

Maternal Autoantibody Triggers De Novo T Cell-Mediated Neonatal Autoimmune Disease¹

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Although human maternal autoantibodies may transfer transient manifestation of autoimmune disease to their progeny, some neonatal autoimmune diseases can progress, leading to the loss of tissue structure and function. In this study we document that murine maternal autoantibody transmitted to progeny can trigger de novo neonatal pathogenic autoreactive T cell response and T cell-mediated organ-specific autoimmune disease. Autoantibody to a zona pellucida 3 (ZP3) epitope was found to induce autoimmune ovarian disease (AOD) and premature ovarian failure in neonatal, but not adult, mice. Neonatal AOD did not occur in T cell-deficient pups, and the ovarian pathology was transferable by CD4⁺ T cells from diseased donors. Interestingly, neonatal AOD occurred only in pups exposed to ZP3 autoantibody from neonatal days 1–5, but not from day 7 or day 9. The disease susceptibility neonatal time window was not related to a propensity of neonatal ovaries to autoimmune inflammation, and it was not affected by infusion of functional adult CD4⁺CD25⁺ T cells. However, resistance to neonatal AOD in 9-day-old mice was abrogated by CD4⁺CD25⁺ T cell depletion. Finally, neonatal AOD was blocked by Ab to IgG-FcR, and interestingly, the disease was not elicited by autoantibody to a second, independent native ZP3 B cell epitope. Therefore, a new mechanism of neonatal autoimmunity is presented in which epitope-specific autoantibody stimulates de novo autoimmune pathogenic CD4⁺ T cell response. *The Journal of Immunology*, 2003, 170: 4656–4664.

Adult autoimmune diseases transmitted to infants by maternal autoantibodies are usually transient in nature. However, defective cardiac conduction and heart block occur in some progeny of lupus women with autoantibody to the Ro52 Ags, and this can be associated with destructive lesions and permanent functional impairment that persists long after the disappearance of maternal Abs (1). In addition, maternal Ab has been recently reported to be required for T cell-dependent diabetes mellitus in nonobese diabetic (NOD)³ mice (2). Therefore, maternal autoantibodies may induce neonatal autoimmune disease by mechanisms besides direct Ab action, for example, by the induction of a pathogenic T cell response.

In vitro studies have shown that an Ab facilitates the uptake of its cognate Ag into APCs by 10³- to 10⁴-fold (3, 4), and that Ab activates Ag-specific T cells through interaction of immune complex with FcγR on dendritic cells, leading to APC activation, cytokine production, and presentation of Ag peptides from the immune complex (5, 6). In this process, the epitope specificity of the Ab can influence the specificity of the T cell response (7); depending on the epitope specificity, the Ab may facilitate or negate a T cell response (7–9). Therefore, an Ab can potentially trigger a T cell response and, by modifying APC function, influence the mag-

nitude and the specificity of the T cell response to its cognate Ag. Similar results have been reported for autoimmune responses. mAbs to murine thyroglobulin facilitated or inhibited the presentation of defined T cell epitopes of thyroglobulin (10). In primary biliary cirrhosis, serum Ab to pyruvate dehydrogenase greatly enhanced presentation of its peptides to T cell clones derived from the patients (11). These studies have raised the intriguing possibility that autoantibodies elicited by self- or cross-reactive foreign Ags are often detected in the patients' relatives (12) and may trigger neonatal or juvenile autoimmune disease by invoking de novo pathogenic T cell response.

Autoimmune ovarian disease (AOD) is a known cause of premature ovarian failure (13) and a component of autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, an autosomal recessive autoimmune disorder that results from mutation of the autoimmune regulatory gene (14). The ovarian disease can occur very early in life (15). We have studied an adult murine model of AOD induced by a peptide of the zona pellucida 3 (ZP3) Ag, ZP3_{330–342}, which contains a native B cell epitope (335–342) that overlaps a pathogenic T cell epitope ZP3_{330–340} (16). To investigate the role of autoantibody in murine AOD, we have used a chimeric peptide consisting of the foreign T cell epitope of bovine ribonuclease_{94–104} linked to the ZP3_{335–342} B cell epitope (17). This chimeric peptide 2 (CP2) elicits a strong Ab response to the native ZP3 without concomitant T cell response to ZP3; despite strong binding of the Ab to the ZP in vivo, adult mice with ZP3 Ab alone do not develop any ovarian pathology (17). The only observable effect of ZP3 Ab in adults, demonstrable in ZP3-specific T cell recipients, is the capacity to retarget the location of ZP3-specific T cell-mediated injury from the interstitial atretic ovarian follicles to the normal ovarian follicles (18).

In this paper we describe a novel murine model of neonatal autoimmune ovarian disease (nAOD) with which we have documented the capacity of ZP3 autoantibody to elicit de novo T cell-mediated AOD. The nAOD is manifested only when FcγR function is intact, and it is induced by autoantibody to ZP3_{335–342}, but not against a second native ZP3 B cell epitope. Remarkably,

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Received for publication December 13, 2002. Accepted for publication March 5, 2003.

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¹ Peptide synthesis was supported by a contract from the Contraceptive Branch of the National Institute of Child Health and Human Development.

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³ Abbreviations used in this paper: NOD, nonobese diabetic; AOD, autoimmune ovarian disease; CP2, chimeric peptide 2; nAOD, neonatal autoimmune ovarian disease; ZP3, zona pellucida 3.

nAOD induction requires exposure to the autoimmune complex within a neonatal time window when mice are known to be more susceptible to AOD induction by thymectomy. On the other hand, functional CD4⁺CD25⁺ regulatory T cells appear to confer AOD resistance in older mice. The study has therefore provided strong evidence for a new mechanism of autoimmune disease pathogenesis applicable not only to neonatal or juvenile mice, but also to older mice deficient in regulatory T cells.

Materials and Methods

Peptides

The following CPs were used to generate Ab to native antigenic epitopes of the mouse ZP3. CP2 (NCAYKTTQANKQAQIHGPR) contained a bovine RNase T cell epitope 94–104 (underlined) linked to the modified native self B cell epitopes of ZP3_{335–342} (QFQIHGPR) in which Phe³³⁶, a critical residue of the T cell epitope, has been replaced by an alanine. CP3 (NCAYKTTQANKFSLRLMEENW) contained a bovine RNase T cell epitope 94–104 (underlined) linked to ZP3_{171–180}. The peptides were synthesized by Multiple Peptide Systems (San Diego, CA) with >90% purity as determined by HPLC.

Mice, immunization, nAOD induction, Ab transfer, and surgery

C57BL/6 (B6), A/J, and (C57BL/6×A/J) F1 (B6AF₁) mice were obtained from the National Cancer Institute (Frederick, MD) and housed in a specific pathogen-free facility under the animal care and use committee guidelines of University of Virginia.

To produce CP Ab in milk, 6- to 8-wk-old B6 or B6AF₁ female mice were injected with 50 nmol of CP2 or CP3 in CFA at one footpad and at the base of the tail. One month after immunization, the mice were impregnated by normal A/J males. For nAOD induction, the fluorescence serum Ab titers of milk donors usually exceeded 1/1000.

mAb or polyclonal Ab was transferred to neonatal mice by an i.p. injection. A 50- to 100- μ l volume of Ab was injected through a 30-gauge needle inserted s.c. from the chest to the level of the abdomen and then into the peritoneal cavity. Following injection, the needle was withdrawn very slowly to avoid leakage of Ab. The mAbs used for in vivo studies included Ab to Fc γ R (2.4G2), CD4 (GK1.5), CD8 (53-6.7), and CD25 (PC61; BD PharMingen, San Diego, CA). Normal rat IgG was obtained from Sigma-Aldrich (St. Louis, MO).

Implantation of adult and neonatal ovarian grafts was conducted as previously described (19). Briefly, under general anesthesia and through a posterior vertical skin incision, the kidneys were exteriorized. The incised renal capsule was lifted to create a subcapsular pocket into which an ovary from a 3-day-old or an 8-wk-old B6AF₁ mouse was carefully inserted, and the various layers of the wound were closed separately.

Induction of nAOD by transfer of CP antiserum and antiserum IgG

To produce serum autoantibodies to the CP, male or female B6AF₁ adults were immunized with CP2 or CP3 in CFA. Sera were pooled from mice with a serum fluorescence Ab titer at or exceeding 1/3000. At this titer the polyclonal ZP Ab consistently induced nAOD. The CP3 antisera used in this study had comparable Ab titers as that of CP2. Serum IgG was isolated from serum by a protein G-Sepharose column (Sigma-Aldrich), and the bound IgG was eluted at pH 3, dialyzed with PBS, and concentrated by Centrifix column (Millipore, Bedford, MA). The IgG concentration was determined by an ELISA. For nAOD induction, 100 μ l of serum or ~0.3 mg of serum IgG (equivalent to the amount of IgG in 100 μ l of mouse serum) was injected i.p. on days 3 and 5 to normal B6AF₁ pups, and the mice were studied on day 14.

Histopathologic grading of nAOD

Mouse ovaries were fixed in Bouin's fixative and embedded in paraffin, and 5- μ m-thick sections were stained with H&E. The severity index of nAOD was determined without knowledge of the identity of the mice under study. A severity grade of 1–4 was assigned to each of the following histopathological changes: 1) oophoritis or ovarian inflammation, 2) depletion of growing oocytes, and 3) depletion of primordial oocytes. An oophoritis grade of 1 denotes one or two foci of leukocyte infiltration, grade 2–3 represents an incremental number of multifocal inflammatory foci, and grade 4 is diffuse inflammation. The loss of each type of oocytes was graded 1 when occasional oocytes were lost from the follicle and was graded 4 when all oocytes disappeared. Grades 2 and 3 represent partial and incremental oocyte loss between grades 1 and 4. Because loss of oocytes results in reduction of ovarian function, their grades were weighed as follows: the grade for growing oocyte loss was multiplied by

1.5, and those of primordial oocyte loss were multiplied by 2. Accordingly, the total disease severity index is calculated as: oophoritis grade + (growing oocyte loss grade $\times$ 1.5) + (primordial oocyte loss grade $\times$ 2.0), and total neonatal AOD scores ranged from 1–18.

Immunofluorescence and immunoperoxidase staining

Ab to the native ZP determinants was determined by immunofluorescence as previously described (20). For indirect immunofluorescence, normal ovarian frozen sections fixed in 95% ethanol were incubated with serum diluted in PBS containing 3% BSA. The ZP-bound Ab was detected by FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). The isotype of IgG bound in the native ZP was determined on ovarian frozen sections from experimental mice stained with FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates). In vivo binding of ZP Ab was detected by direct immunofluorescence.

Immunoperoxidase staining was used to identify the ovarian infiltrating cells as previously described (21). In brief, 5- μ m-thick ovarian sections that had been fixed in 4% paraformaldehyde were incubated with primary Ab to CD5 (53-7.313) for T cells, MHC class II (M5/114.15.2), B220 (RA3-3A1/61), or macrophages (F4/80). After washing in PBS, the sections were stained with biotinylated rabbit anti-rat Ab. Bound Ab was detected by avidin-biotinylated enzyme complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), followed by diaminobenzidine substrate (Biogenex Corp., San Ramon, CA). The sections were subsequently counterstained with methylene blue, rinsed with water, and dehydrated with ascending concentrations of ethanol.

Cell enrichment, cell transfer, and in vivo cell depletion

For adoptive transfer of nAOD, CD4 T cells were isolated from the spleens of 2-wk-old mice previously treated with CP2 antiserum. CD4 T cells were enriched on the CD4 T cell enrichment column (R&D Systems, Minneapolis, MN) or by MACS magnetic column with anti-mouse CD4 Ab-coated beads (Miltenyi Biotech, Auburn, CA). CD4⁺ T cells with a purity >90% were injected i.p. into 3-day-old normal B6AF₁ pups at 2×10^6 cells/mouse. The cell recipient was studied 11 days after cell transfer. To deplete cells or to block functional molecules in vivo, the appropriate mAb was injected i.p., 20 μ g/injection, every 2–3 days starting from day 0 or 3. The degree of cell depletion was verified by flow cytometric analysis (FACS), using mAb against the independent epitope of the molecule.

To isolate CD25⁺ T cells from lymph node cells of adult mice, RBCs were lysed and passed through the T cell enrichment column (R&D Systems). The T cells were subsequently incubated with biotinylated CD25 mAb (7D4; BD PharMingen) at 15 μ g/10⁸ cells in 1 ml for 15 min at 4°C and then with PE-conjugated streptavidin (Rockland Immunochemicals, Gilbertsville, PA) at 7.5 μ g/10⁸ cells for 15 min at 4°C. The T cells were labeled with anti-PE Ab-conjugated MACS microbeads (Miltenyi Biotech) and separated through the autoMACS (Miltenyi Biotech). Cell purity was determined by flow cytometry. To study the effects of CD25⁺ and CD25[−] T cells on nAOD induction, 10⁶ CD25⁺ or CD25[−] T cells were injected i.p. into each 3-day old pup, which was then fostered by CP2-immunized dams from day 5. The mice were studied on day 16.

Flow cytometric analysis

A single-cell suspension was prepared from the spleen or lymph nodes. Contaminating RBCs were lysed with ammonium chloride. After two washes, the Fc γ R/III were blocked by incubation with 2.4G2 Ab at 4°C for 20 min. The cells were incubated for 20 min on ice with the desired fluorochrome-conjugated mAbs or isotype control Ig at 0.5 μ g/10⁶ cells. The cells were washed twice with PBS/1% BSA, fixed in 2% paraformaldehyde, and analyzed by FACScan (BD Biosciences, San Jose, CA) using the CellQuest program (BD Biosciences).

Ovarian oocyte quantitation and fertility study

The total oocyte number per ovary was determined as previously described (22). Briefly, the oocytes in every fifth 5- μ m section of the entire ovary, stained with H&E, were counted, and the number was multiplied by 5 to obtain the total oocyte number per ovary. Ovarian function was determined by the fertility of the female mice at 6 wk of age. Each female was mated with a proven fertile CD1 outbred male for 5 wk, and the number of pups born in this period plus the number of implantation sites at the end of the fifth week provided the cumulative litter size.

Results

Induction of nAOD

The murine ZP3 glycoprotein is the autoantigen of the nAOD model. ZP3, the primary sperm receptor in the ZP, is associated with all ovarian growing and mature oocytes that are alive or dead (atretic) (23). The ZP3_{330–342} peptide (NSSSSQFQIHGPR) contains a native B cell epitope 335–342 (underlined) that overlaps with the nested N-terminal T cell epitopes (16). Injection of the ZP3_{330–342} peptide in CFA elicits AOD in adult B6AF₁ mice, and the ZP3-specific CD4⁺ polyclonal or cloned T cells readily transfer AOD to normal recipients (16, 21). In this study we investigated a foreign Ag that elicited ZP3 Ab without a concomitant T cell response. The Ag was a chimeric peptide (CP2) that contained a foreign T cell epitope from bovine RNase_{94–104} and the modified ZP3_{335–342} B cell epitope (Phe³³⁶, critical for T, but not B, cell recognition, was replaced by alanine) (17). CP2-specific T cells did not recognize the ZP3_{330–342} peptide and vice versa (24). All adult C57BL/6 (B6) or (C57BL/6×A/J)F₁ (B6AF₁) mice injected with CP2 in CFA produced Ab to the CP2 that reacted with native ZP3. Although the Ab bound to the adult ovarian ZP in vivo, the ovaries were completely free of pathology (17). Thus, the ZP3 autoantibody is nonpathogenic in adult mice.

Unexpectedly, although the adult ovaries were normal, most B6AF₁ progeny of the CP2-immunized females developed severe nAOD in 2 wk. Thus, of 79 B6AF₁ female progenies from B6 female adults injected with CP2 and CFA, nAOD was found in 82%, with a mean disease severity index of 4.8. In contrast, only 6% of 79 pups from females injected with CFA had focal oophoritis. At autopsy of mice with nAOD, the histopathology was limited to the ovaries (data not shown).

Pathology, progression, and clinical consequence of nAOD

In the progeny of CP2 Ab-positive dams, IgG immune complex was detected on the ovarian ZP 1 day after birth (Fig. 1A). However, the ovaries were normal until days 7–8 when three of 11 mice had low grade inflammation (Fig. 2A). Thereafter, nAOD severity progressed and peaked at 2 wk (Fig. 2A). Thus, a latency period of 1–2 wk exists between Ab binding to endogenous ovarian Ag and onset of ovarian inflammation.

The predominant infiltrating cells in the ovaries with nAOD were lymphocytes/monocytes and occasional clusters of neutrophils/eosinophils, as illustrated in Fig. 1, E and F, respectively. These cells infiltrated the interfollicular space and penetrated the growing follicles that were devoid of oocytes (Fig. 1). Of 79 mice with nAOD studied on day 14, 58% had oophoritis alone, and 32% had oophoritis with partial loss of growing and/or primordial oocytes; in the remaining 10%, oocyte depletion was complete (Fig. 1D). Thus, neonatal AOD can lead to severe organ destruction.

To study the clinical effect of nAOD, the total oocyte number of one ovary from randomly selected pups with nAOD was determined on day 21. At 6 wk the unilaterally ovariectomized mice were mated with fertile males, and their fertility rate was determined between 6 and 11 wk. Compared with control pups, mice with nAOD had a significant reduction in oocyte numbers (Fig. 2B), and only three of eight mice with nAOD became pregnant (Fig. 2C). Importantly, mice with nAOD had an average cumulative implantation site or litter size of 1.5 compared with 15.5 in the control mice ($p = 0.0004$; Fig. 2C). Therefore, severe nAOD invoked by maternal autoantibody to ZP3_{335–342} can inflict sufficient oocyte loss to cause partial or complete premature ovarian failure.

Neonatal AOD was induced by maternal ZP3 autoantibody in milk

To determine whether nAOD was induced by maternal ZP3 autoantibody that crossed the placenta, fetuses from CP2-immunized dams, delivered by caesarian section on gestation day 20, were fed milk from CFA-immunized dams and studied on day 14. These pups had a low incidence of nAOD that did not differ from the control pups fed milk from CFA-injected dams (Table I). In contrast, 73% of 66 pups of untreated dams fed CP2 Ab-positive milk from days 3–14 developed severe nAOD (Table I). Moreover, serum Ab to ZP was not detected in seven pups from CP2-immunized mothers delivered by cesarian section, whereas 100% of 10 age-matched pups with nAOD had serum ZP Ab with a mean titer of 1/300. Thus, milk is the major route of maternal ZP3 Ab transfer.

To rule out the requirement of maternal lymphocytes and pregnancy-associated factors in nAOD induction, serum or purified serum IgG from adult B6AF₁ male or female mice immunized with CP2 in CFA were transferred to neonatal mice on days 3 and 5 at 100 μ l of serum or 300 μ g of IgG/injection. On day 14 the serum and serum IgG recipients developed a high prevalence of severe nAOD compared with recipients of serum from CFA-injected mice ($p < 0.0001$; Table I and Fig. 1D). Importantly, the CP2 antisera from male mice induced nAOD with similar efficacy (data not shown). Thus, autoantibody to ZP3_{335–342} B cell epitope can trigger severe and frequent nAOD, whereas maternal lymphocytes or pregnancy-associated factors are not required.

Immunohistology of nAOD

The ovarian IgG immune complexes in nAOD (Fig. 1A) also reacted with mAb to mouse IgG1, IgG2a, and IgG2b, but not IgG3. Despite IgG2a and IgG2b deposition, C3 was not detected (data not shown). The finding is similar to adult females injected with CP2 or pZP3 in CFA, where the ovarian immune complexes were also devoid of C3. The ovaries of 14-day-old normal B6AF₁ mice (or progeny of CFA-immunized dams) were free of infiltrating T or B lymphocyte (Fig. 1J). A few F4/80⁺ cells scattered outside the ovarian follicles (Fig. 1G) stained negatively for MHC class II (Fig. 1H). In contrast, numerous F4/80⁺ cells were detected inside and outside the ovarian follicles of ovaries with nAOD (Fig. 1J), and many stained intensely for MHC class II (Fig. 1K). In addition, numerous T cells (Fig. 1L) and B cells (data not shown) infiltrated the ovaries.

The histology and cellular infiltrates of nAOD closely resembled those of the AOD in adult recipients of pZP3-specific T cells (25) and the 3-day-old recipients of T cells transferred from day 3 thymectomized donors (26, 27). These immunopathologic findings along with the latency period noted between immune complex deposition and ovarian pathology prompted an investigation on neonatal T cell requirement in nAOD pathogenesis.

Neonatal T cells are required for nAOD development

Pooled CD4 (GK1.5) and CD8 (53-6.7) mAbs (20 μ g each) were injected i.p. every 3 days from birth to deplete neonatal T cells (control mice received normal rat IgG). The mice were simultaneously fed CP2 Ab-positive milk from days 0–14. When they were studied on day 14, most lymph node T cells were depleted by this treatment (data not shown) and, as shown in Fig. 3A, except for one pup, the T cell-deficient recipients of CP2 Ab-positive milk were free of nAOD (Fig. 1B). In contrast, 89% ($n = 9$) of control mice developed severe nAOD (Fig. 3A).

CD4⁺ T cells from nAOD mice adoptively transferred nAOD

We next determined whether neonatal mice with nAOD possessed pathogenic T cells that adoptively transfer nAOD. To avoid participation of maternal lymphocytes, nAOD of the T cell donors

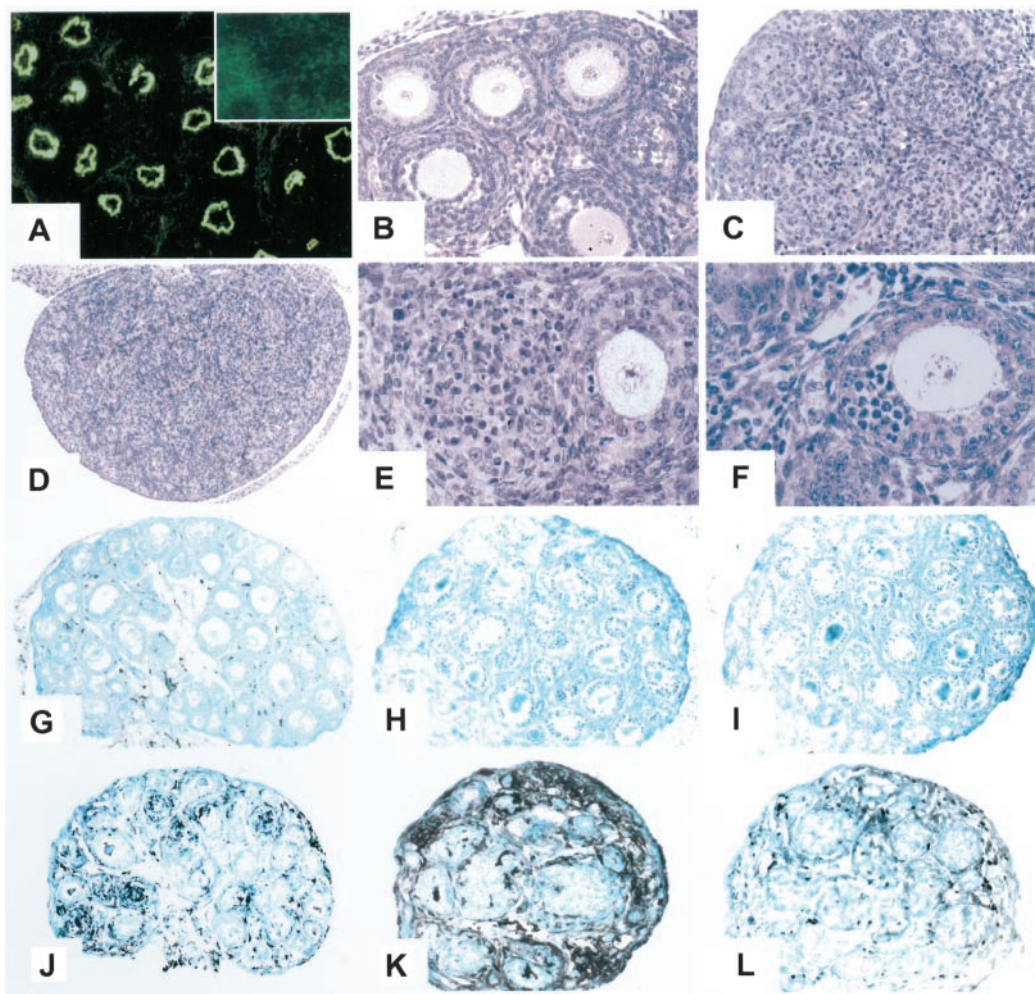


FIGURE 1. Illustrations of ovarian immunopathology of nAOD in B6AF₁ mice induced by CP2 autoantibody with or without additional manipulations. A (inset), B, G, H, and I show ovaries without nAOD, whereas the remaining frames illustrate ovaries with nAOD. A, In vivo IgG binding to ovarian ZP in progeny of CP2 Ab-positive dam (direct immunofluorescence, $\times 100$). The inset shows negative IgG binding to ovarian ZP in control ovary). B, The normal-looking 14-day old ovary with growing follicles from a neonatal female treated with mAb to CD4⁺ and CD8⁺ and receiving ZP Ab-positive milk from day 0 to day 14 ($\times 200$). C, Ovary with heavy mononuclear infiltration from a neonate treated with CD25 mAb, followed by feeding of CP2 Ab-positive milk from days 9–20 ($\times 200$). D, Completely atrophic ovary, from the recipient of serum IgG isolated from CP2 Ab-positive sera ($\times 50$). E, The monocytic nature of the follicular inflammation from a neonatal recipient of CD4⁺ T cells from donors with nAOD ($\times 400$). F, A cluster of neutrophils or eosinophils adjacent to an oocyte in a pup with nAOD ($\times 400$). In G–L, frozen ovarian sections were stained with macrophage or dendritic cell marker F4/80 (G and J), MHC class II (H and K) and T cell marker CD5 (I and L). In the normal 14-day-old B6AF₁ ovaries (G–J), a few extrafollicular F4/80⁺ macrophages were detected without MHC II expression, and T and B cells were absent. In contrast, ovaries from 14-day-old mice with nAOD (J–L) contained numerous F4/80⁺ macrophages, many inside the ovarian follicles (J), and were intensely MHC class II positive (K). In addition to F4/80⁺ cells, many T cells (L) and B cells (B220⁺; not shown) were detected.

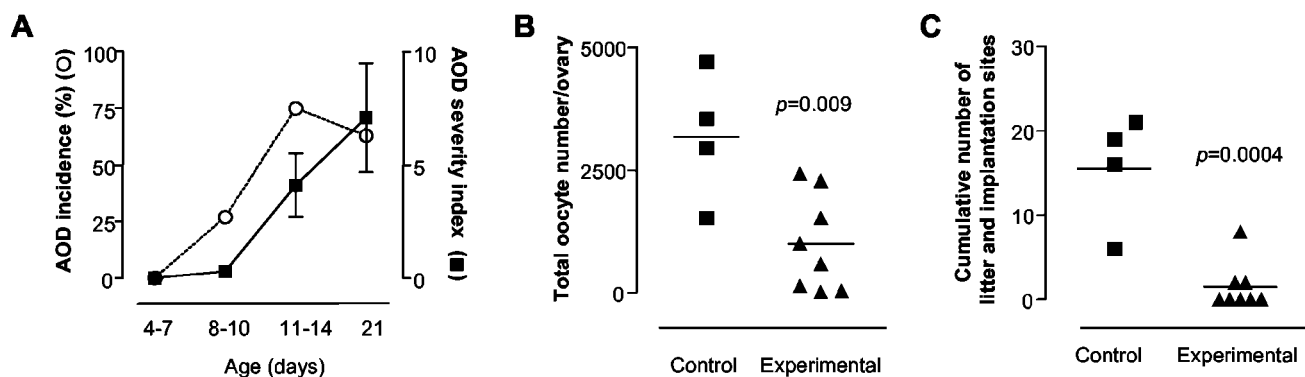


FIGURE 2. Time course of nAOD development and effect on ovarian function. A, Neonatal AOD incidence and severity were determined in 13 mice on days 4–7, in 11 mice on days 8–10, in 12 mice on days 11–14, and in eight mice on day 21. Neonatal AOD began around day 8 and plateaued 4–6 days later. B, The total oocyte numbers per ovary of mice with nAOD were significantly lower than those in control mice on day 21. C, The cumulative litter sizes and implantation sites of mice with nAOD were significantly reduced compared with controls during a mating period from 6–11 wk.

Table I. Induction of neonatal AOD

Treatment of Milk or Serum Donors	Method of nAOD Induction	Neonatal AOD	
		Incidence % (n)	Severity of affected ovaries (mean ± SE)
CFA	Serum transfer ^a	17 (29)	1.7 ± 0.2
	Milk feeding ^b	0 (9)	
CP2 in CFA	Serum transfer ^a	87 (55)	9.5 ± 0.8
	Serum IgG transfer ^a	64 (22)	8.5 ± 1.1
	Milk feeding ^b	73 (66)	5.3 ± 0.6
	Transplacental transfer ^c	20 (10)	1.2 ± 0.2
CP3 in CFA	Serum transfer ^a	7 (14)	2.0 ± 0.0
	Milk feeding ^b	21 (14)	2.7 ± 0.3

^a Normal neonatal B6AF₁ mice were injected i.p. with pooled sera or serum IgG from immunized female or male mice on days 3 and 5 and were sacrificed on day 14.
^b Normal neonatal B6AF₁ mice were fed milk from immunized females mice from days 3–14.
^c B6AF₁ fetuses from the uteri of pregnant females immunized with CP2 and CFA, removed surgically on day 20 of gestation, were foster-fed milk from CFA-immunized, postpartal dams.

was induced by CP2 Ab-positive mouse sera. At 2 wk the donor splenic CD4 T cells, isolated by positive or negative selection to a purity exceeding 90%, were injected i.p. in normal 3-day-old B6AF₁ pups. Eleven days later 65% of the cell recipients develop nAOD with a mean severity index of 3.5 (Figs. 1E and 3B), and the cell recipients' inflammation was limited to the ovaries (data not shown). In contrast, only one of nine recipients of CD4⁺ T cells from control pups that received CFA immune sera had mild nAOD. Therefore, nAOD development is critically dependent on the presence of T cells in neonatal mice, with generation of CD4⁺ pathogenic T cells that induces ovary-specific inflammation in normal mice.

Requirement for neonatal FcγR in neonatal AOD induction

Immune complex can facilitate the uptake and presentation of Ag by the APC while simultaneously activating APC by signaling via their FcγR (5). We therefore blocked FcγR by injecting 2.4G2 mAb at 20 μg/pup every 3 days from day 3. The pups were simultaneously fed CP2 Ab-positive milk from days 3–14. As shown in Fig. 4, nAOD was completely inhibited by the FcγR Ab. In contrast, 80% of 15 control recipients of CP2 Ab-positive milk plus rat IgG developed severe nAOD. Therefore, FcγR is pivotal in the pathogenesis of nAOD, and this strongly suggests participation of the ZP3 immune complex and FcγR⁺ leukocytes in triggering the ZP3-specific T cell response.

Neonatal AOD was not induced by Ab to a second ZP3 B cell epitope

Because induction of T cell response to Ag in immune complexes is influenced by the epitope specificity of the Ab, we compared

FIGURE 3. Neonatal AOD induction depends on pathogenic ZP3-specific T cell response in neonatal mice. A, Neonatal B6AF₁ pups that were treated in vivo with CD4 (GK1.5) and CD8 (53-6.7) mAbs did not develop nAOD when fed CP2 Ab-positive milk. B, CD4⁺ T cells isolated from spleens of 14-day-old B6AF₁ mice with nAOD transferred a high incidence of nAOD to normal B6AF₁ pups.

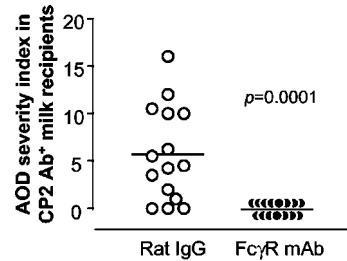
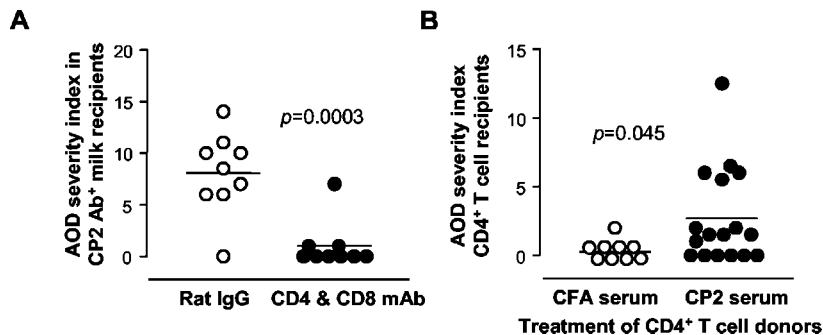


FIGURE 4. Neonatal AOD induction is dependent on FcγR. Neonatal AOD, induced by CP2 Ab-positive milk feeding from days 3–14, was inhibited by injection of mAb to FcγR (2.4G2).

monospecific polyclonal murine Abs to two distinct native murine ZP3 B cell epitopes. CP3 is a chimeric peptide that induces polyclonal Ab to the native B cell epitope, ZP3_{171–180} (24). The ZP3_{171–180} epitope of CP3 does not cross-react with the ZP3_{335–342} epitope in CP2 (24), but CP2 and CP3 share common biological activities in the context of adult AOD (18; see Discussion).

Adult female B6AF₁ mice immunized with CP3 and CP2 produced Ab of comparable IgG isotypes that reacted strongly with the ZP in neonatal ovaries without concomitant C3 binding (data not shown). However, in contrast to CP2 Ab, CP3 Ab did not induce nAOD (Table I). Only one of 14 (7%) recipients of CP3 Ab-positive serum, and three of 14 (21%) B6AF₁ recipients of CP3 Ab-positive milk developed nAOD (compared with CP2 Ab effect: $p = 0.001$ for serum transfer and $p = 0.004$ for milk feeding). Indeed, the nAOD frequency of the CP3 Ab recipients was similar to that of the recipients of Ab to CFA (Table I). Therefore, of two biologically active ZP3 Abs that formed immune complex with endogenous ZP3, only one elicited nAOD. This finding suggests that ZP3 autoantibody's capacity to elicit pathogenic T cells is epitope specific.

Neonatal AOD induction is dependent on exposure to ZP3 Ab within a neonatal time window

Ab to ZP3_{335–342} induced AOD in neonatal, but not adult, mice. To define the age of transition in AOD susceptibility, cohorts of normal B6AF₁ female pups of different ages were fed milk from CP2 Ab-positive dams and studied 11 days later. As shown in Table II, pups fed CP2 Ab-positive milk from day 3 or 5 of age developed peak incidences and severity of nAOD. When CP2 Ab exposure was limited to neonatal days 1–6, 92% of the pups also developed severe AOD 2 wk later (Table II). Finally, although pups delivered by caesarian section from CP2 Ab-positive dams and fed milk of CFA-immunized dams did not develop AOD (Table I), 64% of the pups fed CP2 Ab-positive milk for only 2 days after birth developed nAOD, albeit the mean disease severity was low (2.1).

In contrast, mice fed CP2 Ab-positive milk from day 7 or 9 developed minimal nAOD (Table II). Therefore, nAOD occurred

Table II. Neonatal AOD is induced only in mice exposed to CP2 antibody in the first 5 days of life

Feeding of CP2 Ab-Positive Milk		Neonatal AOD			
Starting age (day)	Ending age (day)	Duration of CP2 Ab Exposure (days)	Age of nAOD evaluation (days)	Incidence % (n)	Severity of affected ovaries (mean $\pm$ SE)
1	6	5	14	92 (12)	4.5 $\pm$ 1.1
3	14	11	14	73 (66)	5.3 $\pm$ 0.6
5	16	11	16	76 (17)	5.6 $\pm$ 1.2
7	18	11	18	12 (8)	2.5
9	20	11	20	9 (11)	1.0

only when neonatal mice were exposed to CP2 Ab that began at or was restricted to the first 5–6 days of life. Between days 5 and 7, something happened to the neonatal mice and rendered the older mice resistant to nAOD.

Resistance to neonatal AOD in 9-day-old mice is abrogated by in vivo depletion of CD4⁺CD25⁺ regulatory T cells

CD4⁺ T cells in normal adult mice that express CD25 play a critical role in maintaining self tolerance and preventing autoimmune disease occurrence. The development of CD4⁺CD25⁺ regulatory T cells has been reported to occur after the development of CD4⁺CD25[−] effector T cells (28, 29). If the belated emergence of CD4⁺CD25⁺ regulatory T cell function accounts for the resistance to nAOD in 7- or 9-day-old mice, then CD25⁺ T cell depletion should enable older mice to develop nAOD.

Injection of mAb to CD25 (PC61) into normal pups on days 3, 5, and 7 led to a reduction in lymph node CD4⁺CD25⁺ T cells from 3.1% to 0.1% (Fig. 5A). When neonates treated with Ab to CD25 were fed CP2 Ab-positive milk beginning on postnatal day 9, 90% of the mice developed nAOD (Fig. 5B). The disease severity was comparable to that found in pups that received CP2 Ab-positive milk from day 3 (compare Fig. 5 with Table I). As controls, nAOD did not develop in mice injected with rat IgG and fed CP2 Ab-positive milk from day 9 (Fig. 5B). Finally, CD25 Ab treatment per se did not cause nAOD (data not shown). Therefore,

CD4⁺CD25⁺ regulatory T cell depletion abrogates resistance to nAOD in mice older than 7 days.

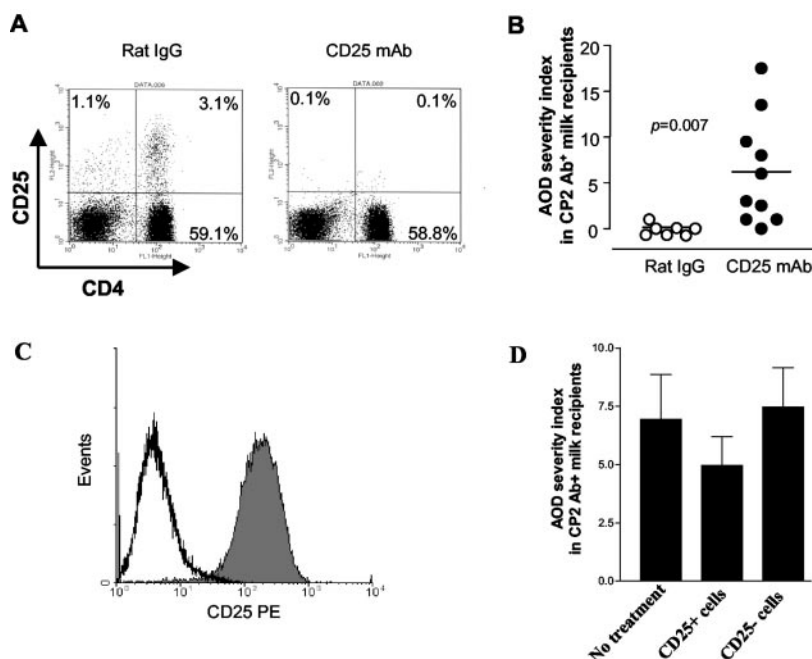
Infusion of adult CD4⁺CD25⁺ T cells to neonatal mice did not prevent nAOD

Neonatal mice have been reported to lack splenic CD4⁺CD25⁺ T cells in the first 3–4 days of life (29). We therefore infused CD4⁺CD25⁺ or CD4⁺CD25[−] T cells isolated from adult female B6AF₁ lymph nodes into B6AF₁ pups that were fed CP2 Ab-positive milk (Fig. 5C). As shown in Fig. 5D, these treatments did not alter the mean severity indexes of nAOD. Moreover, the incidences of nAOD in recipients of CD25⁺ T cells (11 of 13, 85%), recipients of CD25[−] T cells (11 of 13, 85%), and those that did not receive any cells (eight of nine, 89%) were indistinguishable. As a control, the adult CD4⁺CD25⁺ T cells were found to inhibit AOD when infused into day 3 thymectomized B6AF₁ mice, and disease inhibition was cell dose dependent (data not shown).

Neonatal ovaries are not more susceptible to immune injury

Finally, we investigated whether neonatal ovaries are generally more susceptible to immune injury than adult ovaries. Twelve ovaries from normal 3-day-old B6AF₁ pups and six ovaries from adult B6AF₁ mice were engrafted under the renal capsule of six postpartal B6AF₁ females previously immunized with CP2 in CFA. Eleven days later, immune complex was detected in the ZP of

FIGURE 5. A and B, CD25⁺ T cell depletion permits nAOD development in mice older than 5 days. Naive pups were injected every 2 days with rat IgG or PC61 (anti CD25) mAb from days 3–7. A, To assess CD4⁺CD25⁺ T cell depletion, lymph node cells from day 9 mice were stained with GK1.5 (anti-CD4) mAb and 7D4 mAb that recognized a CD25 epitope independent of PC61 mAb and were analyzed by FACS. B, Following rat IgG or PC61 Ab treatment, mice were given CP2 Ab-positive milk starting on day 9; on day 20 ovarian pathology were determined. C and D, Infusion of adult CD25⁺ T cells or CD25[−] T cells into 3-day-old mice did not affect the development of nAOD. C, FACS profiles of unseparated T cells (open peak) and the isolated CD25⁺ T cells (filled peak). D, Mice that did not receive any cells had similar severity of nAOD as recipients of CD25⁺ or CD25[−] T cells.



ovarian grafts, but all the grafts were free of AOD. In contrast, normal B6AF₁ pups that were fed milk from the same cohort of postpartal B6AF₁ ovarian graft recipients developed nAOD (64% ($n = 11$); mean $\pm$ SEM severity index, 3.6 ± 1.6). Therefore, neonatal ovaries are not uniquely prone to AOD. Instead, the predisposition to nAOD depends on the unique neonatal environment.

Discussion

This study, based on a new model of nAOD, has documented that a murine organ-specific autoantibody severely injures the organ in neonatal or progenic mice, but spares the same organ in the adult. Neonatal AOD is inducible by Ab in milk and is transferable by serum IgG from immunized adult male or female donors; thus, autoantibody alone is sufficient to trigger the neonatal disease and does not require other factors associated with the pregnancy state. The autoantibody rapidly forms immune complex with endogenous Ag in the neonatal ovaries, and after a period of 7–14 days brings about frequent and severe ovary-specific inflammation. In >10% of mice with nAOD, complete depletion of ovarian oocytes results in premature ovarian failure. In this study the maternal autoantibody was triggered by the foreign CP2 peptide containing a B cell epitope that mimics endogenous ZP3.

The occurrence of nAOD does not depend on the propensity of neonatal ovaries to immune injury but results from a novel neonatal response driven by the CP2 Ab. We have provided evidence that the ZP3 autoantibody mediated nAOD by triggering a *de novo* neonatal pathogenic T cell response. First, nAOD does not occur in T cell-deficient neonates. Second, CD4⁺ T cells from neonatal mice with nAOD adoptively transfer ovary-specific inflammation to normal neonatal recipients. Third, nAOD occurs only in pups that are exposed to the autoantibody in the first 5 days of life. This corresponds precisely with the neonatal time window when B6AF₁ mice are prone to T cell-dependent AOD following pZP3 injection (30) or thymectomy (26, 29, 31–35). Finally, the most severe nAOD occurred in inbred mice that express the IA($\alpha^k\beta^b$) haplotype, a dominant MHC for pZP3-specific T cell response (36) (Y. Setiady and K. S. Tung, unpublished observation). To our knowledge, this is the first *in vivo* documentation that an autoantibody can induce *de novo* pathogenic autoimmune T cell response, leading to severe organ-specific autoimmune disease with ablation of organ function.

The pathogenic T cell response is likely triggered by endogenous ZP3 Ag in the ovarian immune complex. Consistent with this, nAOD is inhibited by Ab to Fc γ R, which is known to facilitate APC in uptake, processing and presentation of Ag to T cells. That not all Abs to ZP3 induce nAOD is also consistent with Ab induction of T cell response observed *in vitro* (7, 9, 10). The endogenous ZP3 Ag most likely comes from dead oocytes generated in the process of oocyte atresia known to occur in neonatal ovaries (37). Because T cell traffic in neonatal mice is different from that of adult mice (38), we cannot yet predict the location(s) where Ag recognition by T cells and T cell activation may be occurring. However, once T cells are activated, they would induce oophoritis by recruiting cells of the innate system, including macrophages and NK cells (Y. Setiady et al., manuscript in preparation).

A direct action of the autoantibody in nAOD has not been formally ruled out in this study. For example, the Fc γ R requirement in nAOD induction may indicate direct leukocytic activation by immune complex through Fc γ R, thereby enhancing leukocytic cytotoxicity and proinflammatory cytokine production, as documented in the Arthus reaction, glomerulonephritis, arthritis, and immune thrombocytopenia (39). However, nAOD does not occur in neonates deficient in T cells, and T cell depletion should not affect the proinflammatory effect of immune complex. Comple-

ment activation is also not pivotal, because C3 is not detected in the ovarian immune complexes, and our preliminary study indicates that nAOD development is not affected by treatment with mAb to complement component C5 (Y. Setiady and K. S. Tung, unpublished observations).

The nAOD findings also support our previous studies based on murine AOD, which has been found to develop more readily in neonatal than adult mice (reviewed in Ref. 35). Indeed, similar observations have been reported for immune response to autoantigens other than ZP3. Autoimmune gastritis is induced by neonatal injection of the gastric parietal cell H⁺K⁺-ATPase in water (40). Lupus autoantibody and nephritis develop in mice injected neonatally with a peptide that mimics dsDNA in IFA (41). In addition, tolerance to the alloantigen is preceded by an early and transient graft-vs-host response to the donor MHC class II alloantigen and by a transient lupus-like disease (42) that is greatly amplified in mice with *bcl-2*-overexpressing B cells (43). In the present study autoantibody was found to spare the ovaries in adults while inducing severe T cell-dependent AOD in neonates.

Neonatal AOD occurs only when neonatal mice are exposed to the CP2 Ab from days 1–5 after birth. On or after day 7 the mice abruptly became resistant to nAOD when they are infused with CP2 Ab. Therefore, two issues need to be addressed. 1) Why are older mice resistant to nAOD? 2) What could be the basis for the neonatal time window of nAOD susceptibility? The fact that CD4⁺CD25⁺ T cell depletion overcomes the resistance of older mice to nAOD is consistent with the emergence of effective regulatory T cell function beyond the neonatal time window. Previous studies have shown that depletion of CD25⁺ regulatory T cells alone is insufficient for autoimmune disease induction unless it is accompanied by a second event, including lymphopenia-induced homeostatic expansion (as in day 3 thymectomy) or by immunization with Ag in incomplete adjuvant (44). In the case of nAOD, the second trigger is provided by stimulation by autologous immune complexes. However, it is less obvious why mice are more susceptible to nAOD in the first 5 days of life. Although it has been reported that the development of CD4⁺CD25⁺ regulatory T cells lags behind that of the CD4⁺CD25⁺ effector T cells, the finding is currently controversial. While one study did not detect CD4⁺CD25⁺ T cells in neonatal spleen until 3–4 days of age (29), others have documented CD4⁺CD25⁺ T cells in the lymph nodes of 2- to 3-day-old mice and that they suppress CD4⁺CD25⁺ T cell proliferation *in vitro* (45, 46) (E. Samy and K. S. Tung, unpublished observations). The late ontogeny in thymic development of CD4⁺CD25⁺ T cells relative to that of CD4⁺CD25⁺ T cells was reported by one group (28), but the result was not supported by another study (47). If the neonatal susceptibility to AOD was merely due to immature or deficient CD4⁺CD25⁺ regulatory T cell function, one would expect the disease to be inhibited by adult CD4⁺CD25⁺ T cells. Our inability to do so indicates that other properties of neonatal mice may not allow CD25⁺ regulatory cells to function *in vivo*, including the unusual neonatal T cell repertoire (48), the unique neonatal T cell traffic pattern (38), and possibly an innate responsiveness that resists CD4⁺CD25⁺ T cell regulation (49). Regardless of the mechanism, our finding indicates that the neonatal mouse is resistant to regulation by CD4⁺CD25⁺ T cells, and that the resistance state wanes after days 5–7.

The results of our studies on neonatal and adult AOD emphasize a tight connectivity between T cell response and Ab response in autoimmune disease pathogenesis. In adult AOD, activation of pZP3-specific T cells alone is promptly followed by spontaneous IgG autoantibody response to distant ZP3 epitopes (24). The attendant autoantibodies produced in this T to B pathway in adult mice (including CP2 and CP3 Abs), although nonpathogenic by

themselves, retarget the location of the T cell-mediated inflammation in adult AOD, leading to ovarian oocyte loss and fertility reduction (18). In the nAOD model we now document the T to B pathway in reverse; namely, the capacity of autoantibody to trigger de novo pathogenic T cell response. These findings suggest a circular loop whereby autoreactive T and B cells mutually and sequentially stimulate each other, leading to induction and modulation of autoimmune pathology. In addition, endogenous Ags from the normal ovaries are responsible for both the T to B (24, 26) and the B to T responses, whereas the B to T response is also influenced by the integrity of CD4⁺CD25⁺ regulatory T cell function. Therefore, factors that govern endogenous Ag output (e.g., target cell apoptosis) and regulatory T cell function can modulate the T and B cell interactive loop, and consequently, the outcome and progression of an autoimmune process.

The nAOD model has important clinical implications. First, the infertile mice with nAOD lack serum autoantibody, and their ovaries are atrophic, with little or no evidence of ovarian inflammation. The finding of infertility in the absence of any clues for autoimmunity at the time of the clinical diagnosis explains the elusive immunologic nature of human premature ovarian failure and other human autoimmune diseases (50). Second, maternal autoantibodies can trigger experimental autoimmune disease in organs besides the ovaries, including tubulointerstitial nephritis (51) and pancreatic β -cells in NOD mice (2). Although the mechanism of these diseases has not been elucidated, nephritis was associated with significant tubular damage and occurred only in neonatal rats with endogenous Ag; whereas in NOD mice, autoimmune insulinitis is a well-established, T cell-dependent process (52). Therefore, autoantibody induction of T cell-dependent autoimmunity is probably applicable to autoimmune diseases besides the ovaries. Third, the clinical manifestations of human neonatal autoimmune diseases are often not explicable by direct Ab action. In myasthenia gravis neonatal Abs were found to recognize epitopes unique to fetal acetylcholine receptors (53). In congenital heart block associated with maternal Ro52 autoantibodies, 50% of the mothers were free of clinical lupus, and none of them developed cardiac conduction defects. Moreover, the fetal cardiac conduction defects commonly emerge in the second trimester, around the 24th gestational week (1). This time window may correspond to the fetal age when intrauterine thymectomy induced autoimmune ovarian failure in primates (54) and to the murine neonatal time window of nAOD susceptibility. The discrepancy between the rare occurrence of congenital heart block and the high frequency of Ro52 Ab is an enigma that can also be resolved by the nAOD paradigm, including the influence of B epitope specificity of the autoantibody on pathogenic T cell generation and the complex genetic regulation of T cell-dependent neonatal autoimmune disease (Y. Setiady and K. S. Tung, unpublished observations). Perhaps most significantly, the identification of human diseases with the pathogenetic mechanism of nAOD will influence disease therapy, since the de novo autoimmune neonatal T cell response is effectively abrogated by T cell depletion or Fc γ R blockade.

Acknowledgments

We are grateful to Melissa Bevard, Joyce Nash, and Sharon Mangawag for expert technical assistance. The histology and immunohistology were conducted in the Molecular Reproduction Research Center. We also thank Angela Thornton at the National Institutes of Health for her suggestion on CD25⁺ T cell purification.

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