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Potential Roles of A Special CD8αα$^+$ Cell Population and CC Chemokine TECK in Ovulation Related Inflammation[1]

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Abstract

It is well known that ovulation may be an inflammatory process. However, it remains elusive how immune cells participate in this process. We have identified a novel CD8αα$^+$ population, which resembles tissue DC, in the theca of antral follicles. We further observed a dramatic influx of the CD8αα$^+$cells into the ovulating follicles. This CD8αα$^+$population was absent in the ovary of estradiol-induced anovulatory C31F1 mice and sub-fertile athymic nude mice. Expression of a CC chemokine TECK (thymus expressed chemokine) has previously been found in the ovary; we further demonstrated that TECK attracted CD8αα$^+$cells into the ovary. Anti-TECK Ab, elicited in the female mice by active immunization, depleted the ovarian CD8αα$^+$ cells *in vivo*. Mice with a high titer of TECK Ab failed to ovulate after super-ovulation induction. More importantly, the immunized mice had a greatly reduced fertility, which was positively correlated with the Ab titers. Ovarian TECK expression was normal in anovulatory C31F1 mice, suggesting that infertility in the immunized mice is due to a block of CD8αα$^+$ cell migration. Finally, the origin of ovarian CD8αα$^+$ cells was explored. Upon being transferred, thymic CD8α$^+$ cells were able to home to the theca of follicles in the recipients. Thus, ovarian CD8αα$^+$ cells, which participate in the ovulation-related inflammation, may originate in the thymus.

Keywords

Reproductive immunology; inflammation; rodents; dendritic cell; chemokine; ovulation

Introduction

The participation of the immune system in ovarian functions has long been recognized. Clinical and experimental observations have linked immunodeficiency with some ovarian dysfunction such as anovulation (1-3). Numerous studies have investigated leukocyte populations in the ovaries of various species ranging from rodents to humans. The ovarian leukocytes can be classified into two categories: residential and infiltrative. The best characterized residential leukocyte population in the ovary is macrophages, which distribute in the interstitium and corpus luteum (4). In addition, a small number of lymphocytes, including CD4$^+$ and CD8$^+$ T cells, have been reported in the ovary (5). It has been shown that a large infiltration of

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leukocytes (macrophages and neutrophils) into the ovary occurs during the ovulatory process, probably in response to LH (luteinizing hormone) surge (6). An equally important influx of monocytes into the corpus luteum characterizes the late post-ovulatory phase (7). One study reported possible involvement of T cells and eosinophils in corpus luteum regression (8).

Several physiological processes in the ovaries, especially ovulation/luteinization, show similarities to the inflammatory process. A growing body of evidence, including discovery of a leukocyte influx into the ovary during ovulation, suggests that the ovulation/post-ovulatory process may indeed constitute a local inflammatory reaction (9-13). However, controversy remains as to which population(s) participate in the processes. Neutrophils and macrophages have been the most common candidates. However, a recent study showed that depletion of peripheral blood leukocytes, mainly neutrophils, did not affect ovulation (14). Although it remains unclear, a few studies do suggest that cytokines released by those leukocytes might be directly involved in the ovulatory or post-ovulatory functions (15,16). As the first step in the inflammatory process, leukocytes are recruited to the future location of inflammation by chemokines. Several studies have suggested that chemokines may be required for the ovulatory process (15). The CXC chemokine IL-8 is the chemokine that has been most studied for its involvement in ovulation (17-19). However, a recent study has shown elevated expression of different chemokines (mainly CC type MCP-1 and RANTES) during the ovulatory process, suggesting their potential roles in ovulation (20,21). Recently, we have detected a transient expression of a CC chemokine TECK (thymus-expressed chemokine) and its receptor CCR9 during hCG (human chorionic gonadotropin)-induced ovulation (22,23). However, it still remains to be determined whether these chemokines are indeed involved in ovulation and which specific leukocyte populations these chemokines recruit.

In the present study, we identified a novel CD8αα$^+$ cell population in the thecae of antral follicles, and further observed its influx into ovulating follicles during ovulation. A lack of the ovarian CD8αα$^+$ cells was associated with sub-fertility or infertility in mice. We further demonstrated that TECK was a chemoattractant for the ovarian CD8αα$^+$ cells. Importantly, depletion of ovarian CD8αα$^+$ cells by TECK Ab resulted in significantly reduced fertility in female mice. Thus, the novel CD8αα$^+$ cell population may play a critical role in ovarian functions such as ovulation.

Materials and Methods

Abs

A set of mAbs to leukocyte markers including CD4 (GK1.5, BD Bio Sciences, San Diego, CA), CD8α(Ly2, BD), CD8α(MCD0817, Invitrogen, Carlsbad, CA, for fixed tissue), CD8β (H35−17.2, BD), CD11c (HL3, BD), CD86 (GL1, BD), TcRβ(H57−597, BD), TcRγδ(GL3, BD), CD3ε(145−2C11, BD), CD45R (B220)(RA3−6B2, BD) and CD5 (53−7.3, BD) was used. In addition, mAbs to pan macrophage marker F4/80 (RM2901, Caltag, Burlingame, CA) and endothelial marker CD144 (11D4.1, BD) were also used. The above Abs were labeled by FITC, PE or biotin, and used for flow cytometry, immunofluorescence and immunohistochemistry. Normal rat IgG and anti-CD32 (2.4G2) Ab were utilized to reduce the background. Isotype-matched irrelevant mAbs from BD served as negative controls.

Generation of anovulatory mice and induction of ovulation

BALB/c, nude (Nu/NuBALB/c), C3JH, 132J, and CD1 mice were obtained from Jackson Laboratory (Bar Harbor, ME), and maintained in the animal facility at The University of Texas Health Science Center at Houston. C31F1 mice were generated by mating C3JH females and 132J males. The neonatal mice C31F1 were used for estradiol injection following an established method (24). Young female mice of 6 to 8 w of age were used for super-ovulation induction.

Briefly, the animals were injected i.p. with PMSG (pregnant mare's serum gonadotropin) (Sigma, St. Louis, MO) at 5 IU/mouse, and were injected i.p. with hCG (5 IU/mouse, Sigma, St. Louis, MO) after 48 hrs. The mice injected with the same volume of PBS served as a control. Ovaries were collected from 3 mice at designated time points. At the same time, fallopian tubes were removed and examined under a dissecting microscope for presence of ovulated eggs to determine the time of ovulation. Eggs were harvested and counted.

Immunization and fertility trail

BALB/c female mice were used at 6 to 8 wks of age. Recombinant mouse TECK (rmTECK) was expressed and purified as described previously (23), adjusted to 3 mg/ml, and was emulsified in an equal volume of CFA. Each mouse received 0.1 ml of the mixture (150 μg of rmTECK) in one footpad and a subcutaneous site. Another group of mice was immunized with CFA only and served as the control. Sera were sampled by tail bleeding for determination of anti-TECK Ab titer. The mice were boosted by rmTECK (15 μg/mouse) in IFA. Western blot was used to determine antibody titer against rmTECK in the immunized mice. The highest dilution of a serum, at which rmTECK remained detectable, was taken as the antibody titer. Fertility trails were established by housing fertile males with two females, one from the rmTECK-immunized group and the other from the CFA group. Mating was confirmed each morning by the presence of a vaginal plug. Upon the observation of a vaginal plug, the mice were bled. Sera antibody titer was determined and taken as “mating titer”. The mating continued for 3 weeks, and then males were removed. Females were allowed to deliver, and litter size was recorded upon completion of delivery.

Isolation of ovarian cells and flow cytometry

Two pairs of ovaries were removed and immediately placed in a DMEM medium, which was supplemented with 10% FCS, 2 mM L-glutamine, nonessential and essential amino acids, sodium pyruvate, 100U/ml penicillin, 100μg/ml streptomycin (BioWhittaker, Walkersville, MD). The ovaries were torn by repeated needle punch, and briefly digested by collagenase IV (1 mg/ml, Sigma). The digested tissues were then ground, passed through a cell constrainer, and washed with the same medium. Dead cells were further removed by Ficoll gradient (density 1.119) centrifugation. As a control, peripheral blood leukocytes or splenocytes were isolated following our previously-published method (23). The cells were doubly stained by various combinations of two antibodies and analyzed by flow cytometry (FACSCalibur, BD, San Jose, CA).

Immunofluorescence

Ovaries were snap-frozen or fixed in 4% paraformaldehyde (PFA). The fixed ovaries were sequentially-transferred to 30% sucrose in PBS and embedded in OCT. Frozen sections were cut at 3 μm of thickness. Sections were blocked in sequence by 10% goat serum, biotin, and avidin (kit from Vector Lab. Burlingame, CA), and incubated with labeled mAbs for 1 h. If biotin-labeled mAbs were used, the sections were further incubated with avidin-FITC or PE for 30 min. When two-color immunofluorescence was performed, PE-labeled anti-CD8α antibody was co-stained with other FITC-labeled Abs. DAPI was used for counter-staining. The sections were observed using conventional or confocal fluorescent microscope (FV 500, Olympus, Melville, NY).

Leukocyte infiltration assay

Chemotactic activity of ovaries undergoing the ovulatory process for peripheral blood leukocyte (PBL) was measured by leukocyte infiltration assay following our previously published method (23). Briefly, the ovaries collected from mice at 8 hours post hCG injection were teased open, creating a chemoattractant source, and placed at the lower chamber of a B-

D Bio Coat Control Cell Culture Inserts system (pore size 3 μ, BD, San Diego, CA). The PBLs were isolated from the same ovary donor mice with Ficoll gradient centrifugation (Histopaque 1.119, Sigma, St. Louis, MO). The assembled sets were incubated at 37°C, 5% CO_2, for 8 h, and the cells in the insert wells were gently and thoroughly washed away with cold PBS. After fixation in 2% PFA for 10 minutes at 4°C, the membrane was stained by anti-CD8 antibody following an established immunoperoxidase staining method (23). In some cases, bioactive TECK (R&D) was added to the lower chamber (10 μg/ml) as chemoattractant. TECK was mixed with undiluted sera (1:1) from the immunized mice for measuring anti-TECK antibody activity in the sera. Sera from CFA-immunized mice or normal mice IgG were used as controls.

Transfer of labeled thymocytes

Thymic CD8α$^+$ or CD11c$^+$ cells were positively selected using CD8α (Ly2) or CD11c (N148) Ab-conjugated magnetic beads (MACS Miltenyi Biotech, Auburn, CA). The cell purity was determined by flow cytometry using anti-CD8 and CD11c Abs. The cells were labeled by CFSE following an established method (25), and were transferred into untreated or TECK-immunized syngeneic mice by i.v. injection (10×10^6 cells/mouse). The cell recipients were sacrificed at 24 hrs post injection, and their organs including ovaries, kidney, lung and liver were removed. After fixation with PFA, serial frozen sections were cut, and observed using a fluorescent microscope. The labeled cells were counted on randomly selected sections, and cell density was calculated (cells/mm^2).

Statistics

For comparisons between experimental groups, un-paired *T*-tests were performed.

Results

Identification of CD8αα$^+$ cells in murine ovaries

A set of monoclonal antibodies to different leukocyte markers were used to characterize ovarian leukocytes of CD1 mice. Antibody staining on ovarian F4/80$^+$ macrophages, which have been well-characterized (4), was used as a positive control. A population of cells, which reacted strongly with antibody to CD8αwas identified (Fig. 1*A*). Further immunofluorescent staining showed that the cells did not react with antibody to CD8β, suggesting that this cell population possessed the CD8αα homodimer (CD8αα$^+$ cells). Morphologic observation using confocal microscopy showed that CD8αα$^+$ cells were elongated in shape, and resembled tissue dendritic cells or the ovarian F4/80$^+$ macrophages (Fig. 1*B*). However, their tissue location was entirely different from F4/80$^+$ macrophages. CD8αα$^+$ cells were restricted to the thecae internae of antral follicles, while ovarian macrophages were present in the interstitial tissues (Fig. 1, *A* and *D*). The presence of the novel CD8αα$^+$ cells in the ovary were observed in all tested mice strains, including CD1, BALB/c, C3JH. The CD8αα$^+$ cells were then characterized by two-color immunofluorescent staining. Except for a weak reactivity with anti-CD11c antibody, the cells failed to react with monoclonal antibodies to F4/80, CD86, TcRβ, TcRγδ, CD3ε, B220 and CD5. Thus, CD8αα$^+$ may not belong to those leukocyte populations such as T lymphocytes and macrophages, but rather to tissue dendritic cells.

To further characterize the cells, we established a special protocol to obtain single cell suspensions from ovaries for flow cytometry analysis, by using combined mechanical and enzymatic methods. The ovarian cells, which were isolated from two CD1 mice, were stained for two-color flow cytometry using a combination of various mAbs. Flow cytometry on the stained ovarian cells revealed a unique CD8αα$^+$ cell population, which accounted for approximately 0.5 % of the isolated ovarian cells (Fig. 1*C*). Two other independent experiments showed similar results. This CD8αα$^+$ population was not from the blood stream that was circulating through the ovaries as evident by the following results. *First*, two distinct CD8$^+$

cell populations, $CD8^+CD3^-$ and $CD8^+CD3^+$, were observed (Fig. 1*C*). The latter were obviously $CD8^+$ T cells, which probably were from the circulation. *Second*, the $CD8^+CD3^-$ cells were very low in number in the peripheral blood of the same mouse with a $CD8^+CD3^-$/$CD8^+CD3^+$ ratio of approximately 1:50. *Third*, no changes in the $CD8^+CD3^-$ cell population were seen in the ovaries after complete perfusion with PBS, whereas perfusion led to a significant reduction in the $CD8^+CD3^+$ population (i. e. $CD8^+$ T cells).

Absence of the CD8αα$^+$ cells in the ovaries of anovulatory or sub-fertile mice

Discovery of the ovarian $CD8\alpha\alpha^+$ cells led us to postulate that the ovarian $CD8\alpha\alpha^+$ cells might be involved in important ovarian functions such as ovulation and/or post-ovulatory luteinization. Therefore, we decided to observe the ovarian $CD8\alpha\alpha^+$ cells in mice with inherent or artificially-induced ovarian dysfunctions. First, we examined ovarian $CD8\alpha\alpha^+$ cells in anovulatory C31F1 mice, an established model for anovulation, which mimics human polycystic ovarian syndrome (PCOS) (24,26). The neonatal C31F1 female mice were either injected with E2 (estradiol) or carrier sesame oil alone, and the E2-treated female mice became anovulatory after 8 w of age. Importantly, neither immunofluorescence nor flow cytometry detected ovarian $CD8\alpha\alpha^+$ cells in all five E2-treated anovulatory C31F1 mice (Fig. 1, *E* and *F*). The lack of ovarian $CD8\alpha\alpha^+$ cells was not due this specific strain, as evidenced by the fact that all five control C31F1 mice, which received sesame oil alone, had a similar number and an identical distribution pattern of ovarian $CD8\alpha\alpha^+$ cells as those in CD1 or BALB/c mice (Fig. 1, *D* and *F*). In contrast, ovarian $F4/80^+$ macrophages were present in both E2-treated C31F1 mice and their sesame oil-control (Fig. 1, *D* and *E*).

We next examined ovarian $CD8\alpha\alpha^+$ cells in female athymic nude mice. Female athymic nude mice are known to be infertile or sub-fertile, while nude males are fertile (27,28). Both immunofluorescence (two ovaries) and flow cytometry (four pairs) demonstrated a complete absence of the ovarian $CD8\alpha\alpha^+$ cells in the ovaries of BALB/cnu/nu female mice (total five) (Fig. 1*F*). In contrast, ovarian macrophages (which bear $F4/80^+$ marker) were present in nude females as their syngeneic BALB/c mouse. Thus, the ovarian $CD8\alpha\alpha^+$ population was missing in both mouse models manifesting ovarian dysfunction.

Influx of CD8αα$^+$ cells into ovaries during hCG-induced ovulation

Although the above observations showed an association between absence of ovarian $CD8\alpha\alpha^+$ cells and ovarian dysfunction, it was unclear whether ovarian $CD8\alpha\alpha^+$ cells were required for ovarian functions, or in which specific ovarian function they might participate. To further explore potential roles of the newly discovered $CD8\alpha\alpha^+$ population in ovarian functions (such as ovulation), we investigated changes in this cell population during estrous cycles. CD1 mice ovaries were sampled daily (three mice/day), and the stage of their reproductive cycles was determined by vaginal smear for over 5 days. No significant changes in ovarian $CD8\alpha\alpha^+$ population were observed, except for the presence of some $CD8\alpha\alpha^+$ cells in the newly formed corpus luteum. We next decided to examine the $CD8\alpha\alpha^+$ cells during ovulation (Fig. 2). Young CD1 female mice were induced to ovulate by PMSG and hCG, and their ovaries were sampled post-hCG injection at one-hour intervals (2 mice/time point). $CD8\alpha\alpha^+$ cells were localized to the thecae of mature follicles similar to those of untreated ovaries at 0 h (Fig. 1*A*). A dramatic increase in $CD8\alpha\alpha^+$ cell number was observed at 4−6 h post-hCG-injection (Fig. 2*B*). The cells mainly clustered around the thecae of mature follicles, in addition to those pre-existing in thecae. The $CD8\alpha\alpha^+$ cells began to invade the ovulating follicles at 8−10 h (Fig. 2, *E* and *F*). Eventually, the $CD8\alpha\alpha^+$ cells reached the oocyte and formed a cluster surrounding the oocyte just before and immediately after ovulation (10−12 h) (Fig. 2*C*). The $CD8\alpha\alpha^+$ cell number rapidly decreased after ovulation (13 and 14 h), and some of them scattered among the granulosa cells, which were undergoing luteinization. After 24 hours, most of the $CD8\alpha\alpha^+$ cells disappeared except for a few in the newly formed corpora lutea (Fig. 2*D*). A similar result for

influx of ovarian CD8αα$^+$ cells during ovulation was also observed in BALB/c mice. The changes of ovarian macrophages during the ovulation were also investigated in parallel to appearance of CD8αα$^+$ cells. In contrast to CD8αα$^+$ cells, ovarian macrophages remained unchanged either in their number or location during the entire ovulatory process (Fig. 2). The macrophages, however, were found to invade the newly-formed corpus luteum at least 24 hours after the ovulation (Fig. 2*D*). The invasion of macrophages into corpora lutea has been reported (29). Those results suggest that macrophages may play roles in luteinization but not ovulation.

Eliciting TECK antibody in female mice leads to absence of CD8αα$^+$ cells in ovaries

The coincident influx of a large number of the CD8αα$^+$ cells into the ovary during the ovulatory process does not prove that their roles were causally-related. However, the timing of these events continues to suggest the possibility of a significant role for the CD8αα$^+$ cells in the ovulatory process. As a first step in clarifying the relationship, we reasoned that if the newly discovered CD8αα$^+$ cells were involved in ovarian functions such as ovulation, the depletion of ovarian CD8αα$^+$ cells should lead to ovarian dysfunction and/or overt infertility.

We have previously discovered a high level, transient expression of chemokine TECK in the ovary during ovulation (22,23). TECK, a new member in CC chemokine family, is reported to be expressed only in the thymus or small intestine with important functions in early lymphocyte development (31,32). The co-incidence of transient TECK expression with influx of CD8αα$^+$ cells prompted us to test if ovaries with peak expression of TECK were able to chemoattract the CD8αα$^+$ cells using the *in vitro* leukocyte infiltration assay. Among the migrated PBL, which had been attracted by ovulating ovaries, about 40% were CD8α$^+$ cells, as revealed by immunoperoxidase staining (Fig. 3*A*). Thus, the CD8α$^+$ population was enriched 9-fold among the migrated leukocytes, as compared to less than 5% in PBL. To further confirm that ovarian TECK was responsible for the migration of CD8α$^+$ cells, we used anti-TECK Ab to inhibit migration of CD8α$^+$ cells. Importantly, anti-TECK Ab inhibited approximately 80% of CD8α$^+$ cell infiltration (Fig. 3*B*). Thus, ovarian TECK appears to be a chemoattractant for CD8αα$^+$ cells. Based on those observations, we hypothesized that anti-TECK Ab inhibited migration of CD8αα$^+$ cells into the ovaries *in vivo* as well.

To test this hypothesis *in vivo*, female BALB/c mice (6–8 weeks) were either immunized with rmTECK or adjuvant CFA alone. Selected sera were tested for their Ab activity against native TECK by *in vitro* leukocyte infiltration assay. Sera Abs from the immunized mice inhibited TECK bioactivity (Fig. 3*C*). As TECK is critical for early T cell development, several organs including thymus and small intestine were first examined for their potential abnormalities. Histological observations showed no significant abnormalities in those organs, although flow cytometry did detect some changes in thymocyte subpopulations in the TECK-immunized mice (data not shown). Ovaries were collected from the immunized mice with an Ab titer higher than 3200 or from the CFA control group (3 pairs / group), and analyzed for ovarian CD8αα$^+$ cells. Importantly, both immunofluorescence and flow cytometry revealed a total absence of CD8αα$^+$ cells in the ovaries from TECK-immunized mice (Fig. 4, *A* and *B*). Thus, anti-TECK Ab inhibited migration of CD8αα$^+$ cells into the ovaries.

Inhibition of migration of CD8αα$^+$ cells into ovaries by TECK Ab reduces fertility in female mice

In vivo inhibition of CD8αα$^+$ cell migration by TECK Ab provided us with an opportunity to determine whether CD8αα$^+$ cells were required for ovarian functions. We first examined fertility of female mice immunized with rmTECK (Fig. 4, *D* and *E*). Four independent experiments were carried out. In the first experiment, of three mice with an Ab titer high than 3,200, two were never impregnated over a 20-d period of mating, and one delivered a litter of only 3. The infertility in those mice was not due to failure of mate, as repeated mating in those

females was confirmed by vaginal plugs. Furthermore, the infertility was also not due to infertility in males, because an individual male mated with two females, one from rmTECK-immunization groups and the other from CFA control. In contrast, three other rmTECK-immunized mice with a titer lower than 3200 delivered litters of 6 to 8, as compared 10−12 in CFA controls (Fig. 4*E*). Similar results were observed in the other three experiments. Figure 4*D* and *E* summarize the results from those experiments. Thus, anti-TECK Ab not only led to depletion of ovarian CD8αα$^+$ cells, but also resulted in infertility or reduced fertility. Reduction in fertility was correlated with anti-TECK Ab titers (Fig. 4*E*). Interestingly, infertility in the TECK-immunized mice was not permanent, because the immunized mice regained fertility 2 months later when the Ab titers dropped below 1:400, and the ovarian CD8αα$^+$ cells reappeared.

It still is not clear which one, TECK or CD8αα$^+$ cells, was directly responsible for infertility in the immunized mice. We observed ovarian expression of TECK in randomly selected five C31F1 E2-treated anovulatory mice (6−8 w). As a control, ovaries were also collected from five un-treated C31F1 mice of the same age. We have shown previously that randomly selected ovaries express variable levels of TECK (23). RT-PCR showed that ovaries from either E2-treated or un-treated group expressed TECK at a comparable levels and pattern (Fig. 5). Thus, it is less likely that TECK expression was directly related to the infertility in rmTECK-immunized mice.

Antibody-induced absence of CD8αα$^+$ cells in ovaries is associated with defect in ovulation

Although the details of the mechanism remain unclear, it is likely that ovulatory and/or post-ovulatory functions were affected by absence of CD8αα$^+$ cells. To test this hypothesis, superovulation was induced in selected TECK-immunized mice (with an antibody titer higher than 3,200), CFA controls, and age-matched mice. The ovulated eggs were significantly lower in number in TECK-immunized mice (2.9±7.6) than in CFA controls (20.0±6.7), while no difference was observed in egg numbers between the CFA control and the age-matched group (25.2±6.4) (Fig. 4C). Interestingly, the TECK-immunized mice with an antibody titer lower than 3,200 ovulated a normal or slightly reduced number of eggs (data not shown). Thus, infertility in the TECK-immunized mice may be due to a defect in final maturation or ovulation.

Homing of transferred thymocytes to theca of antral follicles

We next investigated the potential origin of theca CD8αα$^+$ cells. We first purified thymic CD8α$^+$ cells from normal female BALB/c mice. Flow cytometry on the purified cells demonstrated a small population of CD8α$^+$CD11c$^+$ in addition to pre-T cells or T cells (Fig. 6*A*). The cells were labeled and injected i.v. into three syngeneic female mice (6w of age) (Fig. 6*C*). In parallel, the labeled thymic CD8α$^+$ cells were transferred into two mice, which had been immunized with rmTECK with an Ab titer higher than 3200. Tissue distribution of the transferred cells was determined 24h later (Fig. 6*D*). Although labeled cells were found in all examined tissues In normal BALB/c, ovaries had a higher density of the labeled cells. More importantly, approximately 65% of the labeled cells were localized to the theca of antral follicles (Fig. 6, *D* and *E*), but the labeled cells were never found in secondary follicles (Fig. 6*F*). Labeled cells also were found in some atretic follicles. On the other hand, the number of labeled CD8α$^+$ cells, which had migrated into the theca, was significantly lower in the TECK-immunized mice than that in normal mice (Fig. 6*D*). Using a similar method, CD11c$^+$ cells were isolated from thymocytes for transfer experiments. The purified CD11c$^+$ cells contained a small population of CD8α$^+$CD11c$^+$ (Fig. 6*B*). After they were transferred into syngeneic mice, a small number of the labeled CD11c$^+$ cells were able to migrate into theca of antral follicles (Fig. 6*H*). Immunofluorescent staining with CD8α mAb further showed that the CD11c$^+$ cells, which homed to the theca, were CD8α$^+$ (Fig. 6*H*).

Discussions

Numerous studies have shown that several ovarian processes such as ovulation/luteinization resemble an inflammatory process (9,13). Discovery of a leukocyte influx into the ovary during ovulation suggests that the ovulatory/post-ovulatory process may constitute a local inflammatory reaction (6,10-12). Despite intense studies in this area, controversy remains as to which population(s) participates in the processes. Neutrophils and macrophages have been the candidates. However, several studies showed that depletion of peripheral blood leukocytes, mainly neutrophils, did not affect ovulation (14). Ovarian macrophages have been postulated as another candidate that may be involved in ovulation (4,29,30). The present study suggests that a unique population of CD8αα$^+$ cells in the theca of antral follicles, but not macrophages, may be a more likely candidate for their involvement in ovulation. *First*, the CD8αα$^+$ cells were absent in anovulatory C31F1 mice and sub-fertile female nude mice, while macrophages were present in the both. *Second*, an influx of CD8αα$^+$ cells into ovulating follicles was observed. In contrast, ovarian macrophages did not infiltrate the ovulating follicles. *Finally*, blocking migration of CD8αα$^+$ cells with anti-TECK antibody led to an impaired ovulation, as well as infertility. One may argue that the infertility caused by anti-TECK antibody may be due to a block of activity of TECK. However, an observation in anovulatory C31F1 mice, which lacked CD8αα$^+$ cells in theca of antral follicles, demonstrated a normal level and pattern of TECK expression in their ovaries as compared to the control group. Thus, absence of the CD8αα$^+$ cells was a more likely reason for the infertility in TECK-immunized mice. In summary, the present study, together with our previous results (22,23), suggest that the ovarian CD8αα$^+$ cells are required for normal ovarian functions, especially ovulation, and this cell population migrates into the ovary as chemotaxis to ovarian TECK. Although ovulation employs an inflammatory response, it is a physiological process. Thus, it is not surprising that the cell populations which are involved in ovulation-related inflammation may be different from those in a conventional inflammatory process.

Our experiments showed that anti-TECK Ab elicited by immunization blocked migration of CD8αα$^+$ cells into the theca of antral follicles *in vivo*. TECK is an important chemokine for T cell development, and it serves as a chemoattractant for multiple cell populations, such as pre-T cells and mucosal lymphocytes in small intestine (31,32). Thus, it raises a question whether elicitation of anti-TECK Ab might also lead to abnormal T cell development or tissue abnormality. We have examined multiple organs including ovaries and intestine, and failed to find significant abnormalities in those organs. It is well known that disturbance of thymus such as thymectomy can lead to abnormal T cell populations and/or autoimmunity. However, this must be done at a very young age (less than 3 days) (35). Since we have used sexually matured mice as experimental animal, eliciting an anti-TECK response may have a less significant affect lymphocyte development. Although anti-TECK Ab affected fertility of the host, the fertility was regained after the Ab titer decreased. This result suggests that at least TECK Ab did not cause permanent damage to the ovaries. Further experiments have been planned to examine whether TECK Ab affects T cell development, especially in small intestine in young mice.

It is important to determine the primary source of the ovarian CD8αα$^+$ cells. Although it still lacks direct evidence, our results argue that this cell population may emerge from the thymus. *First*, absence of ovarian CD8αα$^+$ cells in female nude mice or anovulatory C31F1 mice is associated with either absence of the thymus or disturbed thymocytes respectively. A previous study reported an abnormality in thymic CD8$^+$ cell populations in anovulatory C31F1 mice (26). Interestingly, male nude mice are perfectly fertile, while female nude mice are infertile or sub-fertile. Endocrine dysfunction of thymus had been suspected to be responsible for infertility/subfertility in female nude mice (28,33,34). However, there still is a lack of convincing evidence to support this hypothesis despite several decades of intense studies. Our study suggests that the novel ovarian CD8αα$^+$ cells may originate from thymus. Thus, a lack

of a certain subset of CD8αα$^+$ cells in athymic nude mice may be responsible for their infertility. *Second*, our experiments showed that transferred CD8α$^+$ or CD11c$^+$ thymocytes were able to home to theca of antral follicles. More importantly, the homing is tissue specific, as approximately 65% of transferred cells found in the ovaries were localized to the theca. However, we must further ask why only a few of them were able to migrate to the theca, although a large number of the cells had been transferred. Flow cytometry on isolated thymocytes demonstrated a very small CD8α$^+$CD11c$^+$ population. This small population may represent the precursor of ovarian CD8αα$^+$ cells. This may also explain why only a few transferred cells were able to home to the theca in our cell transfer experiments. More studies are needed to characterize this specific thymocyte population.

PBL contains a relative small percentage (0.1%) of leukocytes, which bear CD8αα (36). Thus, the number of CD8αα$^+$ cells, which may infiltrate the ovaries, is limited as compared with other leukocytes such as neutrophils. It will be important to ask why a small number of CD8αα$^+$ cells are able to mediate an inflammation-like ovulatory process. The CD8αα$^+$ cells are most likely to be involved in initiation of this type of inflammatory response in a mature follicle. Thus, CD8αα$^+$ cells alone may not be sufficient for ovulation to occur, and participation of other inflammatory leukocytes as described by numerous previous studies may also be required.

Abbreviations used in this paper

E2, estradiol; hCG, human chorionic gonadotropin; LH, luteinizing hormone; PMSG, pregnant mare's serum gonadotropin; TECK, Thymus expressed chemokine.

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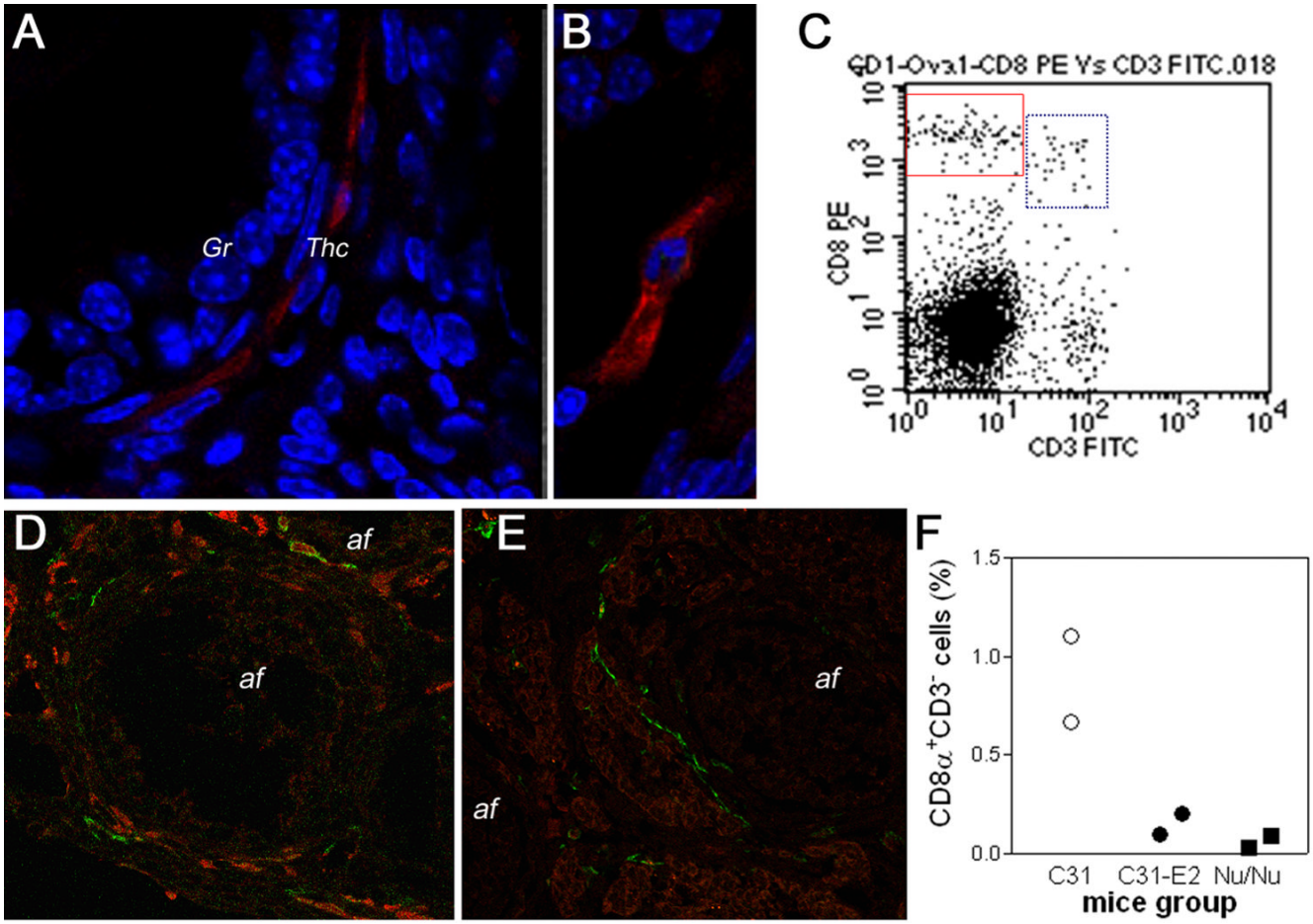

FIGURE 1.

Detection of CD8αα$^+$ cells in the theca of the ovarian follicle and their absence in anovulatory C31F1 and nude mice. *A.* Confocal image of an ovarian section after staining with PE-CD8α mAb (red) in BALB/c mice; nuclei are counterstained with DAPI (blue) (x300). *Gr*, granulosa; *Thc*, theca. *B.* Confocal image of elongated CD8αα$^+$ cells at a high magnification (x600). *C.* A representative flow cytometry on ovarian cells isolated from BALB/c mice after staining with Abs to CD3 and CD8α in order to distinguish CD8αα$^+$ cells from CD8$^+$ T cells. CD8α$^+$CD8$^-$ cells (red line) and CD8$^+$CD3$^+$ (blue dashed line) are gated. *D* and *E*, A pair of ovarian sections of two-color immunofluorescence show presence of both CD8αα$^+$ cells (red) and macrophages (green) in control C31F1 (*D*), but absence of CD8αα$^+$ cells in E2-treated anovulatory C31F1 (*E*). *af*, antral follicle. *F.* Size of ovarian CD8α$^+$CD3$^-$ population (expressed as percentage of total cells) in different groups based on flow cytometry analysis (see red gate in *C*). Each symbol represents one sample, which is the mixture of cells from two pairs of ovaries.

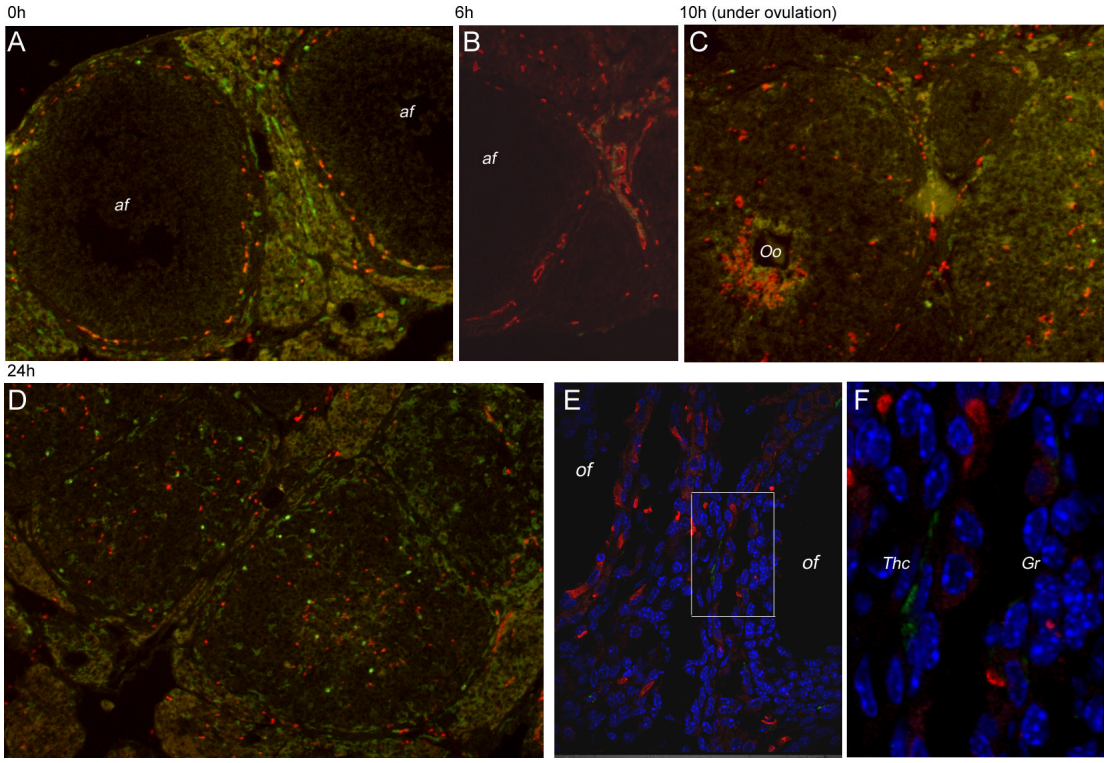

FIGURE 2.

Influx of CD8αα$^+$ cells into ovulating follicles after hCG-induced ovulation, as revealed by two-color immunofluorescence (red for CD8αα$^+$ cells, and green for F4/80$^+$ macrophages). *A.* Antral follicles (*af*) at hCG injection with CD8αα$^+$ cells in the theca and F4/80$^+$ macrophages in the interstitium. *B* and *C*, CD8αα$^+$ cell accumulation (6 h) and their invasion into follicles undergoing the ovulatory process (10 h). Note that numerous CD8αα$^+$ cells are surrounding an oocyte (*oo* in *C*). *D.* Two newly-formed corpora luteum with CD8αα$^+$ cells and macrophages (24 hrs). *E.* Confocal micrograph of CD8αα$^+$ cells (red) which are invading granulosa of two ovulating follicle (*of*) at 8h (x200). *F*. High magnification of the gated area in *E* shows some invading CD8αα$^+$ cells which have left the theca (*Thc*) and mixed with granulosa cells (*Gr*) (x600). Nuclei are counter stained by DAPI.

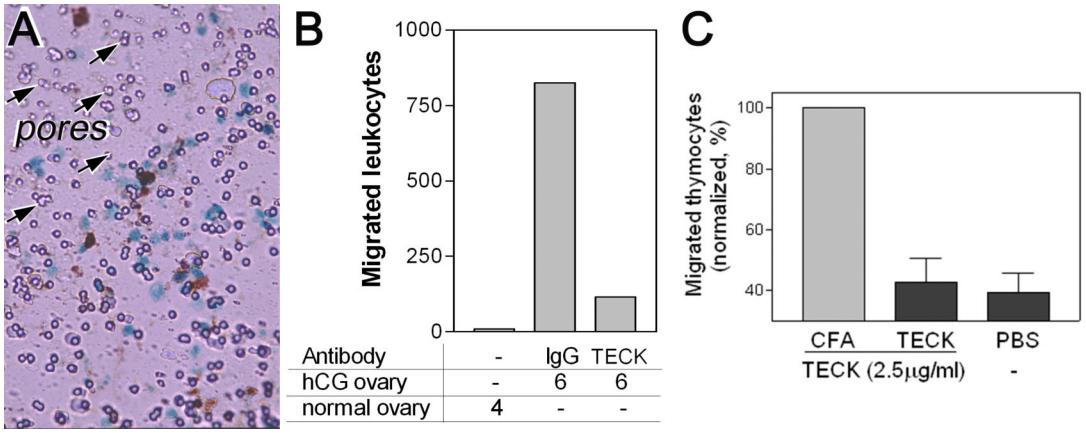

FIGURE 3.

Chemotaxis of CD8α$^+$ cells toward ovarian TECK, as determined by *in vitro* leukocyte infiltration assay. *A.* Immunoperoxidase staining shows CD8α$^+$ cells among PBLs migrating toward ovaries with TECK expression after PMSG and hCG injection. Cells are counter-stained by methylene blue. Numerous pores through which leukocytes could migrate are seen (arrows). *B.* Effect of TECK antibody (TECK) on chemotactic activity of hCG-treated ovaries. Normal mouse IgG (IgG) is used as a control, and untreated ovaries are used as a negative control for chemotactic activity. *C.* Sera antibody from the TECK-immunized mice inhibits TECK activity as demonstrated by *in vitro* infiltration assay. Thymocytes were used as testing cells.

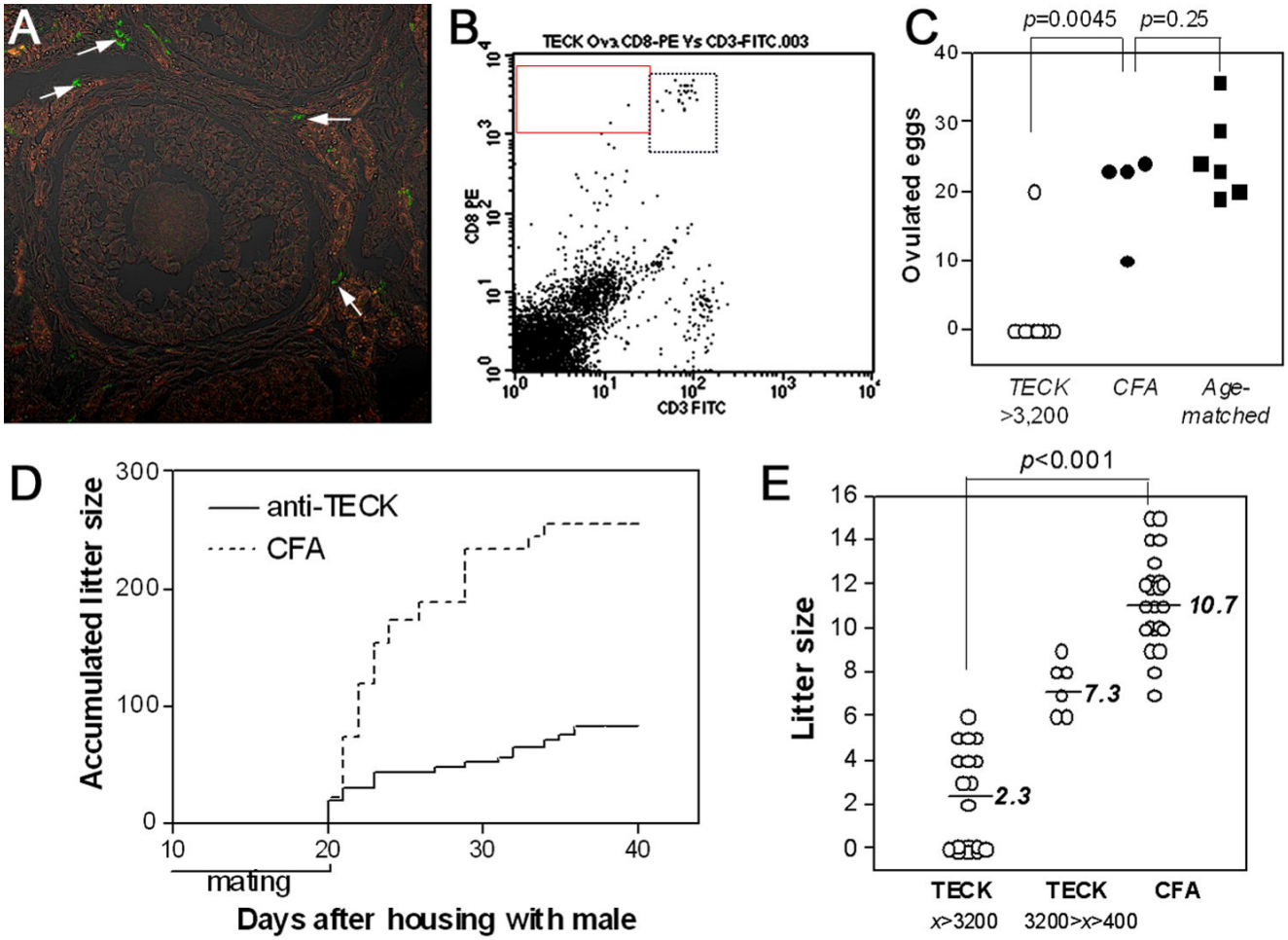

FIGURE 4.

Absence of ovarian CD8αα$^+$ cells and reduced fertility in BALB/c mice immunized with rmTECK. *A.* Confocal micrograph reveals an absence of CD8αα$^+$ cells in the theca of an antral follicle, while macrophages (green, arrows) are present in the interstitium. *B.* A representative flow cytometry of ovarian cells shows an absence of CD8αα$^+$ cells (gated by red line) in TECK-immunized mice, while CD8$^+$CD3$^+$ T cells are present (gated by blue line). *C.* Ovulation capacity of different groups of mice after PMSG/hCG induced superovulation. The capacities are expressed as numbers of ovulated eggs harvested from both oviducts. Each symbol represents one individual. *D.* Overall fertility in rmTECK-immunized group and CFA control (four experiments, total 24 mice/group). Fertility is expressed as a cumulative litter size in each group. *E.* Correlation between litter size and TECK Ab titer. Each circle represents one individual. rmTECK-immunized mice are divided into two groups according to their Ab titers. Group averages are indicated by horizontal lines.

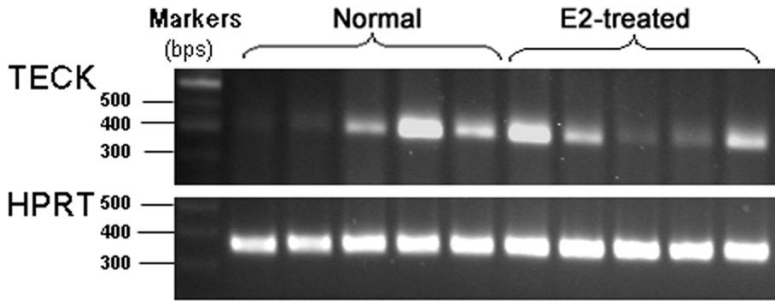

FIGURE 5.
RT-PCR detection of TECK expression in ovaries of E2-treated or untreated C31F1 female mice. Five ovaries were randomly sampled from each group; each lane represents one individual. A house-keeper gene HPRT was used as a control.

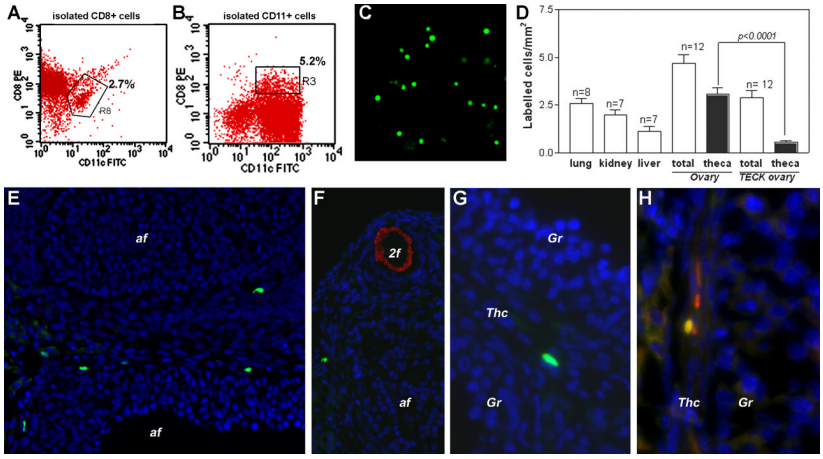

FIGURE 6.

Homing of transferred thymic CD8α$^+$ or CD11c$^+$ cells to theca of antral follicles. *A.* Flow cytometry shows purified thymic CD8α$^+$ cells. A small CD8α$^+$CD11c$^+$ population is gated. *B.* Flow cytometry shows purified thymic CD11c$^+$ cells. A small CD8α$^+$CD11c$^+$ population is gated. *C.* Fluorescent micrograph shows CFSE-labeled thymic cells before transfer. *D.* Tissue distribution of transferred thymic CD8αα$^+$ in normal mice or mice immunized with rmTECK (*TECK*). The results are expressed as labeled cell density (cells/mm^2). *n* indicates number of sections used for counting the cells. *E* and *F*. Fluorescent micrographs show labeled CD8α$^+$ cells in the theca of antral follicle (*af*). Note the absence of labeled cells in the theca of a secondary follicle (*2f*), which is counter-stained by ZP3 mAb (red) (*F*) (x200). *G*. High magnification shows a labeled cell in the theca (*Thc*) (x400). *Gr*, granulosa. *H*. Fluorescent micrograph shows a CFSE-labeled CD11c$^+$ cell (yellow) in the theca, which reacts to CD8α Ab. Note the presence of several endogenous theca CD8α$^+$ cells (red). (x600)