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*J Immunol* 2014; 193:161-169; Prepublished online 23 May 2014;
doi: 10.4049/jimmunol.1400381
http://www.jimmunol.org/content/193/1/161

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Unique Temporal and Spatial Expression Patterns of IL-33 in Ovaries during Ovulation and Estrous Cycle Are Associated with Ovarian Tissue Homeostasis

Colin I. Carlock,* Jean Wu,* Cindy Zhou,*,1 Kiana Tatum,* Henry P. Adams,† Filemon Tan,‡ and Yahuan Lou*

Ovaries are among the most active organs. Frequently occurring events such as ovulation and ovarian atresia are accompanied with tissue destruction and repairing. Critical roles of immune cells or molecules in those events have been well recognized. IL-33 is a new member of the IL-1 cytokine gene family. Recent studies suggest its roles beyond immune responses. We systemically examined its expression in ovaries for its potential roles in ovarian functions. During ovulation, a high level of IL-33 was transiently expressed, making it the most significantly upregulated immune gene. During estrous cycle, IL-33 expression levels fluctuated along with numbers of ovarian macrophages and atresia waves. Cells with nuclear form of IL-33 (nIL-33) were mostly endothelial cells of veins, either in the inner layer of theca of ovulating follicles during ovulation, or surrounding follicles during estrous cycle. Changes in number of nIL-33 cells showed a tendency similar to that in IL-33 mRNA level during estrous cycle. However, the cell number sharply declined before a rapid increase of macrophages and a surge of atresia. The decline in nIL-33 cell number was coincident with detection of higher level of the cytokine form of IL-33 by Western blot, suggesting a release of cytokine form of IL-33 before the surge of macrophage migration and atresia. IL-33 Ab, either by passive transfer or immunization, showed a limited effect on ovulation or atresia. It raises a possibility of IL-33’s role in tissue homeostasis after ovarian events, instead of a direct involvement in ovarian functions. The Journal of Immunology, 2014, 193: 161–169.

INTERLEUKIN-33, which was discovered in 2003, is the newest member of the IL-1 cytokine gene family (1–3). Because it was first discovered in nuclei of endothelial cells of the high endothelial venules (HEV) of lymph nodes, IL-33 was originally named NF-HEV (1). Structurally, IL-33 contains two domains: a histone-binding domain and an IL-1–like cytokine domain (4). Thus, newly synthesized IL-33 will be translocated into nucleus. Although the caspase system cleaves the protein, the generated fragment has no typical IL-1 cytokine activity, which is unusual among members in this family (5). A recent study showed that cleavage of IL-33 was through a different mechanism, and this cleaved fragment showed activity (6). Numerous studies have been devoted to investigating its role in immunoregulation. Those results demonstrated a regulatory role in innate immunity, as well as in Th2 T cell response, with ST2 as its receptor (7–10). More recently, IL-33 has been shown to play a role as an alarmin during viral infection (11). It has been reported that the cytokine form of IL-33 may be released from necrotic cells to induce immune response, and acts as a “danger signal” (12). In contrast, mounting evidence has suggested its role beyond immune responses (13). For example, its expression has been detected widely in many human and mouse tissues (14, 15). However, it remains unclear what the role of widely expressing IL-33 is, and whether it plays a role in physiological processes.

Ovaries are among the most active organs with frequent occurrence of physiological events such as ovulation, ovarian atresia, and luteolysis (16). All these events are accompanied with tissue destruction and massive cell death. Multiple events such as ovulation have been reported to resemble inflammatory response (17–19). The involvement of macrophages or other leukocytes in luteinization and ovulation has been well demonstrated (20, 21). Unlike pathological inflammations, such “physiological” inflammations must be well controlled to avoid pathological consequences such as autoimmunity. Therefore, a different set of immune molecules or cells is most likely to be involved in such ovarian processes. As an essential step to understand how the immune system is involved in those physiological processes in ovaries, many studies have been devoted to examining various leukocyte subsets such as macrophages, dendritic cells, and T cells, and related immune molecules such as cytokines and chemokines (22–26). Huge progress has been made in the last decade. However, the functions of those immune cells or related molecules largely remain to be explored. In addition, the discovery of new immune molecules will surely add more dimensions for exploration.

Immune molecules, which include cytokines, chemokines, adhesion molecules, and others, have been shown to play critical roles in ovarian functions (27–29). Cytokines or growth factors may be directly involved in ovarian functions such as follicle development or apoptosis (29). In contrast, leukocyte trafficking-related mole-
cules such as chemokines and adhesion molecules recruit special immune cells during physiological processes such as ovulation (28). However, expression of these trafficking-related molecules is often regulated by cytokines. Thus, investigation of ovarian expression of cytokines is especially important. Many immune molecules including cytokines have been investigated (27–31). As a newly discovered cytokine, as well as its wide expression in nonlymphoid tissues or organs, we have realized the necessity of systemic examination of expression of IL-33 in ovaries during different ovarian events. With a focus on ovulation and estrous cycle, this study has investigated its temporal and spatial expression pattern in ovaries. Our results revealed a transient high-level expression of IL-33 during ovulation and a unique fluctuation of IL-33 expression during estrous cycle. Those observations suggest IL-33’s involvement in those ovarian processes.

Materials and Methods

**Mice and their treatment**

BALB/c females (4–18 wk) were purchased from Harlan (Indianapolis, IN). Mice of 15–17 wk were used for most experiments if their ages are not mentioned. The mice were maintained in the animal facility at the University of Texas Health Science Center at Houston and allowed to acclimate for a minimum of 7 d. All animal procedures in this study were approved by institutional animal welfare committee. In some experiments, estrous cycle was determined in experimental mice by vaginal smear sampled at 6:00 AM following a published method (32). Ovaries were harvested and fixed in PFA or snap-frozen in liquid nitrogen. In some cases, ovaries were used for isolation of total RNA with a kit from Ambion (Austin, TX). For induction of superovulation in mice, a previously published method was adapted (26). In brief, the animals were injected with equine chorionic gonadotropin (pregnant mare’s serum gonadotropin; Sigma-Aldrich, St. Louis, MO) at 5 IU/mouse i.p. After 48 h, the mice were injected i.p. with human chorionic gonadotropin (hCG; 5 IU/mouse; Sigma-Aldrich). For injection of IL-33 Ab, each mouse was injected through tail i.v. at a dose of 0.1 mg/mouse.

**Antibodies**

The following Abs were purchased from BD Biosciences (San Jose, CA): biotin-labeled anti-mouse MHC class II molecules IA/IE (rat IgG2a, 2G9), Alexa 647-labeled anti-mouse CD11b/Mac-1 (M1/70), and FITC-labeled anti-mouse CD31 (clone 390). Alexa 488-labeled anti-mouse F4/80 (rat IgG2a, BMS) was obtained from Biolegend (San Diego, CA). Biotinylated goat anti-mouse IL-33 Ab and rat anti-mouse IL-33 mAb (neutralizing; clone 396118) were from R&D Systems (Minneapolis, MN) or ProSci (Charleston, SC). Another anti–IL-33 mAb (Nessy-1) was from Alexis (New Hyde Park, NY). Goat antimouse IL-33 Ab and rat anti-mouse IL-33 mAb (neutralizing; clone 396118) were from R&D Systems (Minneapolis, MN) or ProSci (Charleston, SC). Another anti–IL-33 mAb (Nessy-1) was from Alexis (New Hyde Park, NY). Anti–CD11b Abs that were directly conjugated to a fluorescent dye were used. If one biotin-labeled Ab was used, this Ab was used for the first staining after blocking with a biotin and avidin blocking kit from Vector BioLab (Philadelphia, PA). Fluorescent dye-labeled streptavidin was then used as secondary reagent. The ovarian sections were observed by a fluorescent microscope (Nikon Eclipse Ti). Fluorescent images were analyzed with NIS Elements software (Nikon, Tokyo, Japan) and digital images were captured and analyzed with NIS Elements software (Nikon, Tokyo, Japan). Western blot

**Quantitative PCR detection of IL-33 mRNA and cloning of ovarian IL-33**

cDNA was synthesized using 1 μg total RNA through a reverse transcription (RT) reaction (RNA PCR Core Kit; Applied Biosystems, Foster City, CA). Conventional PCR was carried out to detect IL-33 mRNA using a pair of primers (5′-CGTGGTGCCTGGTTGACACCATT-3′ and 5′-CTTAGGAGGCGACCACTCCAC-3′), which resulted in a 202-bp product. PCR was carried out under the following conditions: preheating at 94°C for 3 min, followed by 35 cycles of PCR (94°C 1 min, 58°C 30 s, 72°C 1 min; GeneAmp9700; Applied Biosystems, Foster City, CA). The products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light illumination. Digital images were captured by an imaging analyzer (Perkin-Elmer, Waltham, MA). Quantitative PCR (Q-PCR) was performed with a pair of primers (5′-CGTGGTGCCTGGTTGACACCATT-3′ and 5′-CTTAGGAGGCGACCACTCCAC-3′) under a similar condition as for conventional PCR using SYBR Green system (SuperArray Bioscience, Frederick, MD) on iCycler-iQ thermocycler (BioRad, Hercules, CA). Relative abundance was calculated as

\[2^{\Delta C_{T}}\] with a sample as standard following a published method (30). A housekeeper gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a control with a pair of primers (5′-CGTGGTGCCTGGTTGACACCATT-3′ and 5′-GGTTAGGAGGCGACCACTCCAC-3′) used for 35 cycles of PCR (94°C 1 min, 58°C 30 s, 72°C 1 min; GeneAmp9700; Applied Biosystems, Foster City, CA).

For PCR cloning of ovarian IL-33 cDNA, a high-fidelity DNA polymerase (Platinum pfu; Invitrogen, Carlsbad, CA) was used with a pair of primers to cover majority of IL-33 transcript (5′-CTACACTGACCTCTTACACTGAAGATGGTGTC-3′ and 5′-GTCGAGGGGCCATATCCACAAACC-3′).

For PCR cloning of ovarian IL-33 cDNA, a high-fidelity DNA polymerase (Platinum pfu; Invitrogen, Carlsbad, CA) was used with a pair of primers to cover majority of IL-33 transcript (5′-CTACACTGACCTCTTACACTGAAGATGGTGTC-3′ and 5′-GTCGAGGGGCCATATCCACAAACC-3′).

**rIL-33 and immunization**

puET101/D-TOPO vector with insert of cDNA of whole open reading frame of IL-33 as described earlier was used for expression of mouse rIL-33 following our previously published method (26). The rIL-33 was further purified with preparative SDS-PAGE. In brief, the whole lysate was loaded to preparative SDS-PAGE gel. Gel was immersed into 4 M sodium acetate to visualize proteins. The rIL-33 bands were sliced out and rIL-33 was eluted from the gel by electrophoresis. After dialyzing against PBS, the protein was lyophilized. The purity of rIL-33 was checked by SDS-PAGE and further verified by Western blot using anti-rIL-33 Ab. For immunization, rIL-33 (5mg/ml) in PBS was mixed with CFA at 1:1. Each mouse received 50 μl of the mixture. Another group of mice was immunized with CFA alone. Mice were bled from tail veins at 24 and 36 d, and their sera Ab to rIL-33 was tittered by Western blot through serial dilution. Those mice with a titer higher than 1:1600 were used for superovulation.

**Immunofluorescence, TUNEL staining, and quantitation of cells on sections**

Ovaries, fixed or nonfixed depending on activity of the Abs to be used, were frozen and 3-μm frozen sections were cut. Before staining, all sections were blocked in 3% BSA withAbs to Cd16/32. For multicolor staining, Abs that were directly conjugated to a fluorescent dye were used. If one biotin-labeled Ab was used, this Ab was used for the first staining after blocking with a biotin and avidin blocking kit from Vector BioLab (Philadelphia, PA). Fluorescent dye-labeled streptavidin was then used as secondary reagent. The ovarian sections were observed by a fluorescent microscope (Nikon Eclipse Ti). Fluorescent images were analyzed with NIS Elements software (Nikon, Tokyo, Japan). Digital images were captured and analyzed with NIS Elements software (Nikon, Tokyo, Japan). Western blot

Ovaries were harvested and immediately homogenized on ice in an extraction buffer containing a protease inhibitors mixture (Sigma-Aldrich). After centrifugation at 10,000 × g for 15 min at 4°C, the supernatant was carefully removed and its protein concentration measured. The ovarian extracts were mixed 1:1 with SDS sample buffer. Ten micrograms proteins was loaded on a 12.5% SDS-PAGE and ran at a constant current. After transfer, the membrane (Immobilon-P PVDF; Millipore, Billerica, MA) was first incubated with biotin-labeled anti-IL-33 Ab followed by incubation with IRDye800CW-labeled streptavidin (LI-COR, Lincoln, NE). The membrane was scanned on an infrared fluorescence scanner (Odyssey; LI-COR).

**Detection of genome-wide gene expression in ovaries**

Three hundred nanograms of Total RNA were amplified and purified using Illumina TotalPrep RNA Amplification Kit (Illumina, San Diego, CA) following kit instructions. RNase H and DNA polymerase master mix were immediately added into the reaction mix after RT and were incubated for 2 h
at 16°C to synthesize second-strand cDNA. In vitro transcription was performed and biotinylated cRNA was synthesized by 14-h amplification with dNTP mix containing biotin-dUTP and T7 RNA polymerase. Amplified cRNA was subsequently purified and concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). An aliquot of 750 ng of amplified products was loaded onto Illumina Sentrix Beadchip Array Mouse Ref_8_v2 arrays, hybridized at 58°C in an Illumina Hybridization Oven (Illumina) for 17 h, washed, and incubated with streptavidin-Cy3 to detect biotin-labeled cRNA on the arrays. Arrays were dried and scanned with BeadArray Reader (Illumina). Data were analyzed using GenomeStudio software (Illumina). Clustering and pathway analysis were performed with GenomeStudio and Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) softwares, respectively.

**Statistics**

t tests (comparison between two groups) and Kruskal–Wallis with Dunn’s posttest (more than three groups) were used. Statistical significances were indicated by *<p < 0.05 or **<p < 0.01. Linear regression test was used for analysis of correlation between ages and IL-33 expression.

**Results**

**A transient high level of ovarian IL-33 expression during hCG-induced superovulation**

Two batches of total RNA from ovaries sampled at 0, 3, 6, 9, and 13 h (immediately after ovulation) post hCG injection from two independent experiments were used for DNA microarray for their global gene expression profiles. In both cases, gene expression pattern analysis showed that the samples from different time points formed distinct clusters, suggesting reliability of those samples (Fig. 1A and 1B). Ranking by significance in upregulation of expression as compared with 0 h shows that IL-33 was the 11th (<p = 3.01 x 10^-6) and 15th (<p = 3.72 x 10^-6) greatest for all genes at 6 h post hCG injection for two sample batches, respectively.

**FIGURE 1.** Ovarian IL-33 expression in mice during hCG-induced ovulation. (A) Clustering analysis based on global gene expression profiles from microarray shows well-clustered samples from different time points post hCG injection. Also note that the gene expression pattern for 6 h is significantly different from other groups. (B) Microarray shows significantly upregulated ovarian IL-33 expression at 6 h post hCG injection as compared with 0 h. Expression levels were expressed as fluorescent intensity. (C) Q-PCR detection of IL-33 mRNA revealed a transient upregulation of IL-33 expression during 3–9 h. A housekeeper gene hprt was used as control. The number of samples at each time point is indicated in parentheses. Kruskal–Wallis test was applied; data for each time point was compared with that for 0 h with Dunn’s posttest. (D) Western blot detection of two forms of IL-33 (30 kDa for nuclear form and 20 kDa for cleaved form) at time point as indicated. Note a higher level of cytokine form of IL-33 at 9 h. This represents similar results from three independent experiments. Statistical significances were indicated by *<p < 0.05 or **<p < 0.01. In both cases, IL-33 was ranked the first for all genes related to the immune system including cytokines, chemokine, adhesion molecules, leukocyte markers, or other related molecules (Table I). However, its significance rank rapidly declined to 438th at 9 h, suggesting very transient expression. Interestingly, gene for ST2, a known IL-33R, was also among top upregulated genes related to the immune system (ninth) at 6 h (Table I). Q-PCR was performed to determine IL-33’s mRNA levels during ovulation in detail. IL-33 expression was gradually upregulated from 0 to 3 h (Fig. 1C). A sharp increase in its expression level was followed and reached the peak at 5 h. At the peak, IL-33 mRNA level was >170-fold greater than at 0 h. Expression levels rapidly decreased from 5 to 9 h. Thus, Q-PCR also revealed a transient high-level expression of IL-33 in the ovaries during a narrow 6-h window (i.e., 3–9 h postinjection). By comparison, expression levels of the housekeeper gene HPRT in ovaries nearly did not change during the entire ovulatory process (Fig. 1C).

We next examined ovarian IL-33 during ovulation at the protein level. Western blots detected two forms of ovarian IL-33, that is, the 30-kDa nuclear form and 20-kDa cleaved cytokine form (Fig. 1D). The nuclear form of IL-33 (nIL-33) at 6 h was more prevalent at 6 h than at any other time points, which matched the IL-33 mRNA peak at 5 h. However, the cleaved form of IL-33 was most abundant at 9 h when IL-33 mRNA had already dropped to a low level, suggesting that the cytokine activity of IL-33 was directly controlled by cleavage and release rather than at a transcriptional level. Immunofluorescence was performed to identify IL-33-expressing cells. This detection was based on the nIL-33. Thus, those cells were designated as nIL-33⁺ cells. During ovulation, nIL-33⁺ cells were present at two tissue locations. First, many nIL-33⁺ cells were found in the interstitial tissues. During the ovulatory process (i.e., 0–13 h), no significant difference was observed based on semiquantitative estimation. Late experiments showed their presence during the nonovulatory period as well; thus, their presence was not related to the ovulatory process. This group of nIL-33⁺ cells will be discussed in detail later. The second group of nIL-33⁺ cells was highly related to ovulatory processes. These nIL-33⁺ cells were only found in ovulating Graafian follicles in a high frequency at 6 h (Fig. 2A). The majority of them were located in the inner layer of theca, and occasionally in the interface between theca and granulosa. Two-color staining revealed that nearly 60% of nIL-33⁺ cells were associated with SM-α-actin or CD31-expressing cells (Fig. 2B and 2C). Thus, nIL-33⁺ cells were most likely endothelial cells in the inner theca layer of ovulating follicles. A rapid reduction in nIL-33⁺ cell number at 9 h was observed. Interestingly, a few cells with cytoplasmic IL-33 were observed. Together with Western blot analysis, the reduction in nIL-33⁺ cell number suggested cleavage of nIL-33 and further release of the cytokine domain of IL-33 at 9 h. As an internal control, nIL-33⁺ cells were never found in the theca of any other follicles, including antral follicles in nonovulating ovaries. A small number of nIL-33⁺ cells (about <30%) were also found to be located in the peripheral of granulosa layer of ovulating follicles at 9 h, a clear distance away from the theca layer (Fig. 2D). Based on the shape of the nuclei, those cells were easily identified to be nongranulosa cells. However, they did not express CD31 or SM-α-actin, or several leukocyte markers. Their identity will need more investigation.

We next cloned ovarian IL-33 to further verify its expression. RNA from ovaries harvested at 6 h post hCG injection was used for the cloning. PCR cloning of IL-33 cDNA was performed. Eight clones from two independent RT-PCRs on two individual mice were submitted for DNA sequencing. Only a single nucleotide mutation was found from G to C, which resulted in a conservative
substitution of valine with leucine at aa 179. No other mutations were found. Thus, cloning and sequencing verified reliability of our PCR detection of IL-33 expression.

**Ovarian IL-33 expression is not correlated with age**

Because we found a group of nIL-33+ cells in nonovulating follicles, we investigated the expression pattern of IL-33 in nonovulating ovaries. IL-33 mRNA was detected in ovaries as young as 4 d by RT-PCR. Its expression in ovaries was compared with those from other organs at 1 wk of age (Fig. 3A). Ovaries of 1 wk after hCG treatment already showed a constant high level of IL-33 expression along with two other organs, the kidneys and lungs. Kidneys showed the highest level of IL-33 mRNA, and ovaries the second highest. IL-33 expression has been reported in the lung in association with allergy (11). However, ovarian IL-33 expression level was higher than that in lung. More than 90% of ovaries randomly sampled after 3 wk also showed significant levels of IL-33 expression. However, the expression levels were irregular and did not show a correlation with aging (Fig. 3B). Irregularity in IL-33 expression level, which was well reflected by a wide range of relative mRNA abundances, suggested a potential correlation with certain ovarian functional processes or events. This prompted us to investigate its expression during the estrous cycle.

**Ovarian IL-33 expression level fluctuates during the estrous cycle**

Female mice were monitored for their estrous cycle by vaginal smear. Three to six mice were used for each stage. Their ovaries were used for analyzing their IL-33 gene expression, the number of nIL-33+ cells, and these cells’ distribution. First, Q-PCR demonstrated a fluctuation of ovarian il33 expression levels. Its expression levels peaked at diestrus, which had a relatively large deviation, and were the lowest at estrus (Dunn’s posttest, estrus versus metestrus, p < 0.01; estrus versus diestrus, p < 0.05 and estrus versus diestrus, p < 0.01; Fig. 4A). We next investigated the quantity of nIL-33+ cells and their tissue locations. To accurately describe the distribution pattern of nIL-33+ cells in the ovaries, immunofluorescent micrographs at ×200 covering a whole ovarian section were merged to form a supermicrograph to identify each nIL-33+ cell (marked as a pink dot) and its location (Fig. 4B). In contrast with during ovulation, nIL-33+ cells were located in the interstitial tissues during nonovulatory phases, but never in any follicles (Fig. 4D). Morphological examination suggested these cells to be endothelial cells of the vein system surrounding developing follicles or in the interstitial tissue (Fig. 4D). Two-color immunofluorescence confirmed that nIL-33+ cells were mostly endothelial cells, because about 70% of those cells expressed CD31 (Fig. 4E). nIL-33+ endothelial cells, which surrounded a developing follicle, did not distribute evenly around the follicle. A cell density gradient was observed, with most at the interior facing toward medulla, and the least facing toward ovarian surface (Fig. 4B). Some nIL-33+ cells were also found in corpus lutea. Density of nIL-33+ cells was calculated on each section. A fluctuation of nIL-33+ cell densities during the estrous cycle was also observed (Fig. 4A). nIL-33+ cells gradually increased their numbers with a tendency similar to IL-

Table I. Most significantly upregulated genes of immune molecules during hCG-induced ovulation based on genome-wide DNA microarray

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Significance Ranking</th>
<th>Full or Other Name</th>
<th>Basic Functions or Associations</th>
</tr>
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<tr>
<td>Il33</td>
<td>11</td>
<td>NF-HEV</td>
<td>Th2 response, alarmin</td>
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<tr>
<td>Clcf1</td>
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<td>Cardiotrophin-like cytokine factor 1</td>
<td>IL-6 expression</td>
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<td>Thy1</td>
<td>194</td>
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<td>Chemokine MCP-3</td>
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<td>TCR</td>
<td>T cells, monocytes</td>
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**FIGURE 2.** Tissue location of ovarian cells with nuclear IL-33 (nIL-33+ cells) in mice during hCG-induced ovulation as detected by immunofluorescence. (A) An ovulating mature follicle shows many nIL-33+ cells (red) around inner layer of its theca. Note that nIL-33 staining (red) is colocalized to the cells, which express SM-α-actin (green). (B) Enlarged view of colocalization of nIL-33 (red) with SM-α-actin (green) cells. (C) nIL-33+ cells (red) in the theca of ovulating follicles also express CD31 (green). (D) A group of nIL-33+ cells (red) are mingled with granulosa cells of an ovulating follicle with a distance from SM-α-actin+ cells (green). Note different nuclear morphology of granulosa cells and nIL-33+ cells. An SM-α-actin–associated nIL-33+ cell is also shown (arrow). Nuclei were counterstained by DAPI. Scale bars, 20 μm (B–D).
33 mRNA levels from estrus to diestrus. Unlike IL-33 mRNA levels, which rapidly descended after diestrus, the nIL-33 + cell number continued to increase until proestrus. A dramatic decline in their numbers to the lowest level was observed between proestrus and estrus (estrus versus proestrus \( p, 0.01; \) Fig. 4A).

Estrus had the lowest nIL-33 + cell density among all stages. However, there were no significant differences among those at metestrus, diestrus, and proestrus (Fig. 4A). Because the volume of an ovary may significantly change during the estrous cycle because of follicular development and luteinization, calculation of cell densities may be affected. However, absolute nIL-33 + cell number was again much lower at estrus than at other stages. Sudden reduction in nIL-33 + cell number suggested a release of cytokine form of IL-33. Western blots of ovarian extracts showed that the cleaved form of IL-33 was detected at a much higher level at estrus than that at metestrus (Fig. 4C), despite the lowest number of nIL-33 + cells at estrus. Like the situation at 9 h post hCG injection, endothelial cells with cytoplasmic IL-33 were frequently found (Fig. 4F). In contrast, cells with cytoplasmic IL-33 were rarely present at other stages (Fig. 4G).

Relationship between IL-33 expression and ovarian macrophages or atresia

Because IL-33 is a cytokine, we investigated its relationship with ovarian macrophages. There are at least five macrophage subsets in the ovaries (25). Using two markers (F4/80 + or IA +, or both), we calculated total macrophage number for each individual at each stage (\( n, 3; \) Fig. 5A). Total ovarian macrophages rapidly increased after estrus and reached the peak at metestrus (\( p, 0.05; \) Fig. 5A). A slight but nonsignificant reduction in their number was observed. Macrophage numbers declined between proestrus and estrus. However, the decline was not statistically significant (Fig. 5A). A similar tendency was observed for IA + macrophages. Unlike those for total macrophages, only a few, if any, of IA + macrophages were found in the ovaries during the estrous cycle.

![FIGURE 3.](image-url) IL-33 expression in mouse ovaries of different ages. (A) A conventional RT-PCR shows IL-33 expression at various organs sampled from a 1-wk-old female mouse. Housekeeper gene HPRT was used as a control. (B) Q-PCR detection of IL-33 expression in ovaries of various ages shows variable but significant levels of IL-33 expression in most of them. Regression analysis revealed no correlation between ages and their ovarian IL-33 expression level.

![FIGURE 4.](image-url) IL-33 expression during estrous cycle in mice. (A) Summary of Q-PCR detection of IL-33 mRNA (black dots and line, left y-axis) and numbers of nIL-33 + cells (red dots and line, right y-axis) during estrous cycle. Estrous stage in an individual was determined by vaginal smear; theoretical duration for each stage follows a published article (32). D, diestrus; E, estrus, M, metestrus; P, proestrus. Cell numbers are expressed as cell density (cell number/mm²). Kruskal–Wallis test was applied; data for each group were compared with that for estrus with Dunn’s posttest. Statistical significances were indicated by * \( p < 0.05 \) or ** \( p < 0.01 \). (B) A representative merged superimage covering a whole ovarian section at diestrus after three-color immunofluorescence is shown. Each nIL-33 + cell is labeled by a pink dot for identification. Cytoplasm of oocytes is stained red. Nuclei were counterstained by DAPI. (C) Western blot detection of two forms of IL-33 (30 kDa for nuclear form and 20 kDa for cleaved form) in the ovaries at estrus (E) and metestrus (M). Note a higher level of cytokine form of IL-33 at estrus as compared with the lowest number of nIL-33 + cells during estrous cycle (A) at this stage. (D) Immunofluorescence shows a group of nIL-33 + cells (red) in the veins that surround various follicles (F). (E) Two-color immunofluorescence shows nIL-33 + cells (red) to be CD31 + endothelial cells (green). A, artery; V, vein. (F) A group of cells with cytoplasmic IL-33 (red) are observed in endothelia of a vein (V) at estrus stage. (G) A cell with cytoplasmic IL-33 (arrow, red) is shown to be among many nIL-33 + cells (red) at other stages. Scale bars, 20 μm (D–G).
macrophages were present in the ovaries at estrus. Thus, estrus had a significantly lower number of IA+ macrophages than other stages (for proestrus versus estrus p < 0.05, for estrus versus metestrus p < 0.01; Fig. 5A).

Atresia is an important ovarian process to eliminate no longer needed follicles during the estrous cycle. This process will ensure enough high-quality follicles can mature to be ovulated. We next investigated a potential relationship between IL-33 expression and ovarian atresia with a focus on developing follicles (referred to as follicles in rest of article). Atretic follicles were classified into initial, early to mid with only a few apoptotic cells and many IA+ macrophages; note that the majority of follicular cells are nonapoptotic. An atretic follicle at late stage with IA+ macrophages. Note absence of apoptotic cells. Two obsolete follicles with remnants of ZP (arrows) are also shown. (H) Fluorescent micrograph shows phagocytosis of follicular cells by CD68+ macrophages (purple) at midstage. Note presence of autofluorescence in the follicles (white-green dots) and collapsed zone pellucida (red). Nuclei were counterstained by DAPI. Scale bars, 50 μm.

IL-33 Ab or immunization with rIL-33 has limited effect on ovulation

A transient high-level expression of IL-33 during ovulation suggested its role in ovulation. We investigated whether IL-33 Ab could interfere with ovulation. A neutralizing Ab was i.v. injected into BALB/c female mice, which have previously received equine chorionic gonadotropin, at 3 h of hCG injection. As controls, mice of the same age were injected with normal IgG. Ovulated eggs were harvested and their numbers were compared. IL-33 Ab–treated mice showed a slightly reduced egg number than those of control (Fig. 6A). However, the difference was not insignificant. We conducted another experiment, except the time for IL-33 Ab injection was delayed to 0 h post hCG injection. A marginal significance (p = 0.041) was observed; IL-33 Ab group showed a slightly lower numbers of eggs (Fig. 6B). To confirm whether...
expression levels highly correlated with or fluctuated during special ovarian processes such as ovulation and estrous cycle. Many cytokines and chemokines have been studied for their roles in ovarian events by studying their expression during those events (16, 26–31, 33). Among all those molecules, IL-33 probably is that most significantly correlated or upregulated during a special ovarian event such as estrous cycle and ovulation. It is worthwhile to remember that IL-33 was the most significantly upregulated immune molecule during ovulation. Second, the level of IL-33 expression was not negligible. Many randomly sampled ovaries expressed a higher level of IL-33 than many other organs, apart from those ovaries sampled at IL-33 expression peak in estrous cycle and during ovulation. Both intensity of fluorescence in microarray or Q-PCR analysis on IL-33 expression suggested its expression to be at least at midrange for all genes. At its expression peak, PCR showed that level for ovarian IL-33 was close to that of a housekeeper HPRT. If taking IL-33’s cytokine nature under consideration, it was a relatively high level of expression. Furthermore, both forms of IL-33 were detectable by Western blot in whole ovarian lysate. Third, we have identified distinct tissue locations and cell types for IL-33 expression. Those locations were also closely associated with special ovarian processes. For example, IL-33 was expressed in the inner layer of theca of ovulating follicles during ovulation, although it was mainly expressed in veins that surrounded follicles during estrous cycle. Multiple studies have reported expression of IL-33 in endothelial cells (1, 34). We also showed that those IL-33–expressing cells were endothelial cells.

What is the function of significantly expressed ovarian IL-33? It is well-known that IL-33 plays regulatory roles in promoting innate immunity or Th2-related T cell response (7, 8). However, recent studies have further suggested that IL-33 may also play roles beyond those immunofunctions (3, 11–15). Mounting evidence supports that IL-33 may be involved in a tissue “guarding” system during both traumas and infections (11–13). As a “guarding member,” it is possible that ovarian IL-33 may simply function as a part of the first line of defense, just as in other organs/tissues. However, it is highly questionable because of IL-33’s unique expression pattern during ovarian events. Although this study did not provide direct evidence supporting any role of IL-33 in ovarian functions, our results do suggest several potential functions of IL-33 in ovaries, which may or may not be related to the “guarding” system. First, a sharp transient expression of IL-33 during 3–9 h after hCG injection and IL-33’s expression location in ovulating follicles strongly suggested its role in ovulation-related inflammation. Detection of the cytokine form of IL-33 at 9 h was coincident with a decline in nIL-33+ cells, indicating a sudden release of the IL-33 cytokine form. Thus, although transcription and translation of IL-33 were initiated at hCG injection, the cytokine domain was released at 9 h, just before initiation of inflammation-like ovulatory processes. If we ignore its function as a nuclear binding protein, IL-33 could fit well into the whole picture of ovulation-related inflammation. However, our efforts to block ovulation using a neutralizing Ab failed. Although our experiment did not address whether the nIL-33 played any role in ovulation, a new study has demonstrated that deletion of the IL-33 gene does not significantly affect female reproduction (7) (Dr. S. Nakae, personal communication). Thus, more investigations are needed to answer the question why IL-33 undergoes a high level of transient expression during ovulation. Interestingly, expression of IL-33 and its receptor ST2 have been detected in multiple organs including reproductive organs such as uteri (35). Their expression is detectable under physiological conditions, suggesting their potential roles in the physiological function of an organ.

**Discussion**

This study has systemically investigated IL-33 expression pattern in mouse ovaries. Ovarian IL-33 expression was highly significant and unique, which is supported by the following results. First, IL-33 was present in experimental mice, their sera after experiments used for ELISA to measure rat IgG. All mice showed presence of rat IgG. We next immunized mice with rIL-33 at 8 wks of age. Another group of mice was immunized with CFA as a control. Serum anti–rIL-33 Ab was measured by Western blot at 12–14 wks, and only those with a high titer (>1:1200) were selected for hCG-induced superovulation. Although a more significant reduction in ovulated eggs was observed (Fig. 6C), immunization with rIL-33 Ab failed to block ovulation. In addition, no significant changes in the ovaries of immunized mice were found by H&E staining. We concluded that IL-33, at least its cytokine form, may not be directly involved in ovulation or atresia.

**FIGURE 6.** Interference with IL-33 fails to block hCG-induced ovulation. (A and B) Results of two experiments in which IL-33 Abs were injected at −6 and 0 h relative to hCG injection, respectively. Results are expressed as ovulated eggs/mouse. (C, left) SDS-PAGE for whole lysate and purified rIL-33 as a 31-kDa protein; (right) Western blot detection of sera anti–rIL-33 Ab from a representative rIL-33– or CFA-immunized mouse. Whole lysate was used as Ags. (D) Summary of hCG-induced ovulation for mice immunized with rIL-33 or CFA. Results are expressed as ovulated eggs/ovary. Two-tailed unpaired *t* test was used for statistical analysis.
For example, abnormal IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss (35). In contrast, ovarian steroids may regulate expression of IL-33 (36). This again suggests that IL-33 plays a role, either directly or indirectly, in reproduction. We will surely examine whether ovarian IL-33 expression is regulated by certain hormones.

Second, fluctuation of IL-33 expression levels during the estrous cycle also suggests its potential roles in the cycle. However, unlike the ovulatory process, multiple ovarian events such as follicle development and ovarian atresia occur simultaneously or overlap each other during the estrous cycle. Therefore, it is nearly impossible to separately examine each individual process, because of complicated interaction among many types of cells and their relationship to other ovarian events. Because of this complicated situation, as well as its cytokine nature, our study first aimed to seek out the relationship between IL-33 expression and ovarian immune cells, especially macrophages. We then could relate the immune cells to an ovarian process, in which the immune cells are known to participate. We focused on ovarian macrophages based on the following reasons. First, numerous studies have demonstrated that macrophages play critical roles in ovarian events ranging from follicle development to luteinization (37). Second, multiple subsets of macrophages in ovaries have been well described by our group (25). Those macrophage subsets display distinct tissue distribution patterns, suggesting their special roles. For example, IA+ macrophages, which express a high level of MHC class II molecule (IA or IIE), are mainly found in the atretic follicles. Our result showed that the number of total and IA+ macrophages changed during estrous cycle in a similar tendency to that of IL-33 mRNA levels. It needs to be pointed out that IA+ macrophages nearly disappeared from ovaries at estrus when IL-33 mRNA level and nIL-33+ cell number dropped to their lowest. We further compared IL-33 expression with ovarian atresia because IA+ macrophages were mainly present in atretic follicles. With a combination of multiple methods for identification of atretic follicles, we were able to describe how atresia progressed, which allowed us to classify atretic follicles into stages. Using this classification, we determined the relative frequency of each stage of atretic follicles for each estrous stage. Unlike IL-33 expression and ovarian macrophages, wave of atresia during estrous cycle was much more subtle, even based on our classified atretic follicles. It is clear, however, that initiation of atresia wave occurred at estrus, because atretic follicles at initial stage (with only apoptotic cells) were mostly found at this stage but not others. It means that the initiation of atresia at estrus was coincident with the lowest points of both IL-33 expression and IA+ macrophages. In other words, the initiation of atresia was before a rapid increase in IA+ macrophages. Because the increase in IL-33 mRNA is parallel but not before migrations of IA+ macrophage, it casts doubt on any roles of IL-33 in the recruitment of IA+ macrophage into early atretic follicles.

To clarify the earlier doubt, we further did the following analysis. Our study also showed a fluctuation of nIL-33 number during the estrous cycle. Through comparison of the fluctuation pattern of nIL-33+ cell number during the estrus cycle with that of IL-33 mRNA level, we found a significant difference in the fluctuation patterns between the two. When nIL-33+ cell number continued to climb to a peak at proestrus, IL-33 mRNA level had already fallen. This may be explained by the time required for translation. However, they both fell to their lowest point at estrus. For IL-33 mRNA, it simply means a downregulation of transcription. A sudden decline in nIL-33+ cell number from peak to valley at estrus, however, suggested a rapid release of cleaved IL-33. This was supported by the following results. First, a higher concentration of cleaved IL-33 was detected at estrus than at metestrus. Second, clustered endothelial cells with cytoplasmic IL-33 were present, whereas such cells were rarely observed at other stages. Thus, release of cytokine IL-33 was coincident with initiation of atresia wave, but before massive IA+ macrophage invasion. Based on these relationships, we are able to propose a hypothetic cascade during atresia: 1) atresia signal triggers apoptosis in follicular cells; 2) simultaneously, nIL-33+ endothelial cells cleave nIL-33 into cytokine IL-33 and release the cytokine; and 3) IA+ macrophages invade the atretic follicles, probably under regulation of IL-33. IL-33 has been reported to induce angiogenesis and permeability of blood vessels (38). It is possible that IL-33 facilitates or promotes invasion of IA+ macrophages into atretic follicles.

Several critical questions remain to be addressed for this hypothetical cascade. The most important question is whether those consecutive events are merely coincidence or a cause–effect cascade. Many cytokines or immune molecules have been detected during ovarian events in the past (26–31, 33). Such coincidence had prompted investigations. However, functions for a majority of those molecules still remain obscure. Like those previous studies, there are several obstacles to be overcome for addressing the cause–effect relationship. Cytokines may have similar activities using a shared receptor unit, and a defective molecule may be compensated by others. Alternatively, multiple molecules with a similar function may be involved in a single event. The function of nuclear binding form of IL-33 may play a role in endothelial cells activation as a transcriptional regulator (39). Fortunately, mice with a disrupted IL-33 gene are available, which will surely facilitate our need to address this critical question. Depletion of the IL-33 gene does not seem to significantly affect female reproduction (7). Thus, we need to carefully observe any changes in the ovaries, and to see whether a similar cytokine compensates IL-33’s function. A second important question is how IL-33 is released. Expression of IL-33 by endothelial or other type of cells has been reported. It is still unclear what the nIL-33’s function is, and when and how they are cleaved and released. Biochemical methods have shown that a typical IL-1 cleavage pathway does not generate an active fragment, whereas other enzymes do (5, 6). It raises a question regarding in vivo cleavage of IL-33. A further study on naturally cleaved IL-33 protein as seen in the ovaries will be necessary to understand this molecule’s function. If IL-33’s major function is the role of a cytokine, regulation of their release is probably more relevant to our study on its function in ovaries.

The majority of studies suggested that tissue injuries or cell necrosis leads to release of IL-33 from the damaged host cells as a “danger” signal for immune cells such as mast cells to initiate tissue repair or immune response (11–14). Our next question is whether the situations in ovarian events are different or mimic a “danger” signal, because the ovarian events are physiological processes with tissue destruction. Finally, it cannot be presently ruled out that IL-33 may directly regulate atresia, because several previous studies have suggested induction of atresia by cytokines such as IL-1β (40).

Acknowledgments
Confocal micrographs were taken at the Microscope Core, Department of Developmental Biology, University of Texas M. D. Anderson Cancer Center at Houston. Global gene expression detection using DNA microarray was performed at the Quantitative Genomics & Microarray Service Center, University of Texas Health Science Center at Houston. We thank Dr. Tuan Tran for technical help and Dr. April Ross for reading the manuscript.
Disclosures
The authors have no financial conflicts of interest.

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