Regulated C-C motif ligand 2 (CCL2) in luteal cells contributes to macrophage infiltration into the human corpus luteum during luteolysis

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Running title: Regulated *CCL2* expression in the regressing human CL

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ABSTRACT:

Intense macrophage infiltration is observed during luteolysis in various animals including women; however, we still do not know how macrophage infiltration into the human corpus luteum (CL) during luteolysis is regulated. In this study, we examined the expression, localization, and regulation of an important chemokine for the recruitment of monocyte/macrophage lineages, C-C motif ligand 2 (CCL2), in the human CL across the luteal phase and in cultured human luteinized granulosa cells (LGCs) with special reference to the number of infiltrating macrophages and luteal cell function. *CCL2* mRNA increased in the non-functional regressing CL during menstruation ($P<0.01$), corresponding to an elevated mRNA expression of a macrophage-derived cytokine, *TNF*, and an increased number of infiltrating macrophages positively stained with a macrophage marker, CD68. CCL2 protein was immunohistochemically localized to the cytoplasm of granulosa-lutein and theca-lutein cells, and *CCL2* mRNA was significantly reduced by hCG both *in vivo* ($P<0.05$) and *in vitro* ($P<0.01$). *CCL2* was also down-regulated by luteotrophic prostaglandin (PG) E ($P<0.0001$), but up-regulated by luteolytic PGF ($P<0.05$) *in vitro*. Administration of TNF significantly enhanced the *CCL2* mRNA expression in cultured LGCs ($P<0.01$). More abundant infiltrating macrophages were found around granulosa-lutein cells lacking 3β-HSD or PGE synthase (PGES) immunostaining. *CCL2* mRNA expression was negatively correlated with both *HSD3B1* and *PGES*, suggesting that locally produced progesterone and PGE suppress macrophage infiltration into the CL. Taken together, the infiltration of macrophages in the human CL is regulated by endocrine and paracrine molecules via regulation of the *CCL2* expression in luteal cells.

Key words: macrophage / CCL2 / human corpus luteum / hCG / PGE / progesterone

Introduction

The corpus luteum (CL) is a temporary endocrine gland formed from the ovarian follicle following ovulation that produces large amounts of progesterone essential for the establishment and maintenance of early pregnancy. During a non-fertile cycle in women, the CL matures structurally and functionally by the mid-luteal phase and then begins to regress in a process called luteolysis. On the other hand, if conception succeeds, the CL is rescued from luteolysis and maintains its function during early pregnancy. Although the molecular mechanisms of luteolysis and luteal rescue are not yet fully understood, human chorionic gonadotropin (hCG) secreted from the developing conceptus is known to be a key regulator of luteal rescue by preventing the CL from undergoing luteolysis (Duncan *et al.*, 1998a).

Invasion of macrophages into the CL is a well-known phenomenon of luteal regression in a variety of mammals, and there is convincing evidence that the number of invading macrophages increases as luteolysis proceeds (Hume et al., 1984; Bagavandoss *et al.*, 1988; Lei *et al.*, 1991; Brännström *et al.*, 1994; Best *et al.*, 1996; Takaya *et al.*, 1997; Penny *et al.*, 1999; Komatsu *et al.*, 2003). Infiltrating macrophages into the regressing CL is likely to be involved in phagocytosis of dying luteal cells and their remnants, and in the production of luteolytic cytokines such as tumor necrosis factor α (TNF), oxygen radicals, or substances that inhibit steroidogenesis (Pate and Keyes, 2001; Shirasuna *et al.*, 2012). In most tissues, macrophages are recruited as monocytes from the systemic circulation and subsequently undergo a process of differentiation and activation for phagocytosis. The monocyte recruitment is facilitated by the coordinated intercellular adhesive interactions and the local elaboration of leukocyte-specific chemotactic factors. C-C motif ligand 2 (CCL2), also known as

72 monocyte chemoattractant protein-1 (MCP-1), is a chemokine that plays a role in the
73 recruitment of monocytes and macrophages. An increased CCL2 expression has been
74 shown in the regressing CL of rats (Townson *et al.*, 1996), cows (Penny *et al.*, 1999
75 Townson *et al.*, 2002), pigs (Hosang *et al.*, 1994), rabbits (Krusche *et al.*, 2002), and
76 women (Senturk *et al.*, 1999). CCL2 has been reported to be produced by a variety of
77 cell types including endothelial cells, fibroblasts, monocytes, and T lymphocytes
78 (Yoshimura and Leonard, 1990). However, the production and regulation of CCL2 and
79 cell types expressing it in the human CL remains to be elucidated.

80 HCG can bind to its receptor, lutrophin-choriogonadotrophic hormone receptor
81 (LHCGR) expressed on luteal cells, and activation of this receptor promotes the
82 production of progesterone and luteotrophic prostaglandin (PG) E. Since we previously
83 reported that hCG prevents macrophage infiltration into the human CL (Duncan *et al.*,
84 1998b), we hypothesized that hCG controls macrophage infiltration by down-regulating
85 CCL2 expression in the human CL. However, we still do not know how CCL2
86 expression is regulated in the human CL. In this study, we analyzed the change in the
87 mRNA expression of *CCL2* in the human CL across the luteal phase, with reference to
88 the number of infiltration of macrophages and the function of luteal cells, especially
89 focused on the ability for progesterone and PGE synthesis. We also examined the
90 regulation of *CCL2* expression by endocrine and paracrine molecules which regulate
91 luteal function, using cultured human luteinized granulosa cells (LGCs).
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Materials and Methods

Ethics

All experiments using human tissues and cells were approved by the Lothian Medical Research Ethics Committee and Hokkaido University Research Ethics Committee, and all women gave informed consent.

Reagents

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X was obtained from Life Technologies Inc. (Carlsbad, CA, USA). Matrigel is a product of BD Biosciences (San Jose, CA, USA).

Collection of the human CL

Human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions. The menstrual stages of the CLs were determined by endometrial morphology and by the concentration of LH in daily urine samples, obtained prior to surgery as reported previously (Dickinson *et al.*, 2008). The collected CLs were halved upon collection; one half was snap-frozen and stored at −80°C, and the other half was fixed with 10% formalin and processed into paraffin embedding. Some women were given daily doubling injections of hCG (Profasi®; Serono Laboratories, Welwyn Garden City, UK), starting at 125 IU from 7 days after the LH surge for 5–8 days until surgery. This regimen has been shown to rescue the CL and mimic the hormonal changes of early pregnancy (Dickinson *et al.*, 2008). Only frozen tissues of the rescued CL were available from hCG-administrated women.

Human LGC culture

Follicular aspirates were obtained from patients undergoing assisted conception. Oocytes were removed and the remaining fluid was centrifuged at 1,800 rpm for 10 min, and cells were re-suspended in a culture medium (DMEM/Ham's F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). Cell suspension was layered over 45% Percoll in culture medium mixture, and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed three times with Dulbecco's phosphate buffered saline supplemented by $MgCl_2$ and $CaCl_2$.

One hundred-thousand viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of the culture medium at 37°C in 5% CO_2 in air. The culture medium was changed every two days, and cells were treated on day 6 of culture for 24 hours with either hCG (100 ng/mL), PGE (10 ng/mL), TNF (10 ng/mL), PI3-kinase inhibitor (LY294002; 20 μM, Cell Signaling Technology Inc., Danvers, MA, USA), Protein kinase C (PKC) activator (Phorbol 12-myristate 13-acetate: PMA; 10 ng/mL, Merck Millipore), or cAMP/Protein kinase A (PKA) inhibitor (H89; 10 μM, Merck Millipore, Nottingham). Other cells were treated with TNF (10 ng/mL) on culture day 2 or day 10, and PGF (10 ng/mL) on culture day 10 for 24 hours. For hCG withdrawal experiment, LGCs were treated with hCG (1 ng/mL) from the next day of plating for continuous 6 days, changed to the media without hCG on day 7, and collected daily until day 11. Each experiment was performed in duplicate and repeated at least three times.

144 *Quantitative RT-PCR (qRT-PCR)*

145 The CLs used for a quantitative gene expression analysis were classified as early-luteal
146 (1–5 days after the LH surge, n=4), mid-luteal (6–10 days, n=6), late-luteal (11–14 days,
147 n=6), menstrual phase (over 14 days, n=3), and rescued by *in vivo* hCG injection (n=4).
148 Total RNA was extracted from the frozen human CLs or cultured LGCs using RNeasy
149 Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer's protocol. RNA (200
150 ng) was used to prepare cDNA using the TaqMan Reverse Transcription regents
151 (Applied Biosystems, Foster City, CA, USA).

152 Primers used for qRT-PCR analysis are listed in Table 1. Primers were
153 pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each
154 reaction buffer contained 5.0 μL 2×PowerSYBR® Green PCR Master Mix (Applied
155 Biosystems), 0.5 μL primer pair (5 μM), 3.5 μL of nuclease free H_2O, and 1.0 μL cDNA,
156 and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted
157 of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec
158 and 60°C for 1 min repeated for 40 cycles), and a dissociation step (95°C, 60°C, and
159 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems).
160 The relative expression levels of each target to the housekeeping gene
161 (*glucose-6-phosphate dehydrogenase*: *G6PDH*), previously validated using geNorm
162 analysis (Primerdesign Ltd, Southampton, UK), were quantified using the $^{\Delta}$Ct or $^{\Delta\Delta}$Ct
163 methods.

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165 *Immunohistochemistry*

166 Fixed human CLs at the mid-luteal (n=4), late-luteal (n=3), and menstrual phases (n=3)
167 were used for an immunohistochemical analysis. The tissues were dehydrated through

ethanol and embedded into paraffin according to the conventional method. Tissue sections (5 μm thick) were de-waxed and washed twice in distilled water, then antigen retrieval was performed for 1 minutes in 0.01 M citrate buffer (pH 6.0) using a pressure cooker. After washing twice in phosphate-buffered saline (PBS), the sections were incubated with 3% hydrogen peroxide for 20 min followed by Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each. After pretreatment with normal goat serum for 60 min at room temperature, they were incubated with mouse anti-human CD68 (1:100; PG-M1, Dako UK Ltd., Cambridgeshire, UK), mouse anti-human CCL2 (1:100; MCA2486, AbD serotec, Raleigh, NC, USA), rabbit anti-3β-HSD (1:1,000; kind gift from Prof. Ian Mason, The University of Edinburgh), or rabbit anti-human prostaglandin E synthase (PGES; 1:250; 160140, Cayman Chemical, Ann Arbor, MI) in goat serum at 4°C overnight. Control sections were incubated with non-immune serum and the disappearance of immunoreaction was confirmed. The specificity of the immunoreaction for CCL2 was confirmed using CCL2-transfected HEK293 cells (Supplementary Figure 1).

After washing twice in PBS, the sections were incubated with biotinylated anti-mouse or anti-rabbit IgG (1:500; Vector laboratories Inc.) for 60 min at room temperature. The reaction sites were visualized using a Vectastain ABC Elite kit (Vector Laboratories Inc.) for 60 min followed by ImmPACT^{TM} DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 1 min. The sections were counterstained with haematoxylin and observed under a light microscope (BX51; Olympus corporation, Tokyo, Japan). Photographs taken from the CD68-immunostained sections were analysed in Image J (http://imagej.nih.gov/ij/) and the number of CD68-positive cells in the CL parenchyma was calculated.

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193 *Dual staining for macrophages and apoptotic cells*

194 Some sections after the reaction with anti-CD68 antibody overnight were washed with
195 PBS and incubated with AlexaFluor 594-labeled anti-mouse IgG (1:200; Life
196 technologies Japan, Tokyo, Japan) for 2 hours room temperature. Apoptotic cells were
197 detected in these sections using ApopTag Fluorescein *in situ* apoptosis detection kit
198 (Merck Millipore, Nottingham, UK) according to the manufacturer's direction.
199 Stained sections were observed under a confocal laser scanning microscope (FV300;
200 Olympus, Tokyo, Japan).

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202 *Statistical analyses*

203 After testing for normality, all statistical analyses were performed using unpaired *t*-tests
204 or one-way ANOVA with pairwise comparison using GraphPad Prism 6 software
205 (GraphPad Software Inc., San Diego, CA, USA), and $P<0.05$ was regarded as
206 significant. Values in the graphs represent mean ± SEM.

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Results

Macrophage increases in number during luteal regression

Macrophages infiltrating into the human CL were identified by immunohistochemistry for a human macrophage marker, CD68. In the functional CL at the mid-luteal phase, CD68-positive infiltrating macrophages tended to localize to the border between granulosa-lutein cells and theca-lutein cells at the periphery of the CL, and scattered within the granulosa-lutein cell layer (Fig. 1A). In the CL at the late-luteal phase, abundant CD68-positive macrophages accumulated around the border between granulosa-lutein cells and theca-lutein cells, and were more dispersed within the granulosa-lutein cell layer (Fig. 1B). The regressing CL collected during menstruation contained much more CD68-positive macrophages throughout the CL parenchyma, especially in the granulosa-lutein cell layer (Fig. 1C); this increase was significant when CD68-positive cells were counted in the sections ($P<0.05$; Fig. 1D). These data would suggest stimulated macrophage chemotaxis into the CL parenchyma as luteolysis progresses.

The chemokine CCL2 increased during luteolysis

We next analyzed the localization and expression of a monocyte/macrophage chemokine, C-C motif ligand 2 (CCL2). CCL2 protein was localized to the cytoplasm of both granulosa-lutein and theca-lutein cells (Fig. 1E) and vascular endothelial cells were also weakly immunoreactive for CCL2 (arrow in Fig. 1E). In contrast, non-steroidogenic cells distributed in the parenchyma of the CL were negative for CCL2 (asterisks in Fig. 1E). The staining pattern and intensity of CCL2 protein did not alter during menstrual phase. On the other hand, the mRNA expression of *CCL2* in the

human CL was particularly conspicuous in the regressing CL collected after menstruation had commenced ($P<0.01$; Fig. 1F) where abundant infiltrating macrophages were observed (Fig. 1C, D). Luteal rescue using exogenous hCG *in vivo* to mimic the changes of early pregnancy significantly decreased the expression of *CCL2* when compared to the late-luteal phase in the absence of hCG ($P<0.05$; Fig. 1G).

CCL2 is regulated by hCG and prostaglandins in luteal steroidogenic cells in vitro

Cultured human luteinized granulosa cells (LGCs) also expressed *CCL2*. *In vitro* treatment of hCG decreased the mRNA abundance of *CCL2* ($P<0.01$; Fig. 2A) and the withdrawal of hCG promoted its expression ($P<0.01$; Fig. 2B). Luteotrophic PGE also suppressed the *CCL2* expression ($P<0.0001$; Fig. 2C) while luteolytic PGF enhanced its expression in LGCs ($P<0.05$; Fig. 2D).

TNF expression correlates with CCL2 expression

TNF is a macrophage cytokine and its mRNA expression in the human CL peaked in the regressing CL collected during menstruation ($P<0.01$; Fig. 2E). The expression of *TNF* was highly correlated with *CCL2* expression in the human CL ($r=0.9774$, $P<0.0001$; Fig. 2F). Like CCL2, *in vivo* hCG treatment to rescue the CL from luteolysis reduced the luteal *TNF* expression ($P<0.05$; Fig. 2G). In cultured LGCs, TNF significantly increased the expression of *CCL2* ($P<0.01$; Fig. 2H).

Signal transduction cascades responsible to regulate CCL2 in LGCs

HCG binds to LHCGR on luteal cells and is known to activate cAMP/PKA pathway. Among four types of PGE receptors (EP1-4), EP2 is predominantly expressed in LGCs

264 and the binding of PGE to EP2 activates cAMP/PKA pathway. The receptor for PGF
265 acts through PKC pathway. There are two types of TNF receptors, TNFR1 and TNFR2,
266 and the latter, which activates PI3 kinase pathway, is predominantly expressed in LGCs.
267 To reveal the signal transduction cascades responsible to the expression of *CCL2* in
268 LGCs, we treated cells with inhibitors for PI3 kinase (LY294002) and PKA (H89) or an
269 activators for PKC (PMA). As expected, PMA enhanced and LY294002 suppressed the
270 expression of *CCL2* in LGCs ($P<0.05$ and $P<0.0001$; Fig. 2I and J), suggesting an
271 involvement of PKC or PI3 kinase pathways in the regulation of *CCL2* expression.
272 Unexpectedly, H89 treatment significantly reduced the expression of *CCL2* in LGCs
273 ($P<0.01$; Fig. 2K).
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275 *Macrophage density correlates to steroidogenic cell function*
276 Light microscopic observation revealed that the degree of macrophage accumulation in
277 the regressing CL was not even, showing clear regional variations (Fig. 3A). When
278 serial sections were stained with 3β-HSD, a key enzyme for progesterone synthesis, an
279 intimate relationship between the accumulation of infiltrating macrophages and 3β-HSD
280 immunoreactivity in granulosa-lutein cells was observed (Fig. 3A–D). As we reported
281 previously (Nio-Kobayashi *et al.*, 2014), granulosa-lutein cells in the regressing human
282 CL collected during menstruation can be classified into intact (i), pyknotic (p), and
283 degenerating (d) cells according to the staining intensity of 3β-HSD and structural
284 characteristics (Fig. 3B). Regions with intact granulosa-lutein cells contained few
285 CD68-positive macrophages (“i” in Fig. 3A, B). More abundant macrophages were seen
286 in regions with pyknotic luteal cells with condensed nuclei and fragmented cytoplasm
287 possessing 3β-HSD immunoreactivity (“p” in Fig. 3A–D). Macrophages were more

numerous in regions with 3β-HSD-negative degenerating granulosa-lutein cells ("d" in Fig. 3A–D). Dual staining for CD68-positive macrophages and apoptotic cells clearly demonstrated an accumulation of CD68-positive infiltrating macrophages in the region occupied with pyknotic (apoptotic) and degenerating granulosa-lutein cells ("p" and "d" in Fig. 4A–F) while few CD68-positive cells were located in regions with intact granulosa-lutein cells ("i" in Fig. 4A–C).

Healthy granulosa-lutein cells actively produce a luteotrophic paracrine molecule, PGE. In other set of serial sections of the human CL at late-luteal phase, the areas with marked immunostaining for the PGE synthetic enzyme (PGES) (arrow in Fig. 5A) had low concentrations of CD68-positive macrophages (arrow in Fig. 5B), while areas of the steroidogenic cell layer with many macrophages (asterisk in Fig. 5B) displayed low levels of PGES immunoreactivity (asterisk in Fig. 5A).

The qRT-PCR analysis demonstrated that *CCL2* mRNA expression in the CL tended to be inversely correlated with the expression of *PGES* ($r=-0.3899$, $P=0.0892$; Fig. 5C) as well as *HSD3B1* ($r=-0.4592$, $P<0.05$; Fig. 5D).

TNF is not associated with a loss of steroidogenic cell function

We finally investigated whether macrophage influx was a cause of a loss of steroidogenic function in granulosa-lutein cells. The addition of 10 ng/mL TNF to LGCs on culture day 6 promoted, rather than inhibited, the expression of a key regulator of steroidogenesis, *STAR* ($P<0.01$; Fig. 6A). TNF also enhanced the expression of synthetic enzymes for luteotrophic PGE, *COX2* ($P<0.0001$; Fig. 6B) and *PGES* ($P<0.01$; Fig. 6C), while reducing the key enzymes involved in luteolytic PGF synthesis, *Aldo-keto reductase family 1 member C1* (*AKR1C1*; $P=0.0503$; Fig. 6D) and *AKR1C3*

312 (P<0.01; Fig. 6E). The effect of TNF on the expression of these genes did not change
313 when cells were treated with TNF on culture day 2 or day 10 (Supplementary Figure 2),
314 suggesting that the age of luteal cells did not influence the luteotrophic action of TNF.
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Discussion

This study demonstrated an increased mRNA expression of *CCL2* in the regressing CL of women, associated with an increased number of infiltrating macrophages into the CL and a loss of progesterone and luteotrophic PGE synthesis in luteal cells. The number of infiltrating macrophages is abundant in regions consisting 3β-HSD- or PGES-negative granulosa luteal cells. The regional difference in the number of infiltrating macrophages in the human CL suggests an intimate correlation between luteal cell function and macrophage influx into the CL. Increased number of infiltrating macrophages in the human CL is associated with an increased mRNA expression of *CCL2*, an important chemokine for the recruitment of macrophages. We also revealed here the regulatory mechanism of *CCL2* expression in human luteal cells. Luteotrophic molecules, hCG and PGE, down-regulate the mRNA expression of *CCL2* in luteal cells but luteolytic PGF promoted its expression. On the other hand, a macrophage cytokine, TNF, strongly enhanced the *CCL2* expression in luteal cells possibly enhancing further macrophage influx into the CL. These data suggest that macrophage infiltration is one of the key events for human luteolysis, and CCL2 regulated by endocrine or paracrine molecules plays an important role in the regulation of macrophage infiltration into the human CL.

Macrophages are major immune cells in the CL and known to possess bimodal roles in the regulation of CL function (Wu *et al.*, 2004). It has been reported that macrophages may facilitate the establishment of the vasculature or an increase progesterone production in the functional CL. The present study confirmed that TNF acts in a luteotrophic manner by enhancing the expression of the enzymes involved in the production of progesterone and PGE, and this action did not alter depending on the age of LGCs. In contrast, Shakil and Whitehead (1994) reported that progesterone

360 secretion is markedly inhibited by co-culturing granulosa cells with peritoneal
361 macrophages. They also noted that degree of the inhibition was dependent on both the
362 number and activation status of the co-cultured macrophages. The number of
363 macrophages in the CL is known to be increased during luteolysis in various animals. In
364 the present study, we confirmed an increased number of macrophages in the regressing
365 human CL collected during menstruation, suggesting its contribution to luteolysis.
366 Although infiltrating macrophages augmented in the CL at late-luteal phase, more
367 marked accumulation is observed in the CL collected during menstruation. This
368 suggests that an accumulation of macrophages is likely important for the structural
369 luteolysis rather than the functional luteolysis. We also revealed in this study that the
370 degree of an accumulation of infiltrating macrophages was different depending on the
371 region in the regressing human CL: Few macrophages were observed in the region
372 occupied with intact granulosa-lutein cells intensely expressing 3β-HSD, whereas
373 abundant macrophages occupied the region with apoptotic or degenerating granulosa
374 luteal cells (Fig. 4G). More abundant macrophages were observed in degenerating cell
375 areas than in pyknotic/apoptotic cell areas, suggesting an intimate correlation of the
376 number of infiltrating macrophages and loss of luteal cell function. The factors that
377 regulate the number of infiltrating macrophages in the CL are therefore of particular
378 importance in the control of luteal structure and function.

379 CCL2 is a chemokine that has a potent chemoattractant effect on monocytes and
380 macrophages. An increased expression of CCL2 in the regressing CL was previously
381 reported in various animals including women (Hosang *et al.*, 1994; Townson *et al.*,
382 1996; Penny *et al.*, 1999 Senturk *et al.*, 1999; Townson *et al.*, 2002; Krusche *et al.*,
383 2002). In the human CL, Senturk *et al.* (1999) reported that the number of macrophages

and the expression of CCL2 were highest in the CL at mid-follicular phase. This largely corresponds to our qRT-PCR analysis showing an increased *CCL2* expression in the human CL collected during menstruation. Because the number of infiltrating macrophages was significantly increased in the CL at menstruation, these data strongly suggest an involvement of CCL2 in the macrophage infiltration into the CL during luteolysis.

CCL2 is produced by a variety of cell types including endothelial cells, fibroblasts, monocytes, and T lymphocytes. The cellular source of CCL2 in the CL has been reported as endothelial cells of women (Senturk *et al.* 1999) and cows (Townson *et al.* 2002), or T lymphocytes of cows (Penny *et al.* 1998) by immunohistochemistry. On the other hand, Hosang *et al.* (1994) reported the significant mRNA expression of *CCL2* in porcine luteal steroidogenic cells. Using immunohistochemistry we clearly demonstrated that CCL2 protein is localized to the cytoplasm of steroidogenic cells, namely granulosa-lutein and theca lutein cells. Although a weak immunoreactivity was found in endothelial cells, non-steroidogenic cells distributed in the CL parenchyma were negative in reaction. Because our analysis of cultured human LGCs showed significant expression of *CCL2* mRNA, it is reasonable to consider that luteal cells are major cellular source of CCL2 in the human CL.

The present study for the first time revealed the regulation of *CCL2* mRNA expression by known endocrine and paracrine factors in the human CL and cultured human LGCs. HCG significantly decreased the mRNA expression of *CCL2* in the rescued CL *in vivo* and in cultured LGCs *in vitro*. In our previous study, administration of hCG to mimic early pregnancy in women resulted in the reduction of the number of infiltrating macrophages into the CL (Duncan *et al.*, 1998b). These findings suggest that

hCG negatively regulates macrophage infiltration via down-regulating the *CCL2* expression in luteal cells. We clearly showed for the first time the suppressive effect of PGE on the expression of *CCL2* in LGCs. PGE is one of the luteotrophic molecules secreted from luteal cells by stimulation of LHCGR. In contrast, PGF, a known luteolytic molecule in various animals, significantly enhanced the *CCL2* expression in LGCs. Up-regulation of *CCL2* expression by PGF was previously reported in the CL of sheep and cows (Tsai *et al.*, 1997). We also showed that a macrophage cytokine, TNF, induced the *CCL2* expression in LGCs, suggesting a possible acceleration of infiltrating macrophages by macrophages themselves. PKC and PI3 kinase pathways, possibly activated by PGF or TNF under the physiological condition, seem to be responsible to up-regulate the expression of *CCL2* in LGCs. Although H89 is generally used as a potent PKA inhibitor, this regent is known to inhibit other signaling pathway such as Rho-associated kinase and ERK1/2 (Murray, 2008). Because ERK1/2 pathway is known to be involved in the up-regulation of CCL2, the suppressive effect of H89 on CCL2 expression may be through an inhibition of ERK1/2 pathway. Taken together, CCL2 is a possible luteolytic molecule which is negatively regulated by luteotrophic molecules but positively controlled by luteolytic molecules.

Although hCG secreted from the conceptus plays an important role in the promotion of luteal function, we still do not know how luteolysis is initiated in a non-fertile cycle. Herein we demonstrated that the number of macrophages was different depending on the region of the CL. The number of infiltrating macrophages is fewer in the regions occupied with healthy granulosa-lutein cells that actively produce progesterone and PGE, suggesting that progesterone and PGE are involved in the suppression of macrophage infiltration. This idea is supported by the previous report

showing that the progesterone negatively regulates CCL2 expression in choriodecidual cells and a breast cancer cell line T47D (Kelly *et al.* 1997), and the present data showing the suppressive effect of PGE on the expression of *CCL2* in human LGCs. As mentioned above, PGE has a luteotrophic effect to enhance the production of progesterone and PGE secretion from LGCs. This suggests that if luteal cells can maintain to produce PGE, the expression of CCL2 in luteal cells and the following macrophage infiltration into the CL could be suppressed. The capacity of the PGE synthesis in luteal cells in response to luteotrophic stimuli may be a key function to rescue the human CL from luteolysis.

In conclusion, we revealed here a possible contribution of CCL2 to macrophage infiltration into the CL during luteolysis. The expression of CCL2 in luteal cells is suppressed by luteotrophic hCG and PGE, but enhanced by luteolytic PGF and a macrophage cytokine TNF. The ability of luteal cells to produce progesterone and PGE may be important to control the local number of infiltrating macrophages. Although our *in vitro* experiments may not reflect luteal cells under the structural luteolysis which was seen *in vivo* during menstruation, CCL2 secreted from luteal cells contributes to the regulation of macrophage infiltration into the human CL.

Acknowledgements

We are grateful to Ms. Lyndsey Boswell, Dr. Fiona Connolly, and Ms. Eva Gay, The University of Edinburgh, for their excellent technical support.

Authors' roles

Study concept and designs of the experiments: JN-K and WCD, Acquisition of data: JN-K, Analysis and interpretation of the data: JN-K, Collecting follicular fluids: MK and NS, Establishment of CCL2-transfected cells: SK, Drafting of the manuscript: JN-K, WCD, and TI.

Funding

This study was supported by the Cunningham Trust to W.C.D. and the Postdoctoral Fellowship for Research Abroad from Japan Society for the Promotion of Science to J. N.-K.

Conflict of interest

None declared.

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599 **Figure Legend**

600 **Figure 1 Expression of *CCL2* in the human CL is correlated to the number of**
601 **infiltrating macrophages.** The CD68-positive infiltrating macrophages are distributed
602 at the border of granulosa-lutein (g) and theca-lutein cells (t) and dispersed in the CL
603 parenchyma at mid-luteal (ML) phase (**A**). CD68-positive macrophages tend to increase
604 in the CL at late-luteal (LL) phase (**B**). Numerous CD68-positive cells are found in the
605 CL collected during menstruation (Men), and the number of CD68-positive
606 macrophages is significantly increased in the regressing CL at this stage (**C, D**). CCL2
607 protein is localized in the cytoplasm of granulosa-lutein and theca-lutein cells in the
608 human CL at ML phase (**E**). Endotherial cells are weakly immunoreactive for CCL2
609 (*arrows* in **E**) while non-steroidogenic cells distributed in the CL parenchyma are
610 negative in reaction (*asterisks* in the insert of **E**). The mRNA expression of *CCL2* peaks
611 in the CL collected during menstruation (**F**) and reduced by *in vivo* administration of
612 hCG (**G**). EL: early-luteal phase, R: the CL rescued from luteolysis by *in vivo*
613 administration of hCG. **P*<0.05, ***P*<0.01.

614

615 **Figure 2 Regulation of *CCL2* mRNA expression in cultured LGCs.** The mRNA
616 expression of *CCL2* is significantly decreased by the treatment of hCG *in vitro* (**A**).
617 Withdrawal of hCG significantly enhances the *CCL2* expression in LGCs on day 8 and
618 9 (**B**). Luteotrophic PGE decreases the expression of *CCL2* (**C**) whereas luteolytic PGF
619 promotes its expression (**D**). A macrophage cytokine, *TNF*, significantly increases in the
620 CL collected during menstruation (**E**) and its expression is positively correlated to the
621 expression of *CCL2* in the human CL (**F**). The expression of *TNF* is reduced in the
622 rescued CL by *in vivo* administration of hCG (**G**). *CCL2* expression is promoted by

TNF treatment in LGCs *in vitro* (**H**). PKC activator (PMA) significantly enhanced the expression of *CCL2* (**I**) while inhibitors for PI3 kinase (LY294002: LY) and PKA (H89) suppressed its expression (**J**, **K**). Cont: control, DMSO: dimethylsulfoxide used as a vehicle for PMA, LY, and H89. EL: early-luteal, EtOH: ethanol used as a vehicle for prostaglandins, LL: late-luteal phase, Men: menstruation, ML: mid-luteal phase, R: rescued CL by *in vivo* administration of hCG. **P*<0.05, ***P*<0.01, *****P*<0.0001.

Figure 3 Regional difference in the number of CD68-positive macrophages in the structurally regressing CL during menstruation. Granulosa-lutein cells in the CL at menstrual phase are classified into three different conditions: intact (i), pyknotic (p), and degenerating (d) by the observation of two serial sections stained with either CD68 (**A**, **C**) or 3β-HSD (**B**, **D**). Intact cell area contains few numbers of CD68-positive macrophages (**A**) but intensely immunostained for 3β-HSD, a key enzyme for progesterone production (**B**). Pyknotic cells contain intense 3β-HSD immunoreactivity (**B**) and there are abundant CD68-positive macrophages in this area (**A**). Numerous CD68-positive macrophages are found in degenerating cell area lacking in 3β-HSD immunoreactivity (**A**–**D)**. C and D are closer views of the areas enclosed by squares in A and B, respectively. Arrows in C and D show the condensed nuclei of pyknotic granulosa-lutein cells. Asterisks are theca-lutein cells. Men: menstruation.

Figure 4 Dual staining for CD68 and apoptotic cells in the regressing human CL at menstrual phase. Dual staining for CD68 (red) and apoptotic cells (green) clearly demonstrates the difference in the degree of macrophage infiltration depending on the condition of granulosa-lutein cells (**A**–**F**). There are few CD68-positive macrophages in

647 intact cell area (i) (**A**–**C**) while abundant macrophages are found in the area occupied
648 with pyknotic/apoptotic cells (p) (**A**–**F**). Marked accumulation of CD68-positive
649 macrophages is observed in degenerating cell area (d) where contains few number of
650 apoptotic cells (**D**–**F**). **G** is a schema showing the relationship between
651 immunoreactivity for 3β-HSD in granulosa-lutein cells and the number of infiltrating
652 macrophages. Arrowheads in A-F represent the border of the different areas. +: positive,
653 –: negative.
654
655 **Figure 5 Relationship between the function of luteal cells and macrophage**
656 **infiltration.** Two serial sections of the human CL at the late-luteal phase were
657 immunostained for a PGE synthase (PGES) (**A**) or a macrophage marker (CD68) (**B**).
658 Healthy luteal cells contained abundant immunoreactivity for PGES (*arrow* in **A**) and
659 there are few number of CD68-positive macrophages around them (*arrow* in **B**).
660 Abundant CD68-positive cells are found in the region consisted with PGES-negative
661 granulosa-lutein cells (*asterisks in* **A**, **B**). The mRNA expression of *PGES* (C) and
662 *HSD3B1* (**D**) tends to be negatively correlated to that of *CCL2* in the human CL.
663
664 **Figure 6 Effect of TNF on the expression of genes related to progesterone and**
665 **prostaglandin synthesis.** TNF treatment on culture day 6 enhances the expression of
666 *STAR* (**A**), *COX2* (**B**), and *PGES* (**C**), key enzymes for progesterone and luteotrophic
667 PGE synthesis. On the other hand, TNF tends to decrease the expression of *AKR1C1*
668 (**D**) and significantly reduces the expression of *AKR1C3* (**E**), which are involved in the
669 synthesis of luteolytic PGF. Cont: control. $^{**}P<0.01$, $^{****}P<0.0001$.
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Table 1. Primers used for qRT-PCR analysis

Gene name	Protein name	Accession no.	Forward primer	Reverse primer	Product size
G6PDH	G6PDH	NM_000402	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	155bp
CCL2	CCL2	D26087	AATCAATGCCCCAGTCACCTGC	CGGAGTTTGGGTTTGCTTGTCC	210bp
TNF	TNF	NM_000594	CCCGAGTGACAAGCCTGTAG	GAGGTACAGGCCCTCTGATG	151bp
HSD3B1	3β-HSD	NM_000862	CCATGAAGAAGAGCCTCTGG	GTTGTTCAGGGCCTCGTT TA	202bp
PGES	PGES	NM_0004878	AGTGAGGCTGCGGAAGAAG	AGGGTTAGGACCCAGAAAGG	181bp
AKR1C1	AKR1C1	NM_001353	ACCAAATTGGCAATTGAAGCT	TGGGATCACTTCCTCACCTG	279bp
AKR1C3	AKR1C3	NM_003739	CTGGGATCTCAACGAGACAA	GCTTTCATGTCCTCTGCAGTC	230bp

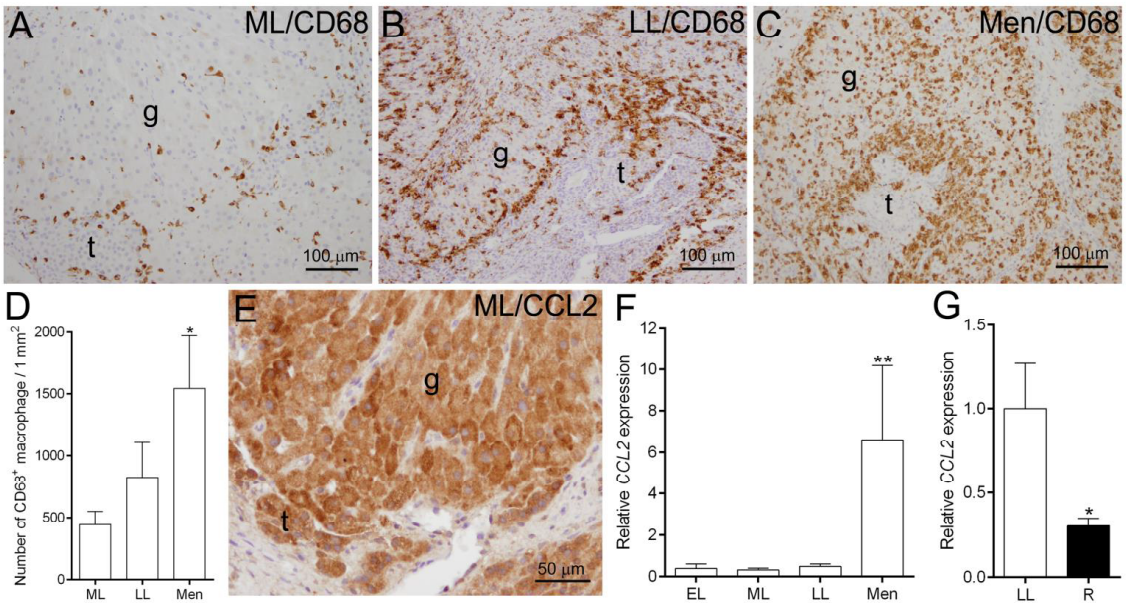

Figure 1 Nio-Kobayashi et al.

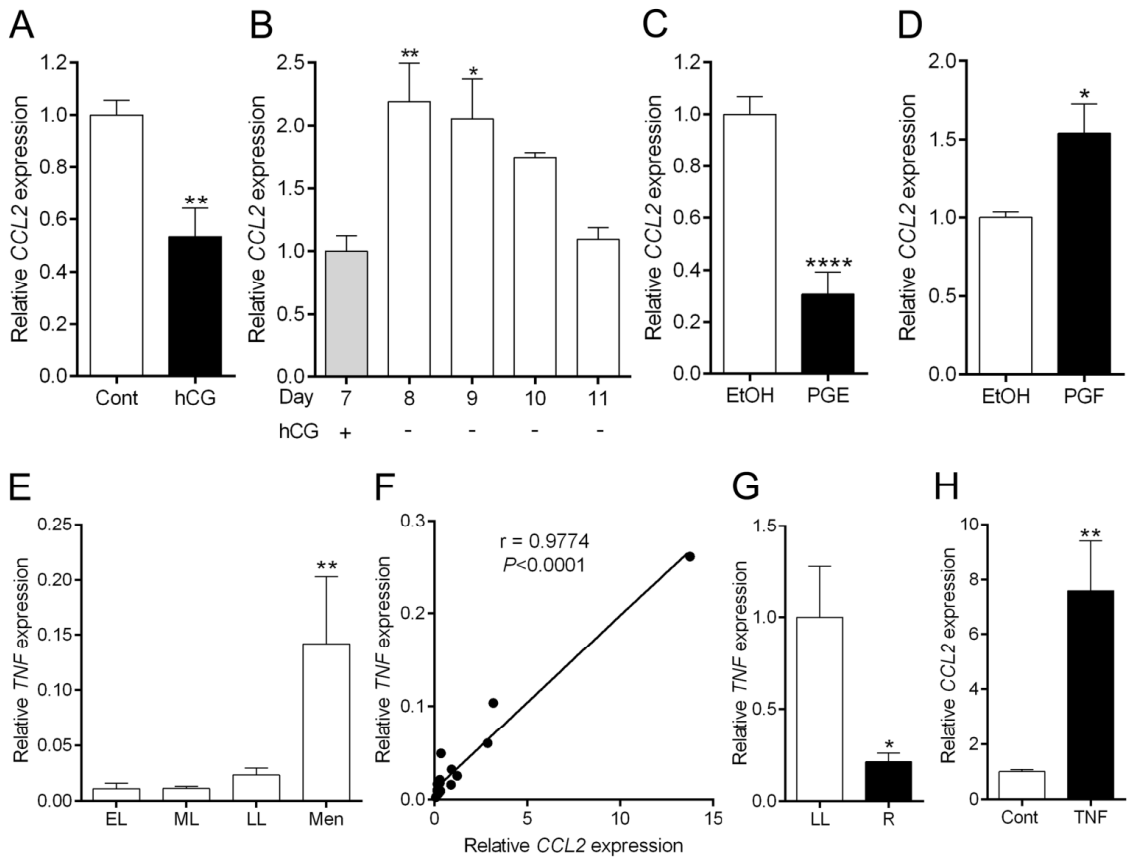

Figure 2 Nio-Kobayashi et al.

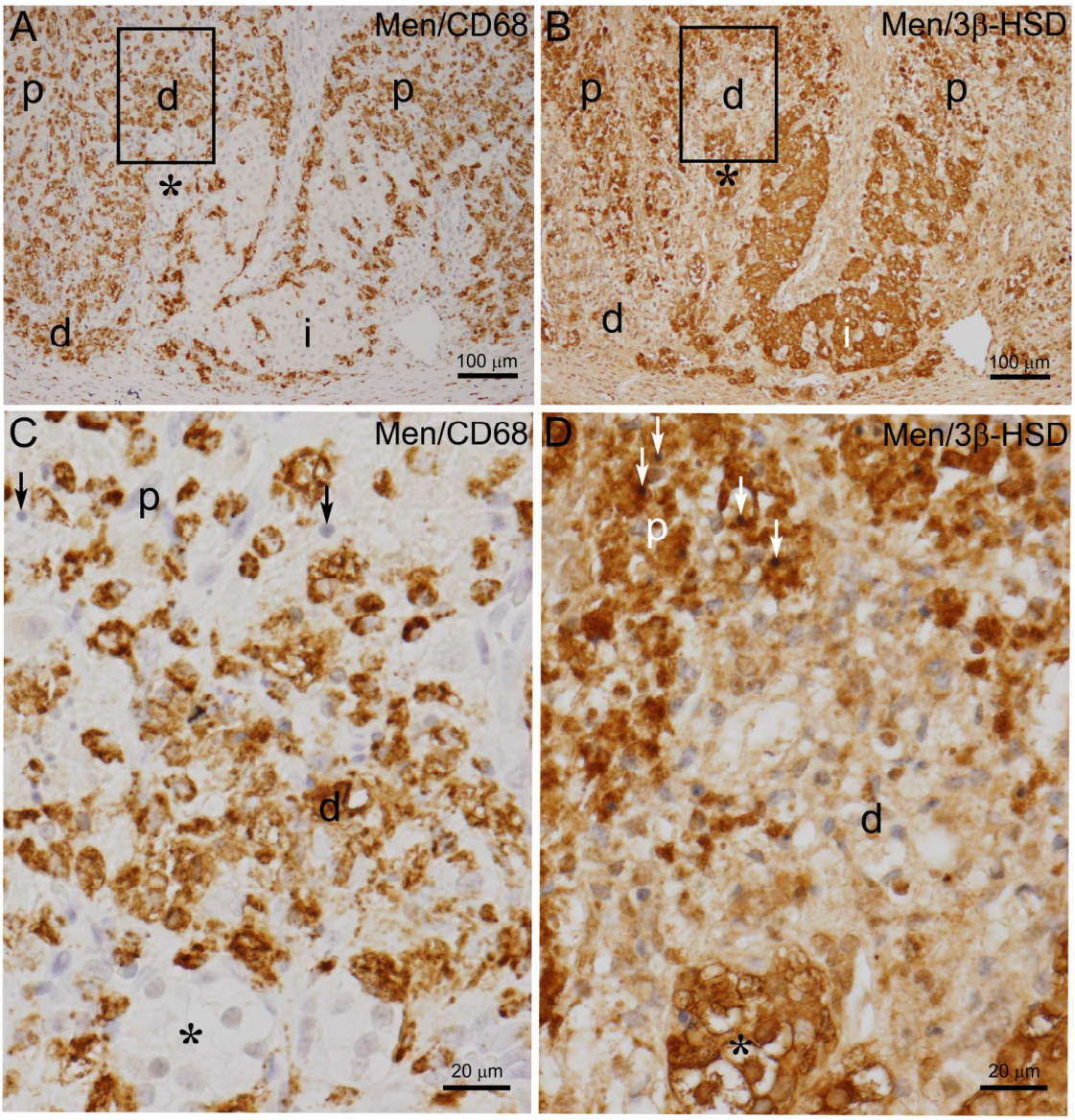

Figure 3 Nio-Kobayashi et al.

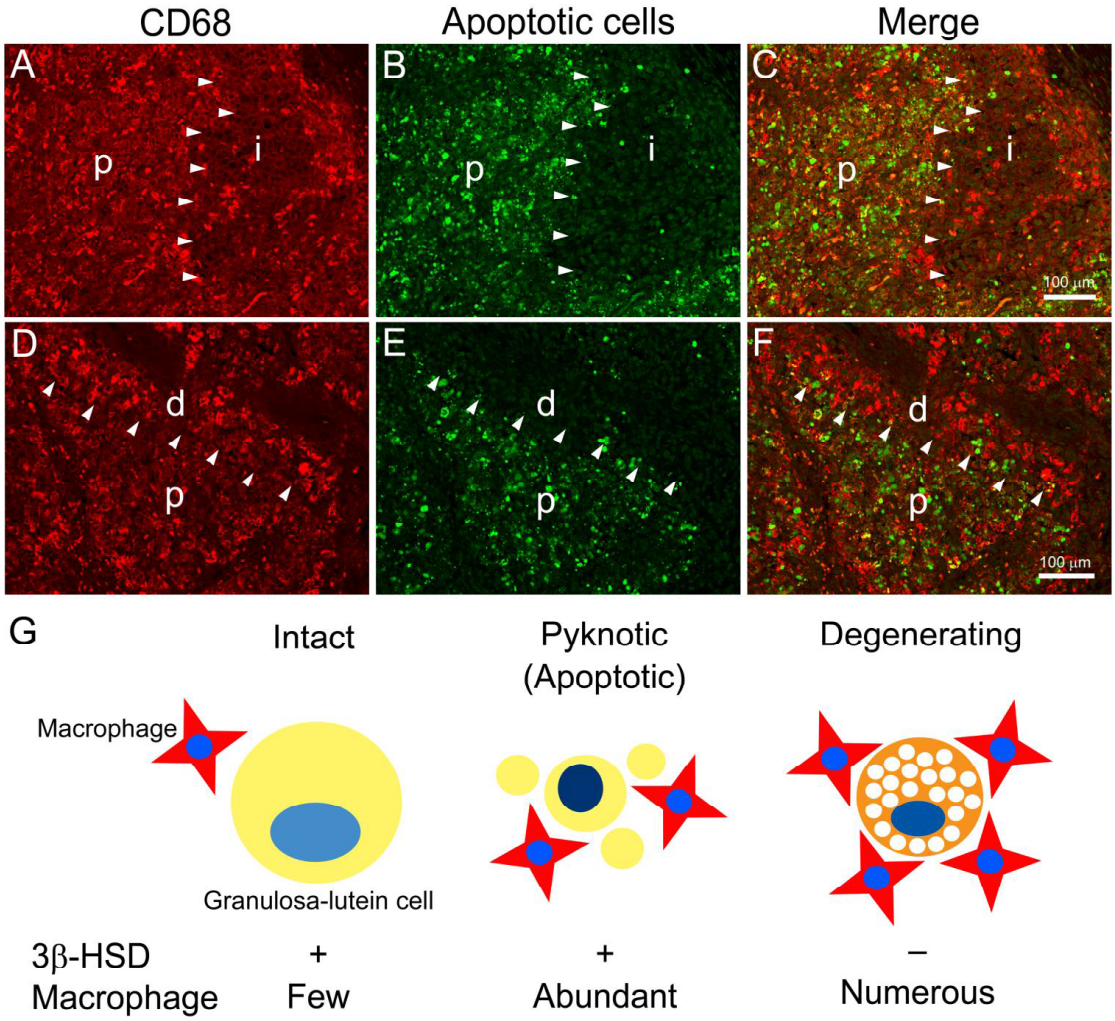

Figure 4 Nio-Kobayashi et al.

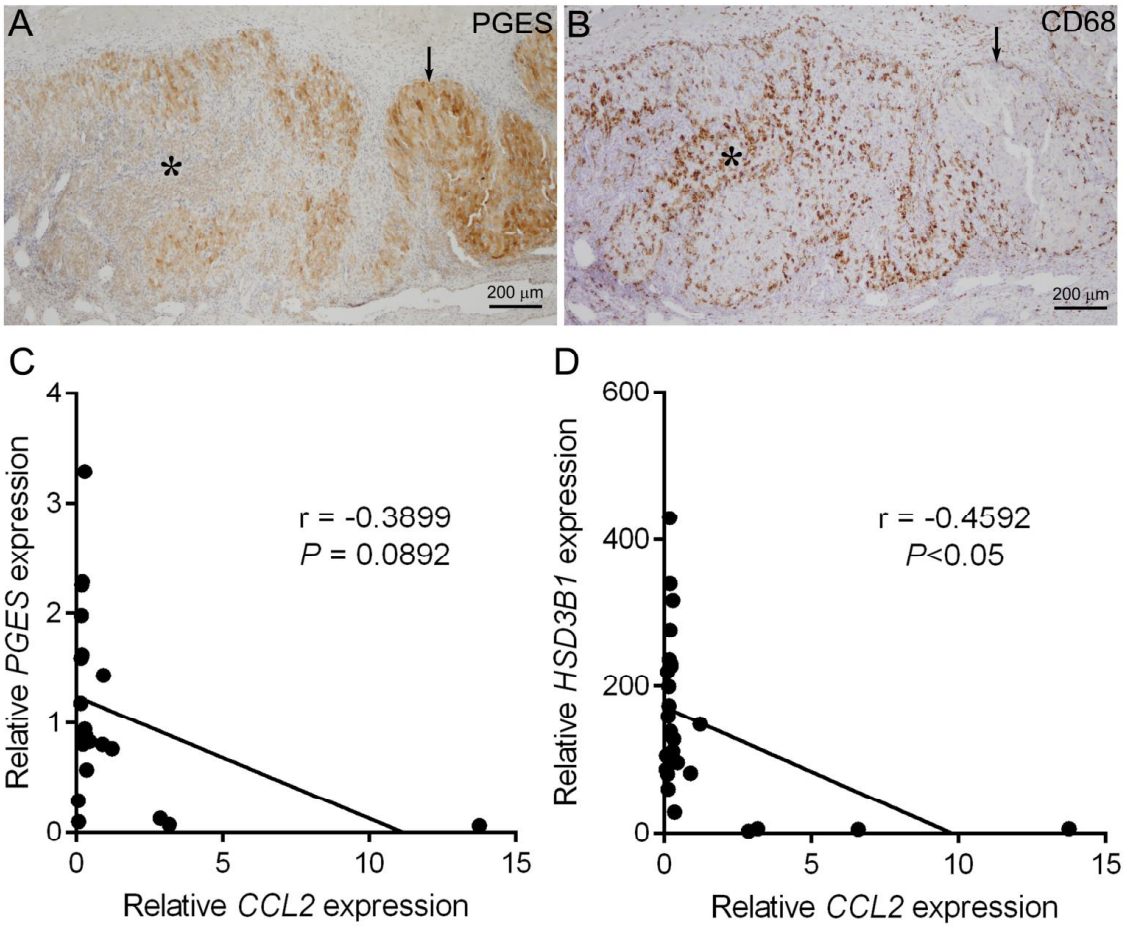

Figure 5 Nio-Kobayashi et al.

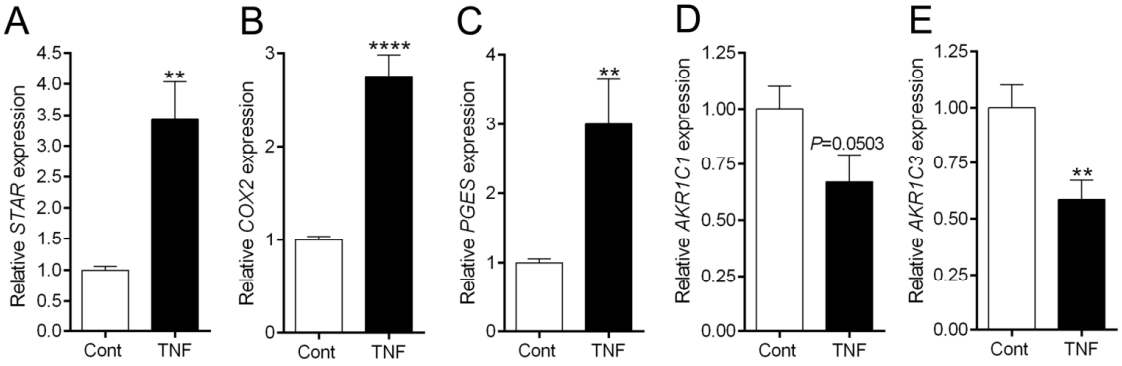

Figure 6 Nio-Kobayashi et al.