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Research Article

Direct role of the C-C motif chemokine receptor 2/monocyte chemoattractant protein 1 system in the feline cumulus oocyte complex†

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Abstract

Studies were designed to (a) evaluate the mRNA expression of the C-C motif chemokine receptor 2 (*CCR2*) and its chemokine ligands, as well as genes related to periovulatory events, within the cumulus oocyte complex (COC) and follicle wall after a luteinizing hormone (LH) stimulus in cultured feline antral follicles; (b) assess the immunolocalization of CCR2 and its main ligand (monocyte chemoattractant protein 1, MCP1) within the feline COC; and (c) examine the direct effects of exogenous recombinant MCP1 on mRNA expression of the *CCR2* receptor and *MCP1* as well as key periovulatory genes in the COC, using a feline COC culture system. Both culture systems were developed by our laboratory and exhibit physiological response to gonadotropin stimuli. In summary, this study demonstrated mRNA expression of *CCR2* receptor and its assessed ligands *(MCP1, MCP2, MCP3,* and *MCP4*) within the feline COC and follicle antral wall, and a significant increase in *CCR2* mRNA by LH within the COC. Also, CCR2 and MCP1 immunoreactivity was observed in the oocyte and cumulus cells of the feline COC. Remarkably, this is the first report, in any species, describing a direct effect of the recombinant MCP1 in the CCR2/MCP1 system within the COC, by increasing the mRNA levels of key genes involved in the ovulatory cascade, as well as its own receptor *CCR2*. Together, these data suggest that CCR2 receptor signaling in the COC may regulate events critical for promoting cumulus oocyte expansion and/or oocyte maturation.

Summary Sentence

MCP1 directly stimulates the mRNA levels of key ovulatory genes and its own receptor *CCR2* within the COC, suggesting that CCR2/MCP1 signaling may regulate events critical for promoting cumulus oocyte expansion and/or oocyte maturation.

Key words: chemokines, CCR2, cumulus oocyte complex, cumulus oocyte expansion, feline, ovulatory cascade genes.

Introduction

Ovulation is a complex, inflammation-like process whereby a fully developed follicle ruptures in response to the actions of the mid-cycle gonadotropin surge, releasing the cumulus–oocyte complex (COC) into the reproductive tract for potential fertilization. Shortly before ovulation, the luteinizing hormone (LH) surge induces processes critical for fertility, including cumulus–oocyte expansion (C-OE), resumption of meiosis, and rupture of the follicle wall. Key components of these processes are temporally expressed and may play a critical role in C-OE and oocyte quality [1]. C-OE is due to a loss in cell-to-cell contacts and formation of a hyaluronic acid (HA)-rich extracellular matrix, resulting in a large increase in area or expansion of the cumulus granulosa cell layer that surrounds the oocyte [2–4]. Hyaluronic acid is the main matrix component of the expanded COC, and the amount of HA synthesized by HA synthase-2 (HAS2) is closely correlated with the degree of expansion [2, 5, 6]. While some of the paracrine-acting factors important for these events have been identified [7], the molecular mechanisms responsible for initiating such complex processes are not fully understood. Thus, understanding the molecular and cellular processes involved in C-OE as well as oocyte maturation would aid in the diagnosis or treatment of infertility and may also identify novel targets for a nonhormonal form of contraception.

Chemokines, also known as chemotactic cytokines, are small heparin-binding proteins classified into four families based on the number and location of N-terminal cysteine residues (CC, CXC, C, and CX3C). Many of these chemokines are expressed in ovarian tissue, and chemokine receptors have been identified in ovaries of many species. The CC chemokines act primarily on monocytes/macrophages and T cells. One important CC chemokine is the monocyte chemotactic protein-1 (MCP1) [8]. Skinner et al. showed that the levels of the mRNA of *MCP-1, MCP-2*, macrophage inflammatory protein-1alpha (LD78β), and chemokine C-C motif ligand-5 (*CCL-5*) increased in bovine granulosa and/or theca cells during antral follicle development [9]. *MCP-1* levels increased significantly during antral follicle development in both cell types [9], and there is a preovulatory upregulation of *Mcp1* in rat follicle [10]. Moreover, chemokine receptors were also shown to be expressed in the granulosa (*CCR1, CXCR3, CCR5,* and *CXCR6*) and theca (*CCR1*) cells, suggesting an autocrine/paracrine role of chemokines in these two cell types. Rodent studies suggest that chemokine signaling regulates the assembly of the cumulus extracellular matrix and that the interaction between prostaglandin and chemokine signaling was required for successful fertilization [11, 12].

The domestic cat (*Felis catus*) serves as valuable model for studying oocyte biology and also for addressing infertility syndromes in women, such as asynchronous oocyte cytoplasmic and nuclear maturation [13]. This is because highly conserved reproductive mechanisms between humans and feline species were recently defined [13]. Interestingly, cat oocytes share several characteristics with human oocytes [14, 15], including the diameter of the oocyte proper and the germinal vesicle, the time to reach the metaphase II (MII) stage of meiosis in culture, and a nuclear configuration with a small nucleolus and a fibrillar chromatin. In contrast, these morphological features are distinct or lacking in the typical laboratory mouse model. In addition to the potential to expand knowledge of feline reproduction, the use of this model system has the advantage of providing an excellent surrogate for understanding events involving human COCs that are necessary for fertility. In our laboratory, we recently established two different feline culture systems, showing that the culture of feline antral follicles was a robust and valuable system to study follicular development, steroidogenesis, and periovulatory events as well as follicle biology in general [16]. Also, we demonstrated that the culture of feline COCs from antral follicles was an appropriate system to study periovulatory events such as CO-E along with oocyte and cumulus cell biology in general [17].

Therefore, studies were designed to (a) evaluate the mRNA expression of the *CCR2* receptor and its chemokine ligands (*MCP-1, MCP-2, MCP-3,* and *MCP-4*), plus genes (*HAS2, TNFAIP6, AREG, PTX3,* and *GDF9*) related to periovulatory events in other species, within the COC and follicle wall after an LH stimulus, using a feline antral follicle culture; (b) assess the immunolocalization of CCR2 and MCP1 within the feline COC; and (c) examine the direct effects of recombinant MCP-1 on mRNA expression of the *CCR2* receptor and its main ligand (*MCP-1*) together with *HAS2, TNFAIP6, AREG, PTX3,* and *GDF9* in the COC, using a feline COC culture.

Materials and methods

Animals

Ovaries from adult female (n = 77) *Felis catus* at different stages of the natural estrous cycle during the breeding season were used. The ovaries were donated following routine spaying procedures conducted at the "Centro de Salud Animal de la Municipalidad de Merlo" (Prov. de Buenos Aires, Argentina). The excised ovaries were immediately transported to the laboratory in chilled physiological solution.

Experiment 1: Follicle culture

Based on preliminary results, antral follicles that measured 0.5–2.0 mm were used in the present study. Follicle isolation from the ovary was performed under a dissecting microscope using 30-gauge needles as previously described [16–18]. Briefly, isolated antral follicles (n = 133) were individually cultured for 6, 12, 24, and 36 h in the presence or absence of recombinant human LH (75 mIU/ml rhLH, Merck Serono) in a 48-well plate containing 300 μl alpha minimum essential medium (αMEM, Sigma) supplemented with 15 ng/ml recombinant human FSH (equivalent to 205 mIU/ml, Merck Serono), 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium (ITS, Sigma), streptavidin/penicillin (100 IU/ml–100 mg/ml, Sigma), and 0.30% BSA (Fraction V, Natocor), as previously reported [16]. At the end of culture, the follicle walls and the COCs were isolated and stored at –80°C for subsequent RNA extraction and RT real-time PCR.

At the end of the culture period, COCs were retrieved from each individual follicle. The morphology and health of the oocyte and its surrounding cumulus cells were observed and analyzed under the dissecting microscope. Images of the individual COCs at 6, 12, 24, and 36 h retrieved post-culture were taken using a digital camera attached to a bright-field microscope to assess the CO-E by an "all or none" response to treatments. The substantial expansion/enlargement of the adjacent cumulus cells of the COC under the dissecting microscope can be easily visualized [16]. CO-E is reportedly the most reliable index of oocyte maturation in cats, since polar body extrusion is difficult to identify in feline oocytes due to the dark appearance of their oocytes [19].

As previously reported [16], even though only healthy antral follicles (devoid of dark follicles/granulosa cells; n = 133) were

isolated and used for this study, at the end of the culture a few (less than 10%) of the retrieved COCs contained naked (n = 8) or dead (n = 1) oocytes. Thus, these samples were excluded for the C-OE analysis.

Experiment 2: COC culture

COCs (n = 143) isolated from antral follicles (0.5–2.0 mm) were cultured, as previously reported [17], in MEM containing Hepes [25 mM, Gibco], L-glutamine (2 mM), sodium pyruvate (1 mM, Sigma), penicillin/streptomycin (100 IU/ml–100 mg/ml, Sigma), and 1% FBS-charcoal/stripped for 3, 12, and 24 h in the presence or absence of human recombinant MCP1 (Life Technologies). Two different concentrations of MCP1 were tested (10 and 100 ng/ml) based on the manufacturer's datasheet. Specificity of the MCP1/CCR2 signaling was assessed in a separate set of experiments (Supplemental Figure S1) by culturing the COCs (n = 3–4/group) in the presence of MCP1 with or without a highly selective CCR2 chemokine receptor antagonist (1 μM, RS 504393, Tocris Biosciences). At the end of culture, COCs were photographed and stored individually at –80°C for subsequent RNA extraction and RT real-time PCR.

mRNA extraction and gene expression analysis

Total RNA was extracted for each individual sample using either TRIzol (Life Technologies) for the follicle walls [20–22] or Absolutely RNA Nanoprep Kit (Agilent) for the COCs [17], as previously described. To synthesize single-stranded cDNA from total RNA, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used following their instructions. RT was performed for 2 h at 37°C using either 344 ng of RNA from the follicle wall or 10 μl from the RNA extraction from each individual COC in a 20-μl reaction volume. After cDNA synthesis, real-time PCR for *MCP1*, *MCP2, MCP3, MCP4, CCR2,* and key genes within the ovulatory *cascade* (such as *HAS2, TNFAIP6, AREG, PTX3,* and *GDF9)* was conducted as previously described [17]. Primers and probes were designed using gene sequences obtained through the feline genome database *(Felis catus)* accessible at the National Center for Biotechnology Information (NCBI). Sequences of all primers and probes are listed in Supplemental Table S1. Relative levels of target gene expression were normalized to *ribosomal protein 18S* levels.

Immunofluorescence and confocal microscopy

A small cohort of COCs was randomly selected during the isolation procedure and fixed in 4% paraformaldehyde for indirect immunofluorescence to localize both MCP1 and CCR2 proteins within the feline COC, as previously described [17, 23]. Details of the primary antibodies are listed in Supplemental Table S2. The fixed COCs were then stored in washing buffer (1% BSA, 0.2% powder milk, 0.2% goat serum, 0.2% donkey serum, 0.1% triton X-100, 0.1M glycine in PBS) at 4°C until use. Briefly, COCs were incubated with primary antibody (CCR2: NBP1-48337, Novus Biologicals; and MCP1: NBP2-22115, Novus Biologicals) for 1 h at 37°C followed by three 10-min washes in washing buffer, and then a 1-h incubation of secondary antibody at 37°C (secondary antibodies conjugated with Alexa Fluor dyes); F-actin was probed with Alexa 488-phalloidin; DNA was labeled with Hoechst 33342. Stained COCs were mounted on slides using 15 μl of VECTASHIELD® Mounting Medium (Vector Laboratories). Optical sections were acquired using the Olympus FLUOVIEW FV1000 confocal laser scanning microscope using two different objectives (PLSAPO 20 × 0.75 DRY CS UV and PLSAPO 60 × 1.35 OIL CS UV). Full Z-stack data sets were collected with the ×60 objective for each COC, with images taken every 0.5 μm.

Statistical analysis

Statistical calculations were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). For parametric data, one or two-way ANOVA (treatment × time) was used to analyze differences, followed by the Newman-Keuls multiple comparison test. For nonparametric data, Kruskal-Wallis and Dunn method tests were performed. Differences were considered significant at $P < 0.05$.

Results

Experiment 1

Expression of key genes involved in the ovulatory cascade within the antral follicle at different time points following LH treatment

The expression of key genes involved in the ovulatory cascade (*HAS2, TNFAIP6, AREG, PTX3,* and *GDF9*) within the follicle wall and COC was assessed by real-time PCR at different time points following LH treatment. Two-way ANOVA analyses (time and treatment) showed significant effects ($P < 0.05$) on many of the mRNA expression assessed within the COC and/or the follicle wall (Figure 1).

This analysis showed a significant increase for *AREG* mRNA expression in the follicle wall 6 h after LH treatment (Figure 1A). In contrast, no significant changes ($P > 0.05$) were observed for *HAS2* (Figure 1B), *TSG6*, *PTX3,* or *GDF9* (Supplemental Figure S2), within the follicle wall.

Furthermore, we observed significant effects by time on many of the mRNA expression assessed within the COC, including *AREG*, *HAS2* (Figure 1C and D), *PTX3,* and *GDF9* (Supplemental Figure S3b and c). The highest expression of *HAS2* and *AREG* in COCs was observed 6 h after LH treatment. No significant changes ($P > 0.05$) of *TSG6* mRNA expression were observed within the COC regardless of treatment or time (Supplemental Figure S3a).

Expression of CCR2 and its chemokine ligands within the antral follicle at different time points following LH treatment

The expression of the *CCR2* receptor and its chemokine ligands (*MCP-1, MCP-2, MCP-3,* and *MCP-4*) was distinguished within the follicle wall and COC (Figures 2 and 3, respectively). The mRNA expression of *MCP1* (Figure 2B) and *MCP3* (Figure 2D) within the follicle wall was affected by time, displaying their highest levels at 6 h (two-way ANOVA; $P < 0.05$). In contrast, the expression of *CCR2* and the chemokines *MCP2* and *MCP4* within the follicle wall did not change regardless of LH treatment or time (Figure 2A, C, and E, respectively).

The expression of the CCR2 receptor and its chemokine ligands within the COC (Figure 3) showed significant differences by treatment and/or time (two-way ANOVA; $P < 0.05$). Importantly, LH treatment significantly increased *CCR2* mRNA expression in COCs after 6 h in culture, compared to controls (Figure 3A). Whereas, *MCP2* mRNA expression significantly changed in time only in the control group (Figure 3C). In contrast, *MCP1* and *MCP3* mRNA expression within the COC (Figure 3B and D) did not show any significant difference either in treatment or time ($P > 0.05$). Finally,

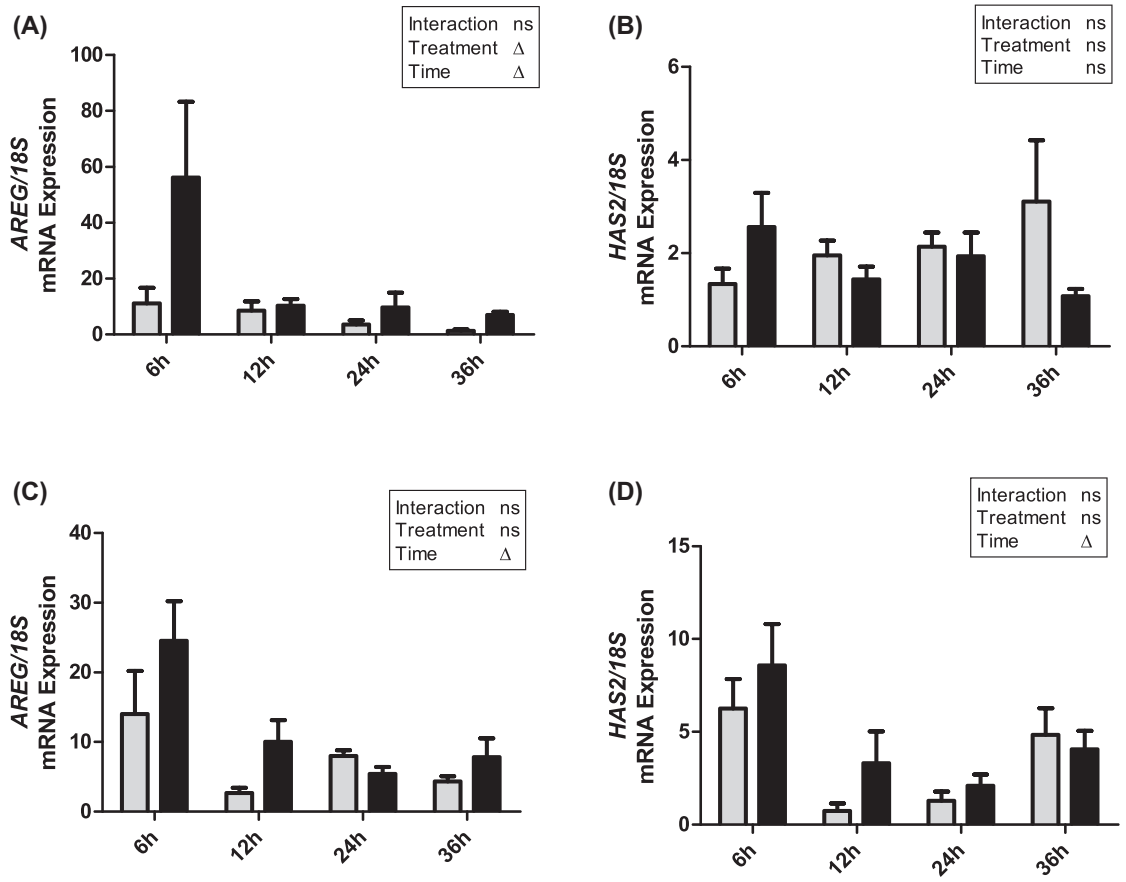

Figure 1. Normalized *AREG* and *HAS2* mRNA expression (mean ± SEM) within the follicle wall (A, B) and the COC (C, D) at different time points (6, 12, 24, and 36 h) in culture in the absence (control, gray bars) or presence of LH (LH, black bars) assessed by quantitative real-time PCR. 18S rRNA served as the invariant control for normalization. (A) Two-way ANOVA analyses (Time × Treatment) showed significant differences ($P < 0.05$) within the follicle wall only for *AREG*. Significant effects were observed by time and treatment for this gene (C6 vs. LH6; LH6 vs. LH12, LH24, and LH36). (B) No significant changes ($P > 0.05$) were observed for *HAS2* mRNA expression within the follicle wall. (C, D) Two-way ANOVA analyses showed significant effects ($P < 0.05$) by time on many of the mRNA expression assessed within the COC, including AREG and *HAS2*. *AREG* mRNA expression was significantly different within the COC at different time points (B: LH6 vs. LH12, LH24, and LH36). Significant effects were observed for *HAS2* mRNA expression (A: C6 vs. C12; LH6 vs. LH12, and LH24).

MCP4 mRNA exhibited very low expression near the limit of detection (data not shown).

Cumulus–oocyte complex expansion following LH treatment

In addition, the proportion of expanded COCs retrieved after the different time points (6, 12, 24, and 36 h) from healthy antral follicles (n = 124) cultured in the presence (LH) or absence of LH (control) were analyzed. The highest percentage of expanded COCs was observed in the LH groups at 12 (94.1%), 24 (100%), and 36 h (85.7%). Whereas, the percentage of expanded COCs in the control groups were only 14.3% (12 h), 5.6% (24 h), and 0% (at 36 h). The proportion of expanded COCs retrieved from healthy antral follicles treated with LH for 12, 24, and 36 h were significantly higher in comparison to their controls ($P < 0.0001$). The only time point where C-OE was very low (around 7%) in either of the groups was 6 h.

CCR2 and MCP1 proteins were immunolocalized to cumulus cells and oocytes

To visualize the CCR2 and its main ligand MCP1 within the feline COC, a small cohort of COCs was randomly selected and fixed during the isolation procedure. All samples exhibited the same immunostaining pattern for both proteins (CCR2 and MCP1), showing immunolocalization within the cumulus as well as the oocyte. A representative picture of the immunofluorescence for CCR2 (Figure 4A and B) and MCP1 (Figure 4C and D) within the feline COC is shown in Figure 4.

Experiment 2

MCP effects on gene expression within the COC using a feline COC culture

To assess a possible direct effect of the CCR2/MCP1 system in the COC, feline COCs were cultured in the presence or absence of recombinant MCP1. COCs cultured for 3 h in the presence of recombinant MCP1 showed a significant increase in the normalized mRNA levels ($P < 0.05$) of all assessed genes involved in the ovulatory cascade (*HAS2*, *AREG*, *TSG6*, *PTX3,* and *GDF9*), as well as, in its own receptor *CCR2* (Figures 5 and 6, respectively). *MCP1* mRNA expression also significantly increased ($P < 0.05$) with its own presence at 100 ng/ml, in comparison to the lower concentration (Figure 6B). Both concentrations (10

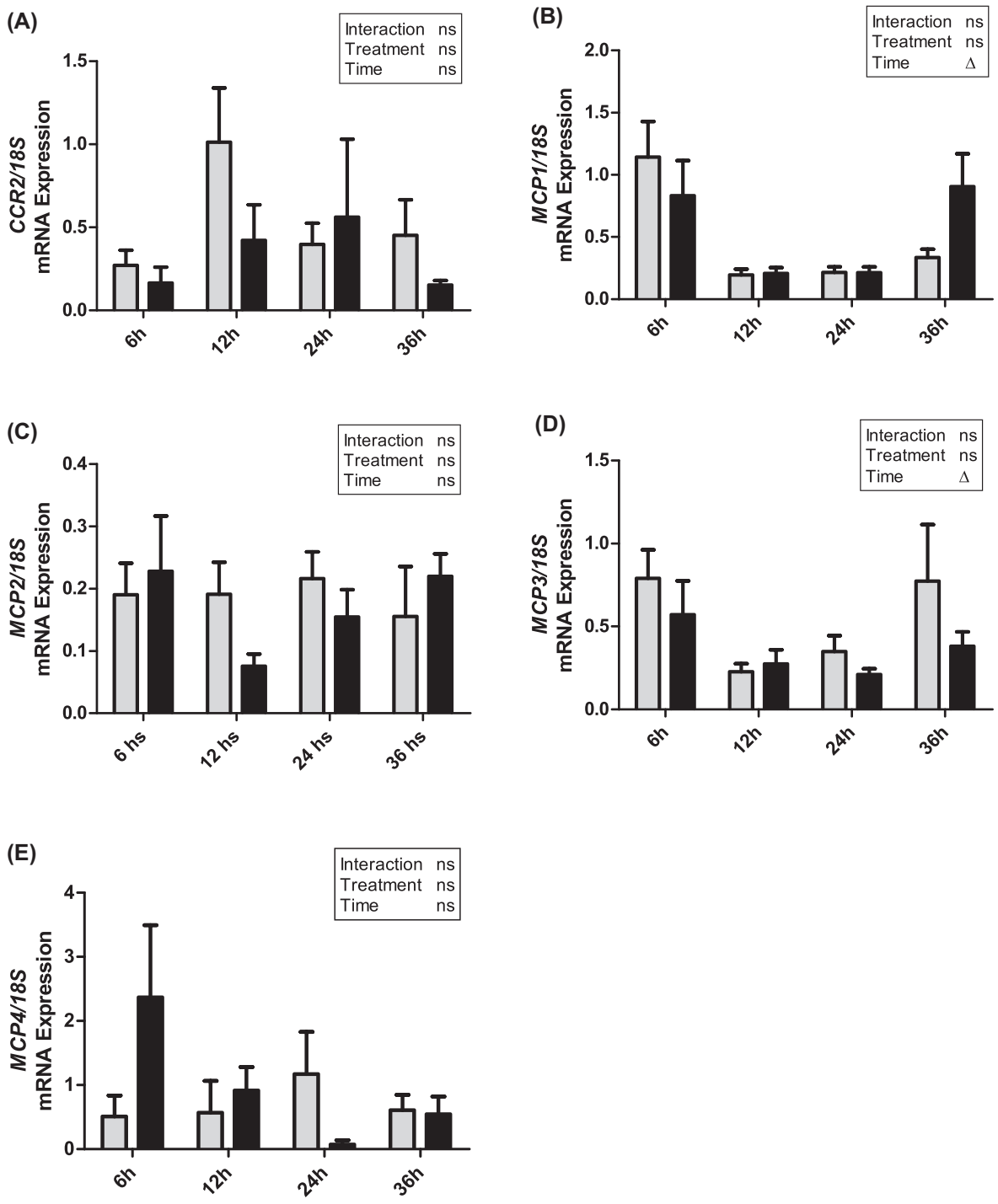

Figure 2. Normalized *CCR2* (A), *MCP1* (B), *MCP2* (C), *MCP3* (D), and *MCP4* (E) mRNA expression (mean ± SEM) within the follicle wall at different time points (6, 12, 24, and 36 h) in culture in the absence (control, gray bars) or presence of LH (LH, black bars) assessed by quantitative real-time PCR. 18S rRNA served as the invariant control for normalization. Two-way ANOVA analyses (Time × Treatment) showed significant difference ($P < 0.05$) among some of the mRNA expression assessed. A significant effect ($P < 0.05$) by time was observed for the mRNA expression of *MCP1* (B: C6 vs. C12, C24, and C36; LH6 vs. LH12 and LH24; LH12 vs. LH36; LH24 vs. LH36) and *MCP3* (D: C6 vs. C12; C12 vs. C36). In contrast, the expression of the receptor *CCR2* and the chemokines *MCP2* and *MCP4* did not change ($P > 0.05$) within the follicle wall, regardless of LH treatment or time (A, C, and E).

and 100 ng/ml) of MCP1 showed a direct significant stimulation on gene expression within the COC; nevertheless, the lowest concentrations seemed to have the maximum effect among most of the analyzed genes.

In agreement with our previous study [17], the mRNA expression of all assessed genes within the COC did not significantly change at 12 and 24 h time points, regardless of treatment (Supplemental Figures S4–S6).

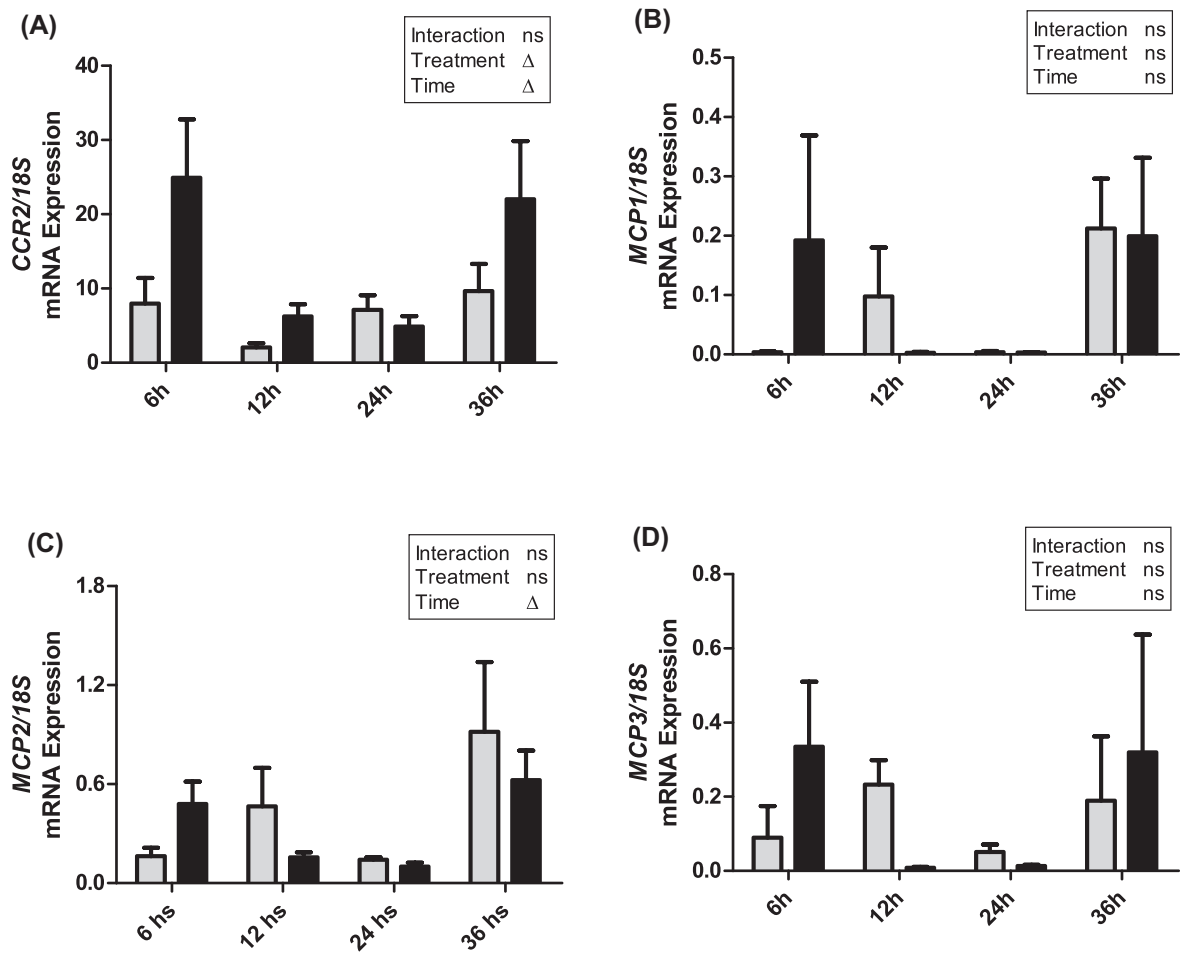

Figure 3. Normalized *CCR2* (A), *MCP1* (B), *MCP2* (C), and *MCP3* (D) mRNA expression (mean ± SEM) within the COC at different time points (6, 12, 24, and 36 h) in culture in the absence (control, gray bars) or presence of LH (LH, black bars) assessed by quantitative real-time PCR. 18S rRNA served as the invariant control for normalization. Significant differences affected by time and treatment of *CCR2* mRNA expression were observed within the COC (A: C6 vs. LH6; LH6 vs. LH12 and LH24; LH12 vs. LH36; LH24 vs. LH36). In contrast, *MCP2* mRNA expression significantly changed ($P < 0.05$) in time only in the control group (C: C6 vs. C36; C24 vs. C36). *MCP1* and *MCP3* mRNA expression within the COC (B and D) did not show any significant difference either in treatment or time ($P > 0.05$).

Discussion

This study reveals, for the first time, the mRNA expression for the chemokine *CCR2* receptor and its assessed ligands (*MCP1*, *MCP2*, *MCP3*, and *MCP4*) within the feline COC and follicle antral wall. Interestingly, a significant effect by LH treatment was observed on the *CCR2* mRNA levels in the COC after 6 h in vitro. In addition, a significant increase by LH in *AREG* mRNA expression in the follicle wall was observed at this time point, together with the highest expression of *HAS2* and *AREG* in the COC in this group. Furthermore, CCR2 and MCP1 immunoreactivity was observed in the cumulus cells and the oocyte of the feline COC. Remarkably, this is the first report in any species describing a direct effect of the CCR2/MCP1 system within the COC, in response to a stimulus with recombinant MCP1. In this regard, MCP1 treatment provoked a significant increase in the mRNA levels of all the key genes involved in the ovulatory cascade assessed (*HAS2*, *AREG*, *TSG6*, *PTX3*, and *GDF9*), as well as in its own receptor *CCR2*.

As expected, *AREG* mRNA expression significantly increased in the follicle wall in response to an LH treatment after 6 h in culture. Similarly, the highest expression of *AREG* within the COC was observed at the same time point in the LH group. Local factors produced within the follicle in response to the mid-cycle gonadotropin surge are critical intermediates in the regulation of C-OE [7]. Park et al. demonstrated that LH stimulation induced the transient and sequential expression of the EGF family members AREG, EREG, together with BTC, and that these EGF-related growth factors were paracrine mediators that propagate the LH signal throughout the follicle to promote periovulatory events in both oocytes and COC [24]. Indeed, EGF-related factors mediate gonadotrophin action through the induction of steroid and prostaglandin production [25, 26]. In primates, AREG is also induced in the follicular fluid of rhesus macaque preovulatory follicles by an ovulatory stimulus, and this EGF-like family member enhances primate oocyte in vitro maturation [27]. In addition, the highest expression of *HAS2* within the COC, together with *AREG*, was observed 6 h after LH treatment. As mentioned before, the amount of HA synthesized through the induction of *HAS2* is closely correlated with the degree of C-OE [2, 5, 6]. The C-OE outcomes from this study showed that the proportion of expanded COCs, retrieved from healthy antral follicles treated with LH, increased after the observed rise for *HAS2*, in comparison to their controls. Levels of all key genes in the ovulatory cascade

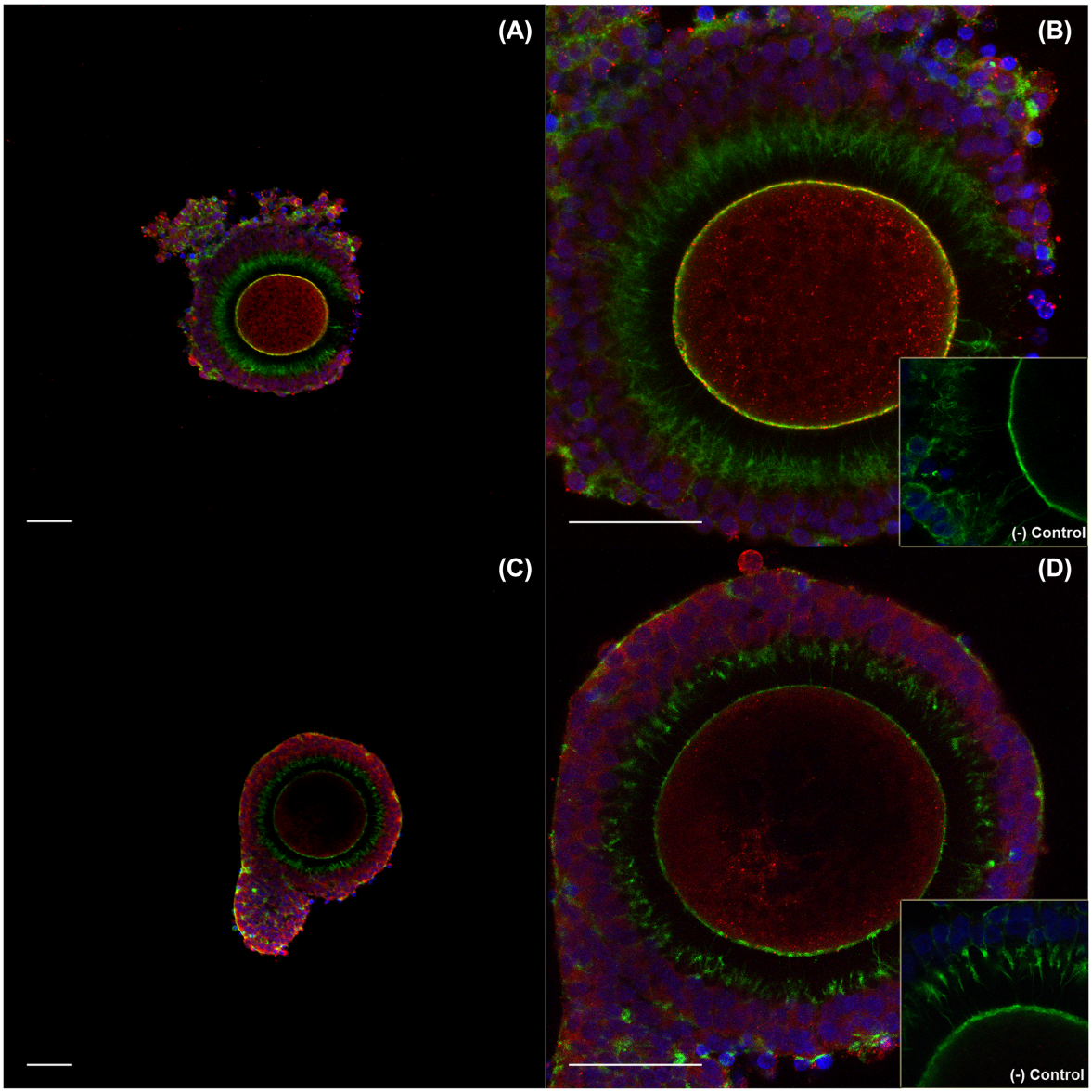

Figure 4. Representative confocal microscopy images ×20 (A, C) and ×60 (B, D) of feline COCs after inmunofluorescensce [blue = Hoechst, green = F-actin, red = CCR2 (A, B) or MCP1 (C, D)]. No staining was observed in the negative control (inserts). The chemokine receptor CCR2 and its principal ligand (MCP-1) immunostaining was observed in the oocyte and the cumulus cells. Bar represents 50 μm.

examined at 12 and 24 h were frequently the lowest in the COC and/or follicle wall suggesting that the action of LH is quite rapid. Additionally, data from 36 h time point showed the most variable results in most of the analyzed genes, probably displaying the differences in capability among the antral follicles to respond to an LH stimulus to ultimately ovulation occurs.

Using this culture system, we evaluated and distinguished the mRNA expression of the chemokine *CCR2* receptor and its ligands (*MCP-1, MCP-2, MCP-3,* and *MCP-4*) within the COC and follicle wall. Our observations are in agreement with data published by other authors demonstrating the expression of immune function genes within follicle cells in different species. For example, Richards and colleagues demonstrated that cumulus cells express chemokine C-X-C motif receptor 4 (*Cxcr4)* mRNA and protein, among others, in the cumulus cells of expanded mice COCs as well as in the granulosa cells [28]. In bovines, the mRNA expression of several chemokines, including *MCP1* and *MCP2*, increased in granulosa and/or theca cells during antral follicle development [9]. Moreover, several chemokine receptors (*CCR1, CXCR3, CCR5,* and *CXCR6*) were also expressed in the granulosa and/or theca cells, suggesting an autocrine/paracrine role of chemokines in these two cell types. Interestingly, in the current study LH treatment significantly increased the mRNA expression of the chemokine receptor *CCR2* in COCs after 6 h in culture compared to controls. This increase occurs at the same time point where the highest expression of *AREG* and *HAS2* in the COC was observed after LH treatment. Although LH treatment seemed to also stimulate *MCP1* and *MCP3* mRNA within the COC after 6 h in culture, comparable to *CCR2* results, it did not reach significance probably due to high variability in the mRNA expression of these chemokines. In other species, *Mcp1* mRNA levels were shown to be upregulated in the rat preovulatory follicle [10], and its protein levels increased in the follicular fluid by hCG treatment

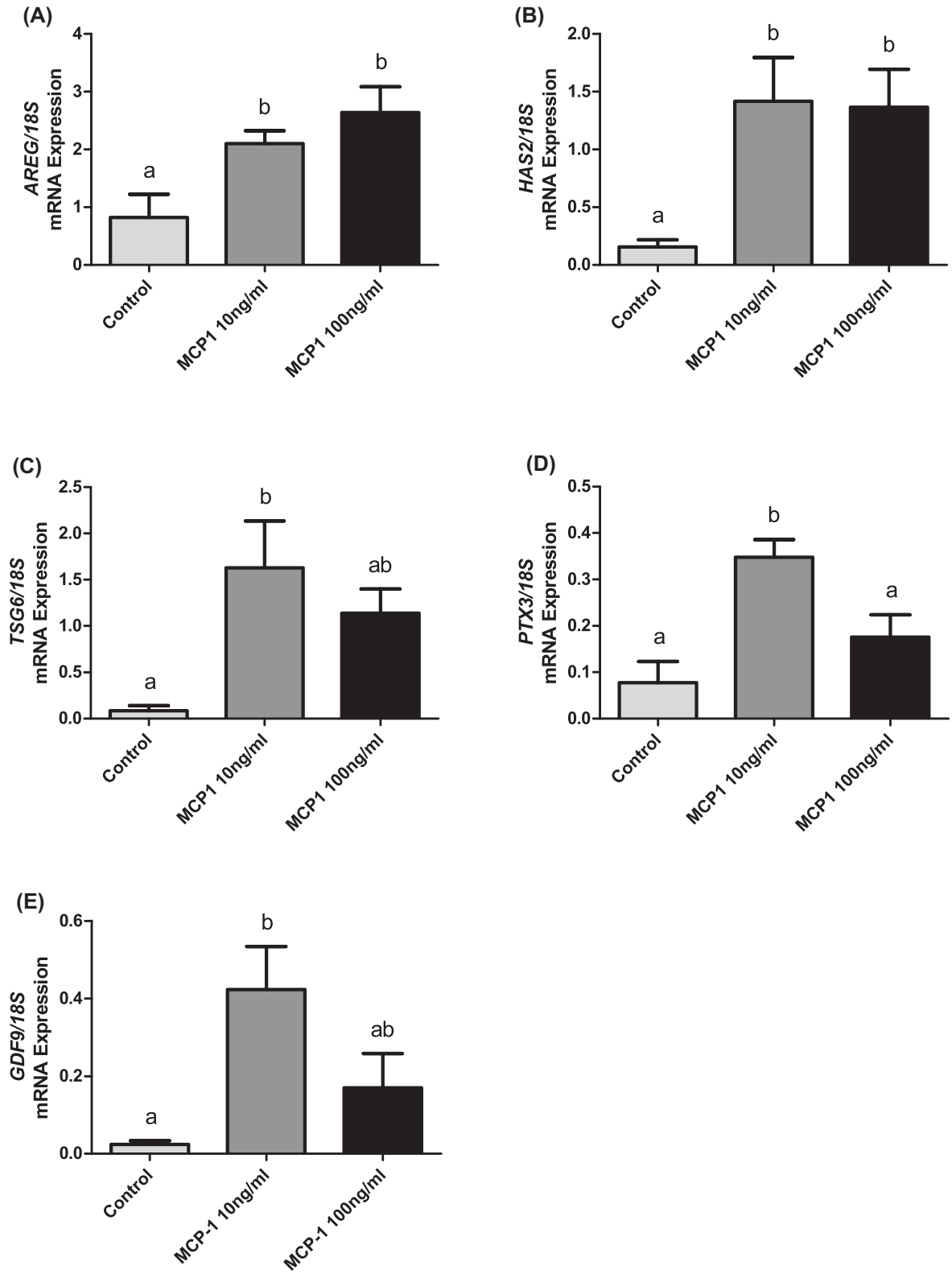

Figure 5. Normalized *AREG* (A), *HAS2* (B), *TSG6* (C), *PTX3* (D), and *GDF9* (E) mRNA expression (mean ± SEM) within the COC after 3 h in culture in the absence (control) or presence of MCP1 (10 or 100 ng/ml). Different letters represent significant differences between groups ($P < 0.05$).

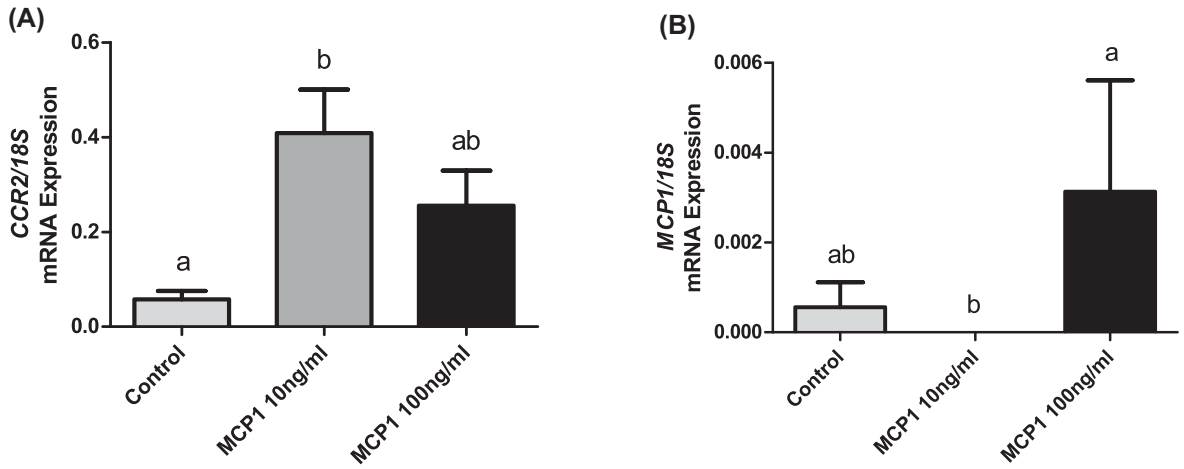

Figure 6. Normalized *CCR2* (A) and *MCP1* (B) mRNA expression (mean ± SEM) within the COC after 3 h in culture in the absence (control) or presence of MCP1 (10 or 100 ng/ml). Different letters represent significant differences between groups ($P < 0.05$).

of women during IVF cycles [29]. Also, it has been reported that the ovulatory LH surge increases the mRNA and protein levels of another chemokine receptor (*CXCR4*) in mice ovarian follicles [28].

CCR2 and its main ligand MCP1 were visualized within the feline COC by immunofluorescence, obtaining a similar immunostaining pattern for both proteins. Specific staining was observed in the oocyte and cumulus cells in all samples analyzed, suggesting a direct effect of MCP-1 on these two different cell types. Recently, we have reported similar results for the receptor CXCR4 and its chemokine ligand SDF1, showing immunolocalization in the oocyte and cumulus cells of the feline COC from antral follicles [17].

A direct effect of the CCR2/MCP1 system within the feline COC was observed in vitro. Whereas, a significant increase was observed in the mRNA levels of all the genes assessed involved in the ovulatory cascade (*HAS2, AREG, TSG6, PTX3,* and *GDF9*), as well as in its own receptor *CCR2* in response to a stimulus with recombinant MCP1 in vitro. But, what could be the direct effect of the CCR2/MCP1 system in the COC? One possibility would be related with the capacity of the COC to migrate. Akison et al. have demonstrated that cumulus cells were able to migrate, in contrast to granulosa cells, and proposed that the expanded COC transition to an adhesive, motile, and invasive phenotype in the periovulatory period may be required for successful release of the oocyte from the ovary at ovulation [30]. Furthermore, it was proposed that cumulus cells were multipotential and appear to function uniquely to ensure that the oocyte is released within a stable protective environment [28]. Since immune cells have been reported present in the ovulated COCs, the idea that these cells can be responsible for these results, or part of them, cannot be fully discarded. However, the number of immune cells relative to the abundance of cumulus cells is remarkably small [31, 32], and according to our IF results, it was well defined that the immunostaining for CCR2 and MCP1 was localized in the cumulus cells and oocyte. Since MCP1 stimulated the expression of several genes involved in C-OE and/or oocyte quality and maturation, the CCR2/MCP1 system can also be implicated in such processes and further studies are warranted to investigate this. Supplemental Figure S7 illustrates the results of this study together with known LH-inducible pathways and "possible" interactions/crosstalk.

It is important to emphasize that chemokines, like MCP1, are frequently elevated in these disorders with a state of chronic low-grade inflammation; including obesity, metabolic syndrome, type 2 diabetes and polycystic ovary syndrome (PCOS). Being PCOS, the most common endocrinopathy of women and it is also closely linked to type 2 diabetes and cardiovascular disease. Elevation of proinflammatory genes including *Mcp1* was observed in ovaries from obese mice [33]. Interestingly, immunostaining of CCR2 was highly observed in several nonimmune cell types (oocyte, theca, granulosa, stromal, luteal, and cumulus cells) in the feline ovaries during the natural estrous cycle [34, 35], suggesting possible roles for this chemokine in the ovary that extends beyond its capacity to serve as a chemoattractant. Consequently, further studies are warranted to assess the physiological processes triggered by the CCR2/MCP1 system in the ovary.

In summary, besides contributing to the knowledge of feline reproduction, the present results further support the value of feline antral follicle and the COC culture systems as good models to study periovulatory events in vitro, providing an excellent surrogate for understanding events involving human COCs that are necessary for fertility. Additionally, the data obtained from the present study indicate, for the first time in any species, a direct effect of the CCR2/MCP1 system in the COC in vitro. The stimulation of the COC with recombinant MCP1 significantly increased the mRNA levels of all the genes assessed involved in the ovulatory cascade (*HAS2, AREG, TSG6, PTX3,* and *GDF9*) and its own receptor *CCR2*. Thus, these studies demonstrate that isolated feline COCs directly respond to MCP1. Together, these data suggest that CCR2 receptor signaling in the COC may regulate events critical for promoting C-OE and/or oocyte maturation, processes that are necessary for ovulation and/or luteinization. A better understanding of this novel and direct role of CCR2 within the COC could eventually aid in the diagnosis or treatment of infertility related to metabolic disorders, for example, or may also identify novel targets for a nonhormonal form of contraception.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Normalized *AREG* (a) and *HAS2* (b) mRNA expression (mean ± SEM) within the COC after 3 h in culture in the presence of MCP1 (10 or 100 ng/ml) with or without

the highly selective CCR2 chemokine receptor antagonist (1 μM, RS 504393, Tocris Biosciences). Different letters represent significant differences between groups ($P < 0.05$).

Supplemental Figure S2. Normalized TSG6 (a), *PTX3* (b), and *GDF9* (c) mRNA expression (mean ± SEM) within the follicle wall at different time points (6, 12, 24, and 36 h) in culture in the absence (control, gray bars) or presence of LH (LH, black bars) assessed by quantitative real-time PCR. 18S rRNA served as the invariant control for normalization. Two-way ANOVA analyses showed no significant effects ($P > 0.05$) by either time or treatment.

Supplemental Figure S3. Normalized TSG6 (a), *PTX3* (b), and *GDF9* (c) mRNA expression (mean ± SEM) within the COC at different time points (6, 12, 24, and 36 h) in culture in the absence (control, gray bars) or presence of LH (LH, black bars) assessed by quantitative real-time PCR. 18S rRNA served as the invariant control for normalization. Two-way ANOVA analyses showed significant effects ($P < 0.05$) by time on some of the mRNA expression. No significant changes ($P > 0.05$) of *TSG6* mRNA expression were observed regardless of treatment or time ($P > 0.05$). *PTX3* showed significant differences in time and treatment (a: LH6 vs. LH36 and C36 vs. LH36). However, for *PTX3* results since the interaction was statistically significant, the *P* values that follow for the time and treatment effects are difficult to interpret.

Supplemental Figure S4. Normalized *AREG* (a), *HAS2* (b), *TSG6* (c), *PTX3* (d), and *GDF9* (e) mRNA expression (mean ± SEM) within the COC after 12 h in culture in the absence (control) or presence of MCP1 (10 or 100 ng/ml). No significant differences at 12 h in culture were observed ($P > 0.05$).

Supplemental Figure S5. Normalized *AREG* (a), *HAS2* (b), *TSG6* (c), *PTX3* (d), and *GDF9* (e) mRNA expression (mean ± SEM) within the COC after 24 h in culture in the absence (control) or presence of MCP1 (10 or 100 ng/ml). No significant differences at 24 h in culture were observed ($P > 0.05$).

Supplemental Figure S6. Normalized *CCR2* (a and c) and *MCP1* (b and d) mRNA expression (mean ± SEM) within the COC after 12 and 24 h in culture in the absence (control) or presence of MCP1 (10 or 100 ng/ml). Different letters represent significant differences between groups ($P < 0.05$).

Supplemental Figure S7. CCR2/MCP1 signaling within the feline COC. MCP1 and CCR2 mRNA levels increase significantly following LH treatment. MCP1 significantly increases the mRNA levels of genes that are involved in ovulation/cumulus–oocyte expansion (AREG, GDF9, PTX3, TSG6, and HAS2), as well as its own receptor (CCR2), suggesting that the MCP1-CCR2 system might regulate key events within the ovulatory cascade (i.e. CO-E, oocyte maturation, etc.) that are necessary for fertility. Black arrows represent known LH-inducible pathways; green arrows show the positive effect of CCR signaling observed in the present study, and red arrows represent "possible" interactions/crosstalk.

Supplemental Table S1. Q-PCR MGB probe sequences and forward and reverse primers.

Supplemental Table S2. Antibodies used for immunofluorescence.

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References

1. McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 2004; **19**:2869–2874.
2. Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev* 1993; **34**:87–93.
3. Hess KA, Chen L, Larsen WJ. Inter-alpha-inhibitor binding to hyaluronan in the cumulus extracellular matrix is required for optimal ovulation and development of mouse oocytes. *Biol Reprod* 1999; **61**:436–443.
4. Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P, Salvatori G, Mahoney DJ et al. PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. *Development* 2004; **131**:1577–1586.
5. Fulop C, Salustri A, Hascall VC. Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex. *Arch Biochem Biophys* 1997; **337**:261–266.
6. Kimura N, Konno Y, Miyoshi K, Matsumoto H, Sato E. Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. *Biol Reprod* 2002; **66**:707–717.
7. Richards JS. Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol* 2005; **234**:75–79.
8. Baggiolini M, Dewald B, Moser B. lnterleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv Immunol* 1993; **55**:97–179.
9. Skinner MK, Schmidt M, Savenkova MI, Sadler-Riggleman I, Nilsson EE. Regulation of granulosa and theca cell transcriptomes during ovarian antral follicle development. *Mol Reprod Dev* 2008; **75**:1457–1472.
10. Wong KH, Negishi H, Adashi EY. Expression, hormonal regulation, and cyclic variation of chemokines in the rat ovary: key determinants of the intraovarian residence of representatives of the white blood cell series. *Endocrinology* 2002; **143**:784–791.
11. Tamba S, Yodoi R, Segi-Nishida E, Ichikawa A, Narumiya S, Sugimoto Y. Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization. *Proc Natl Acad Sci USA* 2008; **105**:14539–14544.
12. Yodoi R, Tamba S, Morimoto K, Segi-Nishida E, Nishihara M, Ichikawa A, Narumiya S, Sugimoto Y. RhoA/Rho kinase signaling in the cumulus mediates extracellular matrix assembly. *Endocrinology* 2009; **150**:3345–3352.
13. Wildt DE, Comizzoli P, Pukazhenthi B, Songsasen N. Lessons from biodiversity-the value of nontraditional species to advance reproductive science, conservation, and human health. *Mol Reprod Dev* 2010; **77**:397–409.
14. Combelles CM, Cekleniak NA, Racowsky C, Albertini DF. Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. *Hum Reprod* 2002; **17**:1006–1016.
15. Comizzoli P, Wildt DE, Pukazhenthi BS. Impact of anisosmotic conditions on structural and functional integrity of cumulus-oocyte complexes at the germinal vesicle stage in the domestic cat. *Mol Reprod Dev* 2008; **75**:345–354.
16. Rojo JL, Linari M, Musse MP, Peluffo MC. Felis catus ovary as a model to study follicle biology in vitro. *J Assist Reprod Genet* 2015; **32**:1105–1111.
17. Rojo JL, Linari M, Young KA, Peluffo MC. Stromal-derived factor 1 directly promotes genes expressed within the ovulatory cascade in fe-

line cumulus oocyte complexes. *J Assist Reprod Genet* 2018; **35**:785–792.
18. Peluffo MC, Hennebold JD, Stouffer RL, Zelinski MB. Oocyte maturation and in vitro hormone production in small antral follicles (SAFs) isolated from rhesus monkeys. *J Assist Reprod Genet* 2013; **30**:353–359.
19. Goodrowe KL, Wall RJ, O'Brien SJ, Schmidt PM, Wildt DE. Developmental competence of domestic cat follicular oocytes after fertilization in vitro. *Biol Reprod* 1988; **39**:355–372.
20. Peluffo MC, Young KA, Stouffer RL. Dynamic expression of caspase-2, -3, -8, and -9 proteins and enzyme activity, but not messenger ribonucleic acid, in the monkey corpus luteum during the menstrual cycle. *J Clin Endocrinol Metab* 2005; **90**:2327–2335.
21. Peluffo MC, Young KA, Hennebold JD, Stouffer RL. Expression and regulation of tumor necrosis factor (TNF) and TNF-receptor family members in the macaque corpus luteum during the menstrual cycle. *Mol Reprod Dev* 2009; **76**:367–378.
22. Peluffo MC, Murphy MJ, Baughman ST, Stouffer RL, Hennebold JD. Systematic analysis of protease gene expression in the rhesus macaque ovulatory follicle: metalloproteinase involvement in follicle rupture. *Endocrinology* 2011; **152**:3963–3974.
23. Peluffo MC, Barrett SL, Stouffer RL, Hennebold JD, Zelinski MB. Cumulus-oocyte complexes from small antral follicles during the early follicular phase of menstrual cycles in rhesus monkeys yield oocytes that reinitiate meiosis and fertilize in vitro. *Biol Reprod* 2010; **83**:525–532.
24. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 2004; **303**:682–684.
25. Jamnongjit M, Hammes SR. Oocyte maturation: the coming of age of a germ cell. *Semin Reprod Med* 2005; **23**:234–241.
26. Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Mol Endocrinol* 2006; **20**:1352–1365.
27. Peluffo MC, Ting AY, Zamah AM, Conti M, Stouffer RL, Zelinski MB, Hennebold JD. Amphiregulin promotes the maturation of oocytes isolated from the small antral follicles of the rhesus macaque. *Hum Reprod* 2012; **27**:2430–2437.
28. Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Mol Endocrinol* 2006; **20**:1300–1321.
29. Arici A, Oral E, Bukulmez O, Buradagunta S, Bahtiyar O, Jones EE. Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells. *J Reprod Immunol* 1997; **32**:201–219.
30. Akison LK, Alvino ER, Dunning KR, Robker RL, Russell DL. Transient invasive migration in mouse cumulus oocyte complexes induced at ovulation by luteinizing hormone. *Biol Reprod* 2012; **86**:125.
31. Piccinni MP, Scaletti C, Mavilia C, Lazzeri E, Romagnani P, Natali I, Pellegrini S, Livi C, Romagnani S, Maggi E. Production of IL-4 and leukemia inhibitory factor by T cells of thecumulus oophorus: a favorable microenvironment for pre-implantation embryo development. *Eur J Immunol* 2001; **31**:2431–2437.
32. Akkoyunlu G, Korgun ET, Çelik-Özenci Ç, Seval Y, Demir R, Üstünel . Distribution patterns of leucocyte subpopulations expressing different cell markers in the cumulus–oocyte complexes of pregnant and pseudopregnant mice. *Reprod Fertil Dev* 2003; **15**:389–395.
33. Ruebel M, Shankar K, Gaddy D, Lindsey F, Badger T, Andres A. Maternal obesity is associated with ovarian inflammation and upregulation of early growth response factor 1. *Am J Physiol Endocrinol Metab* 2016; **311**:E269–E277.
34. Linari M, Rojo JL, Dascal E, Venara MC, Peluffo MC. CCR2 expression and Its ligands in the feline ovary during the natural estrous cycle. SAB. Chascomus, Buenos Aires, Argentina: 2014.
35. Linari M, Rojo JL, Murphy MJ, Hennebold JD, Jaworski JP, Peluffo MC. MCP1 in the cat ovary. 49th Annual Meeting of the Society for the Study of Reproduction (SSR). San Diego, CA, EE.UU.: 2016.