considering that MHC-TCR interactions could neutralize, mask or stimulate male female communication, the present study examined the presence of these polymorphic elements mainly in the female reproductive tract, which comes into contact with sperm, leading to fertilization and embryo development. The results presented herein show that such polymorphic elements could indeed be detected throughout the reproductive tract of mice, affect male/female interactions and consequently interfere with fertility.

Materials and methods

Animals and animal manipulation

BALB/c (H-2^d) and CBA/J (H-2^k) inbred mice were purchased from Charles River and bred in the animal facility of the Department of Biology at the University of Crete (Crete, Greece, EL91-BIObr-09) under standard conditions of temperature (18–25°C), humidity (45–50%) and photoperiod of 12 h light and 12 h dark. Males and females 6–14 weeks of age (20 ± 0.3 g) were handled according to the international and national bioethical rules and conformed to the bioethics regulations following the EU Directive 2010/63/EU for animal experiments.

Vaginal fluid and urine sample collection was performed during two estrus cycles for each of the three mice tested. Vaginal cells were processed for Giemsa staining and immunofluorescence experiments, while vaginal fluid and urine were submitted to enzyme linked immunosorbent assays (ELISA). In addition, samples were isolated from three mice during the 12th day of pregnancy and two days after labor, considering as day 0 the day the vaginal plug was observed after overnight caging with a fertile male.

For oocyte collection, female mice were superovulated by intraperitoneal injections of pregnant mare serum (PMSG, 5 IU/mice, Sigma, G4877) and human chorionic gonadotropin (hCG, 5 IU/mice, Sigma, CG5-1VL) at an interval of 47 h. The time of administration of gonadotropins was based on the light cycle used. Oocyte-cumulus complexes were collected 15–17 h post-hCG injection.

For fallopian tube epithelial cell and oviduct fluid collection, females were killed by cervical dislocation. For the oviduct fluid collection, mice were cycle synchronized following the superovulation protocol, where estrus was obtained 10–11 h post-hCG injection. In some cases, mice were caged with males and oviduct fluid was also collected on days 0, 1 and 2 of pregnancy.

Sperm and seminal fluid was isolated from the epididymis of fertile males upon killing by cervical dislocation. Sperm was submitted to sperm quality evaluation, immunofluorescence and motility experiments, while seminal fluid was submitted to ELISA.

Antibodies

Monoclonal anti-mouse H-2K^d (hybridoma HB159TM, ATCC, Rockefeller University, NY) was purified from culture supernatants, used at the concentration of 0.1 µg/mL in ELISA experiments and of 1 µg/mL in immunofluorescence experiments. Fluorescein-conjugated mouse anti-IA^d monoclonal antibody (Becton Dickinson, New Jersey, USA) was used at the concentration of 1 µg/mL in immunofluorescence experiments. Mouse anti-IA/IE mAb (HB-225TM hybridoma: *Mus musculus* (myeloma), hamster, Armenian B cell, reacts with a monomorphic determinant on the I-A and I-E region, IgG isotype, generous gift from Dr R Steinman, (Rockefeller University, NY) was purified from culture supernatants and used at the concentration of 0.1 µg/mL in ELISA experiments. PE-conjugated TCR α/β (anti-mouse, beta TCR PE conjugated, Immunotools 22155214, Friesoythe, Germany) or FITC-conjugated TCR γ/δ (anti-mouse, gamma/delta TCR FITC conjugated, Immunotools 22155223) were used at the concentration of 1 µg/mL in immunofluorescence experiments. TCR β (Armenian hamster anti-mouse, BioLegend, B167344, San Diego, CA,) or TCR γ/δ (Armenian hamster anti-mouse, BioLegend, B272223) were used at the concentration of 0.1 µg/mL in ELISA experiments. Secondary antibodies IgG Fab Specific (anti-mouse, Peroxidase-conjugated, Sigma, Ronkonkoma, NY) or IgG-FITC (goat anti-mouse, Sigma), were used at the concentrations of 0.1 µg/mL or 1 µg/mL in ELISA and immunofluorescence experiments, respectively.

Vaginal cell and fluid collection

Collection of vaginal cells and fluids was performed during natural cycle at the same time every day, following the technique described by [Cora et al. \(2015\)](#). Briefly, upon immobilization of the mouse in the palm, by holding the back skin using the index finger and thumb, 10 µL of phosphate buffer saline (PBS, Sigma) were placed to the vagina opening at 1–2 mm depth, and smoothly washed 3–5 times with the same buffer ([Caligioni 2009](#), [Byers et al. 2012](#), [Cora et al. 2015](#)). The content of the last wash was collected in an eppendorf (Sarstedt, Numbrecht, Germany) containing 90 µL of PBS to a final volume of 100 µL. The samples were centrifuged for 15 min at 1100 rpm, and while supernatants were collected to clean tubes until tested for the content of immune polymorphic molecules by ELISA, the cell pellet was resuspended in 100 µL PBS and processed either for Giemsa staining and immunofluorescence experiments.

Urine collection

Holding the mouse at the base of the tail, the vagina opening and urethra become obvious. If urine is not produced during animal handling, this could be achieved by a slight pressure to the bladder. Urine samples were collected in eppendorfs, centrifuged for 3 min at 2000 rpm to eliminate any debris or mucus, transferred to clean tubes and stored at 4°C until tested ([Mestecky et al. 2015](#)).

Oviduct fluid and oocyte collection

Oviducts were isolated from cycle synchronized mice at each stage of the estrus cycle, and flushed with PBS using a mouth pipette. Oviduct fluid was collected in a final volume of 600 µL, centrifuged for 5 min at 4000 rpm to eliminate any debris, transferred to clean tubes and stored at 4°C until tested. During ovulation, oocytes can be distinguished in the swollen ampulla, from where, using a mouth pipette, were flushed out ([Kyvelidou et al. 2016](#)). Oocyte-cumulus complexes were isolated using a stereoscope, transferred to droplets of PBS supplemented with 0.1% bovine serum albumin (BSA, Sigma) and processed for immunofluorescence experiments. In other experiments, epithelial fallopian tube cells were aspirated from the tube using a mouth pipette ([Hirose et al. 2003](#)), washed in HBSS and cultured in DMEM medium (Biosera) supplemented with 10% fetal bovine serum (FBS, Biosera) at the concentration of 1×10^6 cells/mL in 24-well plates (Sarstedt) for 48 h.

Sperm and seminal fluid collection

After isolating clean cauda epididymis and performing a small cut at the edge, soft squeezing of the tube in 1 mL HBSS (Biosera, Kansas City, USA) released spermatozoa and seminal fluid ([Anyfantaki et al. 2018](#)). The sample was then transferred to eppendorfs, centrifuged for 6 min at 2500–3000 rpm. The supernatant seminal fluid was transferred in clean tubes and stored at 4°C until tested, while the cell pellet containing spermatozoa was resuspended and either capacitated using PBS-BSA 2% for evaluating sperm motility or fixed in 4% paraformaldehyde (PFA, Sigma) for immunofluorescence experiments. In some experiments, the effect of antibodies against MHCI, MHCII, TCRαβ and TCRγδ on sperm motility was evaluated. To this extend, 60 µL of sperm were added to 30 µL of test antibody, or 0.1% PBS-BSA as control, and 6 µL of capacitation buffer. In other experiments, upon capacitation spermatozoa were added to a vaginal cell layer in the presence or not of vaginal fluid and sperm motility was evaluated.

Sperm motility evaluation

In order to definitely quantify sperm motility under the different conditions and timing tested, 10 s recordings of three different microscope fields were performed using a digital microscope imager (Celestron, Torrance, California). In all cases, 10 µL of capacitated sperm in the presence or not of antibodies, vaginal cells or oviduct fluid were placed on a slide, covered with a cover slip and motility was recorded. Recordings were thereafter analyzed and sperm motility, as to the number of spermatozoa with grade a to d motilities, was evaluated ([Cooper et al. 2010](#)). Thus, progressive motility (grade a) includes the strongest and swim fast in a straight line motility, nonlinear motility (grade b) includes curved or crooked motion, in nonprogressive motility (grade c) spermatozoa do not move forward, even if they move their tails, whereas immotile (grade d) spermatozoa fail to move at all. In the results presented here, only spermatozoa with progressive motility are shown. Sperm mobility video was edited using the Final Cut Pro software.

Giemsa staining

Vaginal cells (50 μ L of the preparations described above) were placed on slides, let to dry, fixed with isopropanol (Sigma) for 5 s, washed with ddH₂O, dried and proceeded to Giemsa staining (Sigma) as described by the manufacturer (Brown *et al.* 1993).

Immunofluorescence staining

Vaginal cells (10 μ L of the preparations described above) were placed on slides, let to dry, fixed with 4% w/v PFA in PBS for 10 min, washed three times with PBS, and proceeded to immunofluorescence staining. In this case, each sample was incubated for 30 min with blocking PBS-BSA 3% solution at room temperature, washed with PBS, incubated with the appropriate antibody diluted in PBS-BSA 1% w/v for 45 min at room temperature and if necessary, after washing, the second antibody was added. After mounting the samples with 30% glycerol (Sigma) and covering with a cover slip, the percent of fluorescent cells was evaluated using a fluorescent microscope (Zeiss, Oberkochen, Germany). A similar procedure was followed for oocytes and sperm, but in this case, oocyte-cumulus complexes were incubated in small droplets, while for sperm all incubations were conducted in small eppendorf tubes and finally the cells were submitted to flow cytometry analysis (FACScan, Becton Dickinson).

ELISA techniques

Indirect ELISA was performed in vaginal (1:50 dilution), oviduct (1:2 dilution), seminal (1:2 dilution) fluids, supernatants of epithelial fallopian tube cell cultures (1:2 dilution) and urine (1:50 dilution) as previously described (Bakela *et al.* 2015).

Statistical analysis

Data were analyzed with two-tailed Paired Student's T-test. *P*-values <0.05 were considered significant (*), values <0.01 were considered very significant (**), and values <0.001 and <0.0001 were considered highly significant (***) and ****). Statistics were performed using GraphPad Prism 6.01 (Graphpad Software, La Jolla, CA).

Results

Following the course of sperm through the female reproductive tract, the presence of membrane or soluble immune polymorphic elements susceptible to molecular interactions were examined in the vagina, oviduct, cumulus cells as well as sperm.

Expression of polymorphic immune molecules in the vagina

Vaginal receptivity to sperm represents the first contact of the female and male counterparts, toward fertilization and pregnancy. In mice, females will only allow

copulation during estrus. Therefore, except from the hormonal changes guiding each reproductive stage, one would expect polymorphic immune molecules, if any, to display differential expression during the different stages of the reproductive cycle.

Thus, female mice were followed through the reproductive cycle and vaginal cells as well as vaginal fluid and urine were examined for the expression levels or presence of MHCI, MHCII, TCR $\alpha\beta$ or TCR $\gamma\delta$, respectively. To identify each reproductive stage, vaginal smears were submitted to Giemsa staining for the detection of leukocytes, (mainly neutrophils), nucleated small or large epithelial cells as well as anucleated keratinized epithelial cells (Cora *et al.* 2015). To this extend, proestrus is mainly characterized by the presence of small, round nucleated epithelial cells of uniform appearance and size; estrus is mainly characterized by the presence of anucleated keratinized epithelial cells and occasionally by nucleated epithelial cells; metestrus is characterized by a combination of anucleated keratinized epithelial cells and neutrophils; diestrus, which is the largest stage of the estrus cycle, is characterized by a decreased number of anucleated keratinized epithelial cells and a moderate to low combination of small or large nucleated epithelial cells (Fig. 1; Cora *et al.* 2015).

Immunofluorescence staining of vaginal smears detected the presence of all immune polymorphic elements tested in the different stages of the estrus cycle (Fig. 1). In all cases a statistically significant decrease

Figure 1 Vaginal cells isolated from the different stages of the estrus cycle of BALB/c mice and stained either with Giemsa or with various antibodies for the detection of MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$ by immunofluorescence. A representative experiment is shown here. Photographs were taken using a 10x objective of an optical fluorescent microscope.

was observed during metestrus, which varied from 40 to 67% as compared to estrus (Fig. 2A). Although the expression levels of MHCI, MHCII and TCR $\alpha\beta$ during proestrus, estrus and diestrus did not show any statistically significant change, the expression of TCR $\gamma\delta$ was observed to increase by 24% ($P=0.0017$) from proestrus to estrus, decrease by 67% ($P<0.0001$) from estrus to metestrus and increase again by 240% from metestrus to diestrus (Fig. 2A).

Following the same rationale, the presence of soluble MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$ was examined in vaginal fluid and urine during the four stages of the estrus cycle, but also on day 12 of pregnancy and two days after labor (Fig. 2B). The results showed that soluble MHCI significantly increased from proestrus to estrus (71%, $P<0.0001$), but stayed at low levels thereafter; soluble MHCII significantly increased from estrus to metestrus (54%, $P=0.0329$), while it significantly decreased during pregnancy (48%, $P=0.0009$) to increase again two days after labor (77%, $P=0.0032$); TCR $\alpha\beta$ retained almost stable levels through the estrus cycle and pregnancy,

but significantly increased two days after labor (87%, $P=0.0006$); finally, the levels of TCR $\gamma\delta$ showed a peak of production from proestrus to estrus (120% increase, $P=0.0009$), which decreased thereafter to levels similar to those during proestrus to increase again two days after labor (60% as compared to day 12 of pregnancy, $P=0.0032$) (Fig. 2B). In urine, only the levels of TCR $\alpha\beta$ were shown to increase from proestrus to estrus (55%, $P<0.0001$), while an increase of TCR $\alpha\beta$ and MHCII levels was also observed on day 12 of pregnancy (37 and 46%, $P=0.0024$ and $P=0.0011$, respectively).

Thus, immune polymorphic molecules seemed to be able to dictate vaginal receptivity, since their expression on nucleated cells and their presence in the vaginal fluid followed a specific pattern.

Expression of polymorphic immune molecules in sperm

Spermatozoa and seminal fluid were also tested for the presence of MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$. Thus, spermatozoa were isolated as described in methods and submitted to immunofluorescence experiments followed by flow cytometry analysis. The results showed that only 8–10% of the cells expressed MHCI and MHCII molecules, while 30–35% expressed TCR $\alpha\beta$ and TCR $\gamma\delta$ (Fig. 3A). By the same token, MHCI and MHCII were detected in the seminal fluid at lower levels as compared to TCR $\alpha\beta$ and TCR $\gamma\delta$ (Fig. 3B).

These results raised many questions as to the role and the origin of these polymorphic immune molecules. As TCR $\alpha\beta$ is expected to interact with MHCII/MHCI and TCR $\gamma\delta$ with MHCI, one would expect a mutually exclusive expression of TCR or MHC, which could also correlate with sperm quality. Indeed, the obtained results were in line with such hypothesis, since the levels of TCR $\alpha\beta$ and TCR $\gamma\delta$ are approximately 200% higher than the levels of MHCI and MHCII. It is interesting to note that bad quality, immotile or with nonprogressive motility sperm, showed the opposite profile, namely, higher levels of MHCI and MHCII and lower or equal levels of TCR $\alpha\beta$ and TCR $\gamma\delta$ both at the cellular (spermatozoa) and seminal fluid level (data not shown). In order to evaluate whether these molecules have a functional role in sperm motility, specific antibodies were added to the sperm sample and motility was evaluated from 2 to 45 min after capacitation (Fig. 3C and Video 1, only straight progression is shown). As expected, sperm motility in all cases decreased with time. Except for the anti-MHCI antibody, neutralization of all other immune polymorphic molecules, decreased progressive motility at all time points tested (Fig. 3C). Forty-five minutes after capacitation, neutralization of MHCI was shown to significantly increase motility by 105% ($p=0.026$) as compared to control progressive motility, while neutralization of TCR $\gamma\delta$ significantly decreased such motility (76%, $p=0.0160$) as compared

Figure 2 Quantification of MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$ expression on vaginal cells of BALB/c mice during proestrus (P), estrus (E), metestrus (M) and diestrus (D) as evaluated by immunofluorescence experiments (A left panel). A closer look for the expression of TCR $\gamma\delta$ on vaginal cells during the four stages of the estrus cycle (A right panel). The results are expressed as percent of positive cells $\pm$ S.E.M. The presence of soluble MHCI, MHCII, TCR $\alpha\beta$ and TCR $\gamma\delta$ was also detected in vaginal fluid and urine by ELISA during the four stages of the estrus cycle as well as day 12 of pregnancy (D12) and 2 days after labor (D2PL) (B). The results are expressed as percent of optical density increase over background $\pm$ S.E.M. In all cases, the results represent the mean of at least three experiments.

Figure 3 Quantification of MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ expression on spermatozoa from BALB/c mice as evaluated by immunofluorescence experiments followed by flow cytometry analysis (A). The results are expressed as percent of positive cells $\pm$ S.E.M. and represent the mean of five experiments. The presence of soluble MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ was also detected in seminal fluid by ELISA (B). The results are expressed as percent of optical density increase over background and represent the mean of five experiments $\pm$ S.E.M. Sperm motility in the presence of monoclonal antibodies against MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ was calculated out of digital recordings of 10 s duration (see Methods) 2, 10, 20, 30 and 45 min after capacitation (C). Only progressive motility is shown here. The results expressed the percent of progressively motile spermatozoa and represented the mean of five experiments $\pm$ S.E.M. A closer look of sperm motility after 45 min of capacitation (D) showed that anti-TCR $\gamma\delta$ caused a coagulation of spermatozoa at the level of the cytoplasmic droplet, (D, photo on top of the TCR $\gamma\delta$ bar), while TCR $\alpha\beta$ showed an agglutination at the level of spermatozoa head (D, photo on top of the TCR $\alpha\beta$ bar).

to control (Fig. 3D). Interestingly, the addition of anti-TCR $\gamma\delta$ caused a coagulation of spermatozoa at the level of the cytoplasmic droplet, which is located at mid-tail in mice (Fig. 3D, photo on top of the TCR $\gamma\delta$ bar), while TCR $\alpha\beta$ showed much less agglutination but at the level of spermatozoa head (Fig. 3D, photo on top of the TCR $\alpha\beta$ bar).

Video 1

Sperm motility in the presence or not of monoclonal antibodies against MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ was digitally recorded with the duration of 10 s 2, 10, 20, 30 and 45 min after capacitation (see Methods). One sample recording out of at least five recordings for each time point from one out of five mice tested is presented here.

As earlier shown, female vaginal cells also express the polymorphic immune molecules and display a specific pattern of expression following the estrus cycle. In order to evaluate whether the vaginal environment could affect sperm motility, capacitated sperm was left to interact with vaginal cells, vaginal fluid or the combination of both and motility was evaluated (see Methods). The

Figure 4 Sperm motility in the presence of vaginal cells, vaginal fluid or the combination of vaginal cells and fluid, all deriving from BALB/c mice, during proestrus (P), estrus (E), metestrus (M) and diestrus (D). The results are expressed as the percent of progressively motile spermatozoa and represent the mean of three experiments $\pm$ S.E.M.

results showed that vaginal cells alone facilitated sperm motility at estrus, vaginal fluid significantly increased sperm motility at diestrus, while the combination of vaginal cells and fluid increased sperm motility mostly during diestrus and proestrus (Fig. 4).

Expression of polymorphic immune molecules in oviduct fluid and ovum

Sperm will meet the ovum in the fallopian tube. During natural fertilization, spermatozoa need to cross zona radiata, reach zona pellucida and penetrate the ovum following the acrosomal reaction. If polymorphic immune molecules guide sperm through such process, one would be able to detect their expression in zona radiata cells and/or oviduct fluid. Therefore, ova were isolated as described in methods and submitted to immunofluorescence staining followed by optical imaging using a fluorescent microscope. Following such experiments, zona radiata cells showed high levels of MHC II and TCR $\gamma\delta$ expression (Fig. 5A).

Oviduct fluid was examined for the presence of MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ during the estrus cycle as well as days 0, 1 and 2 of pregnancy. A peak of expression of these molecules was detected during estrus (Fig. 5B), with TCR $\alpha\beta$ showing the highest concentration of all. It is interesting to note that once pregnancy was established (day 0, 1 and 2), these molecule were almost undetectable in the oviduct fluid (Fig. 5B).

In an attempt to examine the origin of the immune polymorphic molecules detected in the oviduct fluid, fallopian tube epithelial cells were isolated and cultured for 48 h and supernatants were tested for the presence of MHC I, MHC II, TCR $\alpha\beta$ and TCR $\gamma\delta$ by ELISA. Although

Figure 5 Immunofluorescence staining of BALB/c oocyte-cumulus cell complexes for the detection of MHC I, MHC II, TCRαβ and TCRγδ (A). A representative experiment is shown here. Photographs were taken using a 10× objective of an optical fluorescent microscope. The presence of soluble MHC I, MHC II, TCRαβ and TCRγδ was also detected in oviduct fluid (B) and supernatants of fallopian tube epithelial cell cultures (C) by ELISA. Oviduct fluid was analyzed during proestrus (P), estrus (E), metestrus (M) and diestrus (D) as well as day 0 (d0), 1 (d1) and 2 (d2) of pregnancy. The results are expressed as percent of optical density increase over background $\pm$ S.E.M. In all cases, the results represent the mean of at least four experiments.

all tested molecules were detected in the culture supernatants of fallopian tube epithelial cells, TCRαβ showed the highest levels of all (Fig. 5C).

Discussion

In various species, including humans, mate selection has been shown to correlate with MHC deviating partners (Bernatchez & Landry 2003, Sommer 2005), where allelic diversity enhances defense to newly arising pathogens, following the generalized environmental evolutionary pathways (Madsen & Ujvari 2006, Piertney & Oliver 2006, Schwensow *et al.* 2007, Alcaide *et al.* 2010, Spurgin & Richardson 2010), while also playing an important role in fet/oembryonic-maternal recognition (Alberts & Ober 1993). To this extend the present study was designed to define, if any, immune polymorphic elements in the female reproductive tract, capable in guiding sperm at the fertilization site and allow ovum penetration for pregnancy initiation. The immune polymorphic molecules studied herein included MHC I, MHC II molecules as well as their receptors TCRαβ and TCRγδ. The results showed that these molecules could be detected either as cell surface markers in vaginal cells, corona radiata cells and spermatozoa, or as secreted molecules in vaginal fluid, oviduct fluid, culture supernatants of fallopian tube cells as well as seminal fluid. The levels of these molecules varied following the estrus cycle, indicating the existence of complicated positive or negative interactions allowing sperm to reach and fertilize the ovum.

Although at the behavioral level, mate choice has been considered to depend on odortypes (volatile MHC forms for sensing MHC polymorphism and appropriate mate selection; Wedekind & Penn 2000), during copulation, sperm will first come in contact with vaginal cells and fluids. In mice, females will only allow copulation during estrus, and therefore it is important

to foresee whether the polymorphic immune molecules could follow a specific pattern of expression according to the estrus cycle. Indeed, the results showed that these molecules were expressed in vaginal cells, and when calculating the percentage of positive cells, estrus and diestrus presented the highest levels of expression. Taking, however, under consideration the absolute number of cells in the different stages of the estrus cycle, where although of variable origin, the numbers of cells follow an approximate analogy of 1:5:30:25 by field area for proestrus: estrus: metestrus: diestrus (data not shown). Such calculations render diestrus the stage of highest cell surface expression of the immune polymorphic markers tested.

Vaginal fluid was also found to contain these immune polymorphic molecules in a soluble form, where TCRγδ was shown to be highly expressed during estrus and correlated to the lowest levels of MHC I and MHC II. Interestingly, during pregnancy, only the levels of MHC II were shown to significantly decrease, while 2 days after labor, the levels of MHC II, TCRαβ and TCRγδ significantly increased, which, although hard to interpret, indicates the establishment of novel regulatory mechanisms in the vagina. Examining the levels of immune polymorphic elements in urine, it was shown that only TCRαβ during estrus was produced at higher levels as compared to proestrus, but in all other cases, the levels did not change during the estrus cycle. Therefore, the results obtained here, were not in favor of the odortype hypothesis for mate selection, where soluble MHC I molecules are at the basis of allorecognition (Wedekind & Penn 2004), except if odortypes are represented by TCRs and not MHCs.

Concentrating on sperm and seminal fluid, it was shown that spermatozoa themselves express lower levels of MHC I and MHC II, but higher levels of TCRαβ and TCRγδ, while a similar pattern could be detected in seminal fluid. In addition, it seemed that neutralization

of these molecules by monoclonal antibodies could significantly affected sperm motility: anti-MHCI could increase motility, while anti-TCR $\gamma\delta$ could significantly decrease motility. In nature, one could hypothesize that seminal plasma MHCII could be 'neutralized' by vaginal soluble TCR $\gamma\delta$ to increase sperm motility during estrus, while vaginal soluble MHCII during proestrus could 'neutralize' seminal plasma TCR $\gamma\delta$ to inhibit sperm motility. Indeed, as it was shown sperm motility could be affected by vaginal cells and vaginal fluid, observation that favors the hypothesis of immune facilitated vagina-sperm interactions.

Similar male-female cross-talk could be detected in the fallopian tube, the site where fertilization will take place. Indeed, cells of zona radiata, as well as oviduct fluid and supernatants of epithelial fallopian tube cell cultures express/produce immune polymorphic molecules. Zona radiata cells mainly expressed MHCII and TCR $\gamma\delta$, while all immune molecules, with special reference to TCR $\alpha\beta$, were highly produced during estrus, and were almost undetectable on days 0, 1 and 2 of pregnancy.

Taking the results together, one could definitely conclude that immune polymorphic molecules are involved in male-female interaction processes toward successful fertilization. These results point to the potential existence of multiple complicated, post-copulatory interactions between male and female immune molecules, but remain difficult to interpret. Thus, TCRs, following their ability for cognate recognition, will interact with the thermodynamically stable antigen-loaded MHC molecules. TCR $\alpha\beta$ at the cellular level is expected to interact with MHCII/II and TCR $\gamma\delta$ with MHCII. Soluble MHCII have been shown to compete with TCR $\alpha\beta$ of CD4-positive cells, indicating that at least soluble MHCII interact with TCR $\alpha\beta$. Such interactions could be expected not only to allow or not spermatozoa to penetrate the female reproductive tract but also to facilitate or not their motility toward the fallopian tube, and guide their path to cross zona radiata cells, reaching zona pellucida for fertilization. On the other hand, since these immune markers were shown to affect sperm motility, one could consider using those as markers for evaluating sperm quality.

It has to be noted that the present study did not include the uterus in the analysis, not because it is neglected, but because of the complicated nature of possible interactions with the various types of cells through the reproductive cycle, that need to be separately and profoundly studied. Indeed, the uterine milieu plays a definite role for sperm to reach the oviduct, but yet, the final act is to be played at the level of the fallopian tube.

In conclusion, the results presented here demonstrated the existence of immune polymorphic molecules as cell surface or soluble proteins, in all sites of the female reproductive tract tested, as well as in sperm. The variation of their expression during the estrus cycle indicates

that they could even control female susceptibility to fertilization. The nonconventional expression of TCRs in cells other than T lymphocytes observed herein is in accordance with recent observations detecting TCR-positive macrophages/monocytes, granulocytes including neutrophils and eosinophils (Puellmann *et al.* 2006, Legrand *et al.* 2009, Fuchs *et al.* 2013, 2015, Chávez-Galán *et al.* 2015). In addition, although the properties and the origin of soluble TCRs have not been sufficiently studied, it has been suggested that they play an amplifying role for some T cell functions (Cone 1996). Understanding the nature of such immune interactions could provide a whole new perspective in the immune regulatory processes, while in reproduction they could offer a solid molecular basis for mate selection and the maintenance of MHC polymorphism in species evolution, while also correlating to cases of infertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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