

REI K08

12138801

Health and Human Services
Health Services

Application OCT

PI: 1 K08 K08
Dual:

Council: 05/2008

Do not exceed character length restrictions indicated

1. TITLE OF PROJECT (Do not exceed 81 characters, including spaces) IRG: ZHD1 SRC(99) Received: 10/10/2007
Antigen Identification in a Novel Model of Spontaneous Autoimmune Ovarian Disease

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION NO YES
(If "Yes," state number and title)
Number: PA-06-512 Title: Mentored Clinical Scientist Research Career Development (K08)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR New Investigator No Yes

3a. NAME (Last, first, middle) 3b. DEGREE(S) MD PhD 3h. eRA Commons User Name

3c. POSITION TITLE Adjunct Assistant Professor 3d. MAILING ADDRESS (Street, city, state, zip code)

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Endocrinology

3f. MAJOR SUBDIVISION Medicine

3g. TELEPHONE AND FAX (Area code, number and extension) E-MAIL ADDRESS:
TEL: FAX:

4. HUMAN SUBJECTS RESEARCH 4b. Human Subjects Assurance No. 5. VERTEBRATE ANIMALS No Yes

No Yes 4c. Clinical Trial No Yes 4d. NIH-defined Phase III Clinical Trial No Yes 5a. If "Yes," IACUC approval Date 5b. Animal welfare assurance no.

4a. Research Exempt No Yes If "Yes," Exemption No. 8/30/2007

6. DATES OF PROPOSED PERIOD OF SUPPORT (month, day, year—MM/DD/YY) 7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD 8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT

From 07/01/08 Through 06/30/12 7a. Direct Costs (\$) \$112,750 7b. Total Costs (\$) \$121,770 8a. Direct Costs (\$) \$563,750 8b. Total Costs (\$) \$608,850

9. APPLICANT ORGANIZATION Name Address 10. TYPE OF ORGANIZATION Public: Federal State Local Private: Private Nonprofit For-profit: General Small Business Woman-owned Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER DUNS NO. Cong. District 12

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE Name Title Contracts and Grants Officer Address Tel: FAX: E-Mail:

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION Name Title Contracts and Grants Officer Address Tel: FAX: E-Mail:

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. (In ink. "Per" signature not acceptable.) DATE 10/2/07

DESCRIPTION: See instructions. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project (i.e., relevance to the mission of the agency). Describe concisely the research design and methods for achieving these goals. Describe the rationale and techniques you will use to pursue these goals.

In addition, in two or three sentences, describe in plain, lay language the relevance of this research to public health. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Autoimmune ovarian disease (AOD) is a poorly understood clinical entity wherein immune-mediated destruction of oocytes leads to premature ovarian failure and infertility. The mechanisms of pathogenesis and antigens targeted remain largely unknown, and directed therapies for intervention are lacking. We have generated a spontaneous disease model to study AOD using mice deficient for the Autoimmune REgulator (AIRE) gene, a gene first identified in the human Autoimmune Polyglandular Syndrome (APS) type 1. Like patients with APS 1, aire-deficient mice develop disease of multiple organs, including the ovary, due to a breakdown in central tolerance. Autoimmunity is manifested by development of antigen-specific autoantibodies, organ infiltration, and transfer of disease by lymphocytes from affected aire KO mice. We have demonstrated that the primary defect in the aire-deficient animals is a loss of the ability of epithelial cells within the thymus to present and tolerize developing T cells to self-antigens. As a result autoreactive lymphocytes can escape into the periphery with potential for auto-reactivation within target organs with destruction of self-tissues. In a susceptible strain of aire KO females, I see 100% incidence of oophoritis on histology as well as circulating autoantibodies to ovarian tissue. This presents a valuable spontaneous disease model in which to identify ovarian antigens. Thus, I hypothesize that AOD arises in aire KO females due to failure of negative thymic selection of ovarian reactive T cells and that these autoreactive T cells reflect a lack of appropriate aire-dependent ovarian antigen expression in the thymus. To investigate this hypothesis, I propose the following specific aims:

- Aim 1: To characterize the humoral and cell-mediated immunopathogenesis of AOD in aire KO mice.
- Aim 2: To identify ovarian autoantigens targeted in aire-deficient mice;
- Aim 3: To establish clinical correlations of disease markers in AOD.

By identifying the ovarian component that initiates attack of self, I will better understand the common mechanisms that maintain immune tolerance and normally protect our tissues from many forms of autoimmune disease. Knowledge of the disease-inciting antigen could enable improved testing for the diagnosis and prognosis of autoimmune ovarian disease. Finally, identifying the causative ovarian antigen may provide a potential therapeutic target for treatment and prevention of a significant cause of human ovarian failure and infertility.

PERFORMANCE SITE(S) (organization, city, state)

Principal Investigator/Program Director (Last, First, Middle):

KEY PERSONNEL. See instructions. Use continuation pages as needed to provide the required information in the format shown below. Start with Principal Investigator(s). List all other key personnel in alphabetical order, last name first.

Name	eRA Commons User Name	Organization	Role on Project
			PI
			Sponsor
			Co-Sponsor

OTHER SIGNIFICANT CONTRIBUTORS

Name	Organization	Role on Project
		Collaborator, Consultant

Human Embryonic Stem Cells No Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: <http://stemcells.nih.gov/registry/index.asp>. Use continuation pages as needed.

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

Cell Line

Use this substitute page for the Table of Contents of Research Career Development Awards. Type the name of the candidate at the top of each printed page and each continuation page.

**RESEARCH CAREER DEVELOPMENT AWARD
TABLE OF CONTENTS (Substitute Page)**

Page Numbers

Letters of Reference* (attach unopened references to the Face Page)

Section I: Basic Administrative Data

Face Page (Form Page 1)	1
Description, Performance Sites, Key Personnel, Other Significant Contributors, and Human Embryonic Stem Cells (Form Page 2)	2
Table of Contents (this CDA Substitute Form Page 3)	4
Budget for Entire Proposed Period of Support (Form Page 5)	5
Biographical Sketches (Candidate, Sponsor(s), * Key Personnel and Other Significant Contributors* —Biographical Sketch Format page) (Not to exceed four pages)	7
Other Support Pages (not for the candidate)	20
Resources (Resources Format page)	21

Section II: Specialized Information

Introduction to Revised/Resubmission Application* (Not to exceed 3 pages)

1. The Candidate

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B. Career Goals and Objectives: Scientific Biography	24
C. Career Development/Training Activities during Award Period	26
D. Training in the Responsible Conduct of Research	27
} (Items A-D included in 25 page limit)	
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I. Consortium/Contractual Arrangements*	n/a
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Appendix (Five collated sets. No page numbering necessary.)

Check if Appendix is included

Number of publications and manuscripts accepted for publication (not to exceed 5)

List of Key Items:

n/a

Note: Font and margin requirements must conform to limits provided in the Specific Instructions.

*Include these items only when applicable.

CITIZENSHIP

- U.S. citizen or non-citizen national Permanent resident of U.S. (If a permanent resident of the U.S., a notarized statement must be provided by the time of award.) Non-citizen with temporary visa (Applicable for only the K99 program)

**BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY**

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: <i>Salary and fringe benefits. Applicant organization only.</i>						
CONSULTANT COSTS						
EQUIPMENT						
SUPPLIES						
TRAVEL						
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES						
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
SUBTOTAL DIRECT COSTS (Sum = Item 8a, Face Page)		112,750	112,750	112,750	112,750	112,750
CONSORTIUM/ CONTRACTUAL COSTS	F&A					
TOTAL DIRECT COSTS		112,750	112,750	112,750	112,750	112,750
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD						\$ 563,750

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel:

1. (Principal Investigator, 9.6 calendar months, 80% effort): As the principal investigator for this application, funds are requested for salary support and fringe benefits for Benefits are calculated according to policy at 17% of salary. will spend a minimum of 80% of her time in laboratory research with clinical duties limited to 10-15%.

2. (Sponsor and Mentor, effort only, no salary support) is an Assistant Professor in Residence of Medicine at the In his role as mentor for this application, will oversee the project and provide scientific and career guidance with a focus on developing expertise in immunology.

3. (Co-Sponsor and Co-Mentor, effort only, no salary support): L is the Director of the

As a leader in research on autoimmunity and immunologic tolerance and an investigator with a history of many successful trainees, will provide valuable mentoring and feedback on scientific and career progress.

(CONTINUED)

Personnel (continued):

4. _____ (Consultant and Collaborator, effort as needed, no salary support): _____ s a Professor in the Department of _____ She also serves as the Director of _____ Endocrine Laboratories, and Director of the K12 Fellowship Program. Her clinical and research interests focus on polycystic ovarian syndrome, the perimenopause, and assisted reproduction. _____ will provide guidance on the clinical aspects of reproductive endocrinology of particular pertinence to _____ studies on ovarian autoimmunity. _____ will also collaborate with _____ in identifying and evaluating patients with premature ovarian failure to facilitate the clinical correlation studies proposed. Finally, she will serve as part of the advisory committee with _____ and _____ to assist in monitoring progress during the award period.

Principal Investigator/Program Director (Last, First, Middle):

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
eRA COMMONS USER NAME mickiec		Fellow, Endocrinology	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
	AB		IV
	MD, PhD		(
	Resident Fellow		I

A. Positions and Honors

Positions and Employment

1993-1994

1996-2000

2002-2004

2004-present

Honors

1994

1994-2002

2006

Certification and Professional Societies

2006 |

2004-present |

2005-present |

e

B. Publications (in chronological order)

Original Research

Principal Investigator/Program Director (Last, First, Middle):

Theses

Reviews and Book Chapters

C. Research Support

Ongoing Research Support

None

**Salary support from training grant T32

Principal Investigator/Program Director (Last, First, Middle):

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
eRA COMMONS USER NAME		Assistant Professor in Residence	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Medicine	BA		Biological Science
Medicine	PhD		Immunology
Medicine	MD		Medicine

A. Positions and Honors

Honors and advisory committees

B. Publications:

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

16.

Principal Investigator/Program Director (Last, First, Middle): _____

17.

C. Research Grants:

CURRENT

Principal Investigator/Program Director (Last, First, Middle): _____

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE		
eRA COMMONS USER NAME				
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>				
	INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
C		B.S.		
Cc		M.S.	1977	
(S		Ph.D.		

A. Positions and Honors:
Research and Professional Experience:

Honors:

B. Publications (Selected from over 300 full-length publications):

Principal Investigator/Program Director (Last, First, Middle):

7.

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26.

Principal Investigator/Program Director (Last, First, Middle).

C. Research Support

Principal Investigator/Program Director (Last, First, Middle): (

Principal Investigator/Program Director (Last, First, Middle): _____

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE		
eRA COMMONS USER NAME cedarsm				
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>				
INSTITUTION AND LOCATION		DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
		BA	1	
		MD		

A. Positions and Honors.
Experience

Honors and Awards

Principal Investigator/Program Director (Last, First, Middle):

B. Selected peer-reviewed publications (in chronological order, selected from 85 Total).

- 1.
- 2.
- 3.
- 4.
- 5.
- 6.
- 7.
- 8.
- 9.
- 10.
- 11.
- 12.
- 13.
- 14.
- 15.
- 16.
- 17.
- 18.
- 19.
- 20.
- 21.
- 22.

Principal Investigator/Program Director (Last, First, Middle):

23.

24.

25.

C. Research Support.

Principal Investigator/Program Director (Last, First, Middle): (

Completed Research

Principal Investigator/Program Director (Last, First, Middle):

OTHER SUPPORT

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

laboratory is housed in 2500 sq. ft. of general research space shared with the laboratory of _____, on the 11th floor of the _____, maximizing scientific interaction and resources. Common shared facilities include two conference rooms, six tissue culture rooms, a dedicated radioactive area, a darkroom, a walk-in cold room, histology facilities, FACS machines, a high-speed cell sorter, and a microscopy core.

Clinical:

_____ sees patients one-half day per week as part of the general _____ y clinics.

Participation in these clinics rotates among the following clinical sites: the _____ Ambulatory Care Center, the Veterans Administration Medical Center, and the _____ General Hospital. Excellent administrative and support staff are in place at each clinic site to handle scheduling and patient calls. Also, a first-year fellow at each site covers emergency calls and inpatient consultations.

Animal:

Mouse facilities are located within the central barrier animal quarters of the _____.

This center is a specific pathogen-free dual corridor barrier facility within which the animals are also kept in cages protected by microisolator tops. In this way, we are able to maintain the mice in a pathogen free environment. Procedure rooms and hoods on each floor also are available for clean animal work.

Computer:

The _____ Center has its own information technology support staff to maintain the several computers and network servers (4). In addition to her own laptop computer, _____ will have access to fully-equipped computers (4) and scanner (1) within the _____ lab and computers (2) dedicated for FACS data analysis.

Office:

_____ has office space adjacent to her bench, including a desk with phone, ethernet, and wireless internet connections. Photocopiers and administrative support from the Diabetes Center staff are available.

Other:

_____ will have access to several _____ core facilities, including: the Cell Sorting Core Facility, the Mass Spectrometry Facility (Biomolecular Resource Core), the Hybridoma Core, and the Histology Core.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The laboratory contains the equipment needed for general immunology, protein biochemistry, tissue culture and microscopy. This includes a multipurpose flow cytometer (BD FACSCalibur), protein electrophoresis and blotting apparatus, a walk-in cold room, laminar flow hoods, CO₂ incubators, a cryostat microtome, and microscopes (dissecting, inverted, light). Also within the shared equipment on the 11th floor are a Sorvall ultracentrifuge, incubators, and more microscopes (phase-contrast, fluorescence, and confocal). There is also access to a shared MoFlo fluorescence-activated cell sorter, an ABI 7700 (Taqman) real-time RT-PCR analyzer for antigen expression assays, and a multi-channel, four-laser flow cytometer (BD LSR II). Equipment for general molecular biology and biochemistry techniques are also available (five PCR machines, spectrophotometer, centrifuges, Speed-Vac, agarose gel electrophoresis apparatus, and tissue homogenizer). Many reagents can also be conveniently purchased at a discounted rate on-site through the _____ Cell Culture Facility.

RESOURCES (

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

The laboratory occupies ~ 800 sq. ft. in the Center, located on the 11th floor of campus. The common shared facilities include two conference rooms, six tissue culture rooms, a dark room, a histology room, a microscopy room, a cell preparation room, a glass washing facility, a chemistry lab, and a shared equipment space.

Clinical:

n/a

Animal:

Mouse facilities are part of the central barrier animal quarters of the We have access to a specific pathogen-free dual corridor barrier facility. Each cage also has a microisolator top. These facilities enable us to maintain our mice free of viral pathogens.

Computer:

The Center has two computer support staff. Computers are recently purchased Dell and Hewlett Packard PC's. PC's, printers, slide scanners and digital projectors are all linked to a 24 hrs. (Ethernet) Network. The Center currently has four network servers, two Unix systems serve email and web-page and the other two Window NT Servers are primarily used for file sharing.

Office:

Principal Investigator gets administrative support from the Center's staff. The staff includes a financial analyst, research services analyst, a human resources manager, an accounting assistant, a purchasing officer, two administrative assistants and a secretary

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each. The laboratory contains tissue culture, general immunology, and biochemistry equipment including laminar flow hoods, CO₂ incubators, and microscopes (inverted, light, phase-contrast, and fluorescence). A harvester is available for collecting cells from microtiter trays in thymidine-incorporation assays. Both beta and gamma scintillation counters are available. A 137 cesium radiation source is available for radiating cells and animals. We also have access to a FACSCalibur and a MoFlo fluorescence-activated cell sorter. Other equipment available for biochemical research includes: an ultracentrifuge, a centrifugal vacuum concentrator for sample concentration, UV-visible spectrophotometer, a pH meter, conductivity meter, fluorimeter, fraction collectors and various kinds of electrophoresis apparatuses for molecular biology and biochemistry (polyacrylamide gel, isoelectric focusing, two dimensional electrophoreses, and western blot analysis). A large walk-in cold room is available. Liquid nitrogen freezers are available for storage of cells and temperature sensitive reagents.

RESOURCES ()

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

A fully equipped 2500 sq. Ft. laboratory is available for these studies. The laboratory contains tissue culture, general immunology, and biochemistry equipment including laminar flow hoods, CO₂ incubators, and microscopes (inverted, light, phase-contrast, and fluorescence). A harvester is available for collecting cells from microtiter trays in thymidine-incorporation assays. Both beta and gamma scintillation counters are available. A 137 cesium radiation source is available for radiating cells and animals. We also have access to a FACSCalibur and a MoFlo fluorescence-activated cell sorter. Other equipment available for biochemical research includes: an ultracentrifuge, a centrifugal vacuum concentrator for sample concentration, UV-visible spectrophotometer, a pH meter, conductivity meter, fluorimeter, fraction collectors and various kinds of electrophoresis apparatuses for molecular biology and biochemistry (polyacrylamide gel, isoelectric focusing, two dimensional electrophoresis, and western blot analysis). A large walk-in cold room is available. Liquid nitrogen freezers are available for storage of cells and temperature sensitive reagents.

Clinical:

Not applicable

Animal:

Mouse facilities are part of the central barrier animal quarters of the IACUC. We have access to a specific pathogen-free dual corridor barrier facility. Each cage also has a microisolator top. These facilities enable us to maintain our mice free of viral pathogens.

Computer:

For data reduction, we have fully networked individual computers and offices with appropriate statistical and data analysis software programs. For manuscript preparation, we have Pentium-based desktop computers

Office:

The Principal Investigator is supported by a secretary and the Diabetes central administrative staff composed of: a financial analyst, business research unit research administrator, a human resources manager, an accounting assistant, a purchasing officer.

Other:

Not applicable

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

All of the equipment is located in our laboratory or in close proximity and are under our control. The laboratory is adjacent to the dark room, secretaries' offices and neighboring several immunologists. An effective working relationship has been established with many members of the immunology community, as reflected by the joint journal clubs and seminars. This facilitates interactions among investigators interested in basic immunology. Specifically, laboratory share a weekly Lab meeting and the immunology program participates in a weekly journal club.

SECTION II.**1. THE CANDIDATE****A. BACKGROUND**

My interest in science began with a youthful awe in the power of biology and genetics to explain and predict human disease. Studying molecular biology grew naturally out of this early interest, but not until my undergraduate thesis work did I experience how basic research was the vehicle to unraveling the mechanisms of normal development and thus of disease states. This experience set me on the course to pursuing a combined MD and PhD degree with the goal of a career as a physician-scientist. My doctoral research honed my expertise in genetics and developmental biology while medical school introduced me to the complexity of disease pathophysiology. The choice to continue my training with a residency in _____ was driven by the desire to gain clinical insight into disease mechanisms as well as to apply the same problem-solving approach gained in my research training to the care of patients. Now in fellowship, I feel I am poised to for the synthesis of these different skill sets into the realization of my goal of a career in academic medicine.

Throughout this process, I have been fortunate to have the guidance and encouragement of several key mentors in both the roles of scientists and physicians. Their example continues to inspire my dedication to the paradigm of the clinician-scientist with a career rooted in basic research and enlightened by observations from patient care.

Given my desire to pursue a career as a clinician-scientist, my choice of mentor embodies the qualities that I wish to achieve through my training. I chose [] as a sponsor because his work combines questions in endocrinology with investigations using basic science. Moreover, [] is a trained physician-scientist and is pursuing questions in endocrine autoimmunity that I feel will become increasingly important as we learn more about how to manipulate the immune system. Projects in his laboratory are still early in development and thus offer the opportunity to establish an independent line of investigation.

Despite [] success as a new investigator, further guidance from an experienced investigator and mentor is a valuable opportunity for trainees. With [] as a co-sponsor, I will benefit from a formidable resource for immunology expertise as well as career development. Furthermore, the choice of [] as a co-sponsor is ideal given the close collaboration shared by the [] labs.

B. CAREER GOALS AND OBJECTIVES

My long-term goal is to build a career as a physician-scientist, investigating mechanisms of disease in endocrinology and reproductive biology through basic research using tools of genetics, developmental biology and immunology. The use of model organisms is a powerful approach to the study of human disease, and as a clinician-scientist, I hope to build research centered on animal models to address the questions and observations arising from patient cases. My initial research experience focused on investigating the genetics of [] development in a mouse model with potential for application to human disease syndromes such as [] disease. This interest in genetics and developmental biology led to my choice of doctoral research in one of the classical systems for such investigations, *Drosophila melanogaster*. During my studies of [] my interest in reproductive biology was born. The decision to pursue a fellowship in endocrinology has allowed me to continue to develop my interests in this area while introducing me to the growing need for research in endocrine autoimmunity. Interactions with patients facing possible infertility as a complication of their endocrine disorders has reinforced my awareness in the lack of understanding of many of these disease states and lack of targeted therapies.

My current research centers on the study of autoimmune ovarian disease (AOD) by utilizing a novel mouse model of spontaneous autoimmune disease, the *aire* knockout mouse. My prior experience in *Drosophila* genetics and developmental biology as well as undergraduate training in mouse genetics has given me a strong background that can be applied to the new questions and area of research at hand. The switch to research in immunology allows me to combine clinical questions in endocrine autoimmunity with basic scientific investigation, an ideal combination for a career as a clinician-scientist. The choice to study endocrine autoimmunity in the context of ovarian biology allows me to integrate my background in genetics and developmental biology with new training in immunology, and I feel that I have a unique perspective to

contribute insights into ovarian disease with this blend of clinical and scientific expertise. I believe that my research in autoimmune ovarian disease addresses questions of immunologic tolerance in endocrine autoimmunity as well as questions of fertility. All of these efforts support my long-term goal of pursuing an academic career grounded in scientific research but fueled by questions in clinical endocrinology.

The primary objective for the training period is to carry out focused disease-oriented basic research, which will result in a work suitable for publication and serve as the basis for future research proposals. To achieve this, I aim to gain expertise in immunology and immunological techniques through both formal and informal training experiences (see 1C). During the training period, I hope to devote a portion of time to the care of patients with autoimmune endocrinopathies and other endocrine conditions to remain mindful of disease pathophysiology and potential translation of this knowledge into improving care. My hope is also to engage in a few teaching activities as an important component in generating new questions in research.

A mentored K08 grant will provide the necessary training, funding, collaboration, and protected environment to make the transition to a successful independent investigator. During this time, I will be able to devote at least 80% of my time to laboratory research with the remaining 20% of time available for coursework, teaching and continued clinical practice in the care of patients with autoimmune endocrine disease (see **Table 1a, 1b** below). This support will allow me to develop an independent line of research which will be competitive for R01 funding.

Scientific Biography

Undergraduate thesis research with My introduction to basic research began with my work on the genetics of using the mouse model of in the laboratory of Using quantitative trait genetics, I helped map several modifiers of and cloned an allele of one of the known white spotting genes, the *steel* locus (*c-kit* ligand). My studies culminated in a senior thesis with departmental honors, *cum laude* in molecular biology. While in's lab, I was also fortunate work under the mentorship of Upon completion of my undergraduate degree, I followed to his new appointment at to continue this work as a laboratory assistant. My work on this project with contributed to two publications.

Doctoral thesis research with My positive undergraduate research experience solidified my plans to begin training as a physician-scientist and led me to enroll in the While at I chose to pursue my doctoral research with Steven Wasserman, working on the regulation of meiotic entry in *Drosophila* spermatogenesis. My thesis work centered on the study of *boule*, a male-sterile meiotic entry mutant with homology to the human male infertility genes *Deleted in Azospermia factor (DAZ)* and *BOULE*. In the midst of my graduate studies, moved to the and I followed him there to complete my work on the intracellular protein localization and structure-function studies of *boule*. Although the move to another institution was disruptive, I benefited from the exposure to another excellent environment for research and collaboration at Continued meetings with my mentor and with my thesis committee members, provided critical guidance. I returned to in 2000 to complete my medical training with a first author publication as the culmination of my research.

Fellowship research with After completing my MD, PhD and internal medicine residency at, I began my fellowship in endocrinology at the During this time, I have been working in the laboratory of PhD to develop a novel mouse model of spontaneous autoimmune ovarian disease. Working in the Center at, I have also been fortunate to benefit from the mentorship and co-sponsorship of With the guidance of these two outstanding scientists and the excellent laboratory environment at, I have been able to generate a sound body of preliminary data which I expect to submit as a first author manuscript. To date, my work with has generated a first author review and contributed to a collaborative clinical publication. Initial interest in my research has also been positive as evidenced by a departmental poster award and invitation for an oral presentation at a recent national immunology meeting. I am optimistic that the aims outlined in this K08 proposal will provide novel insight into ovarian disease and serve as the foundation for future independent investigation.

C. CAREER DEVELOPMENT ACTIVITIES DURING AWARD PERIOD

My previous research experience has focused on techniques in genetics, molecular biology, immunohistochemistry, and basic protein biochemistry. Acquiring a knowledge base and technical proficiency in immunology will be important for the proposed research plan, and I will avail myself of several formal and informal training opportunities to develop this (see below). I have already completed two courses in advanced immunology offered by the American Association of Immunology (AAI) and the Federation of Clinical Immunologic Societies (FOCIS). Guidance on immunologic techniques will be available from my mentors as well as their many lab members, who have expertise in the approaches outlined (e.g. fluorescence-assisted cell sorting (FACS), intracellular cytokine staining, adoptive transfer, T cell expansion and recall assays). I expect that the outlined training experiences to constitute approximately 10% of my effort (see **Table 1a**). A timeline of training activities is also outlined below (**Table 1b**).

1) Formal Training Opportunities. During the K08 training period, I will take advantage of the following formal venues for advancing my training. The coursework listed below will be of particular assistance in providing better background in the techniques and topics addressed in my specific aims.

- Coursework

- Macromolecules (I

- I will audit this course to gain insights into protein purification strategies and of mass spectrometry methods. This knowledge will be vital for Aim 2.

- Advanced Topics in Immunology (I

- To increase my background in immunology, I will audit this seminar-oriented course. Topics cover diverse aspects of immunology. This course will be of assistance for Aims 1 and 3.

- Principles of Biological Technologies (I

- To augment the informal technical training provided the _____ lab, I will audit this course on basic techniques in immunology. This course will be vital to Aims 1 and 3.

- Assisted Reproductive Technologies (I

- I will audit this course to gain familiarity with the literature in basic and clinical science underlying assisted reproductive technologies. This course will be important in better understanding the challenges faced in the therapy of premature ovarian failure and possible therapeutic targets.

- Immunology Seminar

- I will attend weekly lectures from leaders in immunology research to develop my knowledge base.

- Immunology Journal Club

- Weekly presentations by members of the department of immunology focus on reviewing current literature. I will attend these lectures to remain abreast of current research in immunology.

- Clinical rotation in Reproductive Endocrinology

- In the past, I have had the opportunity to participate in the reproductive endocrinology clinic at _____ and have arranged to rotate in their clinic in the beginning of 2008. The clinic is headed by _____, who is a collaborator and member of my proposed advisory committee. During the award period, I plan to continue periodic participation in their clinic as part of my allotted time for clinical duties. In this way, I will be able to assist in care of patients with premature ovarian failure and infertility as well as identify potential candidates affected with autoimmune ovarian disease.

- Endocrine Grand Rounds

- Weekly lectures from leaders in basic and clinical research are presented during the academic year on the latest topics in endocrinology. I will attend these to further my fund of knowledge in endocrinology during the award.

2) Informal Training Opportunities

- _____ lab meetings

- Weekly meetings are held jointly with the members of the two laboratories. I will present my research there every 8-12 weeks.

- Combined lab meetings

Principal Investigator/Program Director (Last, First, Middle):

Weekly meetings are held with members of the I labs. is Professor and Chair of the Department of Pathology and a renowned immunologist. I will present my research in this forum twice a year.

- lab journal club

A weekly review of recent relevant literature in immunology is presented by lab members. I will attend this and also participate with presentations once every 6-8 weeks.

3) Training in Scientific Writing and Communication

To enhance my ability to effectively present and communicate my research, I will attend the following courses available at I

- Scientific Writing (). This course focuses on developing skill in writing manuscripts by providing individualized feedback on works in progress.
- Art of Lecturing (). Small group meetings and feedback on individual presentations are used to develop skill in oral presentations of data.
- NIH grant writing workshop. This workshop is offered by the Career Development Office and will be used to facilitate submission a successful R01 application as my work progresses.

4) Mechanism to Review Career Progress

To monitor my progress and assist in the training process, meetings with faculty at several levels will take place.

- I will meet weekly with to review my data and discuss my scientific progress.
- I plan to meet with an advisory committee comprised of). The addition of to my mentoring committee will provide expertise in the field of reproductive endocrinology and valuable consultation on applications of my research to premature ovarian failure (see letter of collaboration). I will meet with these committee members on annual basis to review progress on my research.
- An annual report to the Office of the Academic Vice Chancellor will be provided as a means to review the progress achieved with my award.

D. TRAINING IN THE RESPONSIBLE CONDUCT OF RESEARCH

(i) Several courses and seminar series are available at to address the responsible conduct of research. One such course, entitled Responsible Conduct in Science (A), is offered during both the winter and spring quarters and consists of a series of eight 90 minute classroom and small group sessions led by faculty and research staff. Topics covered include publication practices, data management and sharing, issues in human and animal research among others. This course will be used to fulfill my instruction in the conduct of science.

(ii) has protocols in place to maintain training in ethical conduct in animal research as well as in the general workplace. I have completed the Animal Welfare Assurance Program's Basic Regulatory and Ethical Requirements Training course and will remain up-to- date on my training. I have also completed the Development and Training department's Ethics Briefing and will remain compliant with further training.

Table 1a: Proportion of effort for training activities during award period

Activity	Percentage Effort				
	Year 1	Year 2	Year 3	Year 4	Year 5
Career development.					
Coursework and Seminars	5	5	5	5	5
Laboratory training	5	5			
Writing an R01 grant application			5	5	5
Laboratory research	80	80	80	80	80
Subtotal	90	90	90	90	90
Patient care	10	10	10	10	10
TOTAL	100	100	100	100	100

Table 1b: Timeline of Training Activities During Award Period

TRAINING ACTIVITY	Already Completed	Year 1	Year 2	Year 3	Year 4	Year 5
Completed Coursework						
AAI: Advanced Course in Immunology	x					
FOCIS: Advanced Course In Basic and Clinical Immunology	x					
Formal Training Opportunities						
<u>Protein Biochemistry (Aim 2)</u>		x				
Macromolecules						
<u>Immunology (Aims 1, 3)</u>						
Advanced Topics in Immunology			x			
Principles of Biological Technologies		x				
Immunology Seminar Series		x	x	x	x	x
Immunology Journal Club		x	x	x	x	x
<u>Endocrinology and Ovarian Biology (Aims 1, 2, 3)</u>						
Assisted Reproductive Technologies			x			
Clinical rotation in Reproductive Endocrinology						
Endocrine Grand Rounds		x	x	x	x	x
Informal Training Opportunities						
Lab Meetings		x	x	x	x	x
Combined Lab Meetings		x	x	x	x	x
Lab Journal Club		x	x	x	x	x
Training in Scientific Writing and Communication						
Scientific Writing			x			
Art of Lecturing				x		
NIH Grant Writing Workshop					x	
Responsible Conduct of Research						
		x	x	x	x	x

4. Statements by Sponsor, Consultants and Collaborators

A. Sponsors Statement

We are delighted to serve as sponsors for [redacted] K08 Mentored Research Scientist Development Award application and think that she is clearly qualified and has great potential as an independent research scientist. [redacted] obtained her BA with honors from [redacted] in Molecular Biology. While at [redacted] performed research in the field of mouse genetics and wrote a senior research thesis on the quantitative trait genetics of melanocyte development under the supervision of [redacted]. After leaving [redacted] enrolled in the [redacted]. She obtained her PhD in the laboratory of [redacted] studying the regulation of meiotic entry in *Drosophila* spermatogenesis and this work led to a first author publication in [redacted]. After completing her MD/PhD at [redacted] stayed on to pursue a clinical residency in [redacted]. She then came to [redacted] in the summer of [redacted] as an [redacted] Fellow. During her clinical endocrine training year here between [redacted] gathered a keen interest into the mechanisms by which autoimmune endocrine diseases occur and has decided to pursue research in that general field in the [redacted] laboratory. She has now been working full time at the bench in the [redacted] laboratory since [redacted]. We have both found her to be extremely hard-working and enthusiastic about her project in the laboratory and she has made enough significant progress to have her own preliminary data which is described in her research plan with this application. In addition, we should also point out that she has written the entire research plan with only limited guidance and again highlights her tremendous potential as a future independent investigator. [redacted] has had limited experience in the field of immunology and we believe she would benefit greatly with an extended training period under the auspices of this K08 application. As outlined in the training plan, we will make every effort to help her gain expertise in the field of immunology and autoimmunity in what we think is an outstanding training environment (and is described in greater detail below our signatures).

As research mentors for her project, we are committed to guiding [redacted] project and career development. Both of us have strong publication records in the field of immune tolerance and autoimmunity. Because [redacted] is a relatively junior investigator here at [redacted], we believe that [redacted] will benefit from having [redacted] as co-sponsor for her training award. [redacted] has a strong track record of training scientists in immunology that now have independent positions around the world and this is described in greater detail below our signatures. As outlined in her application [redacted] has a detailed plan of career development and training activities that will help improve her gaining the expertise to become an expert in the field of autoimmune disease research. She will have 200 square feet of research space in [redacted] laboratory in the [redacted] Center at [redacted] and this is also outlined by [redacted] institutional letter of support. We will meet with [redacted] on a weekly basis to discuss the details of her project and also to help guide her in her career development program. She will take coursework to improve her knowledge and skills with grant and manuscript writing, advanced coursework in immunology and reproductive technologies. [redacted] has also laid out a plan with [redacted] here at [redacted] who runs a large reproductive endocrinology clinic to study patients with premature ovarian failure in an important effort to translate her bench findings back to the clinical arena. [redacted] will be a regular participant in our weekly laboratory meetings, journal clubs, and seminars. [redacted] will benefit from being brought into a very strong immunology program at [redacted], where she will participate in weekly journal clubs and seminars and an annual retreat. We expect that [redacted] will attend at least one national meeting per year at which she will present her research findings. During her training we expect [redacted] to devote at least 80% of her time to the research and training described in this project and this is also supported by her institutional commitment letter from the [redacted] Department of Medicine.

[redacted] is a very talented and hard working physician scientist in training and we believe she shows tremendous promise as future independent investigator. She has developed a unique line of research in the [redacted] lab that has clear implications for the important clinical problem of premature ovarian failure and it is our expectation that she will continue with this work in the future for her own independent research program. We expect that [redacted] will publish several first author papers in high level immunology or endocrinology journals during her training program. In this regard, she has already demonstrated her development in

Name of Applicant (Last, First, Middle):

scientific writing in the field of autoimmunity by recently authoring an excellent review on the role of the Aire gene in autoimmunity in the widely read journal of *Immunity*. In addition, she was also a co-author this year on a clinical paper from the *Journal of Clinical Investigation* lab that appeared in *Journal of Clinical Investigation*. In short, we believe that *Journal of Clinical Investigation* shows tremendous promise as a future independent scientist and we are extremely enthusiastic about her application and pleased to serve as her sponsors.

Sincerely yours,

Environment

will be in an outstanding environment for her training during the award period. The *Journal of Clinical Investigation* and *Immunity* laboratories are located next to each other and *Journal of Clinical Investigation* will be able to interact with postdoctoral fellows and graduate students from both groups on a daily basis. The *Journal of Clinical Investigation* and *Immunity* labs hold both weekly lab meetings and journal clubs together and Dr. *Journal of Clinical Investigation* will be a regular participant in both of these activities. In addition, the *Journal of Clinical Investigation* laboratories are located one floor away from over 10 faculty members' laboratories in the *Journal of Clinical Investigation* Immunology Program. The *Journal of Clinical Investigation* Immunology Program has over 30 faculty members with active research programs in a wide number of fields within immunology. *Journal of Clinical Investigation* is a center for Immunology/Infectious disease and includes several Howard Hughes Investigators and one member of the National Academy of Sciences. *Journal of Clinical Investigation* will be an active member of the Immunology Program at *Journal of Clinical Investigation* as outlined above and will be consistently exposed to research work in the immunology and autoimmunity fields. She will also have access to state of the art research equipment for her work during this training program as outlined below:

Research Facilities

Laboratory:

The *Journal of Clinical Investigation* laboratory occupies ~ 800 sq. ft. in the *Journal of Clinical Investigation* Center, located on the 1st floor of the *Journal of Clinical Investigation* campus. The *Journal of Clinical Investigation* laboratory occupies ~ 1600 sq. ft. immediately next to the *Journal of Clinical Investigation* laboratory. The common shared facilities include two conference rooms, six tissue culture rooms, a dark room, a histology room, a microscopy room, a cell preparation room, a glass washing facility, a chemistry lab, and a shared equipment space.

Animal:

Mouse facilities are part of the central barrier animal quarters of the *Journal of Clinical Investigation*. We have access to a specific pathogen-free dual corridor barrier facility. Each cage also has a microisolator top. These facilities enable us to maintain our mice free of viral pathogens.

Computer:

The *Journal of Clinical Investigation* Center has two computer support staff. Computers are recently purchased Dell and Hewlett Packard PC's. PC's, printers, slide scanners and digital projectors are all linked to a 24 hrs. (Ethernet) Network. The Center currently has four network servers, two Unix systems serve email and web-page and the other two Window NT Servers are primarily used for file sharing.

Principal Investigator:

Medical Center

Dear

I am pleased to write this letter of support to affirm my commitment to collaboration in your studies on ovarian autoimmunity, as proposed in your **K08 application**. The AIRE knockout mouse model of autoimmune ovarian disease presents a tremendously exciting opportunity to study the basic mechanisms of a poorly characterized clinical spectrum of premature ovarian failure. The preliminary data you have generated to date is a promising model of spontaneous ovarian autoimmunity with much potential impact for translation into the human disease state.

My expertise in reproductive endocrinology and interest in ovarian aging, especially as it relates to infertility, will be of assistance in the studies of clinical correlation of the animal model that you have proposed. Moreover, as a clinician in an academic medical center, I see a number of patients with premature ovarian failure who may be amenable to participation in your studies. Indeed, I have collaborated with other basic scientists in their studies of both male and female infertility and reproductive biology bringing clinical perspectives complementary to the basic research methods. It is the goal of our Division to build a strong translational program with a focus on multi-disciplinary approaches. Your project fits quite well with our current program.

Finally, as the director of the ~~reproductive endocrinology~~ ~~reproductive endocrinology~~ I have much experience in the mentorship of trainees as they make the transition to independent investigators. I am currently the mentor for two development awards and look forward to working closely with you. I will be pleased to participate as a member of an advisory committee in conjunction with your sponsors, ~~and~~ and D: ~~to~~ to review your progress in these endeavors. I am confident that our collaboration will lead to novel insights into mechanisms of ovarian biology and autoimmunity and be productive for both of us. I look forward to working with you on this exciting project.

Sincerely, 

3. ENVIRONMENT AND INSTITUTIONAL COMMITMENT TO THE CANDIDATE

a. Description of the Institutional Environment

The University () has a well-established record of excellence in basic and clinical research and a strong tradition of training young scientists to become independent investigators. The array of scientific expertise among the faculty combined with the collegial community and well-equipped research centers provide the ideal environment to support recipients of Mentored Career Development awards. In particular, the Department of Medicine, headed by , has a long track record of commitment to the training of physician-scientists.

Within the Department of Medicine, the manifests unwavering support for the training of fellows. Under the leadership of the Chair of the Division, Dr. D , fellows are encouraged to pursue independent research in an area of their choice with 80% protected time from clinical duties following the initial clinical year. Additionally, the fellowship program director, formerly Dr. , and now, Dr. M , meets with trainees at least annually to check on their progress and offer mentoring and career guidance.

Finally, the Center provides an ideal setting for D 's training due to the well-appointed facilities, collaborative environment, and focused expertise in both immunology and endocrinology. The availability of a wide range of core facilities is also of great assistance. Several forums for scientific exchange, such as combined lab meetings, departmental seminar series, and an annual retreat, promote discussion and development of research. Overall, the technical, academic, and administrative resources available provide a well-supported environment for the launching of academic careers.

b. Institutional Commitment to the Candidate's Research Career Development

L has a longstanding tradition of commitment to the development of young investigators. The institution has demonstrated its commitment to L 's continued training by an anticipated faculty appointment with at least 80% protected time for research to begin in . Please see the attached letter from the Chair of the Department of Medicine affirming this commitment and the details of the support provided.

S

Scientific Review Administrator
National Institute of Child Health and Human Development
Center for Scientific Review
National Institutes of Health
6701 Rockledge Drive, Room 1040, MSC 7710
Bethesda, MD 20892-7710

Re: Statement of Environment and Institutional commitment to the candidate

To Whom It May Concern:

I am delighted to provide this statement of institutional support for [redacted] as she submits her application for an NIH Mentored Clinical Scientist Award (K08).

A fellow in our esteemed Division of [redacted], [redacted] has a strong commitment to both clinical medicine and basic research. Her aptitude for research is evident in both undergraduate and doctoral studies resulting in several publications. Prior to coming to the U [redacted], she completed her internal medicine training at the highly competitive residency program at the U [redacted] Center.

Mentors and Training Support

At [redacted] has chosen to conduct her research in autoimmune ovarian disease under the mentorship of Drs. [redacted]. As well-recognized leaders in the field of autoimmune disease and immune tolerance, they will provide exceptional guidance and support in her career development.

The Department of Medicine at [redacted] has a rich and longstanding tradition of training physician-scientists and provides a fertile environment for young scientists to make the transition into independent investigators. The University provides numerous formal and informal venues to stimulate interaction and exchange ideas in the advancement of science. Additionally, training in the responsible conduct of research and a career development programs in training future faculty ensure that we are preparing young investigators for the many roles they will play in academic medicine.

Protected Time and Academic Appointment

At least 80% of her time will be protected for laboratory research. While [redacted] may spend some time instructing trainees in endocrinology, her clinical responsibilities will be limited to 10-15% and her other University service will be minimal.

As an expression of our commitment to Dr. _____, we anticipate her appointment as an Assistant Professor of Medicine at the completion of her fellowship in J. _____. This is a full time entry-level faculty position on our Adjunct track series.

Institutional Support

During the five-year period of the K award, _____ ng will be assigned 200 square feet of research space including individual office space in the I _____ Center. The facilities in the _____ s Center include access to a state-of-the-art modern animal facility and all the equipment she will require to complete here experiments. The Department will also provide administrative and grants management support throughout the duration of the award.

Dr. _____ has demonstrated excellence throughout her educational career and we expect that her accomplishments will continue and will be facilitated by a K08 award. Dr. _____ is committed to a career in academic medicine and she is an outstanding candidate for a Mentored Clinical Scientist Development Award (K08). One of the primary goals of the Department of Medicine and the _____ Center is to make the best possible resources available to exceptional candidates, such as Dr. _____, to help them develop independent academic research programs. This application, which will help ensure that this goal is realized, has the strongest endorsement of the Department of Medicine,

Sincerely yours,

4. RESEARCH PLAN

A. SPECIFIC AIMS

Autoimmune ovarian disease (AOD) is a complex clinical disease wherein the immune-mediated destruction of oocytes leads to premature ovarian failure and infertility. Disease pathogenesis is not well understood, and existing animal models rely on an artificial induction of oophoritis. We have generated a novel spontaneous model to study AOD using mice deficient in *Autoimmune REgulator (AIRE)*[1], a gene critical for immunological tolerance first identified in humans as the cause of Autoimmune Polyglandular Syndrome (APS) type 1. Like patients with APS 1, *aire*-deficient mice develop autoimmune disease of multiple organs, including the ovary, due to a breakdown in central tolerance. Autoimmunity is manifested by the development of antigen-specific autoantibodies, organ-specific infiltration, and immune-mediated destruction of self-tissue. We have demonstrated that the primary defect in *aire*-deficient animals stems from a breakdown in thymic education due to the inability of epithelial cells within the thymus to tolerize developing T cells to self-antigens. As a result, autoreactive lymphocytes escape into the periphery and target organs for destruction.

Genetic background critically affects penetrance of organ-specific autoimmunity in *aire* KO animals [2]. BALB/c *aire* KO female mice develop oophoritis with 100% incidence and a high frequency of ovarian autoantibodies. This creates a novel spontaneous autoimmune disease model to identify key ovarian antigens responsible for AOD. Characterizing the antigens and pathogenesis of AOD will uncover the mechanisms leading to breakdown of central tolerance that we strongly believe are shared among several autoimmune diseases. **Thus, I hypothesize that AOD arises in *aire* KO females due to failure of negative thymic selection of ovarian reactive T cells and that these autoreactive T cells reflect a lack of appropriate Aire-dependent ovarian antigen expression in the thymus.** To investigate this hypothesis, the following specific aims will be addressed:

Aim 1. Characterize the humoral and cell-mediated immunopathogenesis of AOD in *aire* KO mice.

Autoantibody production and lymphocytic infiltration are pathogenic hallmarks of autoimmune disease. To examine whether B or T cell-mediated mechanisms generate AOD, I will characterize the autoantibody response and cellular infiltrates in oophoritis. Kinetics of histologic oophoritis and autoantibody production will define disease onset. Autoantibodies will be assayed to identify the range of autoantigens and their utility as a disease marker. Immune phenotyping and subset analysis of lymphocytes will define the major cell types in ovarian infiltrates and the effector T cell response. I will also define the pathogenic cell type by adoptive transfer using purified cell subsets from affected animals.

Aim 2. Identify ovarian autoantigens targeted in *aire* KO mice.

The focus of this aim is to isolate the ovarian self-antigens recognized by autoantibodies and to demonstrate their thymic regulation by Aire. Ovarian antigens will be identified by mass spectrometry following immunoaffinity purification using *aire* KO autoantibodies. I will test putative autoantigens for specificity by recombinant protein expression. In addition, antigens will be tested for Aire-dependent thymic expression to define their requirement in central tolerance. Finally, a critical role for ovarian antigen-specific autoreactive T cells in AOD pathogenesis will be determined. Adoptive transfer of ovarian disease via T cells from affected ovaries of *aire*-deficient females will identify autoreactive populations and verify cognate T and B cell antigen recognition.

Aim 3. Establish clinical correlation of disease markers in AOD.

To date no antigens have been convincingly linked to AOD in humans. Using this spontaneous disease model of autoimmunity, I have the unique opportunity to identify common targets of AOD. I will characterize ovarian function in *aire*-deficient females by monitoring of estrus cycles and hormone profiling. Ovarian function will be correlated to the onset of AOD as assessed by autoreactivity and organ infiltration to better establish a clinical assessment of autoimmunity. I will also determine whether similar antigens are recognized in human autoimmune ovarian disease. Serum samples from patients affected with ovarian failure and APS 1 or other autoimmune endocrinopathies will be tested for reactivity to putative ovarian antigens to establish clinical correlation of my disease model.

Together, these studies will test the specific hypotheses that autoimmune ovarian disease results from a breakdown in central tolerance and lack of tissue specific antigen expression in the thymus. The results will provide important insights into the pathogenesis of autoimmune ovarian disease, mechanisms of central tolerance, and potential targets for novel therapeutic approaches to treat autoimmune ovarian disease.

B. BACKGROUND and SIGNIFICANCE

B1. Human autoimmune ovarian disease (AOD)

Premature ovarian failure (POF), defined as cessation of menses in women less than 40 years of age, is a notable cause of infertility affecting ~1.5 million women in the US. Multiple etiologies lead to POF, but the majority of cases are found to be idiopathic after excluding genetic, structural, and iatrogenic causes. Many of these idiopathic cases may be attributable to autoimmune ovarian disease (AOD). Indeed, organ-specific autoantibodies are detected in 50-70% of POF patients [3, 4]. Ascertainment of AOD is difficult since definitive diagnosis requires ovarian biopsy to document tissue infiltration and destruction. Given the variable clinical course of AOD, correct diagnosis of this subset of POF patients can be critical since some AOD patients can conceive with glucocorticoid therapy. Furthermore, detection and prediction of AOD in patients with known autoimmune disease or unexplained infertility would be valuable prognostic information, allowing changes in family planning or assisted reproductive interventions. To date, treatment is limited to general immunosuppression with glucocorticoids and is being studied in an ongoing prospective clinical trial based at the NIH (Trial ID NCT00001306).

Assisted reproductive methods frequently rely on in vitro fertilization (IVF) and ovulation induction. With improvement in these technologies leading to their wider practice, more outcome data on IVF patients has accumulated. Some studies suggest that anti-ovarian antibodies can be induced by repeated attempts at IVF or hormonal stimulation [5, 6]. Additionally, a higher prevalence of such antibodies in IVF failures than those with successful IVF suggests that anti-ovarian antibodies may affect embryo development or implantation [7, 8]. Thus, better understanding of the pathogenesis as well as sensitive screening for AOD would be important in patients considering assisted reproduction or with unexplained failure with IVF.

More often, the diagnosis of AOD is inferred by its association with other autoimmune conditions. Among the most frequent associations are the Autoimmune Polyglandular Syndromes (APS), type 1 and 2, as well as isolated Addison's disease. These polyendocrine syndromes are relatively rare but exhibit a significantly increased prevalence of AOD, reaching 72% in APS 1 females by age 40 [9]. Although the recognized link between AOD and other autoimmune conditions dates back as early as the 1950's, little is known about the mechanisms that lead to disease nor have the means for diagnosis improved greatly.

Earlier attempts to understand the antigenic targets of AOD have relied primarily on indirect immunofluorescence staining of tissue with serum from affected patients. This approach has revealed steroid cell autoantibodies (SCA), which include the P450-17 α -hydroxylase and P450-side chain cleavage enzymes, as well as reported antibodies to follicle stimulating hormone (FSH), the FSH receptor, the zona pellucida, and the oocyte itself (reviewed in [10-12]). However, description of anti-ovarian antibodies is often confounded by discordance in detection methods, variability in tissue sources, and heterogeneity of the patient groups. Furthermore, the known antibodies have low specificity and little predictive value for development of POF outside of limited clinical scenarios. Therefore, experimental animal models provide a valuable mechanism to dissect and understand the immunopathology of AOD.

B2. Experimental animal models of AOD

Early studies induced oophoritis by immunization with various ovarian extract preparations or treatment with cyclosporine (reviewed in [4, 11]). The primary animal model for oophoritis for over 20 years has been the mouse neonatal thymectomy (NTx) model. In multiple strains of inbred mice, removal of the thymus at day 3 after birth induces a variety of autoimmune diseases targeting the ovaries, testes, prostate, stomach, thyroid, and lacrimal gland, notably with development of oophoritis in BALB/c and A/J mice [13-17]. Autoimmunity develops through an imbalance between regulatory and effector T cells. Disease arises from the relative depletion of CD4⁺ CD25⁺ regulatory T cells (Tregs), which begin to exit the thymus after day 3, and replacement of Tregs in NTx mice prevents autoimmune disease [18, 19]. The generation and progression of AOD requires continued exposure to endogenous antigen as illustrated by prevention of AOD in NTx mice that undergo oophorectomy [20]. Moreover, the ability of polyclonal Treg populations to suppress autoimmunity is disease-specific and dependent on the presence of endogenous autoantigen [21, 22].

A marker of autoreactivity in NTx mice is the presence of oligoclonal anti-ovarian autoantibodies in 70-90% of females [15, 20, 23]. Characterization of a predominant 110-kD antigen in this model led to the identification of a novel oocyte-specific protein, MATER (*Maternal Antigen That Embryos Require*) [23]. Inactivation of the mouse *Mater* gene caused embryos from *Mater* null mothers to arrest at the 2 cell stage and eventually degenerate, indicating that *Mater* is a maternal affect gene required for embryonic development after fertilization [24]. The human homolog for mouse *Mater* has been identified and also shows oocyte-restricted expression [25]. However, no data demonstrating a causal link to either mouse NTx-induced AOD or human AOD have been demonstrated to date. Given that the NTx model relies on experimental induction of disease, it is difficult to predict whether the same pathogenic mechanisms will be responsible for spontaneous AOD.

B3. A new model for AOD: Aire-deficient females develop spontaneous oophoritis

A novel model of autoimmune disease has been recently generated in our lab by inactivation of the mouse *AIRE* (Autoimmune REgulator) gene. This gene was identified from patients with an autosomal recessive disorder known as Autoimmune Polyglandular Syndrome (APS) type 1. Affected patients develop spontaneous autoimmune disease targeted primarily at endocrine organs, including the parathyroids, adrenals, thyroid, ovaries or testes, and pancreatic islets. Positional cloning efforts in human subjects led to identification of the *AIRE* locus on chromosome 21q22.3 by two independent groups [26, 27].

AIRE encodes a 545 amino-acid protein with several domains that suggest it functions as a transcription factor (reviewed in [28]). *In vitro* studies have shown that Aire can bind DNA [29], activate transcription [30, 31] and act as an E3 ubiquitin ligase [32]. Aire is expressed primarily in the thymus and to a lesser extent in lymph nodes and spleen [1, 33, 34]. Within the thymus, Aire is restricted to a subset of medullary thymic epithelial cells (MTECs) and dendritic cells [34].

aire-deficient mice, similar to their human counterparts, **spontaneously** develop autoantibodies and immune infiltrates in multiple organs as they age, including the eye, salivary gland, stomach, liver, thyroid, and ovary [1, 35, 36]. Immunological analysis of *aire* KO mice did not reveal profound defects in the major immune compartments of bone marrow, lymph node, spleen or thymus. Observation of a defect in the thymic stromal compartment in the KO mice along with localization of Aire expression to MTECs suggested that the critical function of Aire lay in these cells. Promiscuous or ectopic expression of self-antigens by MTECs has been described in association with development of autoimmune syndromes [37-39]. Aire-deficient MTECs exhibit reduced or absent expression of a number of genes, including several tissue-specific self-antigens when compared to wild-type MTECs [1, 40]. Additionally, transfer of Aire-deficient thymic stromal cells into athymic *nude* recipients recapitulates the organ-specific autoimmune disease seen in *aire* KO animals. These findings support a model in which Aire acts in MTECs to regulate the expression of a subset of thymic self-antigens to allow negative selection of autoreactive T cells generated during thymocyte development (see **Figure 1**).

During normal T cell development, thymocytes migrating through the thymic cortex begin to express functional T-cell receptors (TCRs) with a diverse range of specificities due to the generation of the TCR alpha and beta chains arising from somatic recombination events. Cells that have undergone successful TCR rearrangement travel into the thymic medulla where they encounter MTECs, cells that are specialized to present an array of self-antigens to the developing T cell. Thymocytes that express TCRs with high-affinity for self-antigens presented by MTECs will then undergo deletion, thus preventing autoimmunity. Aire mediates the expression of a subset of these self-antigens in MTECs, such that in the absence of Aire, presentation of some self-antigen by MTECs is reduced or absent, leading to persistence of self-reactive T cells. Although APS 1 is a relatively rare disorder, it is likely that similar mechanisms are in play for more common autoimmune disorders. Perhaps, the best evidence for this is seen in the case of Type 1 diabetes and insulin (reviewed in [41]). Absence of *insulin-2* expression in the thymus leads to accelerated autoimmune diabetes in the NOD mouse [42, 43]. Moreover, a significant disease risk locus in type 1 diabetes is the VNTR of the insulin promoter, and protective alleles of VNTR are associated with increased levels of thymic insulin expression [44-46].

AIRE and APS 1 are a rarity in the world of autoimmune disease: a pleiotropic phenotype of multi-organ specific disease arising from a single gene defect. The mouse model provides a powerful tool to identify autoantigens with high potential relevance to the human disease state. For example, preliminary studies indicate that APS 1 patients with uveitis show similar patterns of autoreactivity to the retinal autoantigen we have identified in the *aire* KO mouse (J [47]). As such, the *aire*-deficient mouse

C. PRELIMINARY STUDIES**C1. *aire* KO female mice develop spontaneous oophoritis.**

aire-deficient animals develop spontaneous multi-organ infiltrates and autoreactive antibodies, including ovarian disease (Figure 2A, [1]). The severity and incidence of histological disease varies among inbred congenic strains with increased susceptibility or resistance to disease in certain organs (Figure 2B, [2]). I have chosen the BALB/c strain to characterize Aire-dependent ovarian disease based on 100% penetrance and consistent severity of oophoritis. Additionally, BALB/c mice also have increased susceptibility to oophoritis generated by NTx, so comparisons with this established model will also be possible and informative.



Figure 2. Ovarian infiltrates develop in *aire*-deficient mice. A. *aire* KO females develop oophoritis. Representative H&E sections from ovaries of *aire* KO (right) or age and sex-matched wild-type control mice (left). Note a marked lymphocytic infiltrate with no intact follicles in the KO (From [1]). B. Genetic background shapes autoimmunity in *aire* KO mice. At far right are percentages of organ-based infiltrates among inbred strains of *aire* KO mice. For example, B6 mice are resistant to oophoritis but prone to prostatitis. Note, BALB/c mice are very susceptible to ovarian disease (From [2]).

	B6 n=12 (F7/M5)	BALB/c n=10 (F6/M5)	NOD n=16 (F8/M7)	SJL n=7 (F4/M3)
retina	87	80	93	NA
cornea	-	60	14	-
stomach	9	100	87	100
salivary gland	42	20	100	100
lung	38	60	100	100
liver	-	40	86	83
prostate	100	100	100	100
ovary	-	100	88	60
pancreas	-	-	100	71
thyroid gland	-	-	54	-

Strain	Histology	AutoAb
B6/NOD	78 (7/9)	9 (4/45)
BALB/c	100 (21/21)	38 (8/21)
NOD	88 (7/8)	38 (3/8)

Figure 3. Incidence of oophoritis in *aire* KO females. Percentage of females positive for histologic oophoritis and autoantibody production (as assayed in C4.2) among mouse strains. Parentheses indicate numbers positive over total examined. B6/NOD are F2 intercross progeny. Females are 20wks (BALB/c), 15wks (B6/NOD), or 5-8wks (NOD) of age. Note the penetrance of histologic ovarian disease in BALB/c females.

As in other models of spontaneous autoimmune disease like the NOD mouse and diabetes, the penetrance of organ-based autoimmunity increases with age in *aire*-deficient animals, though the severity and onset of disease varies among strains. Since BALB/c *aire* KO mice are long-lived, 20 week-old mice were examined and determined to have moderate to severe oophoritis by histology 21/21 and 38% autoreactivity (Figure 3).

C2. Ovarian infiltrates are comprised primarily of T cells in Aire-mediated oophoritis**C2.1 CD4+ and CD8+ T cells infiltrate the affected ovaries.**

To define the composition of ovarian infiltrates in *aire* KO females, I used immunohistochemistry to characterize the primary immune cell types involved. The infiltrates are comprised primarily of CD4+ and CD8+ T cells with very few B cells (Figure 4A). Relative numbers of CD4+ or CD8+ T cells vary with severity of oophoritis between individual KO animals; however, samples consistently demonstrate presence of both cell types with generally more CD4+ cells. The predominance of CD4+ and CD8+ cells suggests that T cells are key mediators of oophoritis

C2.2 CD4+ T cells predominate in intra-ovarian lymphocytes and display a Th-1 like phenotype.

Ovarian infiltrates were examined to determine the T helper activation profile of infiltrating cells. Ovarian tissue was mechanically dissociated into single cells by microdissection or incubation with collagenase. *aire* KO female ovaries were thus examined by flow cytometry for CD4, CD8, CD19, CD45, IL-4, IL-17 and IFN- γ . CD4+ T cells were identified as the largest proportion (36%) of ovarian infiltrate (Figure 4B). Further characterization suggests polarization of CD4+ cells to a Th-1 like phenotype based on increased production of IFN- γ (Figure 4C). These results suggest that Th-1 mediated autoimmune attack causes the ovarian disease

in *aire* KO females. Both immunohistochemical and FACS analysis of affected ovaries support the hypothesis that oophoritis arises from autoreactive T cells that persist in the absence of Aire, infiltrate the ovaries, and participate in end stage tissue damage.

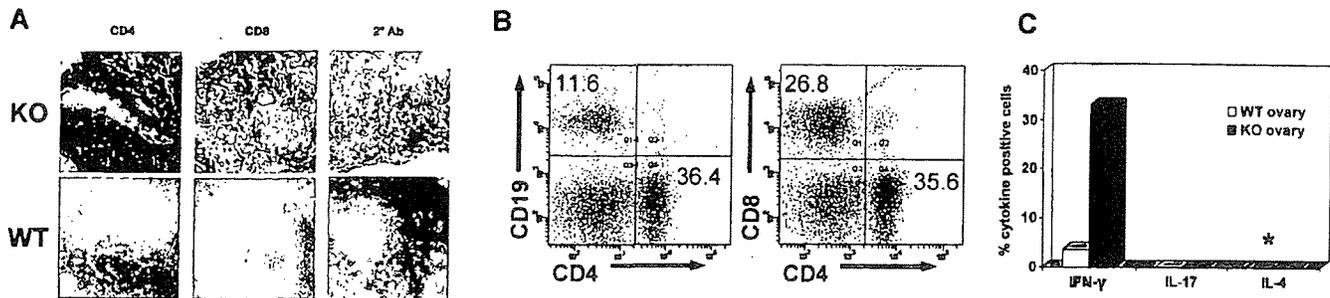


Figure 4. Ovarian infiltrates in *aire* KO females contain CD4⁺ and CD8⁺ cells. A. Immunohistochemistry of CD4⁺ and CD8⁺ cells. Above right are sections from *aire* KO (top row) and wild-type (bottom row) BALB/c females. Images acquired at 400X. 8 μ m frozen sections were stained with CD4, CD8, or secondary antibody alone, developed with DAB, and counterstained with hematoxylin to aid visualization. IgD staining did not reveal positive cells above background levels (not shown). Note the atrophic appearance and absent follicles in the ovarian tissue in the KO compared to wild-type. B. CD4⁺ cells predominate in oophoritis. At center are FACS data showing numbers of B cells (CD19⁺), CD8⁺, and CD4⁺ cells isolated from the ovaries of a 6wk old NOD *aire* KO female. Percentages based on gating for CD45⁺ lymphocytes. About 36% of lymphocytes are CD4⁺. C. Infiltrating CD4⁺ T cells are Th-1 like. At right is a bar graph showing intracellular cytokine staining of cells harvested from ovaries of a 5-6wk old *aire* KO or wild-type NOD female. Gating on CD4⁺ CD45⁺ lymphocytes, numbers are expressed as percentage of cells with positive staining above isotype control. Activated CD4⁺ cells isolated from KO ovaries produce more IFN- γ , consistent with a Th-1 phenotype. Column with * indicates sample not done.

C3. Ovarian disease can be transferred by immune cells from affected *aire*-deficient females

Given the likely role of autoreactive T cells in the pathogenesis of oophoritis, I predicted that adoptive transfer of immune cells would cause ovarian disease. Preliminary results in **Figure 5** indicate oophoritis can be transferred by polyclonal lymphocytes from an affected *aire* KO female. In this study, *aire*-KO splenic cells were transferred to SCID recipients and after 2 months assayed for disease development. Histology reveals ovarian infiltrates and tissue destruction in all recipient females (n=3), and immunohistochemistry demonstrates oophoritis with CD4⁺ cells (**Figure 5**), demonstrating that ovarian disease can be transferred from a polyclonal pool of lymphocytes from *aire*-deficient donors. Future work will determine the critical cell type responsible for disease using lymphocyte subsets (see D1.2 below for further details).



Figure 5. Adoptive Transfer of Oophoritis with cells from an *aire*-deficient female. At left are representative sections from a SCID female host transferred with splenic cells from an *aire*-deficient female donor. Notably, the donor was also affected with oophoritis. Images acquired at 100X. Sections show evidence of CD4⁺ and CD8⁺ staining consistent with transfer of oophoritis.

C4. *aire*-deficient mice develop ovarian-specific autoantibodies

C4.1. Autoreactive antibodies to oocyte cytoplasm and follicular cells develop in *aire*-deficient mice

Given the presence of immune infiltrates within the ovary, I tested *aire*-deficient females for tissue-specific autoantibodies. Autoantibodies to the oocyte cytoplasm and the follicular cells were detected in the serum of *aire*-deficient female mice but not in serum from wild-type females using indirect immunofluorescence staining (**Figure 6**). Interestingly, not all *aire* KO mice were serum positive, and this correlates with presence of autoreactivity by immunoblot (discussed below in C4.2). Furthermore, for those animals with autoreactivity, intensity of staining on indirect immunofluorescence correlates with that by immunoblot, indicating that disease severity may be predicted by intensity of autoantibody response.

Name of Applicant (Last, First, Middle): _____

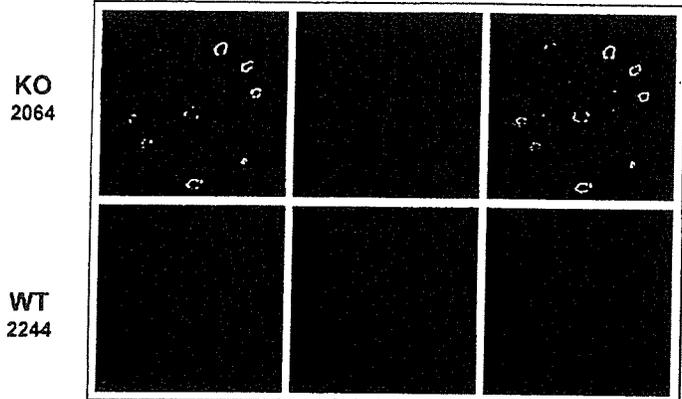


Figure 6. Ovarian autoantibodies develop in *aire* KO females. Frozen sections from wild-type female ovaries were sectioned at 8µm, fixed, and incubated with serum from either an *aire* KO (top row) or wild-type female (bottom row) diluted 1:200. FITC-conjugated secondary antibody was used for detection (right column), and DAPI staining was used to visualize cell nuclei (middle column). Merged images are shown in the left column. All images acquired at 100X under matched exposures for each channel.

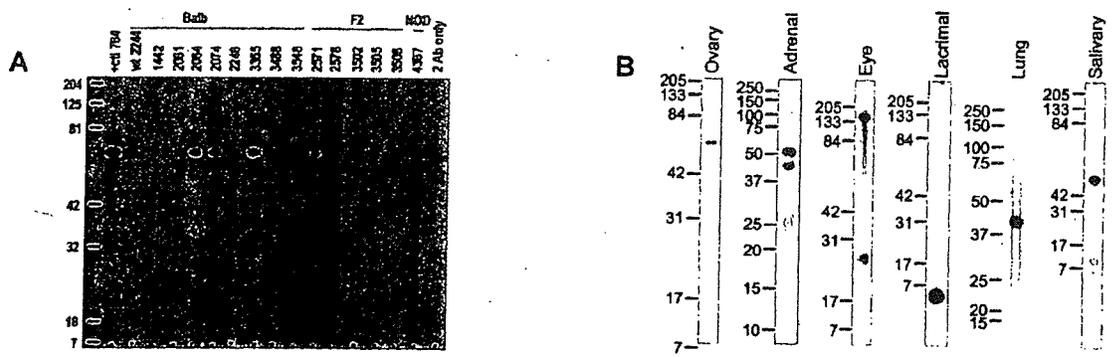


Figure 7. Autoantibodies in *aire* KO females recognize a 60-kD ovarian antigen. A. *aire* KO females produce antibodies to a 60-kD ovarian antigen. Above is a representative immunoblot illustrating reactivity to a 60-kD band in serum from *aire* KO females of several strains. F2 mice are from an intercross of B6 and NOD strains. Samples were screened using a BioRad Mini-PROTEAN II Multiscreen Apparatus, which allows probing with 20 different antibody samples on a single blot. Wild-type ovarian extract was resolved by SDS-PAGE using a multi-well preparative gel comb prior to transfer and immunoblotting. Note absence of reactivity in the wild-type control (lane 2) and secondary antibody alone (lane 20). B. The 60-kD autoantigen is found distinctly in ovarian tissue. Shown are single lanes from several independent multiscreens of various organs extracts run as in A. All lanes shown were probed with serum from an NOD *aire* KO female (ID#4358) affected with ovarian disease. Distinct antigens are recognized in each tissue with a possible similarly sized band in adrenals.

C4.2. Immunoblot analysis of ovarian extracts reveals a putative autoantigen in *aire*-deficient females

Using immunoblot screening of serum from *aire*-deficient females against mouse ovarian extracts, I have identified a unique anti-ovarian autoantibody response common to several *aire* KO mice (Figure 7A). This single band is ~60 kD, and is recognized by *aire* KO serum from females bred in B6/NOD, NOD, and BALB/c backgrounds but not by serum of wild-type females from these strains. The pattern of autoreactivity seems to be most prevalent in the BALB/c strain with 4/8 shown below positive on immunoblot. Notably, the observed 60-kD autoantibody response is distinct from prior profiles demonstrated with the NTx model of oophoritis and likely represents a novel autoantigen.

To determine whether the 60-kD autoantigen is unique to the ovary, I probed multiple organs extracts. Immunoblot of serum from individual females reveals distinct oligoclonal autoreactivity in each organ. Comparison of immunoblots indicates that the 60-kD band occurs uniquely in ovarian tissue (Figure 7B). A similarly sized band may also be seen in adrenal extracts. Precedence for shared antigens is known in the example of SCA, which is found in patients with autoimmune adrenal or ovarian disease (see B1). Indeed, a positive SCA in patients precedes autoimmune adrenalitis and often predicts subsequent ovarian failure [7]. Notably though, BALB/c mice are not affected with adrenalitis. Shared antigens will also be of great interest given the broader implications for the pathogenesis of multiple organ-based diseases. Our examination of autoantibodies to multiple organs suggests that the ovarian autoantigen is largely distinct from other tissue antigens.

At first, the lack of a uniform detection of autoantibodies seems at odds with the 100% penetrance of oophoritis by histology. Further examination of ovaries from autoantibody-negative BALB/c females revealed atrophic organs with complete lack of follicles, indicating severe, end-stage disease. Autoantibody production wanes with age and progressive disease as more antigen is depleted by tissue destruction. Thus, the autoantibody-negative animals may have been positive earlier in the course of disease, but are negative by the 20-week time point of this experiment. A careful kinetic examination will investigate this (See D1.1).

C4.3. Ovarian autoreactivity is detectable as early as 10 weeks of age in *aire* KO females.

Initial assessment of disease kinetics by autoantibody production suggests that ovarian disease begins at 8-10 weeks of age in *aire*-deficient BALB/c females. Serum samples from cohorts of *aire* KO females of 5, 8-10, or 18-20 weeks of age were screened by immunoblot as above (see C4.2) for ovarian autoantibodies. Reactivity to the 60-kD autoantigen is detectable starting at 8-10 weeks of age with increasing frequency of affected females by 18-20 weeks of age (Figure 8). Preliminary analysis of ovarian histology from *aire* KO females also shows some evidence of mild CD4+ lymphocyte infiltration of the ovaries as early as 5 weeks of age (data not shown), however this remains to be confirmed in larger numbers of animals.

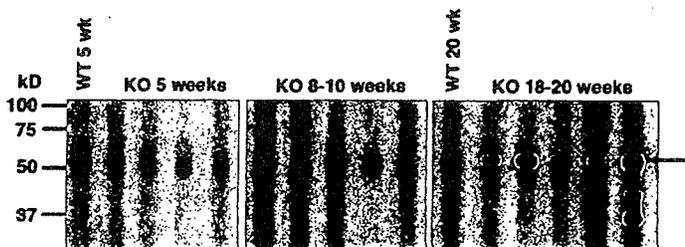


Figure 8. Autoantibodies are detectable by 10 weeks of age in *aire* KO female. Kinetic analysis of sera from *aire* KO females aged 5, 8-10, or 18-20 weeks are shown by immunoblot against ovarian extract as described in Figure 7A. Similarly aged wild-type female sera are included as controls. At 8-10 weeks of age, 3/5 KO females show evidence of autoreactivity to the 60-kD antigen with increased frequency of affected females by 18-20 weeks. Red arrow indicates 60-kD band. Note one female with multiple bands recognized, suggestive of epitope spreading that can occur with increasing age.

C5. Immunoaffinity chromatography purifies the 60-kD species from ovarian lysates.

I utilized the highly specific autoantibody response seen in *aire*-deficient females to isolate ovarian antigens with a column purification strategy. Sera pooled from *aire* KO females with the strongest patterns of ovarian autoreactivity (as tested in C4.2) were used to maximize efficiency of antigen binding. Serum autoantibodies were covalently coupled to Protein-G conjugated agarose beads. Column chromatography with elution under a low pH gradient was used to immunoprecipitate antigen from a large-scale preparation of mouse ovarian lysate. Ovaries from SCID or RAG females were used to prevent interference by endogenous IgG. As a control, tissue extract was pre-adsorbed against a similarly prepared column of wild-type serum samples. Elutions from both the KO and wild-type columns were resolved by SDS-PAGE and confirmed for reactivity by immunoblotting with the original *aire* KO serum used to generate the column (Figure 9A). I detect the 60-kD species specifically in elutions from the KO column. Though the yield from a single elution is low, I can pool and concentrate repeated elutions from the same column to obtain quantities sufficient for further analysis.

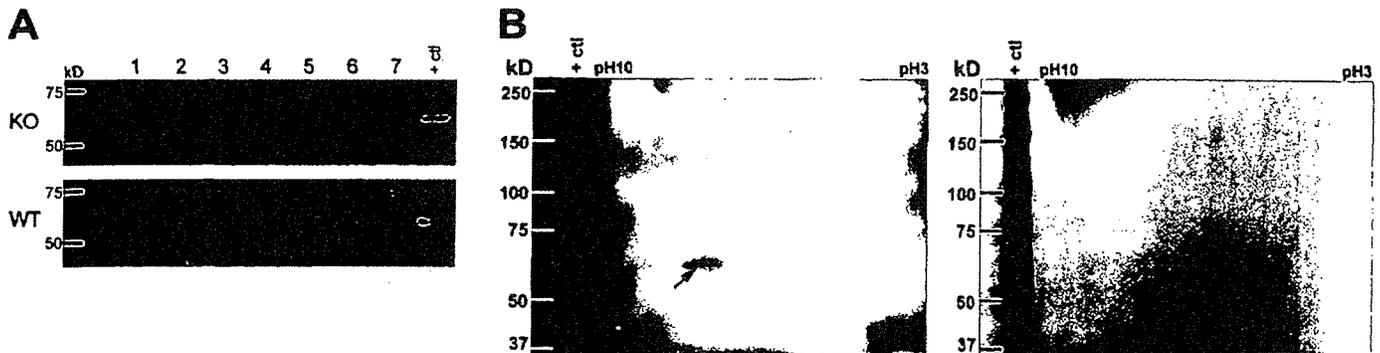


Figure 9. Isolation of a putative 60-kD autoantigen from ovaries. A. Immunoprecipitation of a 60-kD antigen. Fractions (1-7) eluted by stepwise addition of pH 2.5 100mM glycine from either KO (top) or WT (bottom) antibody columns were probed with autoreactive KO sera. Last lane in both panels includes crude ovarian extract as a positive control. B. 2D-gel analysis of KO elutions reveals a 60-kD antigen. Pooled, concentrated KO elutions were resolved by 2D gel, then probed with KO sera as in A. Immunoblot (left), Coomassie stained gel prior to transfer (right). Ovarian extract is resolved in the 2nd dimension only at the left of each gel. Arrows indicate the 60-kD protein. Red dot shows the 60-kD band in the positive control (ovarian lysate).

Complexity of the purified 60-kD species is unlikely to be problematic based on ability to resolve both whole ovarian extract and purified elutions by 2D-gel analysis. When pooled, concentrated elutions are resolved by 2D analysis, only a limited array of autoreactive protein spots are seen on immunoblot with a tight cluster of spots migrating at ~60 kD (Figure 9B). The pattern of autoreactivity correlates to faint but visible protein spots on Coomassie staining, which is important for later larger-scale purification plans. Estimate of migration on isoelectric focusing suggests a pI of ~6.5 for the putative autoantigen. Together, these initial results indicate likely success for antigen identification by this approach with regard to yield and resolution of complexity.

C6. Conclusions. In summary, our preliminary data indicate that I have an excellent novel model for the study of spontaneous autoimmune ovarian disease in the *aire*-deficient mouse. *aire* KO females on the BALB/c background have highly penetrant ovarian disease with spontaneous immune infiltrates of the organ. The ovarian disease appears to be T cell-mediated and is transferable by lymphocytes from affected donors into immunodeficient hosts. Finally, autoantibodies develop in *aire* KO females that seem to target specific ovarian antigens as demonstrated by the restricted reactivity to the oocyte cytoplasm and follicular cells as well as the single prominent protein antigen detected by immunoblot of ovarian extracts.

D. RESEARCH DESIGN AND METHODS

D.1. Aim 1. Characterize the humoral and cell-mediated immunopathogenesis of AOD in *aire* KO mice.

Rationale: Organ-specific autoimmunity has been broadly characterized by detection of tissue-restricted autoantibodies and lymphocytic infiltrates in *aire* KO animals. Further analysis of these features within the ovary will define the tissue-specific humoral and cell-mediated mechanisms of disease. Since *aire* primarily affects the negative selection of T cells, I expect that autoreactive T cells will be the primary mediators of oophoritis. Autoantibody responses may result directly from activated autoreactive CD4 T cell help or may represent a later event due to epitope spreading. Whether or not autoantibodies initiate pathogenesis, their identification may serve as a diagnostic or prognostic disease marker. Moreover, targets of the autoantibody response often correlate with those recognized by autoreactive T cells, as in the example of insulin and GAD reactivity in NOD mice and humans with Type 1 diabetes [41]. Additionally, our laboratory has established that this approach is valid with the successful identification of a key autoantigen in another autoimmune disease in the *aire* KO model [47]. Examination of the lymphocyte infiltrates and their cytokine profile in the affected organ along with adoptive transfers of cell subsets will identify which cells are necessary or sufficient to initiate disease and the effector mechanisms they employ.

By characterizing the autoantibody response and immune infiltrates mediating ovarian disease, I hope to define the pathogenic mechanisms by which tolerance is broken. This knowledge will direct future studies aimed at the development of T cell or B cell targeted therapies in AOD. **I hypothesize that *aire* KO mice develop an oligoclonal autoantibody response and autoreactive T cell population against tissue antigens that reflect the failure of Aire-dependent expression of selected antigens in the thymus.**

Experimental Plan

D1.1. Characterize the humoral responses in AOD mediated by autoantibodies.

Kinetics. I have established that autoantibodies to a single ovarian antigen develop in *aire*-deficient females. To determine whether autoreactivity to the 60-kD species correlates with onset of ovarian pathology, I will sacrifice *aire* KO mice at 5, 10, and 15 weeks of age and assess ovarian disease by histology and autoantibody reactivity on immunoblot (methods C1, C4). Preliminary analysis of kinetic cohorts suggests that AOD begins at 8-10 weeks of age with detection of autoantibodies and low-grade lymphocytic infiltrates (see C4.3). Further analysis of more animals is needed to better characterize progression of disease. Additionally, I will follow a cohort of mice to determine the kinetics of the autoantibody response in individual females by bleeding each mouse at 5, 10, and 15 weeks prior to sacrificing the animal at 20 weeks. Serum from sequential bleeds will be analyzed for autoantibody reactivity by immunoblot or indirect immunofluorescence. A closer timeline with weekly assessments can be made once the initial kinetic analysis is completed so that I can narrow the window in which disease begins. This approach will allow us to detect variation in the onset and progression of disease among animals and to estimate sensitivity and specificity of autoantibody

production. The results of these experiments will help predict disease onset after lymphocyte transfers and identify a therapeutic window for disease modulation.

Serum transfers. Although our preliminary data support a central role for autoreactive T cells in breakdown of tissue-specific tolerance, I cannot exclude that autoantibodies contribute to disease initiation or progression. To address whether autoantibodies are sufficient to initiate disease in the presence of a normal T cell repertoire, I will perform adoptive transfer of serum from affected *aire*-deficient females into syngeneic female hosts. Multiple injections of two different doses (150 or 200 μ l) of serum will be administered by intraperitoneal injection as per the methods shown to be effective in the K/BxN model of arthritis [48]. Recipients will be aged 10 weeks and then sacrificed for examination of tissues by histology. Transfers with mouse Ig and serum from wild-type females will serve as controls. In addition, as an alternative approach to address the role of B cells (and thus of autoantibodies) in disease pathogenesis, I will genetically delete B cells by crossing the *aire* KO allele into μ MT KOs, a B cell deficient line. Double KOs can then be analyzed for oophoritis in the absence of B cells.

D1.2. Identify cell mediated responses in oophoritis

Identification of cell subsets within affected ovaries. Our initial data suggest that T cells are key mediators of oophoritis (C2, Figure 5). CD4⁺ T cells were visualized in affected *aire* KO ovaries by immunohistochemistry and quantitated by FACS analysis of ovaries from an *aire* KO female. Future work will identify activation states of CD4⁺T cells (CD62L, CD25, CD69, CD44) as well as the presence or absence of CD4⁺ Foxp3⁺ regulatory T cells among the CD4⁺ T cells identified. Cells harvested from ovaries of multiple females will be pooled for staining and analysis by flow cytometry as described in C2. Analysis of infiltrates by flow cytometry will also be used to enhance our kinetic studies outlined in D1.1 to examine cell populations within the ovary at different time points. My preliminary data indicates that the predominant cell type in infiltrates are CD4 T cells, suggesting that these cells will be the major pathogenic population, but do not exclude a possible contribution of CD8⁺ cells. This question will be further tested in D1.3 below. Nevertheless, the ability to quantitatively identify CD4 T cells (or CD8 cells) with this method will allow future sorting of autoreactive T cells from diseased ovaries and draining lymph nodes.

Intracellular cytokine staining. Preliminary analysis of ovarian infiltrates from *aire* KO females affected with oophoritis suggests Th1 polarization of CD4⁺ cells based on increased production of IFN- γ (see C2). To determine the mechanism by which CD4 T cells provoke ovarian destruction, I will expand my initial intracellular cytokine staining studies. As in C2, infiltrating lymphocytes will be harvested from ovaries of multiple *aire* KO mice. Pooled cells will be activated with PMA and ionomycin in the presence of the Golgi inhibitor monensin. After 4-6 hours, cells will be fixed and permeabilized to allow intracellular cytokine staining for IFN- γ , IL-4, IL-6, IL-10, IL-17, and TNF- α . Surface staining of cells for CD4, CD8, and CD45 will allow identification of T cell subsets by flow cytometry. Once the T cell polarization and inflammatory mediators have been identified, future experiments can test whether neutralization of cytokines will abrogate development of oophoritis.

D1.3. Adoptive transfer of lymphocyte subsets.

As noted in C3, transfer of splenic lymphocytes was capable of inducing oophoritis, suggesting that a polyclonal repertoire of ovarian-reactive cells can be found in peripheral immune organs. Ongoing experiments in our laboratory aimed at defining the lymphocyte populations required for transfer of autoimmune disease are in progress. I will continue to define the pathogenic cell types by analysis of the adoptive transfers of lymphocyte subsets derived from the spleen or pooled lymph nodes of *aire*-deficient animals. Antibodies to mouse CD3, CD4, CD8, and B220 have been used in a series of purifications or depletions with magnetic beads and flow cytometry to sort T cell and B cell populations from *aire* KO donors. Specifically enriched or depleted subsets were transferred to immunodeficient SCID recipients. Recipients will be aged 10 weeks and sacrificed for analysis of tissue and serum reactivity. Oophoritis will be assessed by histology and immunohistochemistry for CD4, CD8, and IgD (as in C2). In preliminary studies, ovarian disease was transferred with polyclonal splenocytes (see C3), suggesting that an ovarian-reactive repertoire circulates in the periphery. Additional studies with donor lymphocytes isolated from affected ovaries will determine if organ-specific disease can be transferred in the absence of the other multi-organ infiltration (see D2.5 below).

Thus, these experiments will help establish which key cell populations are necessary and sufficient for induction of oophoritis.

D1.4. Summary: Further characterization of the autoantibody and immune infiltrates will define the tissue-specific oligoclonal response in oophoritis. I will determine if autoantibody production occurs early or late during disease pathogenesis. I predict that the kinetics and profile of autoantibody production will correlate with disease severity. Determination of cell subsets and cytokines will provide future targets for therapeutics, and identification of the humoral and cellular effectors of oophoritis will help us understand mechanisms that are activated following the breakdown of central tolerance.

Potential pitfalls and future opportunities:

- *Transfer of serum fails to produce oophoritis.* Initiation of disease by serum transfers may be limited by the titer of autoantibodies in the donor mice or the limited half-life of antibodies. To address this, will use pool serum from mice with higher autoreactivity assessed on immunoblot. I will also give multiple injections at two different dosage levels to compensate for lower donor titers or clearance of autoantibodies by the host.
- *Cell purification or depletion is not efficient.* I will monitor recipients for maintenance of injected cell ratios. If I determine presence of contaminating cell populations, *in vivo* treatment will be administered to deplete unwanted cell types post-transfer. Alternatively, I can genetically delete various cells by crossing the *aire*-deficiency into genetic models lacking lymphocyte subsets and assess for oophoritis in double KOs (e.g. B-2M KO, CD4 KO, TCR- α KO or SCID). Both strategies can identify the lymphocyte populations required for disease pathogenesis.
- *Adoptive transfer of lymphocyte subsets is not effective.* I have already demonstrated that polyclonal splenocytes can adoptively transfer oophoritis (C3), thus it is likely that transfer of one or more subsets will generate AOD. To confirm effective transfer of cells, I will confirm oophoritis by histology and perform immunohistochemistry on ovarian sections to verify identity of the infiltrating cells.

From my preliminary data, I anticipate that these studies will define a central role for autoreactive CD4+ T cells in the pathogenesis of AOD. Identification of the pathogenic cell population will guide the rationale for future treatment designs in targeting T or B cells. Furthermore, characterization of disease progression through kinetic studies will define a potential therapeutic window for such treatments for experiments in the induction of tolerance.

D2. Aim 2: Identify the ovarian autoantigens targeted in *aire*-deficient mice.

Rationale: I hypothesize that autoreactive T cells play an important role in mediating AOD pathogenesis based on detection of T cells in immune infiltrates and adoptive transfer of disease by lymphocytes (see C2, C4). Furthermore, I predict that an oligoclonal repertoire of autoreactive cells produces ovarian disease based on the restricted pattern of ovarian tissue reactivity by immunostaining and immunoblot. Autoantibody production in the *aire* KO model is likely driven by autoreactive T cells through cognate help. Because T cell specificities are restricted to peptide epitopes, the use of autoantibodies which recognize the cognate antigen provides a more effective approach to identifying antigens. Such an approach has been used in multiple autoimmune diseases [49, 50]. Discovery of the ovarian antigen(s) being targeted will help define the specificity of the autoimmune response. Moreover, identification of the ovarian autoantigen could enable development of an assay for improved clinical screening or of potential antigen-directed therapies in AOD. Lastly, determination of self-antigens will allow me to confirm that their expression in the thymus is Aire-dependent and is consistent with the model of Aire-mediated central tolerance. Thus, I propose to identify ovarian autoantigens by immunoaffinity purification and establish T cell recognition of ovarian-specific antigens by transfer of antigen-specific lymphocytes and antigen recall assays.

Experimental Plan

D2.1. Isolate self-antigens recognized by autoantibodies

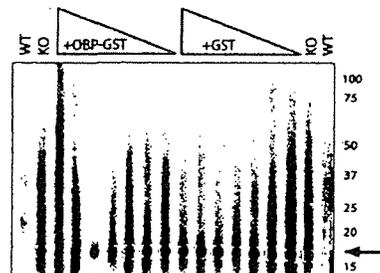
Immunoaffinity purification. I will exploit the highly specific autoantibody response seen in *aire* KO females to isolate ovarian antigens with a column purification strategy. As shown in C5, I am able to successfully immunoprecipitate the autoreactive 60-kD species from ovarian lysates using this method. Serial

immunoprecipitations and elutions using the KO antibody column will be performed as described in C5, and elution fractions will be pooled and concentrated for analysis by mass spectrometry. Elutions from both the KO and wild-type columns will be resolved by SDS-PAGE and confirmed for reactivity by immunoblotting with the original *aire* KO serum samples used to generate the column. Again, immunoblotting with wild-type serum and analysis of elutions from the control column will serve to screen out nonspecific protein binding.

Once reactivity has been confirmed, the eluted fraction will be resolved by SDS-PAGE and undergo excision, digestion, and extraction in preparation for mass spectrometry. I will utilize the expertise of the Biomolecular Resource Core Facility at ~~MSU~~ to prepare the sample for analysis by peptide mass fingerprinting. In addition to data acquisition, the facility will also assist with the data processing required for peptide identification.

Confirmation of autoantibody antigen-specificity. To verify that a protein identified by mass spectrometry is a true positive (i.e., a specific target of the autoantibodies), I will test reactivity of the autoantibodies against the recombinant purified protein. I will purchase commercial preparations of the protein if available or synthesize the protein of interest in a tagged bacterial expression system. To produce recombinant antigen, I will synthesize cDNA from mouse ovarian RNA and design primers to amplify the gene of interest for cloning into a GST or His-tagged expression vector. I will transform *E. coli* with the construct and purify the fusion protein from large volume bacterial cell lysates via columns that recognize the epitope tag. Recombinant protein will be used for confirmation of reactivity when probed with *aire* KO serum by both ELISA and immunoblotting. Also, I will use recombinant protein in immunoabsorption experiments to demonstrate antigen specificity by dose-dependent competitive inhibition of autoantibody reactivity on immunoblot or immunostaining of ovarian tissue. Similarly, I can use known antibodies to the antigen of interest in competition or immunoabsorption assays with ovarian extracts to titrate out the autoantibody response. Our lab has used these techniques successfully to confirm specificity of a lacrimal autoantigen, OBP, identified by immunoaffinity column purification (see ~~results~~ results).

Figure 10. Competition assays demonstrate specific reactivity to the lacrimal autoantigen OBP. Recombinant OBP-GST fusion protein was generated in our lab for these studies. Sera from *aire*-deficient animals was preincubated with increasing (from right to left) concentrations of purified, full-length recombinant OBP (odorant binding protein) or GST prior to use as primaries on immunoblot against lacrimal tissue extract (performed as in C4.2). With decreasing concentrations of recombinant OBP, autoreactivity to the 17-kD band increases (indicated by red arrow). Note, GST does not compete out reactivity.



Demonstrate Aire-dependent expression of autoantigens in the thymus. Since Aire-mediated autoimmunity arises as a result of defective thymic selection, I expect not only that disease will be T cell-mediated but also that expression of the antigen will be Aire-regulated in the thymus. Antigens that do not exhibit Aire-dependent thymic expression may represent later targets recognized as epitope spreading occurs. Thus, I plan to examine the thymic expression pattern of potential autoantigens found in D2.1. I will purify thymic medullary epithelial cells as previously described [1] for analysis by real-time PCR in *aire*-deficient and wild-type thymic cells. qRT-PCR allows exquisitely sensitive detection of even low copy transcripts in a quantitative fashion. Aire-regulated antigens will be detectable in wild-type MTECs but absent in the MTECs from *aire* KOs. Thymic medullary epithelial cells, based on the markers CD45⁻, CDR1^{int}, G8.8⁺, CD80⁺, will be purified on a Mo-Flo cell sorter, and sorted cells will be checked for purity by repeat analysis prior to RNA preparation. Purified RNA will then be used as a source for cDNA synthesis followed by quantitative PCR with Taqman primers and probes. Self-antigens for which autoreactivity by antibody or T cell assays is noted earliest in the evolution of the disease will be given priority. As controls, I will also run real time assays for a known Aire-dependent gene, insulin, and an Aire-independent gene, GAD67, both of which are detectably expressed as self-antigens in MTECs. Importantly, our lab has successfully used this technique to show the retina-specific antigen, IRBP, is expressed in MTECs in an Aire-dependent fashion [47].

D2.2 Demonstrate a critical role for ovarian antigen-specific autoreactive T cells in AOD pathogenesis

T cell recall and ELISPOT assays. Preliminary data in **C3** demonstrating transfer of ovarian disease by polyclonal cells suggest that populations of autoreactive cells circulating in the periphery are capable of producing organ-specific disease. Examination of multiple tissues in **C4.2** indicates though that largely distinct antigens are recognized in each organ (**Figure 7B**). To address whether ovarian-specific antigen recognition occurs, I will perform T cell recall experiments to putative ovarian antigens identified in **D2.1**. This approach will verify tissue-specific T cell recognition, immune specificity, and pathogenicity of putative autoantigens. *aire* KO or wild-type ovary and the draining lymph node cells will be cultured with whole ovarian extract, putative autoantigens, or anti-CD3 as a positive control. Following antigen stimulation, cells will be assayed for proliferation, IL-2 production, and IFN- γ production.

In addition, I will compare the frequency of antigen-specific T cells in *aire* KO versus wild-type mice by ELISPOT assays. ELISPOT analysis is exquisitely sensitive allows detection down to the level of single cells. T cells will be isolated from *aire* KO or wild-type females and cultured with APCs loaded with putative ovarian antigens. Cells will then be analyzed by ELISPOT assays for IL-2 and IFN- γ production. In addition to addressing organ-specificity of autoreactive T cells, these experiments will also confirm that ovarian antigens identified in **D2.1** are recognized by endogenous autoreactive T cells.

Adoptive transfer of ovarian-specific T cells. In the *aire*-deficient mouse, organ-based autoimmunity is thought to arise from persistent autoreactive T cells that recognize a repertoire of tissue-specific self-targets due to defective thymic education. My data support a critical role for T cells in the pathogenesis of AOD (**C2**, **C3**) and predict the presence of ovarian-specific T cells responsible for disease. To begin to address pathogenicity of ovarian antigen-specific T cells in AOD, I will conduct adoptive transfer experiments with T cells from affected *aire* KO or wild-type females that have been expanded by culture with antigens identified in **D2.1**. Cells will be harvested from the ovaries of affected *aire* KO females as described in **C2**. T cells will be purified by sorting over magnetic beads coupled to CD45 antibody (from Miltenyi Inc.) followed by expansion in culture with anti-CD3 and anti-CD28 coated beads. Cells from wild-type ovaries will be included as a control for purification and expansion of T cells.

Expanded cells will then be stimulated in culture with purified antigen and IL-2 for multiple rounds to generate enough cells for adoptive transfer. This method of establishing antigen-specific T cells lines is a standard technique that has been used successfully in multiple systems [51-53]. To monitor proliferation of T cells with antigen stimulation, proliferation assays measuring incorporation of ^3H -thymidine will also be conducted. To control for nonspecific activation, stimulation with GST and IL-2 will be conducted for both expanded wild-type and KO T cells. Following transfer of cells into SCID females, recipients will be aged and analyzed by histology after 10 weeks. As another approach, if antigen-specific T cells cannot be generated, I will transfer ovarian T cells without further antigen stimulation. Cells isolated from wild-type females will be used as negative controls in this instance.

In the absence of identification of ovarian antigens, several alternatives are available. Since I can successfully immunoprecipitate the autoreactive species from the ovary, this immunopurified material can be used for testing in T cell recall assays as well as the antigen source for expansion of T cells prior to adoptive transfer. Ovarian T cells from *aire* KO or wild-type females can be isolated as described above and expanded in culture with immunopurified material or whole ovarian extract. Importantly, the _____ lab has successfully used this approach to transfer autoimmune peripheral neuropathy from infiltrated sciatic nerves of neuropathic NOD mice (data not shown).

D2.3. Summary: My goal is to identify tissue-specific self-antigens that are the key mediators of ovarian disease. I expect that a limited set of antigens will be found since Aire affects the transcription of only a subset of genes within the thymus. Antigen identification will allow us to assess corresponding T cell reactivity as well as for the possibility of epitope spreading by testing antigens for Aire-dependent thymic expression.

I predict that autoreactive T cells with restricted antigen-specificity mediate development of oophoritis. I expect that the autoantigens recognized by these cells will also reflect the cognate antigens recognized by developing autoantibodies. Determination of the autoantigen and pathogenic T cells in AOD will help us understand the mechanisms that initiate ovarian tissue destruction and provide us with a tool to further dissect the breakdown of tolerance in *aire*-deficient mice. Moreover, with a known antigen, the potential to develop improved diagnostic assays and therapeutics grows significantly. TCR transgenic mice, specific for the autoantigen, can be generated in which to better model disease. Trials of selective tolerance induction with

anti-CD3 treatment [54] or antigen-coupled cell tolerance [55] would also be possible with a known ovarian antigen.

Potential pitfalls and future opportunities:

- *Immunoaffinity purification fails.* This approach can be limited by the repertoire of autoantibodies and quantity of target antigen. However, autoantigen is detectable in as little as ~5ug of ovarian extract shown in positive controls in **Figure 9A**. Even immunoblot of ~200ug whole ovarian extract resolved by 2D gel shows a restricted set of autoreactive spots despite multiple spots visible on Coomassie staining (data not shown). Additionally, complexity, solubility, and stability of protein samples can be problematic. Though potentially challenging, our lab has successfully used this method to isolate the retinal autoantigen IRBP as well as putative thyroid and salivary antigens ([47], unpublished results). Other protein purification techniques can assist in initial enrichment for low affinity or low level antigens. Protein precipitation with an ammonium sulfate gradient can both fractionate and concentrate the initial ovarian lysate. Also, anion exchange chromatography can be used to enrich for the protein of interest based on initial determination of its pI on 2D-gel analysis (**Figure 9B**). Protein fractions from either approach can subsequently be screened for presence of the autoreactive species by immunoblot.
- *The eluted fraction has high complexity on mass spectrometry.* The detection of a single autoreactive species at 60 kD suggests that protein complexity is less likely. Alternatives include: 1) a proteomic approach with 2D-gel electrophoresis followed by immunoblotting and peptide analysis of reactive spots or 2) an expression screen using phage display. However, both approaches face similar difficulties with complexity and detection limits as well as the potential for false positives.
- *False positives are found.* I will prevent false positives in any of these approaches by confirmatory testing of recombinant protein as successfully demonstrated for the retinal autoantigen IRBP [47]. I will also use qRT-PCR to confirm expression in the thymus is Aire-dependent.
- *I do not identify ovarian-specific autoreactive T cells.* The isolation of antigen-specific autoreactive T cells has been historically difficult in the example of the NOD mouse. Though primary culture of T cells can be difficult due to need for periodic stimulation, this approach has been successful in several autoimmune models [56]. I will use the **lab** as a resource since they have successfully isolated and expanded nerve-specific T cells that are capable of transferring autoimmune neuropathy.

Alternatively, with identification of the antigen, I can immunize mice with recombinant purified protein and increase the precursor frequency of autoreactive T cells specific for the antigen in question. This will improve the ability to purify antigen-specific T cells for adoptive transfer experiments. This approach has also proven successful in the EAE model of multiple sclerosis (reviewed in [57]).

D3. Aim 3: Establish clinical correlation of disease markers in AOD.

Rationale: Antigens in human AOD remain poorly characterized and largely unknown. With the link to a known human autoimmune syndrome, APS 1, the *aire* KO model of autoimmunity provides a unique opportunity to identify common disease targets that may be shared between the mouse model and patients. To better model AOD in the mouse, further delineation of the clinical aspects of disease is needed. Charting the clinical course of ovarian disease in *aire*-deficient females with the timing and severity of immune destruction will be important in understanding ovarian function and in determining a therapeutic window for disease intervention. These studies will be a useful tool in assessing AOD without termination of the animals. Beyond characterizing ovarian reserve, comparison of autoantibody reactivity will be important in establishing the clinical relevance of my model. Screening of serum samples from patients with AOD or APS1 for similar reactivity to putative ovarian antigens will help bridge the findings in my animal model of oophoritis to a broader arena. **Thus, I will test the significance of the immune targets and mechanisms in the generation of clinical AOD.**

Experimental Plan

D3.1 Characterize ovarian function in *aire*-deficient females with ongoing AOD.

Estrus cycle monitoring. The normal mouse estrus cycle is well characterized [58-60] and can be easily monitored by examination of the external genitalia combined with vaginal smear cytology. Estrus cycles last 4-

6 days in mice with well-defined changes in each stage of the cycle. Cohorts of both wild-type and *aire*-deficient females of the BALB/c strain will be examined daily for one week to determine the presence of estrus cycles at 5 weeks, 10 weeks, and 15 weeks of age. In this way, noninvasive monitoring of ovarian function can be accomplished as the animals age. Cycle data will be compared to histologic examination of the ovaries at each stage and autoantibody production as determined in earlier kinetic studies (**Aim 1.1**).

Hormonal profiling. Premature ovarian failure in patients is accompanied by a characteristic elevation in serum gonadotropins, indicating the loss of ovarian cycling. With loss of ovarian feedback signals in the case of end-organ failure or gonadectomy, luteinizing hormone (LH) and follicular stimulating hormone (FSH) levels rise due to loss of sex-steroid suppression of gonadotropin releasing hormones. Female mice that have undergone gonadectomy also have a marked and persistent elevation of FSH and LH and lose cycling of gonadotropins. As another method to chart the ovarian function, measurements of serum FSH and LH will be made during the proestrus and the diestrus stages of the cycle. Female mice in the cohorts described above will be bled by tail vein sampling during the appropriate day of the cycle. Serum samples will be sent to the Ligand Assay Core of the Specialized Cooperative Center for Research in Reproduction at the University of Michigan. This facility performs well-standardized assays for LH and FSH using sensitive radioimmunoassay protocols. Correlation of LH and FSH levels with evidence of autoimmune oophoritis will also better define the clinical course and provide an additional disease marker.

Improved functional markers of AOD in the *aire* KO model will enable me to follow the disease course without termination of the animals. Establishment of clinical phenotyping and noninvasive monitoring will be critical to enable future studies of therapeutic interventions. Thus, these studies will not only inform us of the changes in ovarian function during AOD but also provide the foundation to assess response in future studies in tolerance induction.

D3.2 Determine whether similar antigens are recognized in human AOD.

Autoantibody screening in AOD patients. Although the need and opportunity for human research in AOD and POF is great, my goal is to take a circumscribed approach to the testing of patient samples for the purpose of establishing relevance of my model. Thus, I do not aim to conduct clinical research on patients with AOD, which is beyond the scope of this proposal, but rather to determine whether the antigens discovered in the animal model will have impact on the known human disease state. Currently, I have samples from 8 APS 1 patients donated from collaborators but more samples need to be obtained for analysis. Serum from female patients affected with APS or with POF and other autoimmune disease will be collected, along with relevant clinical data under the guidelines of the NIH Human Subjects Research Policy (detailed in section **E**). As an initial screen, samples will be tested on immunoblot for reactivity to whole mouse ovarian extract and examined for reactivity to the putative 60-kD antigen (as in **C4.2**). Samples will then be screened on immunoblot against the purified elutions of the putative antigen (from **C5**) to confirm reactivity to the 60-kD species. Alternatively, once the 60-kD antigen has been identified, samples can be screened against recombinant purified protein. Recombinant protein will enable testing by both immunoblot and ELISA assays, which are rapid and sensitive. As a complementary approach to immunoblot analysis, serum samples of patients will also be tested by indirect immunofluorescence staining for similar reactivity as seen in *aire* KO females (see **C4.1**). Serum samples from normal healthy women without known infertility will serve as controls.

D3.3 Summary

Characterization of ovarian function as it correlates to immune-mediated pathology will allow me to establish a more complete disease model for AOD. Assessments of ovarian cycling and hormone production will assist in timing disease onset as well as defining a potential therapeutic window. The functional assays described can be used to generate a clinical score to grade disease activity and allow relatively noninvasive monitoring of disease progression in viable animals. This will also allow for measures to follow in future attempts at therapeutic interventions or tolerance induction.

More importantly, comparison of mouse and human autoreactivity in AOD will determine whether antigens in the animal model are applicable to the human syndrome. The future implications for a common antigen are many. A shared antigen will allow development of specific testing for AOD in patients, improving our diagnostic and prognostic abilities. Most importantly, if antigens are shared between the mouse model and human disease, therapeutic interventions, such as antigen-targeted tolerance induction, would gain increased significance. I would indeed have a unique and powerful tool to intervene in the pathophysiology of AOD.

Potential pitfalls and future opportunities:

- *aire* KO females do not exhibit clear loss of estrus or hormonal cycles. This scenario is unlikely to occur given the significant end-organ destruction and follicular loss notable on ovarian histology (as shown in Figure 2) and the observation that *aire* KO females are infertile. However, should the phenotype be subtle, I will pursue more detailed analysis of ovarian function by assessment of antral follicle and corpus lutea counts in wild-type and KO females as an alternative assessment of ovarian reserve.
- *Paucity of patients with APS 1 or AOD available for testing.* is a large tertiary referral center with several different clinics throughout including a well-established Reproductive Endocrinology clinic. Collaboration with , who serves as director of the clinic, will assist with patient referrals. sees POF patients in her practice and has ongoing collaborations with other investigators who have collected cohorts of these patients. She will serve as both a valuable advisor and consultant in the recruitment of these patients. Additionally, as a leading figure in the study of APS 1 and *aire*, has developed collaborations with several clinicians wishing to refer APS 1 patients for further testing. I currently have samples from eight APS 1 patients available for testing and expect to obtain further samples upon IRB approval of our protocol.
- *Patients with AOD do not share similar autoreactivity to ovarian antigens.* Many mouse and human proteins share significant homology, however differences may occur at the epitopes recognized by human autoantibodies. With identification of the mouse ovarian antigen, the human homologue may be identified. To address possible protein epitope differences between mouse and human proteins, patient samples can be screened against recombinant human protein for presence of autoantibodies. Another alternative is to obtain human cadaveric ovarian tissue, which is commercially available, for use in autoantigen screening. Given that the *aire* KO mouse model reflects a similar disease phenotype to that seen in APS 1 patients (deficient for AIRE), it is likely that similar antigens will also be targeted. Nonetheless, although screening may reveal that targets of the ovarian disease differ between the animal model and humans, the mechanisms of pathogenesis will likely be shared and give insight into the progression of clinical disease.

Conclusion

With a known antigen, I will have the opportunity to develop a clinically applicable autoantibody assay to aid the diagnosis of AOD. Equally important, experiments to test whether antigen-directed therapy can prevent or treat AOD will be possible. Together, these studies will characterize autoimmune ovarian disease in *aire* KO mice, creating a model to study spontaneous autoimmune oophoritis. In addition, this project will identify key antigens required during central tolerance in the thymus. The results will provide important insights into the pathogenesis of autoimmune ovarian disease, mechanisms of central tolerance, and potential targets for novel therapeutic approaches to treat autoimmune ovarian disease.

Timeline for Completion of Specific Aims

	Year 1	Year 2	Year 3	Year 4	Year 5
Aim 1: Mechanisms of immunopathogenesis					
1.1 Humoral					
Kinetics	x				
Serum transfers		x			
1.2 Cell-mediated					
Identification of cell subsets	x				
Intracellular cytokine staining	x				
Adoptive transfers of cell subsets		x	x		
Aim 2: Ovarian antigen identification					
2.1 Antigen isolation					
Immunoaffinity purification	x	x			
Verification of ovarian antigen specificity		x			
Demonstration of Aire-dependent thymic expression of antigens					
2.2 Ovarian-antigen specific T cell activity					
Adoptive transfer of ovarian-specific T cells			x	x	
T cell recall assays				x	x
Aim 3: Establish clinical markers of disease					
Estrus cycle monitoring			x	x	
Hormone profiling			x	x	
Testing of patient samples for autoantibodies				x	x

E. HUMAN SUBJECTS RESEARCH: Scenario D – Clinical Research
Protection of Human Subjects

1. Risks to the Subjects

a. Human Subjects Involvement and Characteristics.

The purpose of the proposed human subjects research is to investigate possible clinical correlation of antigens found in the *aire* KO mouse model of autoimmune ovarian disease. Currently, the diagnosis of clinical autoimmune ovarian disease (AOD) and remains poorly defined with a lack of effective screening tools. To this end, I am interested in screening serum samples of patients with potential or documented AOD for autoantibodies to the ovarian antigens isolated in the mouse model.

The involvement of the human subjects will be limited to: 1) a brief interview to obtain clinically relevant data regarding autoimmune conditions and infertility and 2) a blood draw to obtain serum samples. Premature ovarian failure is defined as cessation of menses at less than age 40. Thus, the study population will include women of reproductive age (18-40 years old) with evidence of premature ovarian failure as well as other signs or conditions of autoimmune disease, which include but are not limited to autoimmune thyroid disease, type 1 diabetes, autoimmune hypoadrenalism, or an autoimmune polyendocrine syndrome (APS type 1 or 2). Given a relatively low incidence of premature ovarian failure (1%) in the US, I anticipate no more than 25 individuals will be recruited into the study and hope for a minimum of 10 patient samples. Although APS 1 patients can sometimes have more severe clinical disease, the majority of POF patients are well, and I expect that the general health status of the patients sampled will be good. A limited number (5-10) of healthy women without evidence of reproductive dysfunction will also be tested as controls.

Clearly in the study of ovarian and reproductive biology, the subjects will be limited to adult females with the exclusions of males and younger children. The research will be conducted at _____, and facilities are available at the _____ campus in the General Clinical Research Clinic (GCRC) and outpatient endocrinology clinic where private interviews and blood draws may be performed.

Additional Protections for Children Involved as Subjects in Research

As only minimal risks are anticipated for the subjects, the inclusion of patients age 18-20 in the study population will require assent of the child and permission of the child's parents or guardian (in accord with CFR Title 45, Part 46, Subpart D). Children of age 18-20 will have sufficient education and maturity to comprehend the risks and benefits of this study and to give affirmative agreement. Informed consent will be obtained with both the child's assent and written permission of the parent or legal guardian after discussing risks and benefits with the child and her family (see 2.a. below).

b. Sources of Materials.

The primary research material in this study will be specimens of whole blood, which can then be processed to collect serum. Clinical data about each patient sample will be obtained from a brief interview and/or review of relevant medical records with the permission of the patient. The clinical data obtained will focus on documenting known autoimmune conditions, family history of autoimmunity, and history of menstrual abnormality or infertility.

c. Potential Risks.

The risks to the subjects are minimal and are largely limited to physical risks associated with phlebotomy (pain at the puncture site, bruising, hematoma). Since the information gained from these studies is not intended to be used in a diagnostic manner, the potential for social, legal, and psychological risks are small. Loss of confidentiality is another potential risk and will be minimized as described above (see 1.b.). The removal of personal identifiers and securing of personal data will ensure patient privacy is safeguarded.

2. Adequacy of Protection Against Risks

a. Recruitment and Informed Consent

Patients will be recruited chiefly by referral from care providers in the endocrine and reproductive endocrinology clinic. With their permission, potential subjects will be contacted by the investigators on this protocol. Prior to participation in the study, patients will be informed of the risks and benefits of participation and the non-diagnostic nature of the testing. This discussion will take place in person whenever possible, but alternatively, can also be conducted over the phone if circumstances (*e.g.* travel) prevent a meeting. Written informed consent will be obtained prior to the clinical interview and sample collection and will detail the risks of phlebotomy and possible risk to confidentiality. Adult study subjects will be limited to females who are competent to provide consent. In the case of children, age 18 or older, informed consent will involve

discussion with both the subject and parents or legal guardian of the risks and benefits. Participation will be limited to individuals who assent to the study and have written parental consent.

b. Protection Against Risk

Data will be kept in a database and coded so that personal identifiers will be removed from the samples. Only the investigators and collaborators named on this proposal will have access to the samples and clinical data. The coding of the database samples and clinical data or medical records bearing personal identifiers will be secured in a locked file at the

3. Potential Benefits of the Proposed Research to the Subjects and Others

The potential benefit to the subjects will be the sense of contribution to the advancement of science in a field where little is understood about the underlying pathogenesis. For those affected with AOD, satisfaction may be had in knowing that they are assisting in research that may someday improve diagnosis of the disease and lead to the development of novel therapies. Although this may not impact the course of their own disease process directly, this knowledge can be used to improve care for other women with AOD and may be applicable to other areas of ovarian function and reproductive health. The minimal risks involved in this study are comparable to those that anyone undergoing routine clinical care would encounter, and thus are quite reasonable relative to the potential yield of the investigation.

4. Importance of the Knowledge to be gained

Currently, testing for autoantibodies in AOD has poor sensitivity or specificity on the order of 50-60% for each. Thus, the diagnosis is often made by clinical inference given other coexisting evidence for autoimmune disease. Testing for autoantibodies occurs in cases that have associated conditions with known correlation, such as with 21-hydroxylase antibodies in autoimmune adrenal insufficiency. Thus, the need for improved, disease-specific testing is clear. With a clear means for diagnosis, preservation of future fertility can be better implemented for affected patients by changes in family planning or oocyte preservation and assisted reproductive techniques. Moreover, once advances have been made in the directed therapies for AOD, definitive diagnostic methods will be invaluable for facilitating earlier and more effective interventions.

Testing of patient serum samples for autoantibodies to antigens identified in our animal studies will allow validation of our model system in human clinical disease and provide a potential diagnostic tool. Additionally, with identification of a shared disease target in human AOD, animal studies to develop specific disease-modulating therapies will gain increased significance. In light of the considerable knowledge and improvement in care to be gained by this minimally invasive study, the risks to subjects seem reasonable and justifiable.

Inclusion of Women and Minorities

Given that this study focuses on autoimmune ovarian disease, subjects will be limited to women only. Patients will be enrolled by referral from care providers in our endocrinology and reproductive endocrinology clinics at which include sites at the Hospital. Since no racial/ethnic association has been demonstrated for AOD, participants of all ethnicities will be included but no specific group shall be recruited. Diversity in the subjects will be achieved based on the differing patient populations at the various clinic sites. For example, as the city and county hospital in the region, the Hospital serves the indigent population and also has a high proportion of Hispanic and Asian patients. From the US Census 2000 data, the city of has a racial and ethnic distribution of white, non-Hispanic or Latino (43.63%), black or African American (7.79%), American Indian and Alaska Native (0.45%), Asian (30.84%), Native Hawaiian and Other Pacific Islander 0.49%, and Hispanic or Latino (14.1%) persons. The subjects in the study group will thus reflect the diversity of the clinic sites and the general population of

Inclusion of Children

Female children, age 18-20, will be included as potential participants in this study. The decision to exclude girls of less than 18 years of age is based on the frequency of menstrual irregularities in teenage girls, which is unlikely to represent early menopause or early signs of AOD. Children of age 18-20 are seen in the general endocrinology clinic at, thus, sufficient expertise is available to deal with this age group.

F. VERTEBRATE ANIMALS

F.1. Animals.

Only laboratory mice (*Mus musculus*) are to be used. Our experiments will include use of numerous inbred strains, primarily of the BALB/c type, and transgenic or gene-targeted derivatives thereof. Mice of both sexes will be employed either as breeders or for experimental purposes with a preponderance of female mice studied. The majority of the work will be performed with adult animals. We estimate that we will use approximately 200 mice per year during the course of the project.

F.2. Justification.

Studies of autoimmune disease in human subjects are by their nature restricted in scope and manipulation. Moreover, study of ovarian disease in humans is particularly difficult given the limited availability of ovarian tissue. Thus, there is no substitute for animal experimentation. The mouse is an ideal choice due to its small size and relatively short reproductive cycle, and because of the large number of mutant and otherwise genetically manipulated strains that exist. Considerable numbers of mice are required for the proposed work. Multiple mice in each experimental group are needed to ensure the accuracy of results in immune transfer or genetic cross experiments based on previous experience. We estimate that we will use approximately 200 mice per year to perform the experiments based on the genetic crosses required and the experimental groups involved.

F.3. Veterinary care, maintenance, and monitoring.

Mice will be maintained at the _____) barrier facility within the Laboratory at _____. This facility has SPF status, and is regularly monitored for health and for the presence of microbiological contaminants (sentinel testing system). Daily assessment is performed by animal technicians from the _____ with the assistance and supervision of qualified veterinarians (Dr C F _____ DVM, _____ director). The _____ animal management program meets NIH standards as set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23 Revised 1985). The institution also accepts as mandatory the PHS Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions and NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Our laboratory animal operating procedures have been positively reviewed by the Institutional Animal Care and Use Committee (IACUC) approval number _____

F.4. Procedures.

Recipient mice in cell transfer experiments will be anesthetized briefly with Isoflurane during the procedure. All mice will be regularly examined by the investigators and animal technicians for signs of distress.

F.5. Euthanasia.

CO₂ inhalation, followed by cervical dislocation will be employed, as recommended by the guidelines and consistent with the Panel on Euthanasia of the American Veterinary Medical Association.

G. SELECT AGENT RESEARCH- None

H. LITERATURE CITED

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I. CONSORTIUM/CONTRACTUAL AGREEMENTS- None

J. RESOURCE SHARING

The *A* laboratories are committed to the sharing and distribution of reagents and mice. As an example, the *aire*-deficient mouse has already been deposited into the Laboratory collection. Details regarding the plans for sharing of our model organism and other resources are reported in the current RO1 grant. In the event that further new model organisms or other potential reagents are generated by this project, I will follow the plan of sharing and distribution outlined in the current grant.

CHECKLIST**TYPE OF APPLICATION** (Check all that apply.) NEW application. (This application is being submitted to the PHS for the first time.) REVISION/RESUBMISSION of application number: _____

(This application replaces a prior unfunded version of a new, competing continuation/renewal, or supplemental/revision application.)

 COMPETING CONTINUATION/RENEWAL of grant number: _____

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS

(Competing continuation/renewal appl. only)

 No Previously reported SUPPLEMENT/REVISION to grant number: _____

(This application is for additional funds to supplement a currently funded grant.)

 Yes. If "Yes," Not previously reported CHANGE of principal investigator/program director.

Name of former principal investigator/program director: _____

 CHANGE of Grantee Institution. Name of former institution: _____ FOREIGN application Domestic Grant with foreign involvement List Country(ies) Involved: _____**1. PROGRAM INCOME** (See instructions.)

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS (See instructions.)

In signing the application Face Page, the authorized organizational representative agrees to comply with the following policies, assurances and/or certifications when applicable. Descriptions of individual assurances/certifications are provided in Part III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects Research •Research Using Human Embryonic Stem Cells •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy •Vertebrate Animals

•Debarment and Suspension •Drug-Free Workplace (applicable to new [Type 1] or revised/resubmission [Type 1] applications only) •Lobbying •Non-Delinquency on Federal Debt •Research Misconduct •Civil Rights (Form HHS 441 or HHS 690) •Handicapped Individuals (Form HHS 641 or HHS 690) •Sex Discrimination (Form HHS 639-A or HHS 690) •Age Discrimination (Form HHS 680 or HHS 690) •Recombinant DNA Research, Including Human Gene Transfer Research •Financial Conflict of Interest •Smoke Free Workplace •Prohibited Research •Select Agent Research •PI Assurance

3. FACILITIES AND ADMINISTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions. DHHS Agreement dated: 12/14/2005 No Facilities And Administrative Costs Requested. DHHS Agreement being negotiated with _____

Regional Office.

 No DHHS Agreement, but rate established with _____

Date _____

CALCULATION* (The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)

a. Initial budget period:	Amount of base \$	<u>112,750</u>	x Rate applied	<u>8.00</u>	% = F&A costs	\$	<u>9,020</u>
b. 02 year	Amount of base \$	<u>112,750</u>	x Rate applied	<u>8.00</u>	% = F&A costs	\$	<u>9,020</u>
c. 03 year	Amount of base \$	<u>112,750</u>	x Rate applied	<u>8.00</u>	% = F&A costs	\$	<u>9,020</u>
d. 04 year	Amount of base \$	<u>112,750</u>	x Rate applied	<u>8.00</u>	% = F&A costs	\$	<u>9,020</u>
e. 05 year	Amount of base \$	<u>112,750</u>	x Rate applied	<u>8.00</u>	% = F&A costs	\$	<u>9,020</u>
						TOTAL F&A Costs	\$ 45,100

*Check appropriate box(es):

 Salary and wages base Modified total direct cost base Other base (Explain) Off-site, other special rate, or more than one rate involved (Explain)

Explanation (Attach separate sheet, if necessary.):

F/A Costs per PA-06-512.

Selection Committee, Career Development Award
Center for Scientific Review
National Institutes of Health
Bethesda, MD 20892-7710

Dear Selection Committee Member,

I am writing to recommend _____ for a Career Development Award. I first got to know _____ in _____ when she carried out a summer rotation in my laboratory _____. She returned to my lab for doctoral research later that year and worked with me, first at the _____, until completing and defending her thesis.

_____ came to my laboratory with an excellent academic background, evident in the courses she took and the grades she received both as a _____ undergraduate and as a medical and graduate student in _____. Whereas most of the MD/PhD students I have supervised have taken a while to settle on a project, _____ immediately seized the opportunity to dissect the function of the *Drosophila* RNA binding protein Boule. We had recently shown that Boule was a direct counterpart to the human DAZ (*Deleted in azoospermia*) and Daz-like male fertility factors. We knew that Boule was essential for spermatogenesis and, more specifically, for the G₂ to M transition at the onset of male meiosis. We knew little, however, about how Boule functioned in the developing germline. Recognizing that the subcellular localization of Boule was likely to provide substantial insight, _____ taught herself to fix and stain testis preparations for immunofluorescence studies. Upon examining the pattern of Boule staining, _____ found something quite informative. She observed that Boule first appeared in the nuclei of developing spermatocytes and was concentrated in a crescent-shaped perinucleolar region. In post-meiotic cells, however, Boule was entirely cytoplasmic. By careful observation of large numbers of testes, she demonstrated that the transition point in this biphasic localization pattern occurred just prior to meiotic entry.

The question raised by _____'s initial experiments was an important one: Was the shift in Boule from nucleus to cytoplasm the trigger for meiotic entry? _____ addressed this problem in an elegant manner. First she demonstrated that RNA mediates Boule nuclear localization in spermatocytes, since RNase treatment resulted in an exclusively cytoplasmic distribution of Boule. Second, she demonstrated that the crescent-shaped region of the nucleus to which Boule localized represented the lampbrush structures associated with the Y chromosome fertility loci. Third, and most significantly, _____ carried out a series of genetic experiments with Y chromosome translocations that simultaneously demonstrated that Boule associated with a particular transcript from the short arm of the Y and bulk nuclear localization was nonessential for the meiotic entry function of Boule. These findings had a substantial impact on the

field, since they indicated that Boule (and by analogy DAZ and Dazl) was stored in the nucleus but acted in the cytoplasm to direct meiotic entry. [redacted] reported these findings in a [redacted] experiments carried out in mice subsequently confirmed that DAZ and Dazl also accumulate in the nucleus and then translocate to the cytoplasm. [redacted] also initiated a domain analysis of Boule and generated a transgene that, unlike previous constructs, was capable for providing full Boule activity. Time ran out, however, before these studies were sufficiently far along to generate further manuscripts.

[redacted] s excellent communication skills will serve her well in an academic medical career. Throughout her time in the lab, she gave research presentations that greatly impressed students and faculty alike. She speaks clearly, organizes her thoughts well, and has terrific instincts about how to make an effective visual presentation. She also writes clearly and effectively. By no means one-dimensional, [redacted] found time in the evenings and weekends during graduate school to be a competitive and successful

[redacted] is excited about immunology and, more specifically, about her work on developing a mouse model for autoimmune ovarian failure. She is committed to the career path she has chosen and she is smart enough to do superbly. I am confident that [redacted] will make an outstanding clinician scientist and I recommend her to you with great enthusiasm.

Sincerely, [redacted]

001 1 0 2001

Pr.

Professor of Medicine

Peer Review Committee
Career Development Award (Series K0
NIH

To whom it may concern:

I am writing in strong support of Dr. _____ who is applying for a Career Development Award. I currently serve as the Chief of the Division _____ here at _____. I have known _____ since she came to _____ as a first year clinical endocrinology fellow in _____. She now works in a neighboring laboratory with _____ in our Division, so I have had ample opportunity to follow her performance in the clinical and research settings. _____ is extraordinarily bright – quick to master seemingly difficult concepts in both the clinical and scientific arenas.

She has carried out basic scientific research at several points during her academic career, including a PhD experience in the laboratory of _____ at the _____ That work, which focused on developmental biology in *Drosophila*, was productive leading to three solid publications, one of which _____

Her work in the _____ laboratory focuses on autoimmune ovarian disease using the AIRE knockout mouse, _____, as a model for the human disease. She has approached this work aggressively, providing both the intellectual input and the "elbow grease" to get the project moving in the right direction. This is an important clinical problem given the devastating consequences of premature ovarian failure in the reproductive years and it is, paradoxically, highly understudied. The work is progressing steadily and _____ hopes to identify murine proteins targeted in the autoimmune response during the coming year. I have no doubt that she will be successful in this endeavor.

_____ is strongly committed to a career in academic medicine. I believe that she has the requisite skills (i.e. intellect, self motivation, drive, perseverance and interpersonal skills) to become an outstanding physician scientist. This award would

provide the cap stone to solidify her launch into an academic career. I recommend her to you without reservation and in the strongest terms.

Sincerely, — —

Department of Health and Human Services
National Institutes of Health
Mentored Clinician Scientist Development Award

Greetings:

I write in strong support of _____ M.D., Ph.D., who is applying for a K08 award to continue her physician scientist career. I served on _____ thesis committee and she did a research rotation in my laboratory. From these interactions I feel I can evaluate well her capabilities as a medical researcher.

_____s record as _____t in our M.D./Ph.D. program was tempered by her advisor's move to _____ in the middle of her research training. This unfortunate circumstance caused a loss of momentum that I don't think she ever fully recovered from. The consequence is that she must be evaluated on her potential rather than demonstrated research productivity, which is not true of the typical K08 applicant.

In my opinion, _____ potential for independent research is excellent. She has a very good pair of hands, thinks critically, and is well organized. She gets along well with people of widely differing personalities and takes critical suggestion to heart. Her works-in-progress seminars were well thought out, she was knowledgeable regarding literature in the field of her dissertation research (_____), and she thinks well on her feet. She authored her _____ and did a good job in assembling a lucid dissertation. This performance and her CV clearly indicate that she has all of the talents and brain power to succeed as a physician scientist. What has been missing, and what she now has as an _____ fellow at _____ chance to apply these gifts. _____nply has not been in the right place at the right time in her career to shine at the bench but I think she has now chosen a superb mentor in _____. The project they propose and its training potential are great.

I recommend highly that you take a chance on her. She will not disappoint in terms of a research performance, and she could explode on the science scene when coupled with a vested advisor whose feet are on the ground. She has demonstrated an extraordinary commitment to a career as a physician scientist.

CAREER DEVELOPMENT AWARD REFERENCE REPORT GUIDELINES (Series K)

Title of Award:

Mentored Clinical Scientist Development Award

Type of Award:

K08

Application Submission Deadline: _____

Name of Candidate (Last, first, middle):

Name of Respondent (Last, first, middle):

The candidate is applying to the National Institutes of Health for a Career Development Award (CDA). The purpose of this award is to develop the research capabilities and career of the applicant. These awards provide up to five years of salary support and guarantee them the ability to devote at least 75–80 percent of their time to research for the duration of the award. Many of these awards also provide funds for research and career development costs. The award is available to persons who have demonstrated considerable potential to become independent researchers, but who need additional supervised research experience in a productive scientific setting.

We would appreciate receiving your evaluation of the above candidate with special reference to:

- potential for conducting research;
- evidence of originality;
- adequacy of scientific background;
- quality of research endeavors or publications to date, if any;
- commitment to health-oriented research; and
- need for further research experience and training.

Any related comments that you may wish to provide would be welcomed. These references will be used by PHS committees of consultants in assessing candidates.

Complete the report in English on 8-1/2 x 11" sheets of paper. Return your reference report to the candidate sealed in the envelope as soon as possible and in sufficient time so that the candidate can meet the application submission deadline. References must be submitted with the application.

We have asked the candidate to provide you with a self-addressed envelope with the following words in the front bottom corner: "DO NOT OPEN—PHS USE ONLY." Candidates are not to open the references. Under the Privacy Act of 1974, CDA candidates may request personal information contained in their records, including this reference. Thank you for your assistance.