Biology of Reproduction, 2020, 103(3), 630–642
doi:10.1093/biolre/ioaa072
Research Article
Advance Access Publication Date: 15 May 2020

OXFORD

Research Article

CCL20-CCR6 axis directs sperm–oocyte interaction and its dysregulation correlates/associates with male infertility‡

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‡Grant Support: This research was supported by the "Forschungskommission der medizinischen Fakultät des Universitätsklinikums Düsseldorf", China Scholarship Council (No.2008616050; Peking, China) and Deutsche Forschungsgemeinschaft (DFG; AL 1219/3-1)
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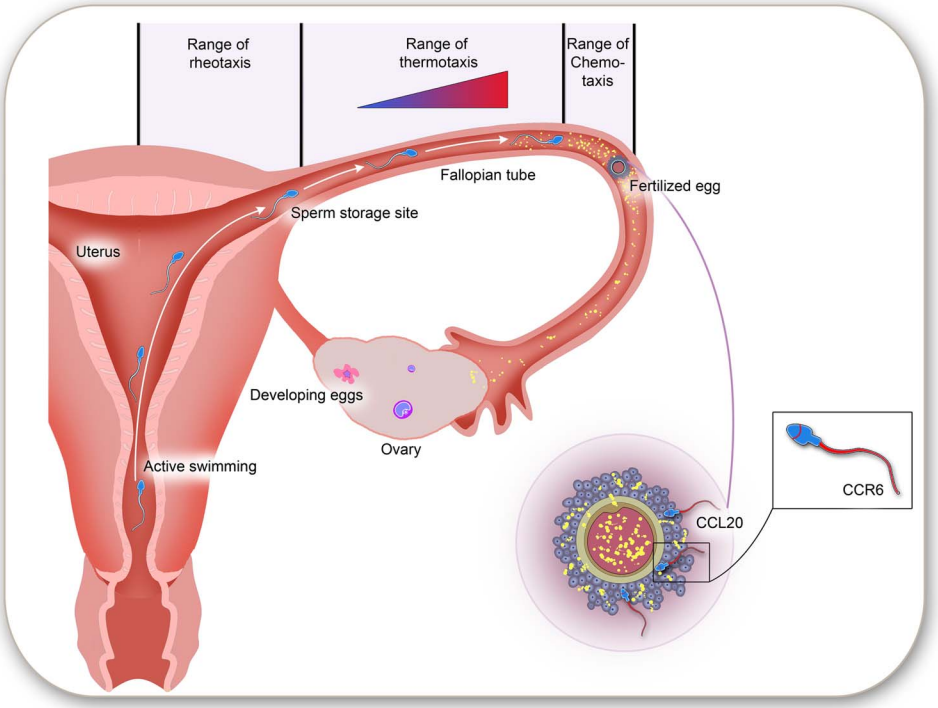
Received 8 November 2019; Revised 28 February 2020; Editorial Decision 9 May 2020; Accepted 12 May 2020

Abstract

The interaction of sperm with the oocyte is pivotal during the process of mammalian fertilization. The limited numbers of sperm that reach the fallopian tube as well as anatomic restrictions indicate that human sperm–oocyte encounter is not a matter of chance but a directed process. Chemotaxis is the proposed mechanism for re-orientating sperm toward the source of a chemoattractant and hence to the oocyte. Chemokines represent a superfamily of small (8–11 kDa), cytokine-like proteins that have been shown to mediate chemotaxis and tissue-specific homing of leukocytes through binding to specific chemokine receptors such as CCRs. Here we show that CCR6 is abundantly expressed on human sperms and in human testes. Furthermore, radioligand-binding experiments showed that CCL20 bound human sperm in a specific manner. Conversely, granulosa cells of the oocyte-surrounding cumulus complex as well as human oocytes represent an abundant source of the CCR6-specific ligand CCL20. In human ovaries, CCL20 shows a cycle-dependent expression pattern with peak expression in the preovulatory phase and CCL20 protein induces chemotactic responses of human sperm. Neutralization of CCL20 in ovarian follicular fluid significantly impairs sperm migratory responses. Conversely, analyses in infertile men with inflammatory conditions of the reproductive organs demonstrate a significant increase of CCL20/CCR6 expression in testis

and ejaculate. Taken together, findings of the present study suggest that CCR6-CCL20 interaction may represent an important factor in directing sperm–oocyte interaction.

Graphical Abstract



Summary Sentence

The chemokine CCL20 is produced by human oocytes as well as surrounding cumulus granulosa cells and induces chemotaxis of CCR6+ sperm. Infertile male donors demonstrate increased levels of CCL20 in testis and seminal fluid, which may negatively influence sperm–oocyte interaction.

Key words: acrosome reaction, capacitation, chemotaxis, cumulus cells, fertility, granulosa cells, male infertility, ovulatory cycle, seminal plasma, sperm capacitation, sperm motility and transport

Introduction

During sexual intercourse, an approximate 250 million human sperms are released into the vagina. Interestingly, only a very low number of ejaculated sperms (80–1400 sperm) ever make their way to the site of the unfertilized oocyte [1, 2]. This limited number of sperms and the anatomic proportions of the fertilization site suggest that precontact guidance mechanisms are considered imperative for a successful fertilization [2–11].

Recent studies and reviews propose mammalian/human sperm guidance as a multi-step process of long- and short-range mechanisms [3, 6, 10–14]. After ejaculation into the vagina, sperms undergo a maturation process referred to as capacitation [15]. The capacitated status is a prerequisite to human sperm in order to be thermotactically and chemotactically responsive, to bind to the oocyte's zona pellucida and to perform the acrosome reaction. Capacitated sperms are subsequently guided from the cooler sperm reservoir's site at the isthmus of the oviduct to the warmer site of fertilization in the tubal ampulla by thermotaxis (long-range guidance) [3, 12, 14, 16–18]. Within immediate distance of the fertilization site, sperms sense gradients of chemoattractants secreted by the oocyte-cumulus complex and navigate to the oocyte by means of chemotaxis (short-range guidance) [3, 6].

Infection and inflammation of the male genital tract are accepted as significant etiologic factors in male infertility. However, difficulties occur in the diagnosis of sub-acute or chronic inflammatory reaction in the male reproductive tract because of its mostly asymptomatic course and unspecific diagnostic criteria [19]. Recent studies demonstrate that induction of testicular inflammation is associated with a Th17-cell-mediated autoimmune response, i.e., disruption of the immune privilege and spermatogenesis [20, 21]. Moreover, elevated levels of several cytokines in semen from men with genital infections suggest that they could be involved in the immune defense of male genital tract; meanwhile, they also might impair semen quality and sperm function [22, 23].

Chemotaxis toward follicular fluids has been described in several mammalian species including the human [24] and the rabbit [13]. Chemotaxis is also well documented in marine invertebrates [3, 6, 25]. In mammalians, at least two different guidance mechanisms have been reported: sperm thermotaxis, defined as the directional

movement of spermatozoa according to a temperature gradient, and sperm chemotaxis, defined as the directional movement of spermatozoa according to a chemical gradient [3, 6, 12, 13]. Human preovulatory follicular fluid (hFF) contains soluble factors produced by the oocyte and/or the granulosa cells [3, 4, 24, 26–29]. Progesterone was proposed as a major sperm-chemoattractant secreted from human cumulus cells [27, 30–33]. Progesterone activates the Catsper channel, which represents a sperm-specific, Ca^{2+}-permeable, pH-dependent, and low voltage-dependent channel that is essential for the hyperactivity of the sperm flagellum, induces chemotaxis, and initiates capacitation as well as acrosome reaction [34–36].

Moreover, recent studies suggest that members of a superfamily of small cytokine-like proteins, the so-called chemokines, are attractive candidates to also interfere in sperm–oocyte interaction [37]. Chemokines play their various biological functions by activating surface receptors of their target cells that are seven-transmembrane-domain G protein-coupled receptors (GPCRs). The binding of the chemokine to their receptors activates signaling cascades that culminate in the rearrangement, change of shape, and cell movement of actin [38, 39]. It is well established that both in humans and mice, chemokine receptors have differences in expression on subsets of effector and memory T cells and provide specificity to cell trafficking both in the steady state and inflammation [40]. Caballero-Campo *et al.* could demonstrate that CCR6 protein is localized on the surface of human sperm and that Ccr6 mRNA is expressed in mouse testis. Moreover, in protein chip analyses, CCL20 was determined in hFF [41].

In the present study, we aimed to systematically analyze the chemokine receptor repertoire of human sperm and evaluate their potential role in sperm–oocyte interaction.

Putative sequence of human sperm guidance mechanisms *in vivo*

Subsequent to ejaculation, human sperms actively swim to the isthmus of the fallopian tube. Here, capacitated sperms sense an ovulation-dependent temperature gradient and travel toward the site of fertilization by means of thermotaxis. Ascending the fallopian tube, sperms sense a gradient of progesterone and CCL20 and are guided to the oocyte-cumulus complex by means of chemotaxis. Next, sperms are guided to the oocyte itself according to the gradient of CCL20 (or a more potent chemoattractant) secreted from the oocyte into the dense cumulus matrix. Finally, the (CCR6+) sperm equatorial segment adheres to the (CCL20+) oocyte, and plasma membrane fusion is initiated.

Materials and methods

Sperm preparation

Human semen samples were freshly obtained, with informed consent, from young healthy donors. Semen samples with normal sperm density, motility, and morphology (according to WHO guidelines; WHO 1999) were separated from seminal plasma by washing and resuspended in sperm preparation medium (Medicult, Jyllinge, Denmark). This medium is based on Earle's Balanced Salts Solution and contains synthetic serum replacement, human serum albumin, HEPES, and Phenol Red. For swim up preparations, washed sperms were centrifuged (150 g, 10 min), and the resulting pellets were overlaid with medium. After 60 min, upper layers were collected and used for experiments. Sperms were capacitated during 2 h incubation at 37 °C in humified air with 5% CO_2. The study was approved by the local ethics committee (Number: 2265).

Human ovary, oocyte, granulosa cell, and follicular fluid preparation

Ovarian tissues were obtained from four donors as described [42]. Follicular fluid and granulosa cells were obtained, with informed consent, from healthy women participating either in *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) programs. Oocytes were retrieved by transvaginal aspiration 36 h after injection of human chorion gonadotropin (hCG). For denudation, oocyte-cumulus complex was incubated in *in vitro* fertilization medium (Medicult, Jyllinge, Denmark) containing 40 IU/ml hyaluronidase (Hylase, Pharma Dessau, Germany) for 30 s. Cumulus cells were removed mechanically.

Quantitative real-time PCR

Total RNA from cells or homogenized tissue samples was extracted and reverse transcribed as described [43]. Complementary DNA was quantitatively analyzed for the expression of human chemokines/receptors by fluorogenic 5'-nuclease PCR assays as reported [43]. Primers and probes specific for the chemokine receptors were obtained from Applied Bisoystems (Foster City, CA, USA). 25 ng of cDNA was assayed per reaction. PCR products were measured continuously by means of an ABI PRISM® 7700 or 5700 Sequence Detection System (Applied Biosystems) during 40 cycles.

DNA microarray analyses

RNA extraction, biotinylation, and hybridization to Affymetrix Human U133 Plus 2.0 GeneChip microarrays (Affymetrix Inc., Mountain View, CA, USA) were performed as described earlier [42]. Expression data were normalized for chip-to-chip variability by applying Robust Multichip Average (RMA).

Flow cytometry

Chemokine receptors on human sperm were stained with the following reagents: PE-labeled antibodies against human CCR1, CCR2, CCR3, CCR5, CCR7, CCR9, CXCR3, CXCR4, CXCR5, CXCR6 (all R&D Systems Inc., Minneapolis, MO, USA), CCR4, CCR6, CXCR1, CXCR2 (all BD PharMingen, San Diego, CA), or CX3CR1 (MBL International Corporation, Woburn, MA, USA), biotinylated anti-human CCR10 (clone 1908; DNAX Research Institute, Palo Alto, CA, USA) and PE-conjugated streptavidin (BD PharMingen), and unlabelled anti-CCR8 (210-762-R100, goat IgG, Alexis Biochemicals, Lausanne, Switzerland) and PE-conjugated swine anti-goat IgG (Caltag, Burlingame, CA, USA). Samples were analyzed using a FACScan (Becton Dickinson, San Jose, CA, USA) and CELLQuest software (Becton Dickinson). Analyses were restricted to sperm swim up preparations to prevent contaminations with other cell subsets (e.g., leukocytes).

Immunocytochemistry

Single human oocytes or cytospins of human sperm and granulosa cells were fixed with acetone and preprocessed with H_2O_2 followed by an avidin–biotin blocking step (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA, USA). Cells were stained with the

following reagents: for granulosa cells or oocytes monoclonal anti-human CCL20 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or appropriate isotype control (Jackson ImmunoResearch, West Grove, PA, USA), for sperm monoclonal anti-human CCR6 antibody (R&D Systems) or appropriate isotype control (BD PharMingen). Development was performed by DAKO AEC-Kits (DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin. For immunofluorescence images, anti-CCR6 or anti-CCL20 antibodies were detected with A488-labeled or Cy3-labeled secondary antibodies, respectively.

Radioligand assay

Ligand binding analyses were performed as previously described with minor modifications [44]. Briefly, following swim-up preparation, 10^6 sperms were incubated in quadruplicates with 0.03 nM of labeled recombinant human [^{125}I]CCL20 (10 µCi; Perkin Elmer, Waltham, MA) and 0.3 nM of competing unlabeled recombinant human CCL20 and CCL21 (R&D Systems, Minneapolis, MN) or anti-CCR6 antibody (20 ng/ml) (BD PharMingen, San Diego, CA) in 250 µl of binding medium (MediCult, Jyllinge, Denmark). After incubation for 30 min at 37 °C, unbound ligands were separated from cells by several wash steps with ice-cold PBS. A final wash step with cold acidic glycine buffer (50 mM glycine, 150 mM NaCl, pH 2.7) was used to remove surface bound ligands. Gamma emissions of cell pellets were then counted on a scintillation gamma counter.

Chemotaxis assays

Capillary assays were carried out as described [24] but with minor modifications. Wells were filled with 100 µl of sperm solution at concentrations of 6–9 × 10^7 cells/ml. Capillaries were filled either with test or control solutions and sealed at one end with wax. The open side of each capillary was inserted into the well. After incubation for 20 min (37 °C in humified air with 5% CO_2) the number of migrated cells was determined by flow cytometry. To determine the total number of migrated cells, a known number of 15 µm microsphere beads (Bangs Laboratories, Fishers, IN) was added to each sample before analysis as previously described [45]. For transwell chemotaxis assays, 24-well cell-culture chambers with 8-µm pore polyvinylpyrrolidone-free polycarbonate filters were used as described [46]. Briefly, 6–9 × 10^7 sperms in 600 µl sperm preparation medium were applied to the lower well of each chamber. Test solutions were added to inserts of transwell systems. After incubation for 30 min (37 °C in humified air with 5% CO_2), migrated cells were determined as described.

Sperm video motion analysis

Videomicroscopic recordings were performed over 25 s, beginning 10–15 min after an assay began, using a SHR-chip digital video camera (IPPI GmbH, Munich, Germany) mounted on an inverted light microscope (Olympus CK40, Olympus, Hamburg, Germany). Sperms were exposed to test and control solutions. Trajectories made by swimming sperm were obtained from areas within 250 µm of capillary tips and displayed using ImageJ software (NIH, USA).

Testicular tissue specimens

Testicular biopsies from adult men undergoing andrological work-up for infertility were retrieved from the archive. According to the histopathological evaluation, specimens were divided into three groups: impaired spermatogenesis with inflammation (ISI, $n = 5$), impaired spermatogenesis without inflammation (ISW, $n = 5$), and normal spermatogenesis (NS, $n = 5$). The specimens were fixed in Bouin's fixative, embedded in paraffin, and processed for histopathological evaluation and immunohistochemical studies. All patients had given their written informed consent prior to surgery, and the evaluation of human testicular biopsy specimens was approved by the local ethics committee (number: 356/16).

Immunohistochemistry of CCR6 and CCL20

Rabbit anti-human polyclonal antibody CCR6 (dilution 1:1000, GTX71397, GeneTex Inc, Irvine, USA) and rabbit anti-human monoclonal antibody CCL20 (dilution 1:100, ab9829, Abcam plc, Cambridge, UK) were used as the primary antibodies for immunohistochemistry. The staining procedure was performed as described previously [20].

Confocal laser scanning microscopy

Fresh ejaculate samples were collected from patients presenting to the andrology unit for routine semen analysis after given informed consent and normal sperm parameters were defined according to World Health Organization criteria [47]. The expression of CCR6 on sperm of normozoospermic samples ($n = 5$) was identified by the immunofluorescent method using confocal laser scanning microscopy as previously described in detail [48]. Briefly, 1 × 10^6 sperm cells were adhered to the slides coated with poly-L-lysine solution (Sigma Aldrich). Cells were fixed by using BD Cytofix/Cytoperm Solution Kit (BD Biosciences, Heidelberg, Germany). The slides were incubated with the primary antibody CCR6 (mouse anti-human monoclonal antibody, dilution 1:50, MAB195, R&D systems, Minneapolis, USA) was performed for overnight at 4 °C. Following a 5-min wash with PBS, slides were incubated with secondary antibody (Cy3-conjugated affinipure goat anti-mouse IgG; Jackson Immunoresearch, USA), and counterstaining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Merck, Germany). All slides were mounted with Fluoromount-G (SouthernBiotech, USA) to preserve fluorescence and analyzed with an Olympus FluoView1000 confocal microscope (Olympus, Hamburg, Germany) equipped with an argon-krypton laser. Images with different dyes were scanned sequentially. All scans were acquired with a Plapo 60×, NA 1.4 oil immersion objective (Olympus). The software FV10-ASW 1.4 (Olympus) was used for picture analysis.

Detection of CCR6 on sperm and CCL20 in seminal plasma, oocyte, granulosa cell, and follicular fluid

Fresh semen samples were collected from patients ($n = 58$) and divided into two groups: (1) inflammation group (IN, $n = 21$): patients showing IL-6 > 30 pg/ml in the seminal plasma were considered to suffer from chronic inflammation of the genital tract (CIGT); (2) control group (CON, $n = 37$): patients with a concentration IL-6 < 10 pg/ml in the seminal plasma. Ejaculate was washed and centrifuged in PBS buffer. The supernatant was discarded, and the pellet was resolved in PBS buffer. Ejaculate measuring 100 µl was incubated with phycoerythrin (PE)-conjugated mouse monoclonal antibody (mAb) against CCR6 (clone 11A9, IgG1, BD Biosciences, Heidelberg, Germany) for 20 min at 4 °C. As the appropriate isotype control, 100 µl ejaculate was incubated with PE-conjugated mAb IgG1 (BD Biosciences, Heidelberg, Germany) for 20 min at 4 °C. After the incubation time, samples were washed in PBS and

analyzed on BD FACSCalibur^{TM} cytometer (BD Biosciences) using Cellquest software (BD Biosciences). Furthermore, cell-free seminal plasma (SP) of these patients was collected. CCR6 and CCL20 concentrations were investigated by ELISA following the instruction manual (Cat.No. DM3A00, R&D systems, USA). Recombinant human CCL20 protein (R&D Systems) was used for standard curves.

Statistical analyses

Data were expressed as mean ± SEM. For statistical evaluation of significances, the Wilcoxon test, Student's t-test (based on the normal distribution of values), one-way ANOVA followed by Bonferroni post-test or Spearman test were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

CCR6 is an abundantly expressed chemokine receptor on human sperm and interacts with CCL20

We systematically analyzed the expression of all known chemokine receptors [49] on human sperm obtained from 26 independent healthy donors. Using flow cytometry and a panel of antibodies directed against 17 different human chemokine receptors, we demonstrated that human sperms express a non-random pattern of chemokine receptors and identified CCR6 as an abundantly expressed member (Figure 1, Supplementary Figure S1) consistent with earlier findings of Caballero-Campo *et al*. [41]. Quantitative real-time PCR analyses on sperm obtained from 5 different healthy donors confirmed flowcytometric results (Figure 1A). Notably, CCR6 cDNA expression in sperm ranging from 290 to 500 fg/25 ng cDNA was comparable to levels seen in peripheral blood mononuclear cells (PBMC), which are well known to express high levels of this chemokine receptor (Figure 1A).

To obtain insights into the microanatomical location of CCR6, we subsequently performed immunocytochemical and immunofluorescence analyses. Herein, we could demonstrate a marked expression of CCR6 protein in the principal piece of the flagellum of human sperm [50]. Conversely, no staining was observed in the mid-piece or the end-piece of the flagellum (Figure 1D and E) [50, 51]. Immunofluorescence also revealed a ring-like staining of CCR6 around the sperm head, which may reflect the equatorial segment (Figure 1E).

Next, we sought to analyze the functionality of CCR6 on human sperm. Therefore, we performed radioactive ligand-binding assays using labeled recombinant human [125I] CCL20 and competing unlabeled recombinant human CCL20, CCL21, or anti-CCR6 antibody. Based on our analyses of human sperm, demonstrating the absence of CCR7 (Supplementary Figure S1), its ligand CCL21 was considered irrelevant for human sperm chemotaxis and used as "negative" control. Binding experiments showed that CCL20 bound human sperm in a specific manner (Supplementary Figure S2). Competition of radiolabeled [^{125}I] CCL20 binding was observed with unlabeled CCL20 and anti-CCR6 antibody but not with CCL21 (Supplementary Figure S2A). Comparable results, however, at a lower intensity, were observed after we performed an additional acidic wash step in order to remove surface-bound ligands (Supplementary Figure S2B). Finally, anti-CCR6 antibody significantly impaired the binding of radiolabeled [^{125}I] CCL20 (Supplementary Figure S2C).

Human ovaries, cumulus granulosa cells, and oocytes are a source of the CCR6-ligand CCL20

Having established the expression of CCR6 on human sperm, we next sought to examine the expression of its physiological ligand CCL20 in the female reproductive system. Therefore, we first determined the expression of CCL20 in human ovaries. Analysis of data sets obtained from genome wide DNA microarray analysis of human ovaries obtained from 4 different donors showed the overexpression of CCL20 during the pre-ovulatory phase of the menstrual cycle, as indicated by the overexpression of the aromatase CYP19 and the luteinizing hormone/choriogonadotropin receptor (LHCGR) (Figure 2A). Notably, both genes indicate the preovulatory phase of the menstrual cycle [52]. We then examined the expression of CCL20 in the cumulus oophorus. The cumulus oophorus represents a complex of closely associated granulosa cells, that surround, protect, and nurse the oocyte. Moreover, the cumulus oophorus has been proposed to induce sperm chemotaxis by the production of follicular fluid, as well as by the radial orientation of the granulosa cells themselves [53]. Quantitative real-time PCR analysis demonstrated that isolated granulosa cells of the cumulus complex produced significant amounts of CCL20 mRNA ranging within levels observed in well-known sources of this chemokine, such as epidermal keratinocytes and activated PBMC (Figure 2B). Immunocytochemical analyses showed that human cumulus granulosa cells abundantly express CCL20 protein (Figure 2C). In addition to granulosa cells, human oocytes also strongly express CCL20 (Figure 2D). These results are consistent with a recent study showing that not only granulosa cells of the cumulus complex but also the mature oocyte itself secretes chemotactic substance [54].

Cumulus cells and oocytes produce human follicular fluid (hFF), considered as a solution containing several chemoattractants such as progesterone [3, 6, 7, 24, 29, 53, 54]. To examine the CCL20 protein content within hFF, we performed ELISA to analyze CCL20 protein concentrations in hFF obtained from 26 different donors. Our data showed concentrations ranging from 4 up to 30 ng/ml. This amount represents approximately 0.007 to 0.03% of total protein concentrations of the tested hFF (Figure 2G).

CCL20 attracts human sperm

To determine the functional implications of our expression analyses, we investigated the effects of CCL20 on human sperm migration. Therefore, we analyzed the accumulation of human sperm cells in capillaries filled with different concentrations of CCL20 (1, 100, 1000 ng/ml) or medium-control (Figure 3A) [28]. Our results demonstrated a significantly higher percentage of migrated sperm accumulating in capillaries that contained CCL20 as opposed to capillaries containing medium alone. Correspondingly, lower (1 ng/ml; *, $P < 0.05$) or higher (1000 ng/ml; *, $P < 0.05$) concentrations of CCL20 showed weaker chemotactic responses. Results were confirmed by additional transwell chemotaxis assays, determining the accumulation of sperm in inserts filled with different concentrations of CCL20 (1, 100, 1000 ng/ml) or medium alone (Figure 3B). As expected, sperms showed a strong accumulation in inserts filled with CCL20, rather than medium only. Again, the highest sperm densities were detected at CCL20 concentrations of 100 ng/ml (*, $P < 0.05$).

Apparent sperm accumulation could result from chemotaxis (movement toward a chemical attractant), chemokinesis (alteration in swimming speed), or trapping (arrest of migrating cells) [4, 5]. To address this question, we additionally performed transwell

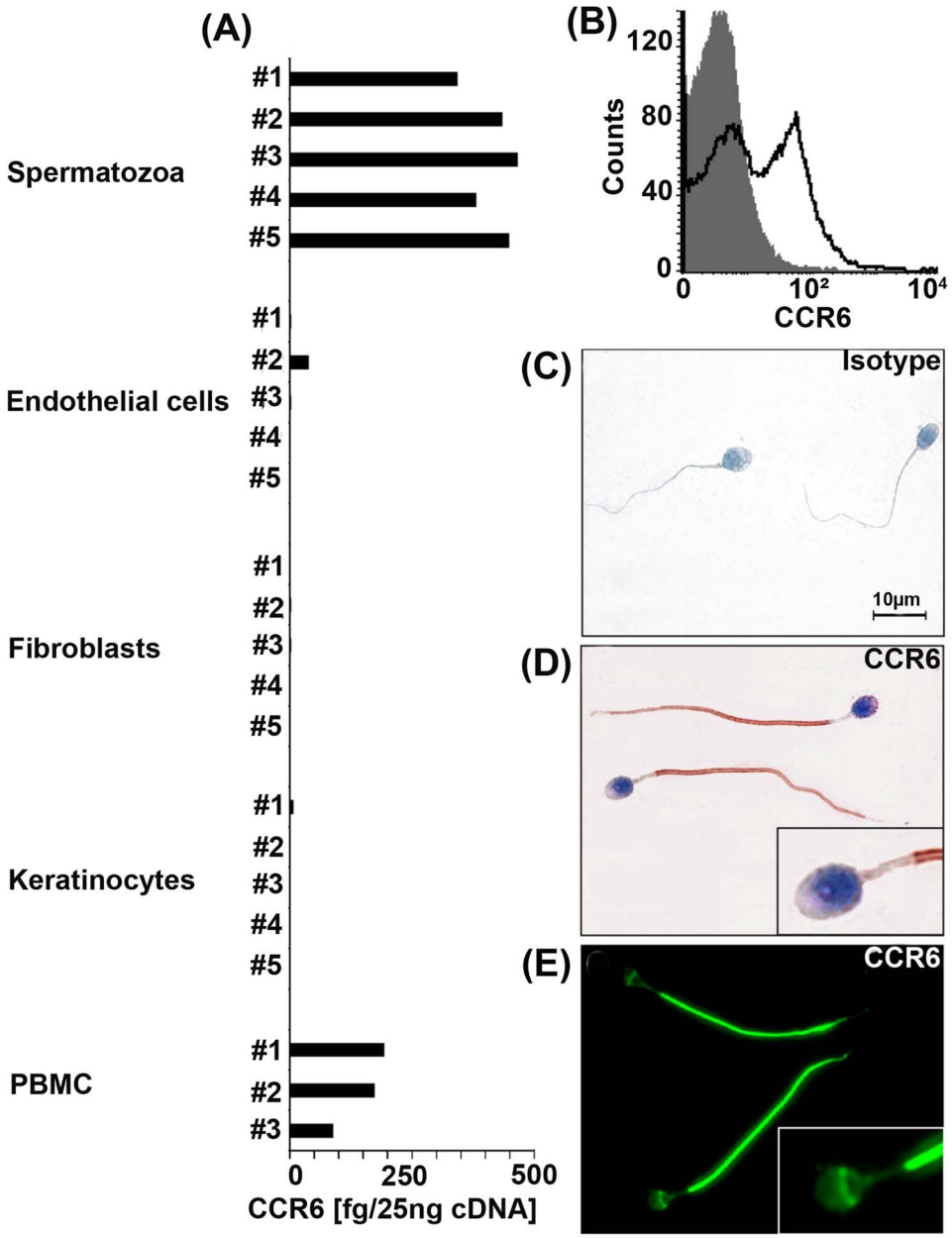


Figure 1. CCR6 expression on human sperm. (A) Quantitative real-time PCR analysis of CCR6 expression in human sperm ($n = 5$ donors) compared with microvascular endothelial cells ($n = 5$ donors), fibroblasts ($n = 5$ donors), keratinocytes ($n = 5$ donors), and PBMC ($n = 3$ donors). (B) Flow cytometric analysis of the cell surface expression of CCR6 on human sperm (representative results of one out of 26 donors). Open histograms represent anti-CCR6 antibody staining; closed histograms represent isotype controls. (C–E) Immunocytochemical analysis of human sperm with anti-CCR6 (D, E) or isotype control antibodies (C). For immunofluorescence images, anti-CCR6 antibodies were detected with A488-labeled secondary antibodies and shown in *green*, nuclei were counterstained with DAPI (*blue*). Original magnification × 1000. *Bar*, 10 µm. Insert areas show the sperm head, the mid-piece, and the proximal part of the principal piece of the flagellum at higher magnifications.

chemotaxis assays determining the accumulation of sperm in inserts presenting not only ascending, but uniform or descending gradients of CCL20 (100 ng/ml; Figure 3C). Indeed, compared to ascending gradients, lower sperm densities were detected in inserts presenting uniform or descending chemokine gradients. Thus, the density of sperm in inserts presenting differentially directed gradients of CCL20 (100 ng/ml) was ranked ascending > uniform > descending, pointing toward chemotaxis as being the most probable cause of sperm accumulation [11, 24]. Descending gradients of CCL20 caused sperm accumulation comparable to the one observed for medium. Consistent with several studies demonstrating that only capacitated sperms are chemotactically responsive [26], we observed that only a fraction (approx. 10–15%) of the total sperm population migrated toward gradients of CCL20.

As solely the directional change of migration toward the source of a chemoattractant is considered as the best criterion for measuring sperm chemotaxis [3, 13], we next examined the swimming behavior of human sperm challenged with ascending

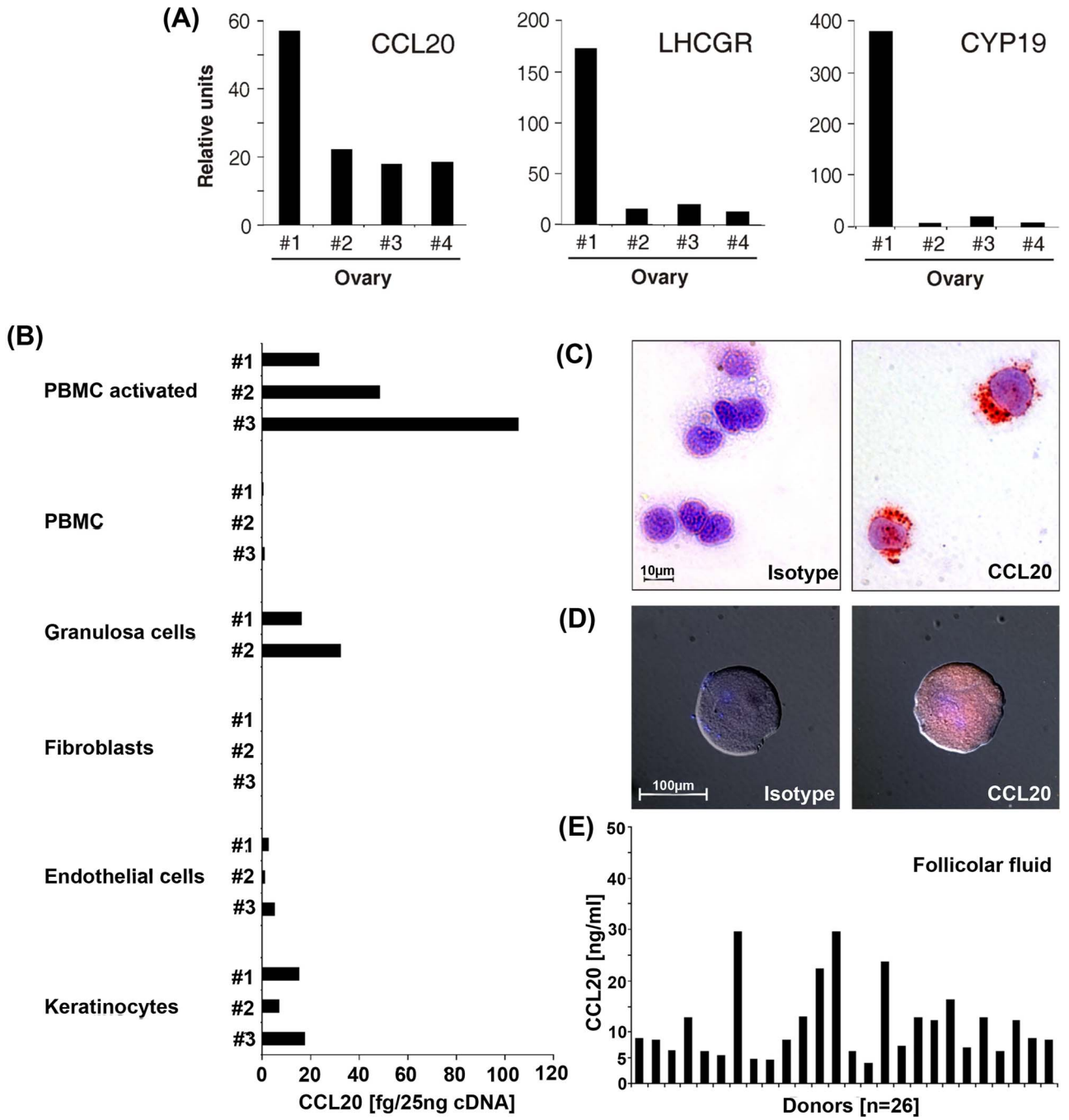


Figure 2. CCL20 production by human ovaries, granulosa cells, and oocytes. (A) DNA microarray analysis of CCL20, LHCGR, and CYP19 expression in human ovaries obtained from 4 different donors. Data are expressed as relative units (average intensity values). (B) Quantitative real-time PCR analysis of CCL20 expression in human granulosa cells ($n = 2$ donors) compared with human microvascular endothelial cells ($n = 3$ donors), fibroblasts ($n = 3$ donors), keratinocytes ($n = 3$ donors), PBMC ($n = 3$ donors), and activated PBMC ($n = 3$ donors). (C) Immunocytochemical analysis of human granulosa cells with anti-CCL20 or isotype control antibodies. Original magnification × 400. *Bar*, 10 µm. (D) Immunofluorescence analysis of human oocytes with anti-CCL20 or isotype control antibodies. Anti-CCL20 antibodies were detected with Cy3-labeled secondary antibodies and shown in *red*; nuclei were counterstained with DAPI (*blue*). Original magnification × 400. *Bar*, 100 µm. (E) CCL20 protein concentration in human follicular fluid ($n = 26$ donors) was determined using ELISA. CCL20 concentrations ranged from 4–30 ng/ml.

gradients of CCL20 (100, 1000 ng/ml) and controls (CCL21 at 100 ng/ml or medium alone). Capillary assays were combined with computer-assisted videomicroscopic motion analysis to obtain trajectories made by swimming sperm from an area within 250 µm of the capillary tip (Figure 3D). Subsequently, sperm trajectories were analyzed relative to the direction of the chemokine gradient using an established directionality-based assay independent of sperm swimming speed and sperm pattern of movement and therefore

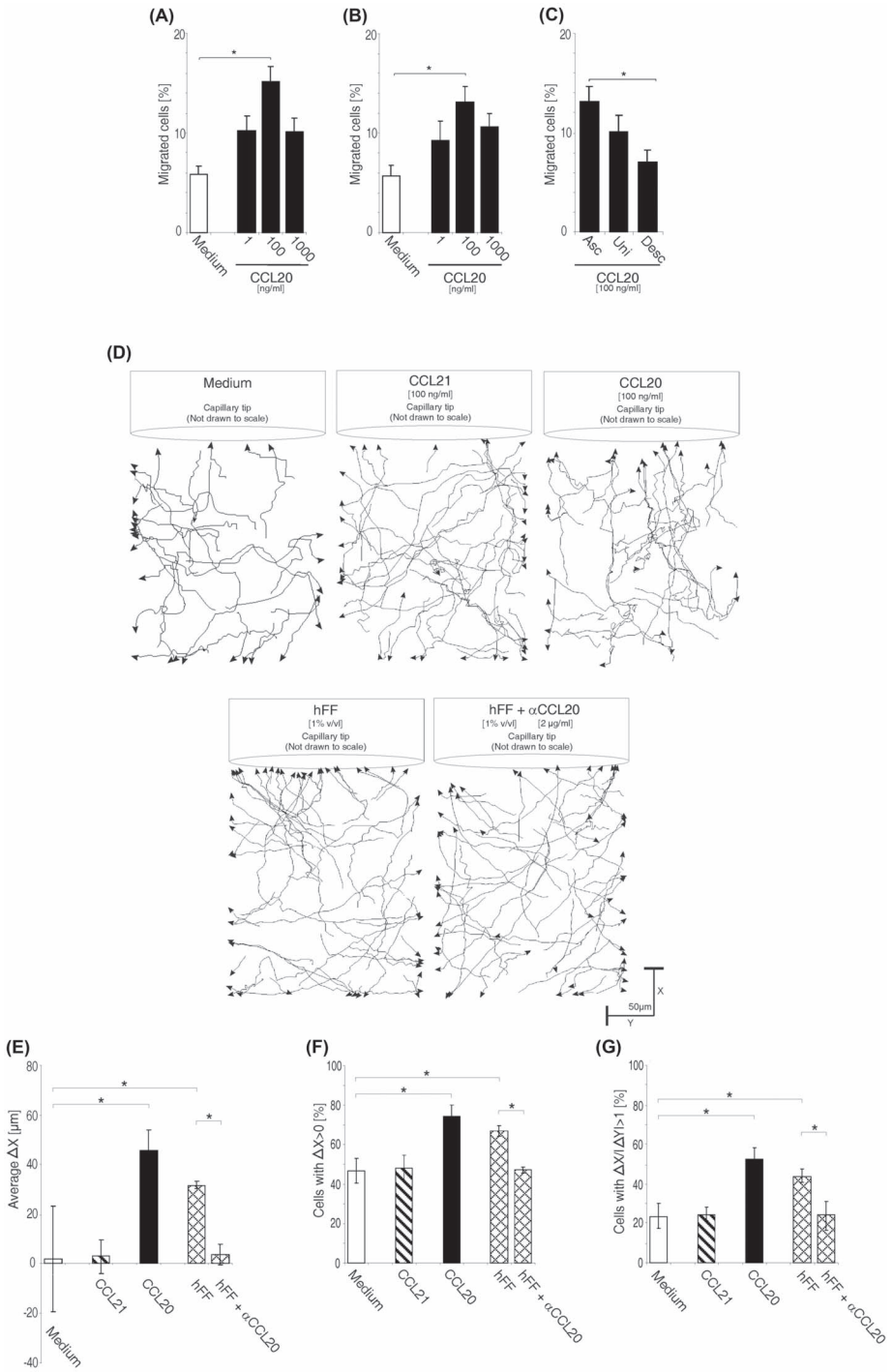


Figure 3. Human sperm chemotaxis to gradients of CCL20. (A) Capillary assay. Accumulation of sperm in capillaries filled with CCL20 (1 ng/ml, 100 ng/ml, 1000 ng/ml) or medium. Results are expressed as percentages of total cells. Values are means ± SEM of 19 different experiments. *Asterisks* indicate significant differences (*, $P < 0.05$) using one-way ANOVA. (B, C) Transwell assays. (B) Accumulation of sperm in inserts filled with CCL20 (1, 100, 1000 ng/ml) or medium. (C) Accumulation of sperm in inserts presenting an ascending, uniform, or descending CCL20 gradient (100 ng/ml). Results are expressed as percentages of total cells. Values are means ± SEM of 10 different experiments. *Asterisks* indicate significant differences (*, $P < 0.05$) using one-way ANOVA followed by Bonferroni post-test. (D) Representative trajectories of sperm imaged near capillaries containing CCL20 (100 ng/ml), CCL21 (100 ng/ml), hFF (1% v/v), hFF (1% v/v) plus a neutralizing anti-human CCL20 antibody (2 µg/ml) or medium. Arrowheads indicate directions of travel for individual cells. Trajectories are representatives for test and control stimuli of at least 3 different experiments. Bars, 50 µm. (E–G) Statistical analysis of sperm trajectories. Each plotted value is a mean (± s.d.) percentage of totally tracked sperm. (E) Average ΔX, mean net distance (µm) travelled along the chemokine gradient (X-axis). (F) $\Delta X > 0$, Percentages of sperm travelling in the direction of chemokine gradients (X-axis). (G) $\Delta X/|\Delta Y| > 1$, Percentages of sperm travelling a longer distance in the direction of chemokine gradients (X-axis) than in the direction orthogonal to the gradients (Y-axis). Cells were imaged for periods of 25 s. A minimum of 25 paths were analyzed for each test or control solution ($n = \geq 5$ different experiments). *Asterisks indicate significant differences (*, $P < 0.05$) using the Wilcoxon test.*

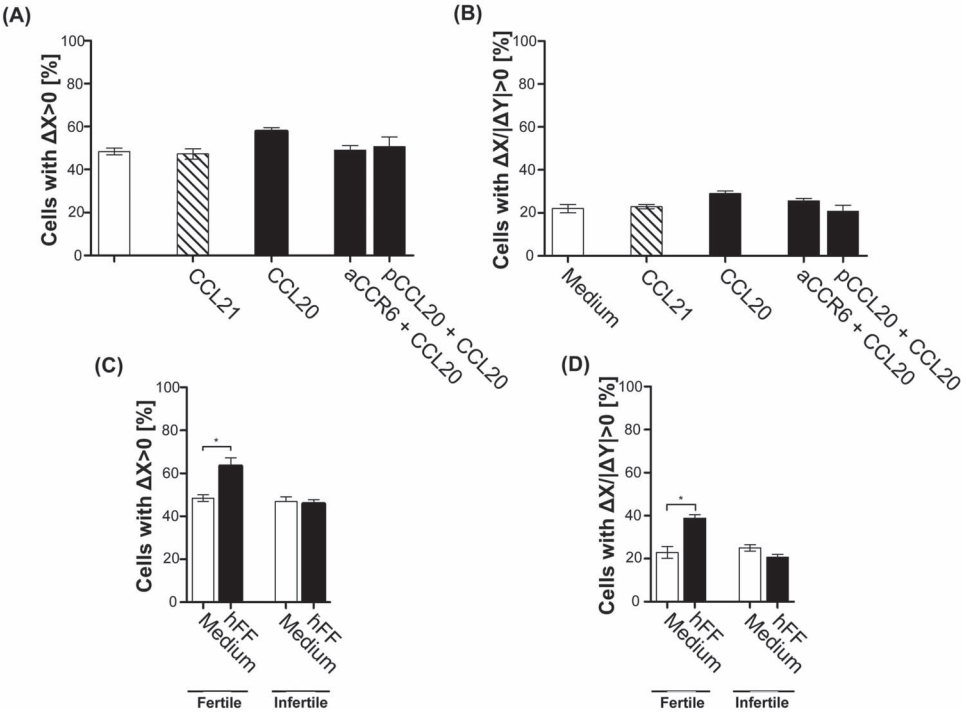


Figure 4. Human Sperm chemotaxis under inflammation conditions. (A + C) $\Delta X > 0$, Percentages of sperm travelling in the direction of chemokine gradients (X-axis). (B + D) $\Delta X/|\Delta Y| > 1$, Percentages of sperm travelling a longer distance in the direction of chemokine gradients (X-axis) than in the direction orthogonal to the gradients (Y-axis). Cells were imaged for periods of 25 s. A minimum of 25 paths were analyzed for each test or control solution. (A-B) Chemotaxis response from sperm from healthy donors toward CCL20 100 ng/ml pre-incubated with neutralized with anti-CCR6 antibodies (αCCR6 + CCL20). Sperm incubated with high concentration CCL20 (100 ng/ml) and chemotaxis toward 100 ng/ml (pCCL20 + CCL20) ($n = 3$). (C-D) Chemotactic responses from sperm of ISI patients toward gradients of hFF ($n = 3$). *Asterisks* indicate significant differences (*, $P < 0.05$) using the Wilcoxon test.

measuring chemotaxis only (Figure 3E–G) [13]. Results confirmed our previous observations, showing concentration-dependent, bell-shaped sperm chemotaxis toward different gradients of CCL20 protein. The strongest chemotactic response was observed at CCL20 concentrations of 100 ng/ml confirming previous observations in capillary and transwell assays. Response rates obtained for CCL21 and medium were in line with values expected for randomly moving cells, hence showing no chemotaxis. Next to CCL20 [41], sperm showed strong migration toward gradients of hFF (1%) [4, 24, 29]. Chemotaxis of human sperm toward hFF (1%) was significantly impaired using neutralizing anti-human CCL20 antibodies. Finally, we next pre-incubated human sperm with high concentrations of CCL20 (100 ng/ml) prior to videomicroscopic motion analysis. Pre-incubated sperm showed a significantly impaired migration toward gradients CCL20 (100 ng/ml) as compared to untreated controls (Figure 4A and B).

In conclusion, these results demonstrate significant concentration-dependent sperm chemotaxis toward gradients of CCL20 and point toward an important role for CCR6-CCL20 in human gamete interaction.

Increased expression of testicular CCL20 in the interstitial of men with impaired spermatogenesis with inflammation

In the next step, we performed immunohistochemical studies in testis specimens of men with impaired spermatogenesis with inflammation (ISI). Our results revealed that the expression of CCL20 was significantly increased in the infiltration area of testicular interstitium (Figure 5A). However, low expression of CCL20 was found in the testicular interstitium of the men with impaired spermatogenesis without inflammation (ISW) and normal spermatogenesis (NS) (Figure 5A). In this context data demonstrated significantly higher expression of testicular CCL20 in the interstitial of ISI compared to the interstitial of lacking inflammatory signs in both ISW and NS groups ($P < 0.01$, Figure 6A). Moreover, expression differences of CCL20 cannot be detected in both ISW and NS groups ($P > 0.05$, Figure 6A).

In view of these data, they indicate that testicular CCL20 could be regarded as a specific marker for testicular inflammation, which facilitates the immune cells (DCs, T cells and B cells, etc.) infiltrate and recruit into the inflammatory infiltration area.

Differential expression of CCR6 in testis from fertile men compared with infertile men with and without inflammation

As the expression of CCR6 was found in interstitial and seminiferous tubules of three groups, the expression level of interstitial CCR6 (iCCR6) and seminiferous tubules CCR6 (tCCR6) was assessed by the stereological technique, respectively. Similar to CCL20 expression, significantly higher expression of iCCR6 was detected in testicular interstitial of ISI in contrast to interstitial of ISW and NS ($P < 0.01$, Figure 5B, Figure 6B). These findings suggest consistent colocalization between CCL20 and iCCR6 in inflammatory conditions with focal lymphocytic infiltrates.

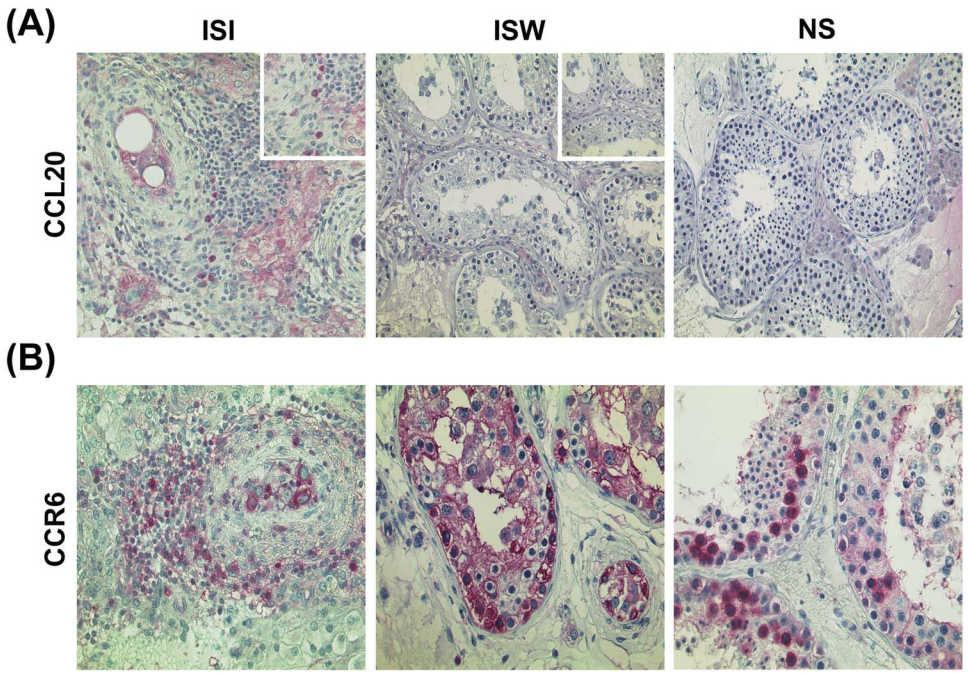


Figure 5. CCL20 and CCR6 in human testis and seminal plasma of infertile men. (A) Immunohistochemical detection of CCL20+ cells in impaired spermatogenesis with inflammation (ISI), impaired spermatogenesis without inflammation (IWI), and normal spermatogenesis (NS) (original magnification, ×200; Inset: ×400) ($n = 5$). (B) Immunostaining for CCR6 in ISI, ISW, and NS (original magnification, ×400) ($n = 5$ donors).

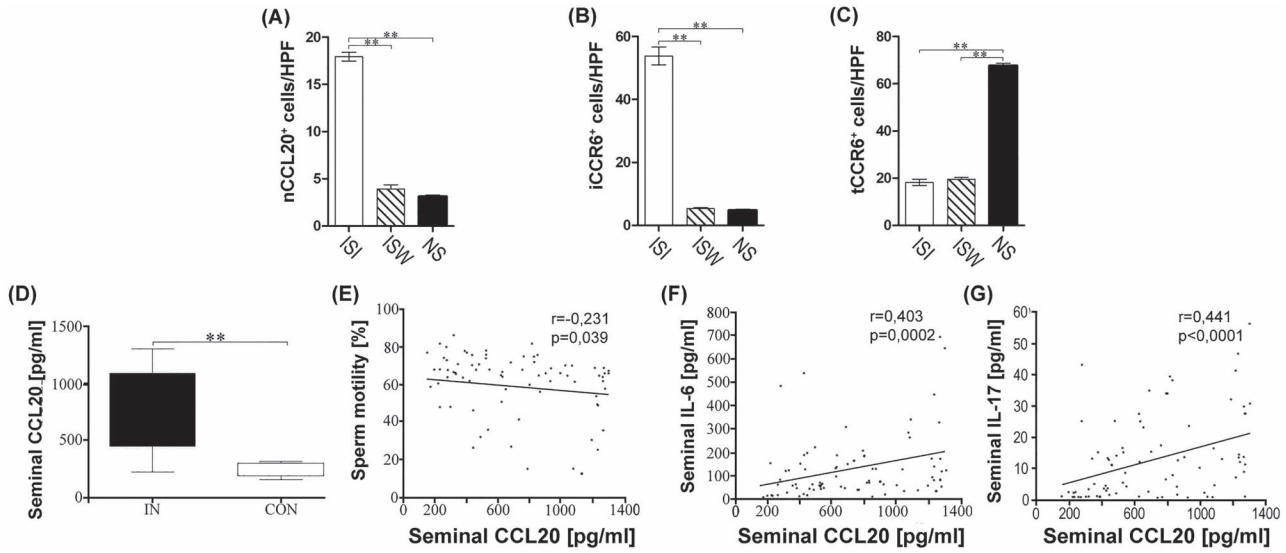


Figure 6. Correlation between CCL20 and CCR6 in infertile men. (A) CCL20+ cells, (B) interstitial CCR6 (iCCR6) and seminiferous tubules CCR6 (tCCR6) in ISI, ISW and NS. *Asterisks* indicate significant differences (*, $P < 0.05$) using one-way ANOVA followed by Bonferroni post-test. (D) CCL20 seminal plasma concentration in patients with chronic genital tract inflammation (IN) and healthy controls (CON).Correlation between seminal CCL20 and (E) sperm vitality, (F) seminal IL-6 and (G) IL-17 concentration ($n = 80$). Data were expressed as mean ± SEM ($n = 5$). Spearman correlation, statistical significance: $P < 0.01$.

On the other hand, the expression of CCR6 was readily detectable in the seminiferous epithelium of all specimens, particularly on spermatocyte and spermatid. As ISI and ISW groups demonstrate impaired spermatogenesis, a significantly lower expression of tCCR6 could be observed in seminiferous tubules revealing the loss of the adluminal compartment of the germinal epithelium and some remaining spermatogenic cells compared with NS ($P < 0.01$, Figures 5D–F and 6C).

From this set of experiments, we conclude that CCR6 not only could regulate the migration and recruitment of DCs, B cells, and T cells during inflammatory and immunological responses but may also be involved in human spermatogenesis.

Expression of CCR6 on sperm and semen parameters in subfertile men

As shown in Figure 1C–E, the positive immunofluorescent signals observed following the use of an anti-CCR6 antibody localized CCR6 to the flagellum of spermatozoa. It is well known that sperm motility is essential for fertilization.

As little is known about CCR6+ Sperm in the human ejaculate, we investigated its presence in the ejaculate of men attending the andrological outpatient clinic for subfertility. In this context, significant numbers of sperm expressed CCR6 (48% +/− SEM 3.41; $n = 58$). Interestingly, sperm from men with chronic inflammation of genital tract (CIGT) displayed significant lower CCR6 expression than sperm from CON (Supplementary Figure S3A and B). Moreover, a significant inverse correlation between CCR6 on sperm and its ligand CCL20 in SP could be demonstrated (Supplementary Figure S3C). Further on, CCL20 also correlated with inflammatory marker IL-6 in SP ($r = 0.403$; $P = 0.0002$; $n = 80$) (Figure 6F). In view of standard semen parameters, a significant correlation between CCR6 on sperm and sperm motility, morphology, and vitality could be detected (Supplementary Table S1). Altogether, CCR6 expression on sperm is influenced by genital tract inflammation and might impact semen quality and sperm function.

Discussion

Most developed countries face the controversial problem of declining birth rates on one and staggeringly high incidences of unplanned pregnancies on the other hand [55, 56]. 100 years ago, Lillie describes chemotaxis by the sea urchin *Arbacia punctulate* [30, 57]. In the last century, *Arbacia punctulata* becomes a role model of chemotaxis. Short peptides like GGGYG-resact were identified at the surface of the egg coat and guanylyl cyclase (GC) in sperm as their receptor [58–60].

In contrast, the precise mechanisms how human sperm reach the oocyte in female genital tract remained elusive for a long time. So far, it has been shown that *in vitro* human sperms are guided by follicular fluid, which is secreted by the oocyte-cumulus complex [3, 6, 12, 17, 54]. Notably, the hormone progesterone represents a relevant chemotactic factor in human follicular fluid [27, 30–32], but it is suggested that additional chemoattractants such as CCL20 of human sperm exist [41]. Finally, sperms are guided through the dense matrix of the cumulus complex to the oocyte itself according to the gradient of a more potent chemoattractant that is secreted from the oocyte [27, 54].

Recent studies demonstrated that the chemoattractant CCL5 (the ligand for CCR3 and CCR5) could play an important role in ejaculated sperm maintaining their motility and acrosome reaction in the female genital tract [61, 62].

In the present study, we systematically screened chemokine receptors on the surface of human sperm (Supplementary Figure S1). Since we consistently detected the expression of CCR6 on human sperm in our donors and also detected the production of its corresponding specific ligand CCL20 during the preovulatory phase of the menstrual cycle in human ovaries (Figure 2A), we chose CCR6 for further detailed analyses.

We demonstrate that the chemokine CCL20 is abundantly present in human follicular fluid and is produced by human oocytes as well as surrounding cumulus granulosa cells. Our analyses of the content of CCL20 protein in human follicular fluid revealed that CCL20 accounted for approximately 0.007–0.03% of total protein concentrations, suggesting an important function of this chemokine for the process of fertilization. These results are supported by previous reports of Kawano *et al.* demonstrating the presence of CCL20 protein in human follicular fluid [63]. Moreover, our analysis of the expression of CCL20 in human ovaries demonstrates a periodical regulation with a peak-expression during the preovulatory phase of the female menstrual cycle. Correspondingly, in human ovaries, CCL20 shows a peak expression in the follicular fluid containing mature oocytes than in follicular fluid containing immature oocytes [63].

The chemokine CCL20 specifically binds to its receptor CCR6. It is interesting that our analysis of the microanatomical expression of CCR6 localized the receptor to the flagellum but also to the equatorial segment of the sperm head. In fact, this segment presents the structure where the first encounter and the fusion of the plasma membranes of sperm and oocyte occur (short-range guidance) [64, 65]. This is in line with previous reports of Caballero-Campo *et al.* demonstrating the location of CCR6 in humans and mice [41]. Diao *et al.* demonstrated in mice that Ccr6 is involved in the chemotaxis of spermatogenic cells *in vitro*, and Ccl20 mRNA was expressed at high levels in murine epididymis [66]. Caballero-Campo *et al.* showed a change in the progressive rapid pattern after incubation with CCL20 for a few minutes. Moreover, they demonstrated a chemotactic effect of CCL20 on human sperm by using a Zigmond-chamber [67].

Using IBIDI chamber videotracking analysis, we demonstrate a dose-dependent, bell-shaped chemotactic response of human sperm toward different gradients of CCL20. [27, 63]. Notably, the most significant chemotactic responses were measured to CCL20 concentrations of 100 ng/ml and weaker responses were observed toward lower as well as higher concentrations (Figure 3). In fact, it is suggested that sperms fail to sense rising gradients of chemoattractants when the concentration of the chemotactic ligand exceeds the level of receptor saturation, hence resulting in random movement [13].

Data of the present study include CCL20 into the list of human sperm attractants including progesterone and CCL5 [27, 54]. The present study demonstrates for the first time that CCL20 is specifically overexpressed in the lymphocytic infiltrates of testicular interstitium of men with impaired spermatogenesis with inflammation when compared with patients of lacking inflammatory sign. The CCR6-CCL20 axis could not only regulate the migration and recruitment of immune cells under testicular inflammatory conditions but also could be involved in spermatogenesis. CCR6/CCL20 expression in ejaculate is influenced by genital tract inflammation, which might influence semen quality and impair sperm function. This is further supported by our finding that a significant positive correlation was found between CCR6 expression and percentage of sperm forward motility, morphology, and vitality. Our previous study demonstrated a high percentage of apoptotic spermatozoa in ejaculates from men with chronic genital tract inflammation, particularly in the swim-up fraction [22]. This is supported by this study, which could demonstrate that sperm from men with chronical orchitis did not react with a chemotactic response toward gradients of CCL20. This is of particular interest, as we could also show that levels of seminal CCL20 were significantly increased in infertile patients with chronic genital tract inflammation. Based upon this data, we develop a model of exhausting human sperm due to the presence of CCL20 in chronical orchitis “at the wrong time in the wrong place”. Chronic orchitis represents a chronic inflammatory condition of the testis, leading to elevated CCL20 levels in human seminal plasma disrupting guidance gradients and finally “blindfolding human sperm”. We propose that the CCL20-CCR6 axis could not only regulate the sperm motility but may also be involved in apoptosis of spermatozoa under normal and inflammatory conditions in the male genital tract.

It has long been recognized that the chemokine receptor CCR6 is abundantly expressed by immune cells such as immature

dendritic cells (DCs), memory T cells, and B cells [39]. Our previous findings revealed that Th17 cells might be critically involved as a regulator in the pathogenesis of male infertility under infection and inflammatory conditions [6], which was confirmed by the mouse model of experimental autoimmune orchitis [8]. In this context, mechanisms guiding the recruitment of immune cells into the immuno-privileged testicular tissue are of great interest.

Based upon the recent knowledge and our own analysis, we propose a novel, modified model (graphical abstract): leaving the vagina, human sperms actively swim by the help of rheotaxis toward the so-called sperm storage site at the isthmus of the fallopian tube [68]. This process is supported by coordinated contractions of vagina and uterus. Having reached the sperm storage site, different subsets of sperm are capacitated in a time-dependent manner. Subsequently, capacitated sperms sense an ovulation-dependent temperature gradient and travel from the cooler storage site toward the warmer site of fertilization by means of thermotaxis (long-range guidance) [3, 12]. Here, sperms sense a gradient of chemotactic factor secreted by the oocyte-cumulus complex, such as CCL20, CCL5, or progesterone, and are guided to the complex by means of chemotaxis (short-range guidance). In reach of the oocyte-cumulus complex sperm guidance factors such as CCL20, CCL5, or progesterone could lead human sperms to the oocyte by a mechanisms like cell-to-cell adherence not yet understood (short-range guidance) [27, 54]. Finally, the CCR6-positive sperm equatorial segment adheres to the CCL20-positive oocyte, and plasma membrane fusion is initiated (short-range guidance) [65].

Taken together, our findings provide new insights into the mechanisms of human gamete interaction and might suggest interesting new targets for non-hormonal contraception, fertility diagnostics, and assisted reproduction.

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