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Autoimmune Targeted Disruption of the Pituitary-Ovarian Axis Causes Premature Ovarian Failure

Cengiz Z. Altuntas,*† Justin M. Johnson,* and Vincent K. Tuohy2*†

Premature ovarian failure (POF) is characterized by amenorrhea and high serum levels of follicle-stimulating hormone (FSH). POF causes female infertility and represents a substantial women’s health risk affecting 1% of women by age 40. Although ovarian autoimmunity has been associated with POF, the identity of ovarian Ags recognized is unknown. In this study, we show that autoimmune-targeted disruption of the pituitary-ovarian axis leads to POF. Immunization of SWXJ female mice with the p215–234 peptide derived from mouse inhibin-α activates CD4+ T cells and induces experimental autoimmune oophoritis with a unique biphasic phenotype characterized by an early stage of enhanced fertility followed by a delayed stage of POF. Affected mice show high serum levels of inhibin-α-neutralizing Abs that prevent inhibin-mediated down-regulation of activin-induced pituitary FSH release. The loss of activin/FSH down-regulation leads to prolonged metestrus-diestrus, superovulation, increased numbers of mature follicles, increased offspring, accelerated depletion of primordial follicles, and ultimately premature infertility. Thus, inhibin-α-targeted experimental autoimmune oophoritis is initiated by CD4+ Th1 T cells that stimulate B cells to produce inhibin-α-neutralizing Abs directly capable of mediating POF and transferring disease into naive recipients. Our inhibin-α autoimmune model of POF shows how premature infertility may develop in the context of elevated FSH levels thereby closely mimicking the hallmark features of human POF.


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2 Address correspondence and reprint requests to Dr. Vincent K. Tuohy, Department of Immunology, Lerner Research Institute, Cleveland Clinic, NB30, 9500 Euclid Avenue, Cleveland, OH 44195. E-mail address: tuohyv@ccf.org
3 Abbreviations used in this paper: POF, premature ovarian failure; FSH, follicle-stimulating hormone; EAO, experimental autoimmune oophoritis; LP, zona pellucida; LH, luteinizing hormone; LNC, lymph node cell; IVF, in vitro fertilization.

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by B cells and Ab neutralization of inhibin-α that prevents regulation of activin-induced FSH release leading to elevated FSH, superovulation, increased production of mature follicles, increased numbers of viable offspring, and ultimately, an accelerated depletion of primordial follicles that leaves affected mice with ovarian failure. Our EAO model of POF shows mechanistically how autoimmune-targeted disruption of ovarian regulation may lead to premature infertility in the presence of elevated FSH levels, the hallmark features of human POF.

Materials and Methods

Mice and immunization

All mice were purchased from The Jackson Laboratory. SWXJ (H-2q,s) mice were generated at The Jackson Laboratory by mating SJL/J (H-2s) males with SWR/J (H-2q) females. All mice were purchased from The Jackson Laboratory. SWXJ (H-2q,s) mice were generated at The Jackson Laboratory by mating SJL/J (H-2s) males with SWR/J (H-2q) females. At 6–8 wk of age, mice were injected s.c. in the abdominal flank with 200 μg of either peptide or the irrelevant OVA control Ag (Sigma-Aldrich), in 200 μl of an emulsion of equal volumes of water and CFA containing 400 μg of Mycobacteria tuberculosis H37RA (Difco). Mice were euthanized by asphyxiation with CO2 followed by cervical dislocation. All protocols were preapproved by the institutional animal care and use committee of the Cleveland Clinic Foundation in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Peptides

Peptides were derived from the known sequence of mouse inhibin-α (27, 28) and were selected based on having the KXXS-tetrapeptide-binding motif for IA* and IA* MHC class II molecules expressed in SWXJ mice (29). Peptides were synthesized by the Molecular Biotechnology Core Facility of the Lerner Research Institute using standard solid phase methodology and F-moc side-chain-protected amino acids. Peptides were purified >97% by reverse-phase HPLC, and amino acid composition was confirmed by mass spectrometry.

Cell culture and proliferation assay

To determine peptide immunogenicity, lymph node cells (LNC) removed 10 days after immunization (10-day-primed LNC) were cultured in 96-well flat-bottom microtiter Falcon plates (BD Labware) at 3 × 10^5 cells/well in DMEM (Mediatech CellGro) supplemented with 10% FBS (HyClone), 5% HEPES buffer, 2% L-glutamine, and 1% penicillin/streptomycin (Invitrogen Life Technologies). Peptides were added in serial 10-fold dilutions to triplicate wells with positive control wells containing 2 μg/ml anti-mouse CD3 (BD Biosciences) and negative control wells containing no Ag. Cells were cultured at a final volume of 200 μl/well. In some experiments, primed CD4+ and CD8+ T cells were purified by negative selection using anti-CD4- and anti-CD8-coated magnetic beads and double passage through a MACS LS column using a MidiMACS cell separator (Miltenyi Biotec). The purified cells were activated with various doses of peptide-containing 3 × 10^5 T cells/microtiter well and 5 × 10^5 gamma-irradiated (2500 rad) syngeneic splenocyte feeders. To measure recall responses to immunogens, spleens were removed 8–9 wk after immunization, mononuclear cells were enriched by centrifugation on density gradient medium Lympholyte-M (Accurate Chemical) and cultured as described above. All cell cultures were incubated at 37°C in humidified air containing 5% CO2. After 96 h, wells were pulsed with [methyl-3H]thymidine (1.0 μCi/well, specific activity 6.7 Ci/mM; New England Nuclear) and harvested 16 h later by aspiration onto glass fiber filters. Levels of incorporated radioactivity were determined by scintillation spectrometry. Results are expressed as mean cpm of experimental cultures with Ag divided by mean cpm of cultures without Ag (stimulation index). In all proliferation assays, mean cpm of cultures without Ag ranged between 500 and 2000 cpm.

Cytokine ELISAs

Cytokine concentrations were determined by ELISA measurement of 48 h supernatants of 10-day-primed LNC cultured in supplemented DMEM at 5 × 10^6 cells/ml in 24-well flat-bottom Falcon plates (BD Biosciences) in the presence of 20 μg/ml Ag in a final volume of 2.0 ml/well. Purified capture/detection Ab pairs and recombinant cytokines were obtained commercially (BD Biosciences) and included anti-mouse IFN-γ (R4-6A2 and biotin (JES6-1A12)); anti-mouse IL-4 (27, 31) and anti-mouse IL-5 (TRFK5 and biotin TRFK4), and anti-mouse IL-10 (JES5-2A5) and biotin SXC-1). Absorbance was measured at 405 nm using a model 550 ELISA microplate reader (Bio-Rad). Standard values were plotted as absorbance vs cytokine concentration, and sample cytokine concentrations were determined as values within the linear part of the standard curve established using known concentrations of each cytokine.

Hormone ELISAs

At 4 and 12 wk after immunization, serum inhibin-A and activin-A were measured by direct ELISA as previously described (30–33). To enhance specificity and sensitivity, all serum samples and standards were pretreated with SDS, heated to 100°C, and exposed to H2O2. Inhibin-A and activin-A were measured using a common solid-phase capture Ab specific for the βA chain and anti-inhibin-α or anti-βA alkaline phosphatase-conjugated detection Ab that, respectively, distinguished inhibin-A from activin-A (SeroTec). Following addition of substrate, alkaline phosphatase activity was determined by detecting absorbance at 620 nm using a model 550 ELISA microplate reader (Bio-Rad). Serum FSH was measured by radioimmunoassay at the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA) as previously described (34).

Immunocytochemistry

Mouse ovaries were fixed in 10% phosphate-buffered Formalin (Fisher Scientific) and embedded in paraffin. For T cell immunostaining, 5-μm sections were sequentially unmasked in 1 m M EDTA (pH 8.0), blocked with 5% normal goat serum (Vector Laboratories), incubated with a 1/10 dilution of rat anti-mouse CD3 (clone NCL-CD3-12; Novocastra Laboratories), incubated with a 1/50 dilution of mouse-adsorbed biotinylated goat anti-rat IgG (BD Biosciences), treated with 1.5% H2O2 in methanol, and developed by sequential treatment with a streptavidin-HRP complex (ABC kit; Vector Laboratories), dianisidine, and H2O2 substrate (BioGenex). Slides were counterstained with H&E (Richard-Allan Scientific), dehydrated in an ascending gradient of ethanol followed by xylene, and mounted in Cytoseal 60 (Stephens Scientific) for examination by light microscopy.

Fertility assessments

To determine fertility phenotypes, test and control female mice were mated serially with the same SWXJ males over six mating periods. All mice were age matched.

Quantification of ovarian follicles

Ovaries were collected at 4 wk, fixed in 10% phosphate-buffered Formalin (Fisher Scientific), and embedded in paraffin. Follicles were counted under light microscopy in every 10th 5-μm H&E-stained section. Small, medium, and large antral follicles were distinguished based on their widest cross-sectional diameter and their morphology (35). Healthy follicles were considered as those with 200–500 cells in the largest cross-section were considered (36). Atretic follicles were distinguished according to the percentage of pyknotic nuclei among the granulosa cells, the presence of neutrophils in the follicles, and the presence of cavities in follicles with fewer than 200 cells in the largest cross-section. Total follicles for each mouse was determined as the sum counted in both ovaries.

 Estrous cycle staging

At 4 wk after immunization, vaginal smears were collected in 20 μl of 0.9% NaCl and transferred to a glass slide daily at the same time each morning. After air drying, samples were fixed in methanol, stained with methylene blue in 9.5% ethanol, washed and coverslipped. The four stages of the estrous cycle were determined as previously described (37, 38) with proestrus showing 100% intact live epithelial cells, estrus showing 100% cornified epithelial cells, metestrus showing ~50% cornified epithelial cells and ~50% leukocytes, and diestrus showing 80–100% leukocytes.

Ab isotyping

Isotype-specific Ab titers to Ino mice were determined in serum samples according to manufacturer’s instructions using the mouse MonoAb ID/SP ELISA kit (Zymed Laboratories).

Passive transfer of EAO

Four weeks after immunization of female SWXJ mice with 200 μg of either Ino 215–234 or OVA as an irrelevant control Ag (Sigma-Aldrich), splenocytes were activated in vitro with 20 μg/ml of each immune Ag in 5 × 10^6 cells/ml in 24-well flat-bottom Falcon plates (BD Biosciences) in a total volume of 2.0 ml/well in DMEM supplemented as described above. After 4 days, 2 × 10^5 activated cells were injected i.v. into naive female recipients. In some experiments, CD4+ T cells were positively purified from activated cultures by incubation of cells with anti-CD4-coated magnetic beads and passage through a MACS LS column using a MidiMACS...
Responses were restricted by IA s because 10 day-cells enriched /H11022 recipients received four 300- and OVA-immunized mice were collected by intracardiac bleeding. Naive peptide immunogen. were tested for recall proliferative responses to each tetrapeptide motif. Ten days after immunization, LNC Th1-like phenotype with elevated production of IFN-

Neutralization experiments using LβT2-immortalized cells

LβT2 putitary cells were provided by Dr. P. L. Mellon (University of California, San Diego, CA) and were grown as previously described (39) in 25-ml T flasks (Corning) to ~50% confluence in DMEM containing 4500 mg/L glucose (Mediatech CellGro), 110 mg/L pyruvate, 548 mg/L t-glutamine (Invitrogen Life Technologies), 10% FBS (HyClone), and 1% penicillin/streptomycin (Invitrogen Life Technologies). Cells were maintained at 37°C in 95% air and 5% CO2. Before testing, LβT2 cells were grown in DMEM growth medium without serum. Cell concentrations were adjusted to 2.5–3.5 × 10^6 cells/microtiter well in a volume of 180 μl. To this volume, 20 μl of varying dilutions of sera were added to a final volume of 200 μl. The diluted sera also contained 25 ng/ml recombinant activin-A (R&D Systems) for inducing FSH production and varying concentrations of recombinant inhibin-A (DSL) for determining whether sera contained neutralizing Ab capable of preventing inhibin-A from inhibiting activin-A-induced FSH production. After 2 days, supernatant FSH levels were measured by competitive ELISA as described previously. A total of 20 μl of PBS and 20 μl of charcoal/dextran-treated FBS (HyClone) were used as additional controls. LβT2 cells showed >90% viability whether cultured in growth medium alone or in growth medium supplemented with 25 ng/ml activin and 25 ng/ml inhibin.

Statistical analysis

The unpaired Student t test was used to analyze differences in fertility, estrous cycle, follicle numbers, and hormone levels between inhibin-α and control-immunized mice.

Results

Induction of EAO in SWXJ mice immunized with In215–234
To identify immunogenic inhibin-α peptides, we applied the -KXXS- tetrapeptide sequence motif associated with IA^α- and IA^β- restricted CD4^+ immunogenicity. Peptides containing a tetrapeptide sequence with a lysine or conservatively substituted arginine residue separated by two irrelevant amino acids from a serine residue have been shown to be immunogenic and capable of inducing several CD4^+ T cell-mediated autoimmune diseases in SWXJ (H-2^k), SWR/J (H-2^p), and SJL/J (H-2^s) mice (29).

We synthesized several -KXXS- containing 20-mer peptides derived from the known sequence of mouse inhibin-α (27, 28) and immunized female SWXJ mice with 200 μg of each peptide in CFA. One of the selected peptides, In215–234 elicited a substantial recall proliferative response from 10 day-primed LNC, whereas the remaining peptides were relatively nonimmunogenic (Fig. 1A). ELISA analysis of 48 h culture supernatants showed that recall responses to In215–234 involved the proinflammatory Th1-like phenotype with elevated production of IFN-γ and IL-2 and minimal production of IL-5 and IL-10. C, Responses to In215–234 were confined to CD4^+ T cells enriched >90% by magnetic bead separation. D, Responses were restricted by IA^α because 10 day-primed LNC from parental SJL/J but not SJL/J mice responded to the peptide. E, Ovarian tissue sections immunostained with CD3 Ab show perifollicular infiltration of CD3^+ T cells 8 wk (arrow, left panel) and 12 wk (arrow, middle panel) after immunization with In215–234. Infiltration of CD3^+ T cells was never observed in any control mice immunized with CFA alone (right panel). All figures are representative of seven to eight mice examined in each group at each time point. Solid bar, 50 μm. All error bars indicate ± SD.

FIGURE 1. In215–234 induces EAO in SWXJ mice. Female SWXJ mice were immunized with selected mouse inhibin-α peptides expressing the -KXXS- tetrapeptide motif. Ten days after immunization, LNC were tested for recall proliferative responses to each peptide immunogen. A, Peptide In215–234 elicited marked dose-response immunoreactivity. B, ELISA analysis of 48-h culture supernatants showed that recall responses to In215–234 involved the proinflammatory Th1-like phenotype with elevated production of IFN-γ and IL-2 and minimal production of IL-5 and IL-10. C, Responses to In215–234 were confined to CD4^+ T cells enriched >90% by magnetic bead separation. D, Responses were restricted by IA^α because 10 day-primed LNC from parental SJL/J but not SJL/J mice responded to the peptide. E, Ovarian tissue sections immunostained with CD3 Ab show perifollicular infiltration of CD3^+ T cells 8 wk (arrow, left panel) and 12 wk (arrow, middle panel) after immunization with In215–234. Infiltration of CD3^+ T cells was never observed in any control mice immunized with CFA alone (right panel). All figures are representative of seven to eight mice examined in each group at each time point. Solid bar, 50 μm. All error bars indicate ± SD.
and IL-2 and minimal production of IL-5 and IL-10 (Fig. 1B). When CD4+ and CD8+ T cells from In215–234-primed mice were enriched >90% by magnetic bead separation and stimulated with peptide on a gamma-irradiated (2000 rad) splenocyte monolayer, recall responses were confined to the CD4+ subpopulation (Fig. 1C). Responsiveness to In215–234 was elicited by 10 day-primed LNC from peptide-immunized SWXJ and SJL/J mice but not from parental SWR/J mice indicating that the response to In215–234 was restricted by IA+ (Fig. 1D).

To determine whether the immunogenic In215–234 peptide was capable of inducing EAO, we immunized female SWXJ mice with 200 μg of the peptide in CFA and assessed disease induction by immunocytochemical analysis of ovarian tissue 8 and 12 wk after immunization. We consistently observed infiltration of CD3+ T cells in the perifollicular granulosa cell region of ovarian follicles 8 wk (Fig. 1E, left panel) and 12 wk (Fig. 1E, middle panel) after immunization with In215–234. In contrast, we never observed ovarian infiltrates of CD3+ T cells in mice immunized with CFA alone (Fig. 1E, right panel). The ovarian T cell infiltrations in In215–234-immunized mice are consistent with an induced autoimmune oophoritis.

**EAO mice have longer estrous cycles**

Vaginal smears show changes in the structure of the vaginal epithelium and follow a regular and predictable sequence across the course of each estrous cycle. To determine the effect of In215–234 immunization on the estrous cycle, vaginal smears were obtained daily and examined for changes in their cellular content over several estrous cycles. The mean duration of each estrous cycle as well as the length of time spent in each stage were determined for peptide (Fig. 2A) and CFA-primed mice (Fig. 2B). Mice immunized with In215–234 had significantly longer estrous cycles (p = 0.017) than mice immunized with CFA alone (Fig. 2C). The mean length of the estrous cycle in In215–234-immunized mice was 8.5 ± 0.441 days whereas the mean duration of each estrous cycle in control mice immunized with CFA alone was 7.275 ± 0.248 days. The lengthening of the estrous cycle in In215–234-immunized mice involved a significant shortening (p = 0.043) of the duration of proestrus-estrus (mean 2.609 ± 0.17 days) compared with mice immunized with CFA alone (mean 3.125 ± 0.179 days, Fig. 2D) and a significant (p = 0.002) lengthening of metestrus-diestrus (mean 5.9 ± 0.416 days) compared with controls (mean 4.15 ± 0.17 days). Thus, EAO lengthens the estrous cycle by prolonging metestrus-diestrus associated with follicle development and shortening proestrus-estrus associated with ovulation and fertilization.

**EAO mice have increased numbers of ovarian follicles**

We next determined the impact of In215–234 immunization on the number of ovarian follicles. Four weeks after immunization

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**FIGURE 2.** Immunization with In215–234 prolongs the estrous cycle and increases FSH and follicle production. Vaginal smears were examined over five estrous cycles starting 4 wk after immunization of SWXJ mice with (A) In215–234 or (B) CFA alone. Each vertical bar represents five estrous cycles of a single mouse with each completed estrous cycle represented by adjoining bars of solid (proestrus-estrus) and diagonally striped (metestrus-diestrus) segments. Horizontal dotted lines are for reference only. C. Immunization with In215–234 increases the duration of the estrous cycle (n = 40; p = 0.17) by (D) shortening proestrus-estrus (p = 0.04) and lengthening metestrus-diestrus (p = 0.002). E. Four weeks after immunization, total follicle numbers as well as small, medium, large, and atretic follicles were significantly increased in In215–234-immunized mice (p < 1 × 10^-6 in all group comparisons). F. During estrus, In215–234-immunized mice had significantly increased activin (left panel, p = 0.01) and inhibin (right panel, p = 0.03) serum levels. Serum inhibin was also significantly elevated during metestrus (right panel, p = 0.03). G. Serum FSH levels were also significantly elevated during estrus at both the early stage (7–9 wk; p = 0.01) and late stage (43–45 wk; p = 0.05) of EAO induced by immunization with In215–234. All error bars indicate ± SD.
with either Inα 215–234 or CFA alone, ovarian tissue sections were examined for the presence of follicles. Follicles were counted under light microscopy in every 10th 5-μm H&E-stained section to avoid counting the same follicle twice. We found that the differences in the mean total number of follicles, as well as in the mean numbers of small, medium, large, and atretic follicles were highly significant ($p < 1 \times 10^{-6}$ in all cases) between mice immunized with Inα 215–234 and mice immunized with CFA alone (Fig. 2E). The mean numbers of total (433.52 ± 12.57), small (305.91 ± 12.1), medium (86.16 ± 6.4), large (41.41 ± 2.1), and atretic (32.41 ± 2.4) follicles in Inα 215–234-immunized mice were higher than the mean numbers of total (214.41 ± 14.13), small (158.25 ± 11.9), medium (36.25 ± 2.6), large (19.91 ± 0.9), and atretic (10.5 ± 1.24) follicles in CFA-immunized control mice. It is worth noting that there were no apparent morphologic differences in the appearance of follicles within each group. Thus, the increase in follicle numbers is consistent with the view that immunization with Inα 215–234 resulted in superovulation.

**EAO mice have elevated serum levels of FSH, activin, and inhibitin**

We next examined whether EAO alters the level of hormones involved in pituitary-gonadal regulation. At different times after immunization, sera were collected from EAO and control-immunized mice at estrus and metestrus. By 4 wk after immunization, serum concentrations of activin-A were significantly higher ($p = 0.01$) in Inα 215–234-immunized mice compared with controls during the estrus stage but not during metestrus (Fig. 2F, left panel). In contrast, serum levels of inhibin-α at 4 wk (Fig. 2E, right panel) were significantly elevated in Inα 215–234-immunized mice compared with controls during both estrus ($p = 0.03$) and metestrus ($p = 0.03$). Serum FSH levels (Fig. 2G) were also significantly elevated during estrus at both the early stage (7–9 wk; $p = 0.01$) and late stage (43–45 wk; $p = 0.05$) of EAO induced by immunization with Inα 215–234. Thus, despite the fact that serum inhibin concentrations were elevated during the entire course of the estrous cycle, their increased levels were unable to prevent the activin-induced FSH surge that was evident during estrus.

**EAO is biphasic with early increased fertility and delayed ovarian failure**

We next examined the reproductive phenotype of female SWXJ mice immunized with Inα 215–234. Test and age-matched control mice were mated to the same males for the same period of time and the number and weight of offspring were determined. We found that mice mated 7–9 wk after immunization with Inα-215–234 showed significantly increased fertility as determined by differences in mean litter size ($p = 0.003$) and mean litter weights ($p = 0.003$) with no significant difference in mean birth weights ($p = 0.10$; Table I, upper). In contrast, when mated at 43–45 wk after immunization, the same Inα 215–234-immunized mice showed dramatically opposite results with significant decreased fertility determined by differences in mean litter size ($p = 0.0002$) and mean litter weights ($p = 0.0004$). Again, there was no significant difference in mean birth weights between both treatment groups ($p = 0.76$; Table I, lower).

When sequential litter sizes are plotted with time after immunization, it is readily apparent that the early enhanced fertility as measured by mean litter size is ephemeral in Inα 215–234-immunized mice and declines gradually with time (Fig. 3A). The early enhanced fertility in Inα 215–234-immunized mice was accompanied by hypertrophic ovaries with increased numbers of follicles and an overall morphology consistent with superovulation (Fig. 3B, upper left). In sharp contrast, ovaries from mice taken 43–45 wk after immunization with Inα 215–234 consistently appeared atrophic with few follicles (Fig. 3B, lower left), a morphology consistent with POF particularly because CFA-immunized control mice showed neither a substantial decline in litter size or observable differences in ovarian morphology over the course of the study (Fig. 3B, upper and lower right).

**Passive transfer of EAO with B cells or sera from Inα 215–234-primed mice**

We next determined whether EAO could be transferred into naive recipients either with activated whole splenocytes, activated CD4+ T cells, nonactivated B cells, or sera. SWXJ female mice were immunized with either 200 μg of Inα 215–234 in CFA or with 200 μg of OVA as an irrelevant control Ag. Four weeks after immunization, splenocytes were activated for 4 days with 20 μg/ml priming Ag and 2–4 × 10^7 cells were injected into naive nonirradiated SWXJ recipients. In some experiments, CD4+ T cells were positively selected from Ag-activated cultures by magnetic bead separation, and the purified (>90–95%) CD4+ T cells were injected at 2–4 × 10^7 cells into naive SWXJ recipients. Similarly, 4 wk after immunization, B cells were purified (>90%) directly from spleens of primed mice without any prior in vitro activation using B220 microbead cell separation on a Midi MACS column (Miltenyi Biotec). The purified B cells were injected at 2–4 × 10^7 cells/naive recipient. Finally, 4 wk after immunization, sera from mice immunized with either Inα 215–234 or OVA were injected into naive recipient mice who received a total of four injections of 300 μl of sera administered every other day. Four weeks after transfer of cells or sera, female mice from test and control groups were mated with the same male mice for determining the impact of immunization on fertility and litter size.

We found significantly increased litter sizes in mice that received peptide-activated splenocytes ($p = 0.003$), nonactivated B cells of Inα 215–234-immunized mice, and anti-Inα 215–234 sera. Interestingly, nonactivated B cells were inoculated at steady state and activated with OVA as control Ag. The increased size of litters in recipients given activated whole splenocytes, activated CD4+ T cells, and nonactivated B cells or sera from Inα 215–234-immunized mice was accompanied by typical atrophic ovaries with few follicles (Fig. 3C, upper left). In contrast, females from control recipients showed neither a substantial decline in litter size or observable differences in ovarian morphology over the course of the study (Fig. 3C, upper right).

### Table I. Inα 215-234-induced biphasic EAO with early increased fertility and delayed POF

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<td>9.5 ± 0.35</td>
<td>15.23 ± 0.82</td>
<td>11.05 ± 0.77</td>
<td>9.5 ± 0.35</td>
<td>9.5 ± 0.35</td>
</tr>
<tr>
<td>Mean Litter Weight (± SD)</td>
<td>p = 0.0002</td>
<td>p = 0.0004</td>
<td>p = 0.0002</td>
<td>p = 0.0004</td>
<td>p = 0.0002</td>
<td>p = 0.0004</td>
<td>p = 0.0002</td>
<td>p = 0.0004</td>
</tr>
<tr>
<td>Mean Birth Weight (± SD)</td>
<td>1.51 ± 0.00</td>
<td>1.49 ± 0.03</td>
<td>1.51 ± 0.00</td>
<td>1.49 ± 0.03</td>
<td>1.51 ± 0.00</td>
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cells (p = 0.003), or sera (p = 0.0009) from Inα 215–234-immunized mice, but did not occur in mice that received Ag-activated CD4+ T cells (p = 0.37; Fig. 4A) from peptide-immunized mice. Our data fulfill Koch’s postulate by showing a classic pattern of humoral-mediated disease thereby indicating that the EAO enhanced fertility induced by immunization with Inα 215–234 is a CD4-initiated event ultimately mediated by inhibin-α autoantibodies.

**EAO results in high inhibin-α Ab titers**

We next characterized the inhibin-α autoantibodies generated during the period of enhanced fertility. Eight weeks after immunization with either Inα 215–234 or CFA, sera were collected at diestrus and serially diluted for determining isotype titers by direct ELISA. High Inα 215–234 Ab titers involving all tested isotypes were evident in Inα 215–234-immunized mice (Fig. 4B, left) but not in CFA control-immunized mice (Fig. 4B, right). Titers were often remarkably high, and in several mice immunoreactivity was clearly detectable at dilutions exceeding 1/64,000. The predominant isotypes responding to Inα 215–234 included IgG2b, an Ab isotype predominantly induced by TGFβ, and IgG1, a Th2-associated Ab isotype predominantly induced by IL-4 and inhibited by IFN-γ. Surprisingly, the Th1-associated Ab isotypes IgG2α and IgG3 known to be induced by IFN-γ and inhibited by IL-4 were somewhat underrepresented in the response to Inα 215–234. Perhaps, the response to this potent self-immunogen was so robust that it induced a broad spectrum of both Th1- and Th2-associated Ab isotypes. Nevertheless, a systemic autoantibody response to Inα 215–234 involving a broad spectrum of Ab isotypes was readily detectable during the period when mice showed enhanced fertility.

**EAO is mediated by Ab neutralization of inhibin-α**

As indicated previously, serum inhibin-α concentrations were elevated during the entire course of the estrous cycle, yet their increased levels were unable to prevent the activin-induced FSH surge that was evident during estrus and the superovulation and increased litter sizes that define the observed enhanced fertility in mice immunized with Inα 215–234. We hypothesized that this apparent paradoxical finding may be due to Ab-mediated neutralization of inhibin-α leading to its failure to down-regulate activin-induced FSH release. Thus, inhibin may be up-regulated and antigenically available for detection but biologically unavailable for antagonizing activin induction of FSH.

To test this hypothesis, we used the LβT2 gonadotroph cells which are mouse pituitary cells immortalized by transformation with a rat LHβ promoter linked to the protein coding sequences of the SV40 T-Ag (Tag) oncogene (40). Inhibin ordinarily inhibits activin-induced secretion of FSH from LβT2 cells (41). LβT2 cells were stimulated with 25 ng/ml recombinant activin in the presence of sera from mice immunized 8 wk prior with either Inα 215–234 or CFA. Increasing doses of recombinant inhibin were added to these cultures to determine the dose at which inhibin inhibited activin-induced FSH release from the LβT2 cells. In cultures containing control sera from CFA-primed mice, increasing doses of inhibin were able to inhibit activin-induced FSH release from LβT2 cells, and complete inhibition of activin-induced FSH production was clearly evident when inhibin concentrations reached 20 ng/ml (Fig. 5). In contrast, increasing concentrations of inhibin up to 25 ng/ml had virtually no inhibitory effect on activin-induced FSH release in cultures containing sera from Inα 215–234-immunized mice. Thus, sera from Inα 215–234-immunized mice contained high...
The importance of our study lies in the fact that the ultimate infertility outcome mimics human POF because these affected mice show decreased fertility in the presence of elevated FSH levels, the hallmark features of human POF. Other models used for human POF include those that target ZP oocyte Ags resulting in autoimmune destruction of oocytes (8, 9, 11–14) and those that involve the immune regulatory failure occurring in athymic or neonatally thymectomized mice (15–17). However, it is currently unclear whether any of these other POF models have documented infertility outcome mimics human POF. It is widely believed that enzymes involved in steroid production may be likely targets in human POF, particularly because the primary targets in human POF appear to be the steroid-producing cells rather than cells of the primordial or secondary follicles. Thus, our inhibin-targeted granulosa cell-targeted EAO model serves as a useful mimic of human POF, a disorder that affects an estimated 1% of women in their childbearing years and is a prominent women’s health risk and cause of human infertility (1, 2, 42, 43).

Despite the fact that inhibin-targeted autoimmunity mimics POF, there are surprisingly no definitive studies that have investigated whether inhibin autoantibodies occur in POF. It is widely believed that enzymes involved in steroid production may be likely targets in human POF, particularly in cases associated with Addison’s disease. Such autoantibodies have been characterized as steroid cell Abs including Abs to P450-17α-hydroxylase and its side chain cleavage product (7, 44). Abs to FSH and LH hormones and to FSH and LH receptors have been reported in POF but only in a small number of patients (45–48). Similarly, autoantibodies specific for ZP proteins occur in <2.5% of POF patients (49). Therefore, it would seem reasonable to determine whether inhibin...
autoantibodies occur in POF sera. These experiments are currently under way in our laboratory.

Mediation of human POF by inhibin autoantibodies has some substantial clinical implications not the least of which is the fact that it implies that the period of infertility is preceded by a period of enhanced fertility. It is possible that this stage of increased fertility may not be detected particularly if the affected women are attempting to have children. Thus, a woman in her 20s may undergo a clinically silent period of superovulation and enhanced fertility that remains undetectable. Upon reaching her 30s, the woman may repeatedly fail to bear children at which time she seeks medical intervention and discovers that her infertility is associated with altered pituitary-ovarian regulation and high serum FSH levels. Given this scenario, human POF may possibly be biphasic with an early clinically silent and undetected period of enhanced fertility followed by a detected later period of infertility with accompanying elevated serum FSH levels. The early period of enhanced fertility would likely be detected only if the woman became pregnant and had multiple births. This scenario provides a most interesting perspective that spontaneous multiple births may in some cases be autoimmune-mediated events.

Another clinical implication of our findings lies in the potential of inhibin Abs to mediate superovulation, the major objective of in vitro fertilization (IVF) programs. Currently, IVF programs achieve superovulation by administering an expensive array of menotropin and gonadotropin hormone treatments over an extended period of time. The hormone treatment approach is effective in stimulating ovaries, yet it is quite costly and it is associated with some health risks, most notably ovarian hyperstimulation syndrome (50). It may be possible to replace the hormone-mediated superovulation approach with an Ab approach particularly one with an inherent or bioengineered short half-life.

Finally, an important question remaining to be addressed is the issue of whether both phases of EAO are mediated by transfer of B cells and/or sera, whereas in the latter case, the development of POF would depend exclusively on the transfer of primed B cells and/or sera, whereas in the latter case, the development of POF would be accelerated in the presence of inhibin-activated CD4+ T cells. Experiments designed to address this issue are currently underway.

In summary, we have found that autoimmune-mediated disruption of the pituitary-gonadal regulatory axis causes a form of murine POF that closely mimics human POF. This finding has substantial implications for understanding human POF and for developing effective alternative therapies for the treatment of autoimmune-mediated infertility.

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Disclosures
The authors have no financial conflict of interest.

References


