Active immunization against the proregions of GDF9 or BMP15 alters ovulation rate and litter size in mice

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Abstract

The transforming growth factor β (TGFB) superfamily proteins bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), are essential for mammalian fertility. Recent *in vitro* evidence suggests that the proregions of mouse BMP15 and GDF9 interact with their mature proteins after secretion. In this study, we have actively immunized mice against these proregions to test the potential *in vivo* roles on fertility. Mice were immunized with either N- or C-terminus proregion peptides of BMP15 or GDF9, or a full-length GDF9 proregion protein, each conjugated to keyhole limpet hemocyanin (KLH). For each immunization group, ovaries were collected from ten mice for histology after immunization, while a further 20 mice were allowed to breed and litter sizes were counted. To link the ovulation and fertility data of these two experimental end points, mice were joined during the time period identified by histology as being the ovulatory period resulting in the corpora lutea (CL) counted. Antibody titers in sera increased throughout the study period, with no cross-reactivity observed between BMP15 and GDF9 sera and antigens. Compared with KLH controls, mice immunized with the N-terminus BMP15 proregion peptide had ovaries with fewer CL ($P < 0.05$) and produced smaller litters ($P < 0.05$). In contrast, mice immunized with the full-length GDF9 proregion not only had more CL ($P < 0.01$) but also had significantly smaller litter sizes ($P < 0.01$). None of the treatments affected the number of antral follicles per ovary. These findings are consistent with the hypothesis that the proregions of BMP15 and GDF9, after secretion by the oocyte, have physiologically important roles in regulating ovulation rate and litter size in mice.

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Introduction

Mammalian ovulation rate, oocyte competence, and fertility are regulated by multiple paracrine and endocrine pathways, including the oocyte-derived transforming growth factor β (TGFB) superfamily signaling molecules, bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9; Eppig *et al.* 2002, Juengel & McNatty 2005). Mouse gene knockout models have demonstrated that GDF9 is essential for ovarian follicular development and fertility (Dong *et al.* 1996, Elvin *et al.* 1999a, 1999b), whereas BMP15 is only partially required for ovulation and fertilization and is not essential for follicular development (Yan *et al.* 2001). In sheep, inactivating mutations in either *BMP15* or *GDF9* result in sterility in homozygous mutants or increased ovulation rate in heterozygous carriers (Galloway *et al.* 2000, Hanrahan *et al.* 2004). In women, *BMP15* mutations lead to premature ovarian failure (Di Pasquale *et al.* 2004, 2006, Dixit *et al.* 2006), while *GDF9* mutations are also linked with sterility (Laissue *et al.* 2006) and dizygotic twinning (Palmer *et al.* 2006).

The TGFB superfamily of proteins are characteristically translated as precursor promature proteins that are enzymatically processed to produce a larger N-terminus proregion or prodomain and a smaller bioactive C-terminus mature protein. Intracellularly, the proregions of TGFB proteins are known to facilitate protein folding, dimerization, secretion, and stability while interacting noncovalently with the respective mature protein (Gray & Mason 1990, Constam & Robertson 1999, Hashimoto *et al.* 2005, Walton *et al.* 2009). Additionally, a wide range of extracellular functions for proregions of several TGFB family members interacting with mature domains has been observed (Bottinger *et al.* 1996, Hill *et al.* 2002, Haramoto *et al.* 2004, Jiang *et al.* 2004, Brown *et al.* 2005, Le Good *et al.* 2005, Anderson *et al.* 2008, Sengle *et al.* 2011). Recent evidence from the crystal structure of TGFB1 supports a number of interactions occurring between proregion and mature proteins following cleavage of the precursor protein (Shi *et al.* 2011). Previously, we reported that transfected human embryonic kidney cells secreted mouse BMP15 and GDF9 as mixtures of processed mature and

proregion proteins, together with varying amounts of unprocessed precursor protein (McIntosh *et al.* 2008). Additionally, the cleaved proregions of BMP15 and GDF9 interacted noncovalently with their respective mature proteins, as well as forming heteromeric complexes between the BMP15 proregion and the GDF9 mature proteins (Liao *et al.* 2003, McIntosh *et al.* 2008). By using a proregion antibody to immunoneutralize BMP15 activity with respect to thymidine incorporation into granulosa cells, we gained further evidence that the BMP15 proregion may have important extracellular functions. Moreover, in ovine follicular fluid, the predominant forms of both GDF9 and BMP15 were the unprocessed precursor proteins (McNatty *et al.* 2006).

To test whether these *in vitro* and *in vivo* findings have physiological relevance, we examined the potential roles of the proregions of BMP15 and GDF9 by immunizing mice against selected proregion peptide sequences or a recombinant full-length mouse proregion GDF9 protein. The end points assessed in this study were ovarian volume, the number of antral follicles and corpora lutea (CL; experiment 1), and corresponding litter sizes (experiment 2). Mouse CL remain visible in the ovary from the four most recent ovulatory cycles, with each cycle taking 4 days (Pedersen & Peters 1968). Therefore, by allowing mice to mate (experiment 2) during the 16 days before the time of ovary collection for histological analysis (experiment 1), we were able to link the CL numbers counted with the corresponding fertility outcome.

Results

Antibody titers

During the experimental timeline (see Fig. 1), antibody titers were measured in the pre-, mid-, and post-immunization sera of animals immunized with the GDF9 full-length proregion or BMP15 proregion N-terminus peptide. Titers were observed to increase consistently throughout the immunization period; for example, titers of post-immunization sera compared with mid-immunization sera significantly increased by 11-fold for the GDF9 full-length proregion ($P < 0.0001$) and by twofold for the BMP15 N-terminus proregion peptide ($P < 0.01$). Post-immunization antisera from all groups were then tested for specificity to the immunization antigen and for cross-reactivity to other antigens. All immunizations with keyhole limpet hemocyanin (KLH)-conjugated BMP15 or GDF9 proregion peptides (see Fig. 2 for peptide sequences) or the full-length GDF9 proregion protein resulted in significantly higher specific antibody titers against their respective antigens than when mice were immunized with KLH alone ($P < 0.0001$, Table 1). No cross-reactivity was observed between BMP15 antisera and GDF9 antigens, or vice versa (Table 1).

Figure 1 Experimental timeline for the immunization experiments. Mice were immunized four times with KLH-conjugated peptides or protein, or KLH as a control ($n = 30$ per treatment group). Each treatment group was divided into two experiments: 1) where ovaries from ten mice per treatment group were collected a week after the final immunization for follicle and CL counts, and 2) where 20 mice per treatment group were allowed to breed and the number of pups born per litter were counted. In experiment 1, visible CL were linked to the last four ovulation cycles before ovary collection. Mating in experiment 2 was therefore allowed only during the same time period of these four ovulation cycles, in order to link the number of CL counted to fertility from the same cycles. Sera were collected after immunization for experiment 1, and pre-, mid-, and post-immunization for experiment 2.

Ovarian volume and number of CL and antral follicles

Ovarian volume, number of CL visible over the last four ovulation cycles and number of antral follicles were analyzed for each mouse and mean values were calculated for each immunization group (Table 2). The mean volume of ovaries from animals immunized against the C-terminus BMP15 proregion peptide was significantly larger than that for the KLH group ($P < 0.05$). No differences were observed in ovarian volumes of animals immunized with other antigens. An increased total number of CL was observed in the GDF9 full-length proregion-immunized group compared with the KLH control group ($P < 0.01$, Table 2). In contrast, total CL number was decreased in the groups immunized with BMP15 proregion C-terminus ($P < 0.01$) and N-terminus ($P < 0.05$) peptides. Since mouse CL are visible in the ovary from the four most recent ovulatory cycles (Pedersen & Peters 1968), the number of CL was compared on the basis of ovarian cycle of origin, where CL1 were from the most recent cycle and CL4 were the oldest CL. CL2 and CL3 were combined for ease of identification because it was not always possible to distinguish between these two groups using morphological criteria. Interestingly, significant differences were observed in the number of CL from ovarian cycles 2 + 3, but not from cycles 1 or 4 (Fig. 3), compared with those of the KLH-immunized group. No differences were observed in the mean total number of antral follicles (Table 2), number of follicles at any individual antral follicular stage, or the number of atretic follicles in any immunized group compared with the KLH-immunized control group (data not shown).

Figure 2 Mouse BMP15 and GDF9 proregion peptides for immunization. N- and C-terminus proregion peptides of mouse BMP15 and GDF9 were selected so that a) C-terminus peptides encompassed a conserved tyrosine residue (underlined) that results in human infertility when mutated in human BMP15 (Di Pasquale *et al.* 2004); b) N-terminus peptides encompassed a hydrophobic motif (underlined) thought to bind to a mature protein sequence that is also the bone morphogenetic protein receptor, type II (serine/threonine kinase) (BMPR2) binding site of the mature protein (Walton *et al.* 2009).

Litter size

Mice immunized with either the N-terminus BMP15 proregion peptide or the full-length GDF9 proregion produced significantly smaller litters compared with the KLH-immunized control group (Table 2, $P < 0.05$ and $P < 0.01$ respectively). To check that the immunizations did not affect mating rate, pregnancy rate, or the number of litters produced in each group, data for these parameters were collected for χ^2 analysis (Table 3). Males were housed with females for mating from the time of cycle 4 (i.e. the first mating cycle). Pregnancy occurred in 35–58% of the mice, similar to Balb/c mice pregnancy rates of 31–44% documented by The Jackson Laboratory, (Bar Harbor, Maine, USA) (<http://jaxmice.jax.org/jax-notes/archive/501d.html>). No significant difference was observed between immunization groups in the proportions of animals that became pregnant or in the number of litters produced by each group per ovulatory cycle ($P > 0.05$, Table 3). A difference between groups in the number of copulatory plugs after mating was identified ($P < 0.05$, Table 3), which was likely due to loss of some plugs in the KLH group, since significance

was lost when data from the KLH group were omitted. As the pregnancy rate for the KLH group was above the mean pregnancy rate for all groups, this difference did not translate into loss of fertility.

Discussion

This study provides physiological evidence to support the hypothesis that the proregions and/or precursor proteins of GDF9 and BMP15 have key regulatory functions within the mouse ovary after secretion from the oocyte, including regulation of ovulation rate as assessed by the number of CL that formed in each ovarian cycle and regulation of litter size. Immunization with the GDF9 proregion was shown to increase ovulation rate, in contrast to a reduced ovulation rate in mice immunized with the BMP15 proregion peptides, indicative of different roles for mouse GDF9 and BMP15 proregions before or during ovulation. These divergent effects are possibly linked to differing abilities of generated antibodies to modulate the concentrations of GDF9 or BMP15. For example, regulation of ovulation rate in sheep by BMP15 and GDF9 is dose dependent (Juengel *et al.* 2002, McNatty *et al.* 2007), with decreasing concentrations of biologically active BMP15 or GDF9 initially linked to increased ovulation rates until a critical threshold is reached, and with decreased ovulation rate predicted when bioactivity is below this threshold (McNatty *et al.* 2004).

Whether our immunizations affect ovulation rate through altering the pattern of follicular growth or through regulating the efficiency of ovulation and luteal formation is difficult to decipher. While no differences were observed in the numbers of antral follicles for any treatment at the time of autopsy, differences might have been present leading up to the cycles forming a different number of CL. The significant difference in the number of CL from cycles 2 and 3, but not from the most recent cycle 1, might have indicated that the antibody titers had decreased by the time of ovary collection. However, the steady increase in antibody titers measured throughout the study indicates that the immunoneutralization effects were likely to be increasingly compensated for over time. Underlying mechanisms for this compensation

Table 1 Optical density (mean $\pm$ S.E.M.) of post-immunization mouse sera following ELISA using specified coating antigens.

Mouse antisera	Coating antigen				
	GDF9 proregion	GDF9 C-terminus	GDF9 N-terminus	BMP15 C-terminus	BMP15 N-terminus
KLH	0.04 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
GDF9 proregion	1.22 $\pm$ 0.19	0.06 $\pm$ 0.03	0.02 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
GDF9 C-terminus	0.81 $\pm$ 0.11	1.86 $\pm$ 0.22	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
GDF9 N-terminus	0.31 $\pm$ 0.05	0.01 $\pm$ 0.00	1.63 $\pm$ 0.19	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
BMP15 C-terminus	0.04 $\pm$ 0.00	0.01 $\pm$ 0.00	0.02 $\pm$ 0.00	1.18 $\pm$ 0.18	0.01 $\pm$ 0.00
BMP15 N-terminus	0.04 $\pm$ 0.01	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.94 $\pm$ 0.16

Values in bold represent $P < 0.0001$ compared with KLH control within column, $n = 28$ –30 mice per group.

Table 2 Effect of immunization treatments on ovarian volume, number of corpora lutea (CL) and antral follicles, and litter size per mouse. Values are mean $\pm$ S.E.M.

Immunization group	Ovarian volume (mm ³)	Number of CL ^a	Number of antral follicles ^a	Litter size
KLH	2.17 $\pm$ 0.12 (10)	55.1 $\pm$ 3.3 (10)	116 $\pm$ 10 (5)	4.30 $\pm$ 0.40 (20)
GDF9 proregion	2.48 $\pm$ 0.12 (10)	79.5 $\pm$ 3.5** (10)	140 $\pm$ 12 (5)	1.75 $\pm$ 0.25** (19)
GDF9 C-terminus	2.25 $\pm$ 0.16 (10)	57.3 $\pm$ 3.3 (10)	171 $\pm$ 15 (5)	3.00 $\pm$ 0.65 (20)
GDF9 N-terminus	1.80 $\pm$ 0.09 (10)	49.2 $\pm$ 3.0 (10)	176 $\pm$ 23 (5)	4.11 $\pm$ 0.72 (19)
BMP15 C-terminus	2.72 $\pm$ 0.14* (8)	41.8 $\pm$ 1.8** (10)	149 $\pm$ 21 (5)	2.88 $\pm$ 0.55 (20)
BMP15 N-terminus	1.65 $\pm$ 0.37 (7)	35.8 $\pm$ 4.3* (9)	90 $\pm$ 22 (5)	2.55 $\pm$ 0.39* (19)

Values in bold represent * P <0.05, ** P <0.01 compared with the KLH control group. The number of mice analyzed in each treatment group is shown in brackets.

^aFor calculation of mean values per immunization group, all visible CL and antral follicles of one ovary were counted for each mouse.

might involve intra-follicular paracrine effects since both the oocyte and the granulosa cells have membrane receptors for GDF9 and BMP15 (reviewed in Juengel & McNatty (2005) and Gilchrist *et al.* (2008)). Further evidence that the decreased number of CL observed in the BMP15 proregion-immunized groups could be related to interference with ovulation/luteinization was shown when normal ovulation and/or CL formation was impaired in ewes passively immunized with anti-plasma to BMP15 or GDF9 just before ovulation (Juengel *et al.* 2002).

Under normal physiological conditions in mice, levels of secreted BMP15 proteins are thought to be low (Hashimoto *et al.* 2005) until the preovulatory gonadotropin surge (Yoshino *et al.* 2006). In this case, it would be unlikely that BMP15 immunization would affect antral follicle numbers. The decreased ovulation rate we observed in mice immunized with BMP15 proregion peptides parallels the reported subfertility of *Bmp15* knockout mice (Yan *et al.* 2001). Our data that GDF9 proregion-immunized mice developed increased CL numbers suggest that the GDF9 proregion might inhibit ovulation in some way, which is consistent with reports that proregions of some other TGF β family proteins inhibit or antagonize their respective mature proteins (Bottinger *et al.* 1996, Hill *et al.* 2002, Jiang *et al.* 2004, Makanji *et al.* 2011). Alternatively, removal of the GDF9 proregion may negatively affect the concentration/dose of bioactive GDF9 mature protein available for signaling. In support of this, mice injected with an Fc fusion protein of the type II BMP receptor bone morphogenetic protein receptor, type II (serine/threonine kinase) (BMPRII) had increased the number of both antral follicles and CL (Myllymaa *et al.* 2010).

A key result from our studies was the decreased litter sizes of mice immunized with either the full-length GDF9 proregion or the BMP15 proregion N-terminus peptide. Since no differences between treatment groups were observed in the proportions of mice that became pregnant, or in the cycles in which fertilization occurred, it seems most likely that the proregion immunizations affected either the number of oocytes available for fertilization and/or the ability of oocytes to be fertilized and undergo normal development. Given the reduced

number of CL observed in mice immunized against the BMP15 proregion N-terminus peptide, the subsequent reduced litter size is likely to be related to a decreased number of oocytes available for fertilization. A smaller litter size despite an increased CL number in mice immunized with the GDF9 proregion is consistent with the notion that some of the follicles and/or oocytes, and therefore the subsequent CL (Juengel *et al.* 2002), were abnormal at ovulation and compromised the pregnancy outcome. Moreover, this is also consistent with the finding that the addition of exogenous GDF9 to mouse oocytes during *in vitro* maturation resulted in an increased number of viable fetuses (Yeo *et al.* 2008).

Figure 3 Number of corpora lutea (CL) according to ovulation cycle of CL origin. (A) Morphological classification of CL into groups to identify the ovulation cycle from which the CL developed. CL1, CL from the most recent ovulation cycle 1; CL2+3, combined CL from cycles 2 and 3 before the most recent ovulation; and CL4, the oldest visible CL from cycle 4. Scale = 50 μ m. (B) Mean number of CL of mice within each immunization group, according to CL group, $\pm$ S.E.M. ** P <0.01 and *** P <0.001 compared with KLH control groups.

Table 3 Effect of immunization treatments on proportion of animals with copulatory plugs, pregnancy rate, and number of litters per ovulation cycle.

Immunization group (n)	Proportion of group with copulatory plugs (%)	Pregnancy rate of group (%)	Number of litters per group, per cycle		
			Cycle 4	Cycles 2 + 3	Cycle 1
KLH (20)	60*	50	4	4	2
GDF9 proregion (19)	75	40	3	4	1
GDF9 C-terminus (20)	95	35	2	4	1
GDF9 N-terminus (19)	79	47	3	4	1
BMP15 C-terminus (20)	85	40	4	3	2
BMP15 N-terminus (19)	95	58	3	6	2

* $P < 0.05$ χ^2 analysis. n, number of animals in each treatment group.

Proregion immunoneutralization of bioactivity is likely to occur through interactions between the proregion and mature proteins. It is anticipated that the antibodies generated by immunization would bind to proregion (free or interacting with mature proteins and other regulatory proteins) and precursor proteins. However, the physiological significance of secreted BMP15 and GDF9 precursor proteins is yet unknown. The N-terminus proregion peptides used for immunization includes a hydrophobic motif identified as binding to the mature protein at the same site that also interacts with BMP2 (Walton *et al.* 2009). Antibodies generated against this region in BMP15 altered CL number, but there was no effect on CL number when mice were immunized with the corresponding GDF9 N-terminus peptide, suggestive of the *in vivo* interactions between the proregion and the mature proteins of mouse GDF9 and BMP15 being functionally different.

In summary, we have shown that active immunization against either the GDF9 or BMP15 proregion protein or peptides altered the number of CL and decreased the number of offspring, providing evidence that the proregions of both factors are important regulators of ovarian function *in vivo* in mice. Discovery of the functional mechanisms of these proregions will be a vital step to understand the full effects of BMP15 and GDF9 on oocyte maturation and gonadotropin responsiveness of both cumulus and granulosa cells.

Materials and Methods

Generation of proregions and proregion peptides

Mouse proregion GDF9 was amplified from the appropriate full-length mouse gene prepared as described previously (McIntosh *et al.* 2008). The *Gdf9* PCR product (using *Gdf9* primers 5'-CACCATGTCTACTGAAGAATCC-3', 5'-TCGACGGCGCCGGGG-3') was blunt-end cloned into the plasmid pET101 Direction TOPO (Invitrogen). The resulting GDF9 proregion protein was purified by immobilised metal ion chromatography (IMAC) and conjugated to equal quantities of KLH (Sigma-Aldrich) using glutaraldehyde, as described previously (Juengel *et al.* 2002). Preparation of a stable, full-length BMP15 proregion was unsuccessful. Proregion peptides

(Fig. 1) were synthesized and conjugated to KLH through a C-terminus cysteine residue by Global Peptide, Fort Collins, CO, USA and GenScript, Piscataway, NJ, USA.

Immunization procedures

All animal immunizations and tissue recoveries were performed under approval of the Animal Ethics Committee of the Wallaceville Animal Research Centre, Upper Hutt, New Zealand, in accordance with the 1999 Animal Welfare Act (Part 6), New Zealand, and by the Victoria University of Wellington Animal Ethics Committee. The experimental design for the immunization experiments is outlined in Fig. 1. For each proregion or peptide antigen, 3-month-old Balb/c female mice ($n=30$ per treatment) were immunized with a total of 0.4 mg conjugated antigen (i.p.) over 6 weeks (0.2 mg in Freund's complete adjuvant, with 2-weekly boosters of 0.1, 0.05, and 0.05 mg in a Span-Tween-Marcol adjuvant). Three separate immunization trials were undertaken, one trial for histological analyses ($n=10$ mice per treatment), and two trials for litter size analyses (data combined, $n=20$ mice per treatment). For histological analyses, ovaries were collected 1 week after the final booster. For litter size analyses, mice were allowed to mate during the 16-day period before immunization week 8, with the males being removed after mating.

Measurement of antibody titers by ELISA

Antisera from mice for litter size analyses were collected before immunization (pre), 2 days after immunization 3 (mid), and at killing (post). Antisera from mice for histological analyses were collected only after killing (post). To test sera ($n=20-30$ per treatment group) for reactivity to BMP15 and GDF9, wells of microtiter plates were coated at 4 °C overnight with 100 ng of unconjugated proregion protein or proregion peptide antigens in coating buffer (50 mmol/l carbonate-bicarbonate buffer, pH 9.6). GDF9 proregion protein was treated with 1% w/v SDS at 56 °C for 30 min before coating. Non-specific binding was blocked by incubation with 0.25 ml coating buffer containing 0.5% w/v gelatin for 30 min at room temperature. Wells were washed (three washes in PBS with 0.05% v/v Tween 20 for 3 min). Mouse sera were diluted 1:1000 in assay buffer (PBS with 0.05% v/v Tween 20 and 0.1% w/v gelatin), and 0.1 ml diluted sera incubated in each of the coated/washed wells of the microtiter plates for 2 h at 37 °C. The wells were washed

and incubated with 0.1 ml HRP-conjugated goat anti-mouse immunoglobulin G (IgG) (1:10 000 dilution in assay buffer; Jackson ImmunoResearch Europe Ltd) for 1 h at 37 °C. Wells were washed and incubated with 0.1 ml of 50 mmol/l citrate buffer containing O-phenylenediamine (0.4 mg/ml; Sigma-Aldrich) and 0.04% v/v H₂O₂ for 30 min at room temperature in the darkness. Reactions were stopped by the addition of 0.05 ml of 2.5 mol/l H₂SO₄, and absorbance measured at 490 nm on a BioTek EL311 microplate autoreader (BioTek Instruments, Inc., Winooski, VT, USA). ELISA results were validated for multiplate assays with the coefficient of variation <6% between assay plates. Antisera from mice immunized with either BMP15 or GDF9 N-terminus proregion peptides were shown to specifically recognize the appropriate 293 HEK cell-expressed proregion and precursor promature proteins (McIntosh *et al.* 2008).

Histological analyses

Ovaries were collected from each treatment group, fixed in Bouin's fluid, serially sectioned at 5 µm, and stained with hematoxylin and eosin. The volume of one ovary per mouse ($n=7-10$ per group) was measured by the Cavalieri principle using the formula $V=\sum ah$, where a , determined by point counting, is the cross-sectional area (µm²) of every tenth section and h is the distance (µm) between sections used to determine a . The number of CL was counted ($n=9-10$ mice per group, see Table 2) by drawing CL outlines of each ovary in every 50th slide using a projection microscope. Counting every 50th slide was valid since the minimum CL size was observed to be >250 µm. In mice, CL progress to form corpora albicans over four consecutive estrous cycles from the most recent cycle (Pedersen & Peters 1968). For the purposes of this paper, all corpora luteal structures regardless of age are referred to as CL. The ovulation cycle of origin of each CL was determined at higher magnification using an Olympus BH-2 microscope (Olympus New Zealand Ltd, Upper Hutt, New Zealand), according to the criteria: CL1, from the most recent cycle, with large luteal cells and significant number of visible blood vessel epithelial cells; CL2+3, from cycles 2 and 3, with smaller luteal cells than in CL1, less vascularization, and presence of macrophages; CL4, from cycle 4, with luteal cells compacted, membranes disintegrating, and increased number of macrophages (Fig. 3A). While CL1 and CL4 were easy to distinguish, it was not always possible to distinguish CL2 and CL3; therefore, in this study, the data for CL2 and CL3 were combined. The number of antral follicles (non-atretic, atretic, or abnormal) in ovaries of five mice per group was measured by counting antral follicles in every tenth section. Follicular stage was classified according to that proposed for mouse follicles (Pedersen & Peters 1968).

Pregnancy and litter size measurement

Five days after immunization 3, female mice were housed with male mice (5:1 ratio). The presence of copulatory plugs was checked daily as an evidence of mating, noting the possibility of false negative data due to potential loss of plugs before observation. After 16 days, males were removed. From 18 days

after immunization 3 onward, females were checked twice daily for parturition, when pups were counted and removed. Mothers were then killed, and post-immunization antisera were collected.

Statistical procedures

When comparing antibody titers, unpaired Student's *t*-tests were performed between each of the treated groups and the control (KLH) group tested with the same antigen. To compare the ovarian effects and litter sizes between the treated groups and the control KLH group, the data were subjected to analysis of variance (ANOVA) and the Dunnett's *post-hoc* test. To test differences between immunization groups for rates of copulatory plugs, pregnancy, or number of litters, data were subjected to χ^2 analysis.

Declaration of interest

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

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