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Estrogen-Induced Modulation of Innate and Adaptive Immune Function

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ESTROGEN-INDUCED MODULATION OF INNATE AND ADAPTIVE IMMUNE FUNCTION

by

FEDA N. MASSEOD

Under the advisement of Dr. Roberta Attanasio

ABSTRACT

Host defense against infection and disease relies on the reciprocal communication between the immune and neuroendocrine systems where sex hormones exert negative and positive feedback actions on immune functions. Indeed, sex hormones have been implicated in gender dimorphic immune response and in the potentiation of immune-related disorders. The female hormone estrogen plays a role as an immunomodulator and may exert immunosuppressive and immunostimulatory effects. Though many studies focus on estrogen's role in immunity within the female reproductive tract and autoimmunity, the modulatory effects of estrogen on vaccine responses are largely unexplored. The insufficient efficacy of some vaccines in certain target populations, as for example the elderly population, is well recognized. Hormones fluctuate throughout an individual's life, and females in particular undergo several necessary reproductive (pregnancy and menopause) and lifestyle (oral contraceptive use) changes which involve sex hormones. Vaccine efficacy might be influenced by endogenous estrogen levels or by exogenous estrogen administration. Therefore, in the pursuit of improved vaccine efficacy, it is necessary to

consider such hormonal factors and their contribution to immune status. We have studied estrogen's role in modulation of vaccine responses using a mouse ovariectomy model where exogenous estrogen delivery can be controlled. Our studies included two different types of vaccines, a bacterial toxoid formulation and a bacterial secreted protein formulation. Results from these studies indicate that estrogen enhances vaccine-specific antibody production by likely supporting a general T_H2 pathway and also modulates expression of genes encoding molecules critical in innate immune signaling and required for development of proper adaptive immune responses and antigen clearance through antibody-mediated mechanisms. The level at which estrogen modulates antibody responses appears to be dependent on the route of vaccine administration. The enhancement of specific humoral responses may involve mechanisms involving TLR2 and antibody Fc receptor expression on macrophages, cells that link innate and adaptive immune responses. Advances in our understanding of the relationship between sex hormones and the immune system may provide new insights into the mechanisms by which hormones act and thus may be exploited to guide the design of future vaccine strategies.

INDEX WORDS: 17 β -Estradiol, Antibody Response, TLR2, Fc γ R, Vaccine Response, Immunomodulator, Sex hormones, Autoimmunity

**ESTROGEN-INDUCED MODULATION OF INNATE AND ADAPTIVE IMMUNE
FUNCTION**

by

FEDA N. MASSEOD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Georgia State University

2008

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CH	Heavy chain constant domain
CRH	Corticotropin-releasing hormone
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
E2	Estradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen response element
Fab	Antigen binding fragment
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
FRT	Female reproductive tract
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HIV	Human immunodeficiency virus

HPA	Hypothalamus-pituitary-adrenal
HPV	Human pappilloma virus
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer
PAMP	Pathogen-associated-molecular-pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pathogen recognition receptor
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

SC	Subcutaneous
SERM	Selective estrogen receptor modulator
SLE	Systemic lupus erythematosus
SRE	Steroid response element
TCR	T cell receptor
T _H	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

CHAPTER 1

Introduction and Study Objectives

The primary function of the immune system is to protect the host from invasion of foreign organisms by distinguishing “self” from “non-self”. While there is a high degree of interconnectivity between its components, the immune system is generally divided into two subsystems, the innate and the adaptive immune systems (Kuby 2007). Innate immunity provides the first line of defense against infection. Its disease-resistant mechanisms are non-specific and though the response is immediate in action, it is short-lived. In contrast, adaptive immunity is much more specific and takes longer to activate. It results in immunological memory which can augment itself to respond more quickly and with greater specificity to future encounters with the same pathogens. To some extent, the distinction between innate and adaptive immunity is vague, because it is now evident that innate immune signaling is required for activation of adaptive immunity (Delves and Roitt 2000). Both systems are highly interactive and cooperative, producing a combined response more effective than either branch could produce on its own, and one that provides protection against a diverse and rapidly-evolving array of pathogens.

The innate immune system consists of molecules and cells that distinguish host components from those of infectious agents, in part by recognizing conserved constituents of microorganisms. Innate immune activation occurs within hours of contact and its efficacy is not influenced by previous exposure. Phagocytes play a pivotal role in the innate immune response. Upon activation by microbial detection, they release cytokines and chemokines which go on to signal a

wide array of functions and responses, including inflammation. The major phagocytic cells include neutrophils and macrophages, which are specialized to internalize and destroy microorganisms, and dendritic cells (DC). DC act as dedicated antigen-presenting cells (APC) that display antigen for recognition by naïve lymphocytes of the adaptive immune system and activate their differentiation into effector cells. Proper activation requires specialized co-stimulatory signals along with antigen recognition; in this way DC provide a crucial link between the recognition of microbial components and activation of adaptive immune responses. Other cells of the innate immune system, such as basophils, mast cells and natural killer (NK) cells, are not phagocytic, and use other mechanisms to kill bacteria. For example, NK cells, which play a role in the host-rejection of both tumors and virally infected cells, kill cells by signaling them to undergo apoptotic cell death.

The interaction between phagocytes and pathogens or foreign substances is facilitated in several ways. Pathogens may be coated with molecules known as opsonins which facilitate phagocytosis. Opsonins include complement proteins of the innate immune system and antibodies, or immunoglobulins, of the adaptive immune system. Another way that phagocytes interact with pathogenic molecules is through direct binding of pattern-recognition molecules to conserved structures on the pathogen. A number of these molecules have been identified, but perhaps the most important of these are the molecules known as toll-like receptors (TLR). TLR are membrane-spanning proteins expressed on a variety of different cell types, including DC and monocytes/macrophages as well as B cells and epithelial and endothelial cells (van Duin and Shaw 2007). They recognize and bind conserved structures on microbial pathogens. The microbial structures, termed pathogen-associated-molecular-patterns (PAMPs), are repetitive structures that include extracellular bacterial structures and nucleic acids. TLR are considered

pattern recognition receptors that, by their recognition and interaction with PAMPs, result in cell activation and thus cytokine secretion and phagocytosis.

Currently, 11 human TLRs and 13 murine TLRs have been described (Table 1.1). Common microbial and synthetic ligands for TLRs 1-9 have been identified in both humans and mice (van Duin and Shaw 2007). Ligands for TLR1 and 2, and 2 and 6, are Gram-positive bacteria and yeast cell wall components, while the predominant Gram-negative bacterial product, LPS, is a ligand for TLR4. Other products such as dsRNA (poly I:C), bacterial flagellin, immiquimod, and CpG oligodeoxynucleotides (ODN) have been identified as ligands for TLR 3, 5, 7, and 9, respectively. The interaction between a TLR and its ligand results in the secretion of anti-bacterial peptides, defensins, and pro-inflammatory cytokines such as TNF- α and IL-6, which initiate an inflammatory response to clear the invading organism. Furthermore, the inflammatory response results in the recruitment of cells of the adaptive immune system, including B and T cells, to initiate clearance of the pathogens by generating a specific immune response. Therefore, TLR-dependent activation of antigen presenting cells represents a crucial step not only for the innate response, but also for ensuing mobilization of the adaptive immune system. Indeed, inherent defects in TLR signaling have been described in humans and are associated with greater susceptibility to bacterial infections (Ku, Yang et al. 2005).

The adaptive immune system is triggered when a pathogen evades the innate immune barriers of protection. As opposed to the innate response, the adaptive immune response is not present prior to encounter with a specific pathogen. Taking several days to develop, it antigen receptor molecule from a pre-existing repertoire recognizing a diverse array of distinct antigen specificities that arises from a recombinant gene rearrangement process (Delves and Roitt 2000).

Table 1.1. Toll-like receptors, their ligands, the pathogens from which they are derived and the phenotype displayed by TLR knockout studies (Carpenter and O'Neill 2007).

TLR	Ligand	Target Pathogen	Phenotypes of knockout mice
TLR1	Triacylated lipoproteins	Mycobacteria	↓ Cytokine production
TLR2	Peptidoglycan, lipoproteins, zymosan	Gram-positive bacteria <i>S. aureus</i> , <i>S. pneumoniae</i> , yeast and other fungi	↓ Cytokine production
TLR3	Double stranded RNA (dsRNA)	Viruses	↓ Cytokine production, ↑ Resistance
TLR4	LPS	Gram-negative bacteria,	↓ Pro-inflammatory cytokines (TNF- α , IL-6, IL-1), ↓ Proliferation of splenic B cells, hypo-responsive
TLR5	Flagellin	<i>S. typhimurium</i> , <i>P. aeruginosa</i>	No obvious phenotype because of TLR4 redundancy
TLR6	Diacylated lipoproteins, zymosan	Mycobacteria, yeast and fungi	↓ Cytokine production
TLR7/8	Single-stranded RNA (ssRNA)	Viruses, HIV, Denge virus influenza	↓ Cytokine release from dendritic cells, CD40 not up-regulated
TLR9	CpG DNA	Bacterial DNA	Dendritic cell maturation, ↓ Splenocyte proliferation, ↓ Inflammatory cytokines
TLR10	Unknown	Unknown	Unknown
TLR11	Profilin, uropathogenic bacteria	<i>T. gondii</i> Uropathogenic bacteria	↓ Survival
TLR12	Unknown	Unknown	Unknown
TLR13	Unknown	Unknown	Unknown

The adaptive immune response has several unique and critical functions. First of all, it has the ability to recognize specific “non-self” antigens in the presence of “self”, within the context of antigen presentation. Second, it is designed to generate responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells, a process achieved by clonal selection. Finally, an additional hallmark of the adaptive immune system is its ability to develop immunological “memory”, which results in the mounting of a stronger and much more rapid response to the same pathogen upon subsequent infection.

Adaptive immunity is mediated by lymphocytes, which fall into two major classes: T lymphocytes and B lymphocytes (Kuby 2007). Lymphocytes constitute 20-40% of the body’s white blood cells and 99% of the cells in the lymph. B lymphocytes, or B cells, originate and mature in the bone marrow and are responsible for the production of antibodies, the major effector molecules of humoral immunity. T lymphocytes, or T cells, originate in the bone marrow and mature in the thymus and are the effector cells of cell-mediated immunity. These circulate in the blood and lymph and carry out their respective functions in the tissues and lymphoid organs.

T cells are further divided into 2 subsets, which are each responsible for distinct roles within the immune system: T helper (T_H) lymphocytes and cytotoxic T (CTL) lymphocytes, distinguished from each other by the restricted surface expression of either CD4 or CD8 molecules, respectively. T_H cells orchestrate the activity of a number of other cells and processes of the immune system depending on the type of cytokine profile that they secrete. There are two main functional categories: T helper 1 (T_{H1}) cells and T helper 2 (T_{H2}) cells. T_{H1} cells secrete cytokines that stimulate an immune response against intracellular pathogens or tumors, resulting

in an activation pathway that favors a strong cell-mediated response. The cytokines secreted by T_H1 cells include IL-2, IL-12 IFN- γ and TNF- α (Salem 2004). T_H2 cells secrete cytokines that stimulate an immune response against extracellular pathogens, resulting in an activation pathway that favors a strong humoral response. Cytokines secreted by T_H2 cells include IL-4, IL-5, IL-10 and IL-13 (Salem 2004). The type of T_H cell response generated (cellular vs. humoral) is dictated by the cytokine milieu produced in response to the signal received upon infection. A third subset of T helper cells, termed T_H17 cells, also exists and is thought to play a role in inflammation, autoimmunity and allergy (Mangan, Harrington et al. 2006; Schmidt-Weber, Akdis et al. 2007). These cells are characterized by expression of IL-17, IL-6, TNF- α and IL-22 (Harrington, Hatton et al. 2005; Park, Li et al. 2005; Chung, Yang et al. 2006; Mangan, Harrington et al. 2006; Zheng, Danilenko et al. 2007). Because of their role in tissue inflammation by supporting neutrophil infiltration and survival, matrix degradation and induction of pro-inflammatory cytokines in structural cells leading to pathology, there is currently a complex topic of controversy regarding whether T_H17 cells are beneficial or harmful to the host (Schmidt-Weber, Akdis et al. 2007).

The other type of effector T cell is the CTL. Pathogenic microorganisms whose proteins gain access to the cell cytoplasm are considered “endogenous” antigens, which stimulate CTLs. Activation of CTLs involves the recognition of antigenic peptides in association with MHC class I molecules on APCs (Kuby 2007). MHC class I molecules are expressed by all somatic cells, excluding red blood cells. Two major mechanisms for target cell killing by CTLs have been described. One mechanism involves the secretion of perforin and its insertion in the plasma membrane of target cells leading to osmotic lysis. Alternatively, CTLs may express Fas ligand on their surface which binds to Fas on the target cell membrane to induce apoptosis. In addition

to killing infected cells directly, CTLs can elaborate a number of cytokines including TNF-alpha and lymphotoxin (Shames 2002).

Humoral immunity is another major component of the adaptive immune system. B cells and immunoglobulins are the main mediators of the humoral response during infection. Immunoglobulins, or antibodies, are proteins present on the B-cell membrane, and can be secreted once the B cell becomes activated or matures into a plasma cell. Specific interaction of membrane-bound antibody with antigen necessarily results in proliferation of antigen-specific B-cell clones with the capability of secreting antibodies which interact with antigen in the blood and tissues. While in circulation, antibodies seek out and neutralize pathogens, thus eliminating them. All immunoglobulins share certain structural features, participate in specific binding to antigen and carry out an array of effector functions that contribute to pathogen clearance. They are composed of 82-96% protein and 4-18% carbohydrate (Spiegelberg 1974). The immunoglobulin protein “backbone” consists of two identical heavy and two identical light chains, linked together by disulfide bonds. Each chain is further divided into domains that make up the variable and constant regions of the molecule. At the amino terminal of the molecule, one variable domain from a light chain and one variable domain from a heavy chain make up the antigen-binding portion. The highly diverse amino acid sequences at these regions confer unique antigen binding properties to the antibody. The rest of the antibody molecule has much less variation than the variable domains and therefore the immunoglobulin domains that comprise these parts of the molecule are called constant domains. Light chains have one constant domain whereas heavy chains have either 3 or four, depending on the type of heavy chain. The COOH-terminal of the constant domains of the heavy chains (CH) contain what is known as the Fc portions. It is through the Fc portion that other immune system components bind

immunoglobulins to initiate immune effector functions. For this reason, receptors that bind this portion of the immunoglobulin are called Fc receptors (FcR) (Ravetch and Kinet 1991).

While the variable regions of antibody are responsible for binding to antigen, the CH regions are responsible for a variety of interactions with other proteins, cells and tissues that result in the effector functions of the humoral response. The major effector functions of antibodies include opsonization, which promotes antigen phagocytosis by macrophages and neutrophils; complement activation, which activates a pathway that leads to the generation of a collection of proteins that can perforate cell membranes; and antibody-dependent cell-mediated cytotoxicity (ADCC), which leads to killing of antibody-bound target cells. These effector functions rely on the interaction between CH regions and other immune cells/molecules. The various antibody classes differ in their CH regions and therefore not all classes of immunoglobulins have the same functional properties.

The binding of IgG to FcR on leukocytes is important for many of the effector functions of IgG. There are four classes of Fc γ R identified in mammals: Fc γ RI (CD64), Fc γ RII (CD32), Fc γ RIII (CD16) (Siberil, Dutertre et al. 2007). Recently, a new member of the mouse Fc γ R family was identified, known as Fc γ RIV, however no human counterpart has yet been identified (Nimmerjahn, Bruhns et al. 2005; Nimmerjahn and Ravetch 2006). Binding of IgG to activating Fc γ R (Fc γ RI and Fc γ RIII) induce antibody-dependent cell cytotoxicity (ADCC), endocytosis of immune complexes followed by antigen presentation, phagocytosis and release of pro-inflammatory cytokines. By contrast, IgG binding to inhibitory Fc γ R (Fc γ RII) regulate immune responses by inhibiting the activation of B lymphocytes, monocytes, mast cells and basophils, induced through activating receptors (Nimmerjahn and Ravetch 2006). The activating and

inhibitory receptors transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM), respectively (Ravetch and Lanier 2000). Besides differences in function, the different classes of Fc γ R vary in cellular distribution and display different affinities for IgG along with characteristic isotype specificities (Figure 1.1). IgG/Fc γ R immune complex formation and the resulting effects are therefore well-regulated processes that provide a means by which antibodies, products of the adaptive immune system, can recruit key cellular elements of innate immunity such as macrophages and NK cells.

Five classes of immunoglobulins present in both humans and mice (IgG, IgA, IgM, IgD, and IgE) have been distinguished on the basis of non-cross-reacting antigenic determinants in regions of highly conserved amino acid sequences in the constant regions of their heavy chains (Ballieux, Bernier et al. 1964; Terry and Fahey 1964; Schur 1972).

IgG is the most abundant class found in serum, constituting about 80% of the total serum immunoglobulin. The IgG molecule is a monomer consisting of 2 gamma heavy chains and 2 kappa or two lambda light chains. This molecule is further broken down into 4 subclasses in humans, designated as IgG1, IgG2, IgG3 and IgG4, based on their relative concentration in normal serum and their frequency of occurrence as myeloma proteins (Kunkel, Fahey et al. 1966). Though mice produce IgG1, IgG2 and IgG3, the presence of IgG4 is strain-dependent (Martin and Lew 1998). Immunoglobulin subclasses are encoded by different germline constant heavy (CH) chain genes whose DNA sequences are 90-95% homologous. The structural characteristics that distinguish these subclasses from each other are the amino acid sequence and size of the hinge region as well as the number and position of the inter-chain disulfide bonds

	FcεRI	FcεRII (CD23)	FcγRI (CD64)	FcγRIIIa (CD32)	FcγRIIb	FcγRIIc	FcγRIIIa (CD16)	FcγRIIb	FcαRI (CD89)	Fcα/μR	FcRH1-6	FcRX FcRY
Chromosomal location	1q32.3	19	1q32.3	1q32.3	1q32.3	1q32.3	1q32.3	1q32.3	19q13.4	1q32.3	1q21-23	1q21-23
Mouse homolog	Yes	Yes	Yes	No	Yes	No	Yes	No	No	Yes	Yes	ND
Signalling motif	γ-chain ITAM β-chain ITAM	C-type lectin	γ-chain ITAM Periplakin	α-chain ITAM	α-chain ITIM	α-chain ITAM	γ-chain ITAM	GPI linked	γ-chain ITAM can activate or inhibit	ND	α-chain ITAM α-chain ITIM	Intracellular
Specificity for human Ig*	IgE	IgE CD21	IgG1=3>4	H ¹⁰¹ , IgG3>1=2 R ¹⁰¹ , IgG3>1>>2	IgG3>1>>2>4	ND	ND	IgG1=3>>>2=4	IgA1=IgA2 SigA1=SigA2	IgA & IgM	FcRH4.5 may bind aggregated IgA & IgG respectively	Ligand(s) unknown
Affinity for monomer Ig	Very high	Low	High	Low	Low	Low	Medium	Low	Low	ND	ND	ND
Cellular distribution	Mast cells Basos Monos Langs Allergic Neuts	B-cells T-cells NK-cells DCs Eos Macs	Monos Macs Neuts & Eos (activated)	Monos Macs Neuts Langs Platelets	Monos Macs B-cells Placenta	Monos Macs Neuts B-cells	Macs NK cells γδ T-cells some Monos	Neuts & Eos (activated)	Monos Some Macs NK-cells Neuts Eos Kupffer some DCs	B-cells Mesangial cells	B-cells	B-cells

Figure 1.1. Human leukocyte Fc receptors.

Relative affinities of various ligands for receptors are indicated in decreasing order, starting with the isotype with the highest affinity. The Ig domains are color-coded according to their subunit homology. Whereas some receptors signal directly through activation (green rectangles) or inhibition motifs (orange rectangles) in their ligand-binding α -chain, others depend on membrane association with the Fc γ -chain to allow signaling through the γ -chain ITAM. Basos, basophils; Eos, eosinophils; Langs, Langerhans cells; Macs, macrophages; Monos, monocytes; Neuts, neutrophils; GPI, glycosylphosphatidylinositol; ND, not determined; S-IgA, secretory IgA (Woof and Burton 2004; Shi, McIntosh et al. 2006).

between the two heavy chains. Though subtle, these differences affect the biological activity of the molecule (Table 1.2).

IgM accounts for 5-10% of total serum immunoglobulin. Monomeric IgM is expressed as membrane-bound antibody on B cells. IgM is secreted by B cells in a pentameric form consisting of five monomeric units held together by disulfide bonds. IgM is the first immunoglobulin class produced in a primary response to antigen and is the first immunoglobulin to be synthesized by the neonate. Because of its high valency, IgM is more efficient than IgG in viral neutralization and complement activation.

IgA constitutes 10-15% of total immunoglobulin in serum, however it is the predominant class present in external secretions such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and digestive tracts. In serum, IgA exists primarily as a monomer although polymeric forms are sometimes seen. The IgA of external secretions is known as secretory IgA because it contains a secretory component that is responsible for transporting polymeric IgA across cell membranes. IgA-secreting plasma cells are present in the mucous membrane surfaces, which are the primary entry way for most pathogenic organisms. Therefore, because of its localization at these sites, IgA can bind such pathogens and prevent attachment to mucosal cells, thereby inhibiting colonization.

IgE exists in extremely low average serum concentration (0.3%), however it plays an important role in mediating the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives and anaphylactic shock. IgE binds to Fc receptors on the surface of blood basophils and tissue mast cells. Cross-linkage of receptor-bound IgE molecules to antigen

Table 1.2. Immunoglobulin classes/subclasses differ in structure and function (Kuby 2007).

	Immunoglobulin Class/Subclass							
	IgM	IgG1	IgG2	IgG3	IgG4	IgA	IgE	IgD
Structure	Pentamer	Monomer	Monomer	Monomer	Dimer	Dimer	Monomer	Monomer
Serum Concentration (mg/ml)	1.5	9	3	1	0.5	3.5	0.0003	0.03
Complement Activation	++	+	+/-	++	-	-	-	-
Placental Transfer	-	+	+/-	+	+	-	-	-
Phagocyte Fc Receptor Binding	?	++	+/-	++	+	-	-	-
Neutralization	+	++	++	++	++	++	-	-
Opsonization	+	+++	-	++	+	+	-	-
Presence in External Secretions	Mucus etc.	Milk	Milk	Milk	Milk	Mucus etc.	-	-

++ = high; + = moderate; +/- = minimal; - = none; ? = questionable

induces degranulation of these cells, resulting in the release of granules and molecules, manifesting in an allergic response.

IgD is the most recently identified immunoglobulin present in serum, constituting about 0.2% of total immunoglobulin in serum. IgD, along with IgM, is the major membrane bound immunoglobulin expressed by naïve B cells. Although its function has not been completely understood, it is thought to play an important role in affinity maturation (Roes and Rajewsky 1993) and B cell activation (Kim and Reth 1995).

Immunoglobulins are made as both secreted antibodies and membrane-bound receptors for antigen displayed on the surface of B cells. The membrane-bound immunoglobulin is one part of a complex that forms the B cell antigen receptor (BCR). The BCR also contains a disulfide-linked heterodimer of two other polypeptides, Ig α and Ig β , which are made only in B-lineage cells. Each of these polypeptides contains a signaling function in their cytoplasmic domains. All five antibody isotypes can be expressed as membrane-bound that also form BCR in this way.

Lymphocyte activation and effector mechanisms

The activation of B and T lymphocytes is mediated by antigen-presenting DC in the B and T cell zones of the secondary lymphoid organs (spleen and lymph nodes) (DeFranco 2007). DC capture antigen at the site of infection or antigen entering the lymphoid tissue by blood or lymph, and then mature and display antigen as peptide fragments bound to surface molecules for recognition by T cells. In the case of B cells, intact antigen is displayed in complexes with complement components or antibodies that bind to receptors on follicular DC surface. These cells play a

central part in stimulating the production of antibodies with increased affinity for antigen (affinity maturation) and reflects a process in which the immunoglobulin genes undergo mutation and B cells with higher affinity immunoglobulin resulting from these mutations are selectively induced to proliferate (clonal selection). IgM and IgD are co-expressed on the surface of mature B cells, possess single antigenic specificity, and act as the initial BCR that binds antigen. After antigenic stimulation, B cells can differentiate into IgM-secreting plasma cells, which is the case in a primary immune response. Other activated B cells undergo a further process of recombination known as isotype switching. This occurs at the immunoglobulin heavy chain gene locus and results in the expression of different classes of antibodies (IgG, IgA or IgE) with the same antigenic specificity, yet able to perform unique effector functions (Kuby 2007).

Differentiation into effector cells requires interaction of naïve lymphocytes with antigen. This recognition occurs via receptors generated through a unique mechanism of genetic recombination that confers virtually unlimited diversity in antigen binding specificity. The major receptors involved are BCR and T cell receptors (TCR). Structurally, each of these receptors contains a highly variable antigen-binding region (V region) and an invariant region known as the constant region (C region), both of which are separately encoded in the germ-line DNA. During lymphocyte ontogeny, the DNA encoding these receptors undergoes programmed rearrangement to generate different segments which recombine to generate a receptor repertoire of vast diversity. After gene rearrangement and receptor expression, but before maturation, antigen binding initiates one of two processes: clonal deletion of cells which bear receptors for self antigens and clonal expansion of lymphocytes with receptors specific for the inducing antigen. This distinction is the basis for antigen-specific adaptive immune responses.

While B cells are capable of recognizing antigen alone, T cells recognize and interact only with antigen that has been processed and presented in association with MHC molecules. MHC molecules (also called human leukocyte antigens [HLA] in humans) are subdivided into class I molecules, which are found on all nucleated cells and class II molecules, which are found on specialized antigen-presenting cells (APCs) such as dendritic cells, macrophages, B cells, and selected activated endothelial or epithelial cells. T_H (CD4⁺) cells only recognize antigen presented by class II MHC molecules and CTLs (CD8⁺) only recognize antigen presented by class I MHC molecules. T-cell activation requires 2 signals. The first signal occurs through antigen/MHC binding to the TCR and is known as “signal 1”. An additional signal, “signal 2”, is provided by interaction between the co-stimulatory molecule CD28 on the T cell and CD80 or CD86 (also designated B7.1 and B7.2 respectively) on the APC. Activation of B cells requires signaling through the BCR and co-receptors such as CD19, CD21, or CD22. The combined antigen receptor- and co-receptor-derived signals define the degree of B cell activation and the strength of humoral immune responses. Activation of naïve T and B cells through this recognition and the subsequent up-regulation of appropriate co-stimulatory molecules is a requirement for differentiation into effector cells and successful pathogen elimination through the adaptive immune response.

T cell activation by recognition of the antigen on the target cell surface triggers the release of soluble mediators that act on the target cell. In the case of CD8⁺ CTL, these are cytotoxic mediators that kill cells infected with intracellular pathogens (e.g. viruses). Activation of naïve B cells occurs when antigen binds to surface immunoglobulin. The antigen is then internalized and presented on the surface where it is recognized by antigen-specific T helper cells which activate the B cell to proliferate and differentiate into antibody-secreting plasma cells. Cytokines secreted

by T helper cells influence not only the antigen binding regions of the antibodies, resulting in affinity maturation, but also direct the effector functions of the antibodies produced by plasma cells, resulting in production of antibodies of classes determined by their constant regions (isotypes). Following activation of naïve B and T cells, specific humoral and cell-mediated immune responses ensue.

B cells need two signals to initiate activation. Most antigens are T-dependent, requiring T cell help for maximal antibody production. With a T-dependent antigen, the first signal comes from antigen cross linking BCR and the second from its interaction with a T cell. When the antigen is first recognized and bound by the BCR, it becomes internalized, processed and presented as a peptide on MHC class II molecules. Recognition of this processed antigen by T_H (CD4+) lymphocytes and the subsequent activation of these cells constitute the critical events in the humoral (antibody-mediated) response. The antigen-MHC II complex forms the epitope that is recognized by antigen-specific T cell receptors on the surface of the CD4+ cells. T helper cells become activated and provide co-stimulatory signals and secrete cytokines (predominantly IL-4, IL-5 and IL-6) that lead to B cell proliferation and differentiation into effector memory cells and antibody-secreting plasma cells.

The ability of the immune system to respond rapidly upon re-exposure to a given antigen relies on long-lived plasma cells and the differentiation of memory cells. Memory responses differ from the primary response to antigen in several ways. First, because the population of antigen-specific cells has already undergone expansion and most of the changes required for effector function, they are much quicker. Second, in the case of B cells, the antibodies have undergone affinity maturation and are more effective than those produced in a primary response (Pulendran

and Ahmed 2006). For these reasons, a subsequent infection with a given antigen usually does not lead to disease. The development of vaccines that safely and efficiently induce immune memory is one of the most important goals of immunological research.

Immune Response to Vaccination

Immunity to a particular pathogen relies on the host's ability to mount a specific response against that pathogen. Immunity can be achieved through passive or active immunization. Passive immunization can occur by the transfer of preformed antibodies to a recipient through natural or artificial means. The goal of active immunization is to elicit protective immunity and immunologic memory so that subsequent exposure will induce a heightened immune response as well as elimination of the pathogen (DeFranco 2007). An example of active immunization is vaccination. Vaccines are designed to direct the immune system towards preventing infection and/or disease by mimicking the immune pathway elicited by a natural infection. Achieving this requires the activation of adequate innate as well as adaptive immune signals.

Mimicking the natural primary response to infection, initiation of a vaccine response relies on neutrophils, macrophages and DC of the innate immune system. These cells coordinate downstream activation of the adaptive response by secreting a variety of inflammatory mediators and cytokines. Upon activation, these cells become potent activators of T cells by virtue of up-regulation of MHC and co-stimulatory molecules on their surface. Activated, or mature, DC are the only APC capable of activating naïve T cells. After antigen processing and presentation through the exogenous pathway by APC, expressing MHC II molecules, CD4⁺ T cells can

specifically recognize the antigen. CD4⁺ cells then become activated and differentiate into either T_H1 or T_H2 subsets.

Immunization also activates the humoral response. The primary humoral response is characterized by the production of antibody-secreting plasma cells and memory B cells. The kinetics of this response varies depending on the nature of the antigen, the route of administration, the dose and the presence of immune enhancers. In general, this response is characterized by a lag phase, during which naïve B cells undergo clonal selection and differentiation into plasma and memory cells (Figure 1.2). Following this is the logarithmic phase, in which serum antibody levels increase until they reach a peak, which then they plateau and then eventually decline. Generally occurring within the first two weeks of immunization, IgM is secreted initially, followed by IgG. The subsequent development of a secondary response relies on the existence of a population of memory B cells and memory T cells. Antigenic activation of these cells generates a secondary antibody response which is characterized by a shorter lag period, more rapid recognition, greater magnitude and longer lasting response as compared with the primary response. In addition, the antibodies secreted in the secondary response have a higher affinity for the antigen and as a result of isotype switching, classes other than IgM predominate. The population of memory B cells in a secondary response is larger and more easily activated as compared with naïve B cells. Upon activation, this memory response continues to increase, reaching maximal levels around six months and persisting for years. Immunization activates a primary response. If an immunized individual is later exposed to the pathogen, the immune system behaves as a secondary response to infection, where plasma cells are already primed to produce high levels specific antibodies. Both activated T cells and

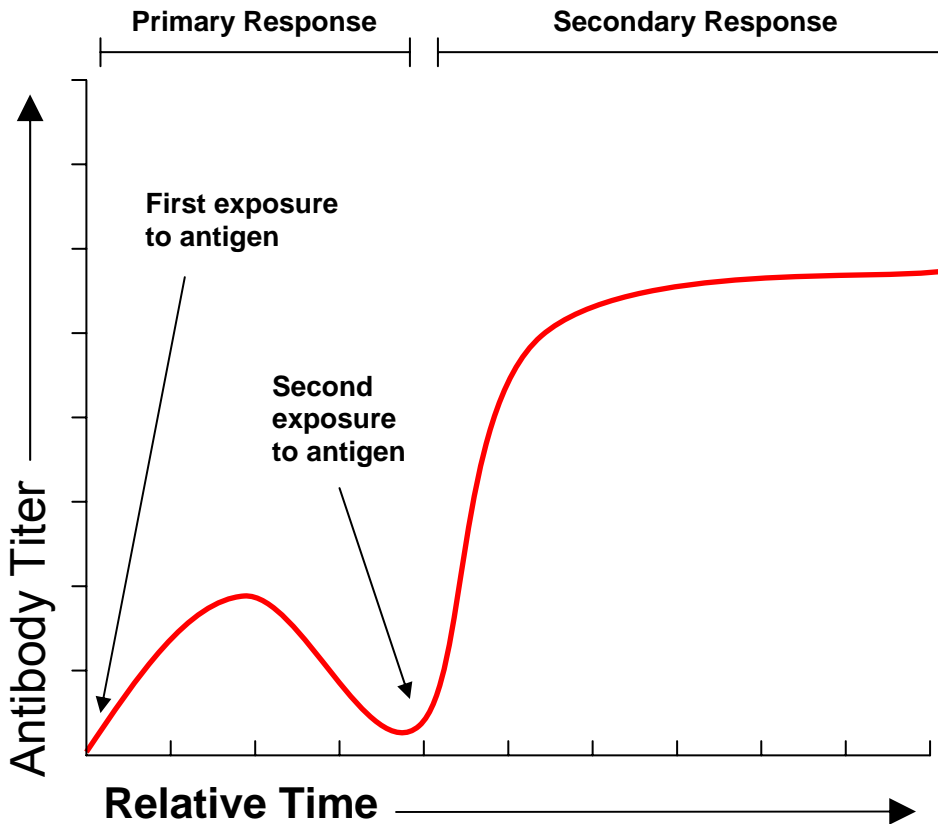


Figure 1.2. Primary and secondary immune responses.

Following initial exposure to an antigen the antibody response develops gradually over a period of days, reaches a low plateau within 2-3 weeks, and usually begins to decline in a relatively short period of time. When the antigen is encountered a second time, a secondary (memory) response causes a rapid rise in the concentration and increased affinity of antibody. The high levels of antibody in the serum may persist for a relatively long period of time.

antibody-secreting plasma memory cells are necessary to achieve an overall response is heightened and more rapid, thus efficiently protecting the body from infection and illness.

Immunization programs have led to the elimination and/or control of several different infectious diseases, including smallpox, polio, measles, mumps, rubella, *Haemophilus influenzae* type B disease, pertussis, tetanus and diphtheria (Rosenthal and Zimmerman 2006). The most successful vaccines are ones mediated through the induction of protective antibodies (Plotkin 2001). Current targets for vaccine development include more challenging infectious agents which require induction of CD4+ and CD8+ cellular responses (Plotkin 2005). These include viral agents such as human immunodeficiency virus (HIV), cytomegalovirus and severe acute respiratory syndrome coronavirus; bacteria such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoea* and *Mycobacterium tuberculosis*; and parasitic diseases such as malaria (O'Hagan, MacKichan et al. 2001; Rosenthal and Zimmerman 2006).

There are currently four types of vaccine formulations that are licensed for humans, including live attenuated, killed, purified subunit and recombinant subunit vaccines (Table 1.3). A live attenuated vaccine relies on decoupling virulence from induction of protective immunity. These are usually effective because they contain the three main elements needed for potent immune induction, the target antigen for induction of specific responses, PAMPs for induction of innate immunity and efficient delivery resulting from the natural invasiveness of the original organism (Ulmer, Valley et al. 2006). The organism is rendered attenuated by being subjected to serial passage in broth or cell culture, followed by selection for reduction of virulence and maintenance of immunogenicity in animal models. One important limitation of live attenuated organism-based vaccines is the risk of the organism reverting to virulence. In a killed vaccine, all of the antigens

Table 1.3. Examples of types of vaccines (Kuby 2007).

Type of vaccine	Disease target
Live attenuated	Mumps, Measles, Polio (Sabin vaccine), Rubella, Rotavirus, Tuberculosis, Varicella, Yellow fever
Inactivated or killed	Cholera, Influenza, Hepatitis A, Polio (Salk vaccine), Rabies
Subunit	Hepatitis B, Pertussis, Streptococcal pneumonia
Conjugate	Haemophilus influenzae type B, Streptococcal pneumonia, Neisseria Meningitidis
Recombinant	Hepatitis A, B
DNA	Tuberculosis, Hepatitis B, HIV (experimental)

of the organisms are available to the immune system, but the organism itself is rendered harmless, usually by treating it with formaldehyde. Because of their inability to replicate in vivo, these are generally less immunogenic than live vaccines, and therefore are often administered with adjuvants to increase their potency. Because of lack of exogenously-administered antigen accessibility to endogenous MHC-I antigen presentation pathways, killed vaccines do not effectively elicit cell-mediated immunity. Therefore, killed vaccines are mostly used for eliciting antibody-mediated protection.

Recombinant vaccines are those in which genes for desired antigens are inserted into a vector, usually a virus. The vector expressing the antigen may be used as the vaccine, or the antigen may be purified and injected as a subunit vaccine. Antigens which do not elicit protective immunity or those which are too toxic can be eliminated from the vaccine, and proteins expressed in a virus, even if it is not the original pathogen, are more likely to preserve their native conformation.

Since the natural immune response has evolved to provide optimal protection against infectious disease, the ideal vaccine should elicit the steps and processes of natural immunity. While the current vaccine approaches are relatively safe and have several protective advantages, a general problem is that the vaccines alone are often poorly immunogenic. Unlike attenuated live vaccines, killed whole organism or subunit vaccines generally require the addition of an adjuvant to be effective (Petrovsky and Aguilar 2004). Adjuvants were originally described as substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone (Ramon 1924). Adjuvants can be used to boost the immunogenicity of vaccines in several different ways. They can (1) increase the immunogenicity of weak antigens; (2)

enhance the speed and duration of the immune response; (3) modulate antibody avidity, specificity, isotype or subclass distribution; (4) stimulate cell mediated immunity, (5) promote the induction of mucosal immunity; (6) enhance immune responses in immunologically immature, or senescent individuals; (7) decrease the dose of antigen in the vaccine and reduce costs; or (8) help to overcome antigen competition in combination vaccines (O'Hagan, MacKichan et al. 2001).

The goal of a vaccine is to trigger the host immune system and mount adaptive immune responses of sufficient magnitude and duration, including B-cell-mediated antibody protection and/or specific T-cell-mediated cellular responses to a protective antigen in order to prevent infection or reduce severity of disease. It is now well-known that to achieve this, a vaccine should contain an adjuvant that efficiently activates the innate immune system to elicit such antigen-specific immune responses. A variety of immuno-stimulatory compounds appear to be TLR ligands and are currently being used experimentally or in clinical trials within vaccine formulations as an adjuvant (Ishii and Akira 2007). However, recent evidence has shown that some conventional adjuvants (Alum, Incomplete Freund's Adjuvant [IFA] and Complete Freund's Adjuvant [CFA]) as well as unconventional adjuvant-containing vehicle activate the innate immune system in a TLR-independent manner (Gavin, Hoebe et al. 2006; Janssen, Tabeta et al. 2006). Such alternative pathways that have been demonstrated to mediate adjuvant-induced innate and adaptive immune responses in a TLR-independent manner include intracellular innate receptors such as NOD-like receptors (NLR) and RIG-like receptors (RLR) (Creagh and O'Neill 2006; Meylan, Tschopp et al. 2006; Ishii and Akira 2007).

Adjuvants ultimately enhance antigen delivery by directly or indirectly facilitating uptake by APC, especially DC. Since these cells have the unique capacity to present antigen to naïve T cells in the lymph nodes, the principal mode of action of many particulate adjuvants (e.g. micoparticles, emulsions, liposomes, Iscoms etc.) are to promote uptake of the antigen by DC at the site of injection. Successful delivery of antigen to the lymph nodes and will not ensure the induction of an immune response because the presence of antigen alone constitutes only ‘signal 1’. Induction of a successful immune response requires the co-presence of ‘signal 2’. Signal 2 is represented by co-stimulatory molecules and cytokines, which are provided by APC and contribute to the priming of T helper cells.

A key issue in adjuvant development is toxicity. Safety concerns have restricted the development of adjuvants since alum was first introduced more than 50 years ago (Edelman 1997). Many experimental adjuvants have advanced to clinical trials but most have proven unsuitable for human use due to local and systemic toxicity (Table 1.4), Despite extensive evaluation of a large number of candidates over many years, the only adjuvants currently approved by the U.S. Food and Drug Administration are aluminum based mineral salts (generally referred to as alum) (Allison and Byars 1991; Brewer 2006; Rosenthal and Zimmerman 2006). Alum up-regulates co-stimulatory signals on human monocytes and promotes the release of IL-4 (Ulanova, Tarkowski et al. 2001) and is thought to act by forming an antigen depot at the site of inoculation (O'Hagan, MacKichan et al. 2001). Alum adsorption may also contribute to a reduction in toxicity for some vaccines, due to the adsorption of contaminating endotoxin (Shi, HogenEsch et al. 2001). Despite these attributes, alum is considered a relatively poor adjuvant in many situations, particularly for antibody induction to protein subunits (Gupta 1998) and for inducing

Table 1.4. Selected examples of vaccine adjuvants (O'Hagan, MacKichan et al. 2001)

Adjuvant System	Example
Mineral Salts	Aluminum hydroxide*, Aluminum phosphate, Calcium phosphate*
Immuno-stimulatory adjuvants	Cytokines (e.g. IL-2, IL-12, GM-CSF)
Microorganism-derived adjuvants	MDP derivatives, Bacterial DNA (CpG oligo's), LPS, MPL and synthetic derivatives
Lipid particles	Emulsions (e.g. Freund's, SAF, MF59*)
Particulate adjuvants	Iscom's, Liposomes, polymeric microspheres, nano-beads, virus-like particles
Mucosal adjuvants	Heat labile enterotoxin (LT), Cholera toxin (CT), Mutant toxins (e.g. LTK63 and LTR72)
Nucleic acid-based adjuvants	

* Currently included in approved vaccines

Many factors may influence the immune response to vaccination. These include the presence of maternal antibody, nature and dose of antigen, route of administration, and the presence of adjuvants. In addition, host factors such as age, nutritional status, genetics, coexisting disease factors and immune status, may also affect the response.

Gender and Hormonal Influences on Immune Function

As mentioned earlier, proper immune function and maintenance of homeostasis relies on the immune system's ability to discriminate between self and non-self as well as the ability to regulate inflammation and therefore tissue damage and pathogenesis. Any malfunction in this regulation may lead to immune dysfunction and, ultimately, disease. One manifestation of immune dysfunction is immunodeficiency. This results when the immune system's ability to fight off disease is either compromised or entirely absent, rendering the host particularly vulnerable to opportunistic infections. Thus, the individual is considered to be "immuno-compromised". In a healthy immune state, self-reactive lymphocytes are deleted via the process of negative selection or made unresponsive to self ligands/antigens through tolerance during development of the immune system. Autoimmunity, another form of immune dysfunction, results from the inability to discriminate "self" from "non-self", which leads to a pathologic inflammatory response and ultimate development of autoimmune disease (Shames 2002). Gender is one of the most important epidemiological risk factors for the development of autoimmune diseases (Da Silva 1999), where the risk is 2.7 times higher in females as compared to males (Jacobson, Gange et al. 1997). Table 1.5 lists data on the prevalence of autoimmune diseases in females as compared to males.

Consistent with differences in prevalence of autoimmune diseases, evidence indicates that even basic immune responses differ between males and females. In response to immunization, females mount more vigorous T cell responses, have higher levels of serum immunoglobulins (Eidinger and Garrett 1972; Weinstein, Ran et al. 1984) and shorter skin allograft rejection time (Grossman 1984; Olsen and Kovacs 1996). Females have higher CD4+ T cell numbers than men (Amadori, Zamarchi et al. 1995) and also mount consistently higher Th2 responses than men (Giron-Gonzalez, Moral et al. 2000).

There is now a large body of evidence suggesting that sex hormones in males and females are significant factors responsible for this gender dimorphism. Since sex hormones differ between males and females as well as within different reproductive stages in females, a lot of research has focused on the detailed effects of sex hormones on immune function. Sex hormones modulate a variety of immune mechanisms including thymocyte maturation and selection, cell trafficking, cytokine and chemokine production, lymphocyte proliferation, and expression of adhesion molecules and HLA class I molecules (Wilder 1995; Olsen and Kovacs 1996). Estrogens have stimulating effects on B cell functions which may be dependent on inhibition of suppressor T cells. Cumulative data follows the general trend that estrogen enhances B cell activity and B-cell mediated diseases but suppresses T cell activity and T-dependent conditions. Testosterone, on the other hand, appears to suppress both B cell and T cell-mediated responses and virtually always suppresses expression of the disease (Da Silva 1999). Physiological levels of estrogens tend to enhance B cell mediated responses and antibody production both in vivo and in vitro, as well as cellular responses such as transplant rejection and tumor-associated immunity, whereas progesterone and testosterone tend to depress these processes (Stoeger, Chiorazzi et al. 1988). Additionally, both the incidence and severity of many autoimmune diseases have been found to

Table 1.5. Gender differences in autoimmune disease prevalence

Disorder	Female: Male ratio
Hashimoto's disease/ hypo-thyroiditis	50:1
Systemic lupus erythematosus	9:1
Sjogren's syndrome	9:1
Antiphospholipid syndrome	9:1
Primary biliary cirrhosis	9:1
Mixed connective tissue disease	8:1
Chronic active hepatitis	8:1
Graves' disease/hyperthyroiditis	7:1
Rheumatoid arthritis	4:1
Scleroderma	3:1
Myasthenia gravis	2:1
Multiple sclerosis	2:1
Chronic idiopathic thrombo-cytopenic purpura	2:1
Type 1 diabetes mellitus	2:1

American Autoimmune Related Diseases Association
(www.aarda.org)

be influenced by a range of female-related endocrinological changes involving sex steroids, such as puberty, pregnancy, menopause, use of oral contraceptives and aging (Da Silva 1999). In conditions such as multiple sclerosis (MS) and rheumatoid arthritis (RA), severity of the disease decreases during pregnancy, particularly during the third trimester, when progesterone and estradiol levels peak. Interestingly, disease severity flares up again during the post-partum, when hormone levels drop (Nelson and Ostensen 1997; Confavreux, Hutchinson et al. 1998). On the other hand, patients with systemic lupus erythematosus (SLE) may worsen or remain unchanged throughout pregnancy (Petri 1994). This is explained by the fact that in pregnancy there is a shift towards T_H2 pathways leading to increased activation of the humoral response and a decreased cellular response. In SLE, humoral responses are a significant pathogenic factor thus disease exacerbation, whereas RA and MS are disorders related to cellular T_H1 pathways (Lahita 1986; Holmdahl 1989).

In human and animal experimental models, the female sex hormones estrogen and progesterone have been shown to regulate both innate and adaptive immunity. Much of the existing data is based on studies which examine immunity within the female reproductive tract (FRT), mainly because this serves as the first site of immunological contact with pathogens such as Chlamydia, HIV and human papillomavirus (HPV) and site of diseases such as gonorrhoea and syphilis. The vagina, cervix and uterus contain the necessary immune cells responsible for both innate and specific immunity, although the numbers and functions of these cells vary significantly throughout the phases of the reproductive cycle. This phenomenon is believed to be correlated with and controlled by changes in the levels of estrogen and progesterone, hence the role of these hormones in susceptibility to infection, gender differences in immune function and development in autoimmune disease.

Primarily due to the female dominance in the incidence of autoimmune disease, estrogen, in particular has attracted significant interest as an immunomodulator. Murine models of autoimmunity have supported this role. Estrogen acts on multiple immune processes including lymphocyte and monocyte development, dendritic cell function, T cell responses, cytokine regulation, B cell expansion and survival, antibody responses, and NK cell activity (Reviewed in (Lang 2004). There is a wealth of clinical and laboratory data generated over the past 20 years demonstrating that sex hormones, particularly estrogen, affect the immune system. However, the mechanisms by which estrogen modulates such processes as well as how its effects are regulated is complex and requires the understanding of the interactions between the immune system with the neuro-endocrine system.

Interaction between the Immune and Neuroendocrine Systems

Hormonal influences on immune function occur through the necessary interaction of the immune system with the neuroendocrine system through common receptors to neurotransmitters, hormones and cytokines. The hypothalamus-pituitary-adrenal (HPA) serves as a key element of this communication pathway. Activation of the HPA axis is known to serve the body's response to stress, which can be defined as any physical or psychological stimulus that disrupts the body's homeostasis.

There are three main glands that comprise the anatomy of the HPA axis. The hypothalamus, located in the lower central part of the brain, functions to regulate metabolism and body temperature. It secretes hormones that regulate the release of hormones in the pituitary gland.

Referred to as releasing hormones, these include corticotrophin releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH) which are secreted and carried directly to the pituitary gland to signal secretion of stimulating hormones. The pituitary gland, located at the base of the brain beneath the hypothalamus, is considered to be the "master gland" of the body because it secretes a range of hormones that control the functions of virtually all physiologic processes in the body. The main hormone secreted by the pituitary is adreno-corticotropin hormone (ACTH), which stimulates the adrenal glands to produce glucocorticoids. Every nucleated cell in the body expresses glucocorticoid receptors; hence the widespread effects of glucocorticoids on most systems of the body, including metabolic, endocrine, nervous, cardiovascular and immune systems. The pituitary also secreted the stimulating hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) which stimulate the gonads to produce and release the male and female sex hormones.

Inflammation resulting from immune aggression during the innate or adaptive immune response involves the release of pro-inflammatory cytokines. These cytokines can activate the HPA axis, resulting in the release of glucocorticoids. In turn, the glucocorticoids exert negative feedback onto immune cells to suppress further synthesis and release of cytokines, thereby protecting the host from detrimental consequence of an overactive immune response (e.g. tissue damage, autoimmunity, septic shock) (Figure 1.3). Cytokine receptors have been detected at all levels of the HPA axis, and therefore, each level can serve as an integration point for immune and neuroendocrine signals. In addition, cytokines are synthesized in the brain, the anterior pituitary and the adrenal gland. The major cytokines that are known to be involved in HPA activation are IL-1, IL-2, IL-6, TNF-alpha and interferon (IFN) (Bumiller, Gotz et al. 1999).

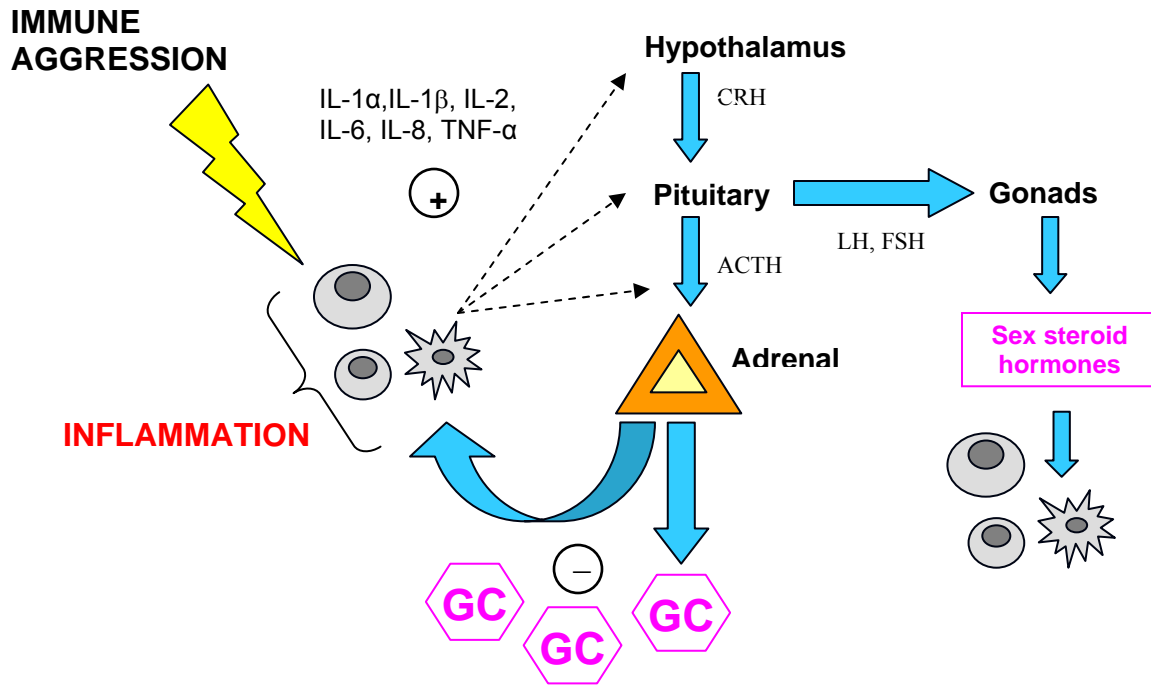


Figure 1.3. Interactions between the immune system and the HPA axis in an inflammatory reaction.

Activation of the immune system through bacterial aggression induced the production of pro-inflammatory cytokines that stimulate the HPA axis, producing the release of immunosuppressive GC, which negatively regulate further pro-inflammatory cytokine production. Gonadal stimulation by pituitary hormones, LH and FSH, results in sex steroid hormone secretion, which can also directly stimulate immune cells by acting on specific hormone receptors in these cell types. GC, glucocorticoids. CRH, corticotropin release hormone; ACTH, adrenocorticotrpin release hormone; LH, luteinizing hormone; FSH, follicular stimulating hormone.

Glucocorticoids regulate a wide range of immunological processes, such as cell proliferation, inflammation, and inhibition of trafficking of T, B, and NK cells, eosinophils, basophils, macrophages, and monocytes and induction of apoptosis in mature T and B cells and peripheral blood lymphocytes. In addition, glucocorticoids may alter downstream adaptive immune responses by causing a shift from cellular (T_H1 /inflammatory) to humoral (T_H2 /anti-inflammatory) type immune responses (Chrousos 2000). These events could partially explain the immuno-suppression that is associated with stress. Therefore, glucocorticoids can modulate immune/inflammatory processes by stimulating as well as suppressing immune function, depending on the type of immune response, the immune compartment, and the cell type involved (Silverman, Pearce et al. 2005). HPA-activated release of glucocorticoids represents a potent negative feedback mechanism which allows the body to have tight control of the local immune response, inhibiting this defense mechanism from endangering the body's integrity (Gaillard and Spinedi 1998). In addition to suppressing HPA axis activating-immune processes, glucocorticoids act on specific receptors within the hypothalamus and pituitary to regulate its own production (Buckingham, Loxley et al. 1996; Da Silva 1999).

In the HPA response to immune stimuli, a gender dimorphism exists (Da Silva 1999). Female rats have higher basal levels of plasma corticosterone compared to males and lower levels of pituitary glucocorticoids receptors, with their expressions being estrogen dependent (Turner 1990). In experimental animals, females also have higher and more prolonged ACTH or stress-induced plasma corticosterone levels than males. Estrogens generally increase HPA responses, whereas androgens suppress it (Da Silva 1999). Gonadectomy in both male and female mice leads to enhanced HPA axis and immune responses to endotoxin, which can be reversed by androgen treatment in both sexes (Spinedi, Suescun et al. 1992). Sex steroid receptors are present

in the adrenals (Gaillard and Spinedi 1998). These observations clearly indicate that sex steroids modulate both immune and HPA axis activities.

Estrogen Mechanism of Action

The major estrogens include estradiol, estrone and estriol. Their effects are mediated through interaction with specific intracellular receptors. These receptors are members of the nuclear receptor super-family of transcription factors. The estrogen receptor (ER) is present in two subtypes, ER- α and ER- β , encoded by two distinct genes (Kuiper, Enmark et al. 1996; Mosselman, Polman et al. 1996; Couse and Korach 1999). They share a high degree of amino acid conservation in their DNA binding domains (97%) and exhibit a significant, but lesser degree of homology in their ligand binding domains (58%). Both receptors are localized in reproductive tissues, as well as non-reproductive tissues including cells of the immune system, cardiovascular system, central nervous system and bone (Lubahn, Tan et al. 1989; Krege, Hodgin et al. 1998; Couse and Korach 1999; Dupont, Krust et al. 2000; Mendelsohn 2000). Within the immune system, ERs are present in B lymphocytes (Suenaga, Evans et al. 1998; Benten, Stephan et al. 2002), T lymphocytes (Cohen, Danel et al. 1983; Suenaga, Evans et al. 1998; Benten, Stephan et al. 2002), monocytes (Weusten, Blankenstein et al. 1986; Suenaga, Mitamura et al. 1996; Suenaga, Evans et al. 1998; Stefano, Prevot et al. 1999), neutrophils (Molero, Garcia-Duran et al. 2002) and NK cells (Curran, Berghaus et al. 2001; Henderson, Saunders et al. 2003).

ER- α is the dominant subtype expressed throughout the FRT and its ablation results in infertility due to defects in sexual behavioral expression, neuro-endocrine gonadotropin regulation, ovulation, uterine function, and post-pubertal mammary gland morphogenesis. The ER- α subtype also plays an essential role in male fertility and mediates many non-reproductive activities of estrogen including regulation of bone-resorption, post-natal bone growth, cardiovascular endothelial regeneration, adipogenesis and sexual behavior (Couse and Korach 1999). In contrast, ER- β ablation results in less severe phenotypic consequences with regard to estrogen signaling. ER- β is expressed in both the male and female reproductive tracts in a pattern which differs from that of ER- α and its ablation results in a sub-fertile phenotype restricted to impaired female ovarian function (Krege, Hodgin et al. 1998). Expression of ER- β has also been detected in non-reproductive tissues including bone-forming osteoblasts, epiphyseal chondrocytes and cardiovascular and central nervous systems (Mendelsohn 2000; Wang, Andersson et al. 2001).

Estrogenic effects are mediated by genomic or non-genomic mechanisms (Figure 1.4). In a genomic mechanism of action, estrogenic ligands activate ER as a transcription factor by inducing a conformational change(s) that leads to nuclear translocation, dimerization, and binding to specific steroid receptor response elements (SRE) in promoters of primary target genes. Activated ER recruits co-activators that are essential for assembly of a productive transcription complex at the promoter. In a non-genomic action of ER, ER associates with cytoplasmic- and/or cell membrane–signaling molecules in an estrogen-dependent manner. This extra-nuclear interaction promotes the Shc-Src-Raf–MAPK kinase (MEK)–MAP kinase phosphorylation cascade. Because MAPK can directly or indirectly activate other transcription factors, this pathway can potentially regulate distinct or complementary sets of genes from those regulated by nuclear ER pathways. As mentioned, ER acts on cells of the immune system as well

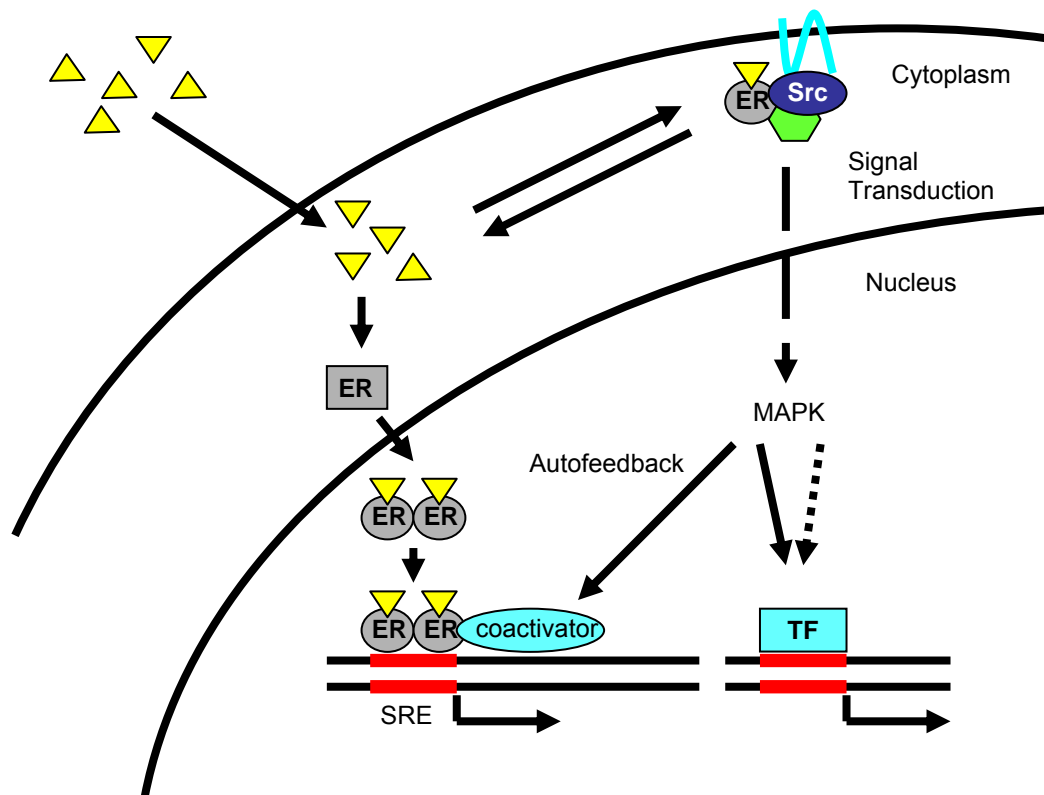


Figure 1.4. Estrogen receptor mechanism of action.

In a genomic mechanism of action, ligands (triangles) activate ER as a transcription factor by inducing a conformational change(s) that leads to nuclear translocation, dimerization, and binding to specific steroid receptor response elements (SRE) the target gene promoter. In a non-genomic action of ER, ER associates with cytoplasmic- and/or cell membrane–signaling molecules in an estrogen-dependent manner. This promotes the Shc-Src-Raf–MAPK kinase (MEK)–MAP kinase phosphorylation cascade. MAPK can directly (solid arrows) or indirectly (dashed arrows) activate other transcription factors (TF). Adapted from (Edwards and Boonyaratanakornkit 2003).

as breast, bone, brain and uterus, where ligand-bound ER functions as a key transcription factor in various molecular estrogen-mediated activities in these tissues. Therefore, modulation of ER expression levels is important in determining tissue-selective action of estrogen. Indeed, despite its localization in several cell types, levels of ER expression is regulated by several factors, which act as ER modulators. Effectors of chromatin structure, hormones, growth factors and a variety of other agents have implicated multiple cellular mechanisms of regulation of ER expression. For example, methylation of the ER gene down-regulates its expression, as does MAPK kinase (MAPKK MEK), a downstream effector of growth factor signaling, and other hormones such as androgens and progesterone (Pinzone, Stevenson et al. 2004).

Animal Models in Immune-Endocrine Studies

Experimental models for human diseases are of crucial importance not only to understand the biological and genetic factors that influence the phenotypic characteristics of the disease but to utilize as a basis for developing rational intervention strategies. Human use in biomedical studies is limited by technical and ethical considerations. And although they have been instrumental in increasing the understanding of the human immune system, studies in nonhuman primates are constrained by high cost, limited availability, paucity of genetic models for human diseases, and lack of genetically inbred strains suitable for stem cell or tissue transplantation. Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its

genome can be manipulated and analyzed. Mice are suitable models for immunological studies, as they demonstrate immunological similarities to humans. They display strong humoral and cellular immune responses to experimental immunization (Gaines, Currie et al. 1965; Pinchuck and Maurer 1965; Braley and Freeman 1971; Cerny, McAlack et al. 1971; Amsbaugh, Hansen et al. 1972; Blomberg, Geckeler et al. 1972; Hellman, Fowler et al. 1972; Ghaffar and James 1973; Heiniger, Taylor et al. 1975). Mice are extensively used in hormone-related studies, and are the most common animal species used for immunological studies. Their immune systems are extremely well-characterized, and biological reagents necessary to perform immunological experiments are commercially readily available.

The use of rodent models in immune-endocrine studies is particularly advantageous because they are short-lived and because of the availability of homogenous laboratory strains which permit controlled research experiments. In addition, rodent transgenic models using genetic manipulation of specific genes such as ER α and ER β as well as gonadectomy models, have contributed greatly to the current general understanding of endocrine function.

Summary and Study Objectives

Estrogen has been shown to play an important role in protection against osteoporosis in postmenopausal women (Turner, Riggs et al. 1994), in the prevention of coronary heart disease (Iafrati, Karas et al. 1997), and in the maintenance of cognitive function (Tang, Jacobs et al. 1996). In addition to the positive effects reproductive and non reproductive tissues, estrogen

plays an important role in the development of uterine cancers and has been implicated in the development of breast cancer (Jordan and Murphy 1990; McKenna, Xu et al. 1999; Peto, Boreham et al. 2000).

The conflict between the positive and negative effects of estrogen has fueled a search for selective estrogen receptor modulators (SERMs) for use in hormone replacement therapies (HRT) that possess the capability of harnessing the tissue-selective beneficial effects of estrogen while lacking adverse activities in breast and uterus. In addition to their use in HRT, SERMs have been studied for their potential therapeutic advances in other areas of clinical practice.

In males, estrogen is produced in small quantities as result of biosynthetic conversion from testosterone and a byproduct of other pathways. In females, estrogen levels fluctuate over a lifetime, from birth, through puberty, pregnancy, and finally menopause. These varying levels have a dramatic impact on health and immunity at a cellular level.

The aim of the present dissertation is to further characterize the immunomodulatory role of estrogen using an *in vivo* vaccine model. This model is designed to determine the influence of estrogen on vaccine responses to commonly administered human vaccine formulations in mice. We have shown that (1) estrogen influences not only specific antibody responses in vaccinated mice, but (2) the mechanism of this effect may occur at the level of innate immunity during initial antigen encounter by macrophage TLR2 and (3) that estrogen influences other important macrophage functions that lead to specific adaptive response by modulating Fc γ R gene expression and function. Therefore, we show that estrogen manipulation in mice impacts both the innate and adaptive immune functions.

Though estrogen's immunomodulatory role has been documented in many different systems, it is still unclear how estrogen may influence induction of immunity through vaccination. Beginning to understand the gender differences and the general role of sex hormones in vaccine responses in human and experimental animal models may be the basis for achieving improved vaccine efficacy in certain target populations based on sex, age and immune status as well as provide new perspectives with which to design future vaccines against infectious diseases.

CHAPTER 2

Experimental Methods

Animals

Female Balb/c mice were housed in standard conditions with food and water provided ad libitum and a constant light cycle of 12 hours (lights on from 8:00 to 20:00). Male mice were co-housed and served as controls. Female and male mice were fed isoflavone-free, soy-free casein diet (Lab Diet, Richmond, IN). All animals were housed and subjected to experimental procedures in accordance to protocols approved by the appropriate institutional review committee.

Ovariectomy

At 6-8 weeks of age, mice were anesthetized with 5% isoflurane gas in 100% oxygen. Ovaries, oviducts and tips of the uterine horns were bilaterally removed via two dorsal intraperitoneal incisions. The incisions were repaired with suture and skin staples. Sham ovariectomies were performed identically except the ovaries were not removed.

Estrogen replacement

Estrogen replacement by subcutaneous implantation of 17- β estradiol (E2) pellets was performed as follows: sterile E2 pellets were inserted through a subcutaneous tunnel made between the scapulae. Mice were given a single (5 mm) subscapular Silastic implant (1.02 mm ID \times 2.16 mm OD) containing either E2 diluted 1:1 with cholesterol (Sigma- Aldrich Corp., St. Louis, MO) or cholesterol only. This pellet size yields E2 concentrations of 85–100 pg/ml, similar to that of intact mice in proestrus (Wersinger, Haisenleder et al. 1999).

Estrogen replacement through the drinking water was performed as follows: treated water was prepared as previously described (Wersinger, Haisenleder et al. 1999), by diluting stock solutions of E2 in 100% ethanol to solubilize. Solubilized estrogen was added to drinking water to produce a final concentration of 1000 nM, as previously described (Mobbs, Cheyney et al. 1985; Wersinger, Haisenleder et al. 1999). The final concentration of ethanol was 0.1%. No adverse effects of this concentration of ethanol or estrogen were reported (Wersinger, Haisenleder et al. 1999).

Isolation of peritoneal macrophages

Prior to euthanization, mice were each injected intraperitoneally with 1 ml 3% sterile Brewer's thioglycollate medium. Five days later, mice were killed by exsanguination under isoflurane as well as by cervical dislocation and a midline incision was made with a pair of sterile scissors. After retraction of abdominal skin, 10 ml sterile cold PBS was injected through peritoneal wall. Mice were gently agitated to dislodge cells bound within the peritoneal cavity. Peritoneal fluid was then taken slowly with a yield of 8-10 ml total volume. Cell suspension was washed twice with PBS, counted, suspended (1×10^6 cells/ml) in complete DMEM media and cultured for 2 h at 37°C and 5% CO₂. Non-adherent cells were rinsed with PBS and the adherent cells were kept in wells to be directly lysed for RNA extraction (see below).

Splenocyte and splenic macrophage isolation

Spleens were removed aseptically and placed into 50-ml conical tube containing cold DMEM medium. The spleens were then gently pressed through a 70 um cell strainer (BD Falcon) using a

10 ml syringe plunger to produce single-cell suspension, and centrifuged at 2000 RPM, at 4°C for 10 min. The erythrocytes were lysed with RBC lysis buffer (Sigma). The remaining cells were then washed, counted, and suspended (1×10^7 cells/ ml) in DMEM medium and incubated in 24-well plates. After 2 h of incubation (37°C at 5% CO₂), non-adherent cells were removed, and the adherent cells were kept in wells to be directly lysed for RNA extraction (see below).

Detection of specific antibody responses

Enzyme-linked immunosorbant assay (ELISA) was used to detect specific antibody responses. Micro-titer wells were coated with the antigen, diluted in coating buffer (KPL, Inc., Gaithersburg, MD), in a volume of 50ul/well. Coated plates were incubated overnight at 4°C. To block nonspecific binding sites, wells were incubated for 30 min at 37°C with 200ul/well PBS containing 5% fetal calf serum (FCS-PBS) (Atlanta Biologicals, Lawrenceville, GA). Plates were washed three times with PBS containing 0.05% Tween-20. To generate titration curves, serum samples were tested by preparing two-fold serial dilutions (1:50-1:25600). Samples were added to triplicate wells in a volume of 50ul/well, and incubated overnight at 4°C. After washing three times with PBS containing 0.05% Tween-20, wells were incubated with HRP-labeled anti-mouse IgG or IgG+A+M (KPL) for 1 hr and 15 min at 37°C. Wells were washed and then incubated with 50ul/well ABTS/H₂O₂ (KPL) substrate for 10 min at room temperature, followed by addition of 50ul/well stop solution (KPL). Optical density (OD) values were assessed at 405 nm using a PowerWave HT plate reader (BioTek Instruments, Inc., Winooski, VT).

Detection of total immunoglobulin production

Total immunoglobulin (Ig) production was assessed by ELISA according to methods similar to those described above, with the following exceptions. Micro-titer plates were coated with unlabeled goat anti-mouse IgG+IgA+IgM (KPL). Individual serum samples were titrated by preparing 4-fold serial dilutions and added to triplicate wells. Plates were incubated overnight at 4 °C, washed as described above and incubated with HRP-labeled goat anti-mouse IgG+IgA+IgM (KPL) for 1 h at 37 °C.

Detection of IgG1/ IgG2a responses

To determine the effects of estrogen on IgG1/IgG2a production, ELISA was used according to methods similar to those described above, with the following exceptions. Plates were coated with purified antigen depending on the vaccine administered. Individual serum samples were diluted at 1:100 and added to triplicate wells. Plates were incubated overnight at 4 °C, washed and incubated with HRP-labeled goat anti-mouse IgG1 or HRP-labeled goat anti-mouse IgG2a (KPL) for 1 h at 37 °C.

RNA extraction, RT-PCR and real time PCR

Total RNA was isolated using the RNeasy MiniKit (Qiagen, Valencia CA) from splenic macrophages and thioglycollate-elicited peritoneal macrophages according to manufacturer's instructions. For plate-adhered macrophages, cells were lysed directly in wells of microplate. RNA samples were quantified by spectrophotometric analysis and/or quantification by gel electrophoresis prior to cDNA synthesis. Total RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit for RT-PCR where AMV reverse transcriptase synthesizes

the new cDNA using random primers according to conditions provided by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). cDNA synthesis reactions were performed with approximately 1ug of total RNA using 4 ug (0.08 A₂₆₀ units) Random Primer p(dN)₆, 10mM of each dNTP, 20 units AMV reverse transcriptase, 50 units RNase Inhibitor and 1 X reaction buffer in a final volume of 20ul. cDNA reactions were incubated at 25° C for 10 min, then at 42° C for 60 min, according to manufacturer's instructions.

Amplification of the following target genes were performed by real time PCR using TaqMan Gene Expression Assays (Applied Biosystems): TLR2, TLR4, FcγRI, FcγRIIB, FcγRIIIA, ER-α. Realtime PCR was performed in a total volume of 25 ul with the following components: 1 X TaqMan Gene Expression Master Mix, 1 X target gene primer/probe mix and 1 X endogenous β-actin gene primer/probe mix (Applied Biosystems). Realtime PCR was performed in an ABI PRISM 7700 Sequence Detection System thermal cycler (PE Applied Biosystems, Foster City, CA) to quantify target genes. Cycle parameters were 50° C for 2 min to activate UNG, 95° C for 10 min to activate Taq, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min. The fold change of target gene mRNA was expressed as $2^{-\Delta\Delta Ct}$ (ΔCt = the difference in threshold cycles for the test gene and β-actin, $\Delta\Delta Ct$ = the difference between the experimental and control mice, or as indicated).

CHAPTER 3

Estrogen-Induced Modulation of Anti-Tetanus Toxoid Antibody Responses in a Murine Model

SUMMARY

It is well established that sex hormones influence the immune response. Estrogen modulates lymphocyte activity and cytokine production and enhances the antibody response to several immunogens. Therefore, estrogen may influence the antibody response to specific vaccine preparations. To test this hypothesis, we used a model consisting of female mice subjected to ovariectomy and immunized with tetanus toxoid (TT) in two experimental protocols, differing for modality of estrogen replacement (either subcutaneous pellet administration or administration in drinking water). In each protocol, mice were assigned to one of three experimental groups. The first group of mice, designated OVEX-E2, was ovariectomized and subjected to estrogen (E2) replacement. The second group, designated OVEX group, was ovariectomized but received no estrogen replacement. The third group remained intact. In the first experimental protocol (pellet-E2), mice were either immunized subcutaneously (SC), or were unvaccinated. In the second immunization protocol (drinking water-E2), mice were immunized either subcutaneously (SC) or intramuscularly (IM) or were unvaccinated. Results from both studies show that, in general, E2 replacement results in higher anti-TT antibody responses. In the drinking water-E2 groups, mice vaccinated by the IM route produced significantly higher anti-TT IgG levels than mice vaccinated by the SC route after the third vaccination dose. OVEX-E2 mice produced significantly higher anti-TT IgG levels than OVEX and intact mice when vaccinated either IM or SC. The anti-TT IgG responses were predominantly of the IgG1 isotype in all groups of mice.

Together, these results show that estrogens may influence the murine antibody response to vaccines routinely administered to human populations.

INTRODUCTION

The major functions of sex hormones relate to sexual differentiation and reproduction. Sex hormones can also interact with the immune system, thus causing a gender dimorphism in immune function. This dimorphism was initially demonstrated by the observation that females produce higher immunoglobulin levels and mount stronger immune responses to immunization or infection than males (Verthelyi 2001; Wierman 2007). Females are also known to show increased susceptibility to autoimmune diseases (Da Silva 1999; Whitacre, Reingold et al. 1999; Verthelyi 2001; Whitacre 2001).

Estrogens can modulate the immune system through direct interaction with estrogen receptors present in immune cells, including lymphocytes, macrophages, dendritic cells (DCs) and natural killer cells (Tornwall, Carey et al. 1999; Curran, Berghaus et al. 2001; Benten, Stephan et al. 2002; Phiel, Henderson et al. 2005) and enhance differentiation and antigen presentation by macrophages and DCs (Tornwall, Carey et al. 1999). Elevated levels of estrogen promote polyclonal B cell activation (Weetman, McGregor et al. 1981; Grimaldi, Michael et al. 2001) and increase production of IgG, including IgG anti-dsDNA by lymphocytes from patients with systemic lupus erythematosus (Kanda and Tamaki 1999). In pregnancy, lymphopoiesis is reduced, as demonstrated by a reduction in the number of B cell precursors in the bone marrow. This reduction appears to be the result of elevated estrogen levels, which impair progression from the pro-B cell stage to the early pre-B cell stage (Smithson, Beamer et al. 1994; Medina,

Garrett et al. 2001). Estrogen may affect humoral immunity via a thymic route of action. For example, *in vitro* stimulation of B cells with ovarian steroid hormones enhances antibody secretion only in the presence of CD8⁺ T cells (Lu, Abel et al. 2002).

Estrogen has modulatory effects on both T_H1 and T_H2 type cells. Estrogen is shown to inhibit the production of T_H1 pro-inflammatory cytokines, such as IL-12, TNF- α and IFN- γ , whereas it enhances the production of T_H2 anti-inflammatory cytokines, such as IL-10, IL-4, and TGF- β (Giltay, Fonk et al. 2000; Harris, Feldberg et al. 2000; Angele, Knoferl et al. 2001; Karpuzoglu-Sahin, Zhi-Jun et al. 2001; Kovacs, Duffner et al. 2004; Salem 2004; Gourdy, Araujo et al. 2005). These modulatory effects may partially explain why estrogen suppresses T_H1-mediated autoimmune diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis and multiple sclerosis (Josefsson and Tarkowski 1997; Bebo, Schuster et al. 1999; Ostensen 1999; Matejuk, Adlard et al. 2001; Voskuhl and Palaszynski 2001; Matejuk, Dwyer et al. 2002; Fu, Jiang et al. 2005; Janssen, Tabeta et al. 2006), and exacerbates T_H2-mediated diseases, such as systemic lupus erythematosus and Sjorgen's syndrome (Roubinian, Talal et al. 1978; Ralston, Russell et al. 1990; Buyon 1998; Ostensen 1999; Bynoe, Grimaldi et al. 2000; Elenkov, Wilder et al. 2001).

Further evidence of estrogen-mediated effects on T_H1/T_H2 cells is provided by results from studies carried out with pregnancy and menopause models. During the luteal phase of the ovarian cycle, when estrogen levels peak, the immune response shifts to favor a T_H2-type response, as reflected by increased IL-4 and TGF- β levels (Faas, Bouman et al. 2000; Ayatollahi, Geramizadeh et al. 2007). Pregnancy is considered to be a T_H2-type phenomenon, which might

play a critical role in maternal acceptance of the fetus (Lin, Mosmann et al. 1993; Wegmann, Lin et al. 1993; Piccinni, Scaletti et al. 2000; Laird, Tuckerman et al. 2003). Pregnancy results in suppression of T_H1-type responses, which may partially explain the spontaneous remission and post-partum relapse of T-cell-mediated autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, observed in women (Fu, Jiang et al. 2005). In menopausal women, there is an increase of T_H1 cytokines relative to T_H2 cytokines. Hormone replacement therapy prevents the T_H1 cytokines increase, thus restoring the T_H1/ T_H2 balance (Kamada, Irahara et al. 2001).

Due to the well-established role that estrogen plays in modulating the immune system, it is reasonable to postulate that estrogen influences the immune response to vaccines. To test this hypothesis, we have compared the murine antibody responses produced in presence or absence of estrogen against the currently available TT vaccine.

MATERIALS & METHODS

Animals

For the entire study, female BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA) were used. The study consisted of two experimental protocols. In the first protocol, animals received E2 as pellet implantation (pellet-E2 replacement). In the second protocol, animals received E2 in the drinking water (oral-E2 replacement). For both experimental protocols, mice were divided into 3 groups. The first group of mice, designated OVEX-E2 group, was ovariectomized and subjected to estrogen replacement. The second group, designated OVEX group, received no estrogen replacement. The third group remained intact. Table 3.1 and 3.2 show the experimental designs for both protocols. Mice were ovariectomized at 8 weeks of age.

Designated mice were anesthetized with 5% isoflurane gas in 100% oxygen, and their ovaries, oviducts and tips of the uterine horns were bilaterally removed via two dorsal incisions. Immediately following surgery, mice in the OVEX-E2 group started E2 replacement. Seven days following surgery, mice were given the first vaccine injection. Blood was collected from each mouse prior to and following hormone manipulation and immunization. To test for uterotrophic responses, uteri were removed and weighed at termination. All animals were housed and subjected to experimental procedures in accordance to protocols approved by the appropriate institutional review committee.

Pellet-E2 replacement and immunization

Sterile E2 pellets were inserted during ovariectomy through a subcutaneous tunnel made between the scapulae. Mice were given a single (5 mm) subscapular Silastic implant (1.02 mm ID×2.16 mm OD) containing either E2 diluted 1:1 with cholesterol (Sigma-Aldrich Corp., St. Louis, MO) or cholesterol only. This pellet size yields E2 concentrations of 85–100 pg/ml, similar to that of intact mice in pro-estrus (Wersinger, Haisenleder et al. 1999). Mice were immunized with a primary (day 0) and secondary (day 28) dose of human TT vaccine, alum adsorbed (Sanofi-Aventis, Bridgewater, NJ). The OVEX and control groups received a third dose at day 125. All injections were given subcutaneously on the dorsal side of the mice. They received 0.05 ml, equal to one tenth the human immunizing dose, given in 0.2 ml saline (0.15 M NaCl), for a total dose volume of 0.250 ml. The immunization and blood collection schedule is shown schematically in Figure 3.1 A.

Table 3.1. Pellet- E2 Study: Experimental Design

Hormone group	Treatment	Vaccine group	No. of mice
OVEX	Placebo pellet	TT	11
	Placebo pellet	Unvaccinated	11
OVEX + E2	E2 pellet	TT	11
	E2 pellet	Unvaccinated	11
Sham OVEX (intact)	none	TT	11
	none	Unvaccinated	11

Table 3.2. Oral-E2 Study: Experimental Design

Hormone group	Treatment	Vaccine group	Route of administration	No. of mice
OVEX	none	TT	IM	11
		TT	SC	11
		Unvaccinated	none	10
OVEX + E2	1000 nM E2	TT	IM	11
		TT	SC	11
		Unvaccinated	none	10
Sham OVEX (intact)	none	TT	IM	11
		TT	SC	11
		Unvaccinated	none	10

TT, tetanus toxoid; SC, subcutaneous; IM, intramuscular

Oral-E2 replacement and immunization

E2 was administered in the drinking water. E2-treated water was prepared as previously described by diluting stock solutions of E2 (Sigma- Aldrich Corp., St. Louis, MO) in 100% ethanol to solubilize. Solubilized estrogen was added to drinking water to produce a final concentration of 1000 nM, as previously described (Levin-Allerhand, Sokol et al. 2003; Fernandez and Frick 2004). The final concentration of ethanol was 0.1%. No adverse effects of this concentration of ethanol or estrogen were reported. The concentration administered results in an ingested dose of approximately 50-70 kg/ug per day, corresponding to about 15 pg/m (Levin-Allerhand, Sokol et al. 2003; Fernandez and Frick 2004). Estrogen levels during the estrus cycle in mice typically range from 10-40 pg/ml (Nelson, Karelus et al. 1995). A 1000 nM dose in the drinking water was previously shown to result in uterine weights that were significantly higher than those of control mice, yet not significantly different from those of mice given either 1500 nM or 2500 nM doses, implying a uterotrophic response that plateaus with increasing E2 doses (Fernandez and Frick 2004). Mice were immunized with a primary (day 0), secondary (day 28) and tertiary (day 84) dose of human TT vaccine. Similarly to animals in the pellet-E2 protocols, mice receiving subcutaneous injections were given 0.05 ml in 0.2 ml saline (0.15 M NaCl), for a total dose volume of 0.250 ml, injected on the dorsal side of the mouse. In mice receiving intramuscular injections, 0.025 ml was injected into each calf muscle for a total dose volume of 0.05 ml. The doses administered are equal to one tenth the human immunizing dose. The immunization and blood collection schedule is shown schematically in Figure 3.1 B.

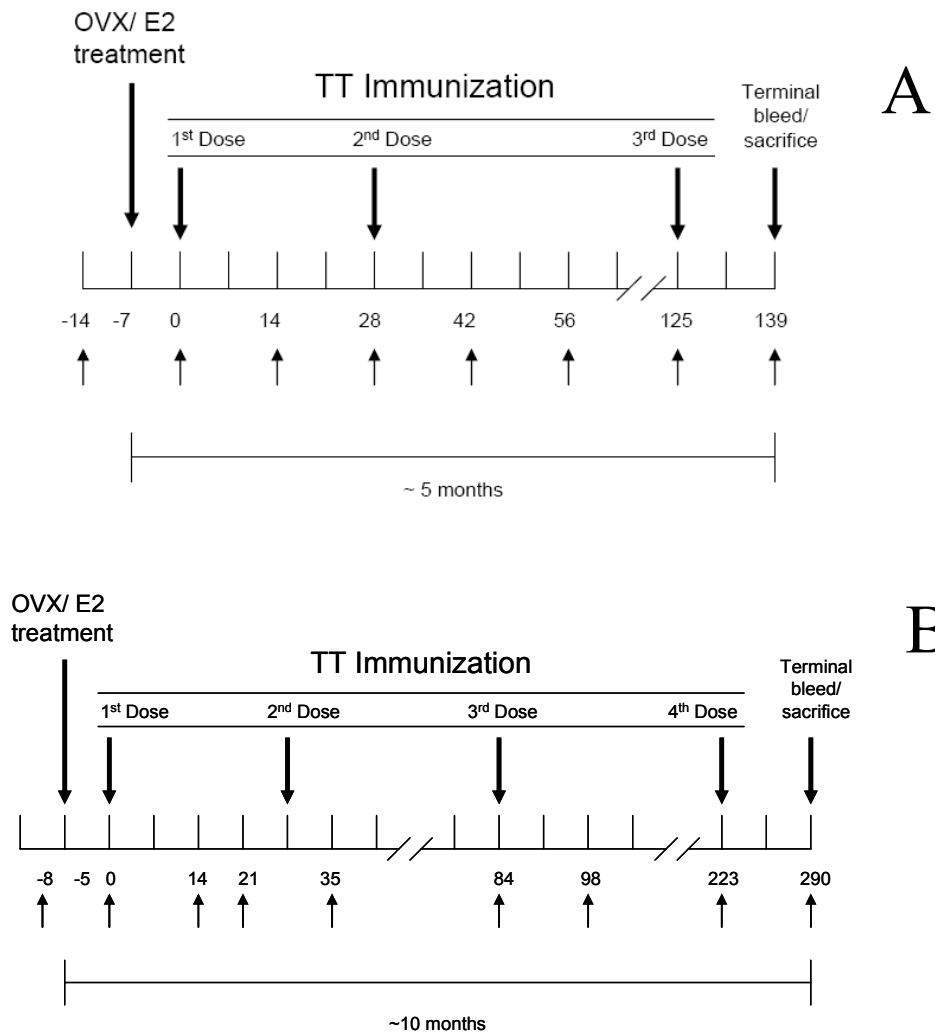


Figure 3.1. E2-TT Study: Hormone treatment and vaccination schedule.

Mice were ovariectomized and mice designated to receive E2 replacement were **A**, implanted with pellets containing E2 or **B**, given E2 orally through the drinking water. Mice were vaccinated on Day 0 with TT. Booster doses are indicated by large arrows. On the days of sacrifice, uteri were removed and weights measured to confirm uterotrophic activity. The small arrows indicate days of blood collection for determination of CFP-specific antibody responses.

Detection of anti-TT toxoid antibody responses

Anti-TT antibody responses were assessed by ELISA. Micro-titer wells were coated with purified TT antigen (Sanofi-Aventis) diluted in coating buffer (KPL, Inc., Gaithersburg, MD). Negative control wells were coated with purified baculovirus-recombinant glycoprotein I from herpes B virus (kindly provided by Dr. Ludmilla Perelygina). To block nonspecific binding sites, wells were incubated for 30 min at 37°C with PBS containing 5% fetal calf serum (FCS-PBS, Atlanta Biologicals, Lawrenceville, GA). Serum samples were diluted in two-fold serial dilutions (1:50-1:25600), added to triplicate wells, and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween-20, wells were incubated with either HRP-labeled goat anti-mouse IgM (KPL) or HRP-labeled goat anti-mouse IgG (KPL) for 1 hr and 15 min at 37°C. Wells were washed and then incubated with ABTS/H₂O₂ (KPL) substrate for 10 min at room temperature, followed by addition of stop solution (KPL). Optical density (OD) values were assessed at 405 nm using a PowerWave HT plate reader (BioTek Instruments, Inc., Winooski, VT). Endpoint titers were defined as the highest serum dilution that yielded an OD value greater than or equal to 3 times the mean values of the same serum samples tested against the negative control coat protein at a 1:50 dilution.

Determination of total immunoglobulin production

Total immunoglobulin (Ig) production was assessed by ELISA according to methods similar to those described above, with the following exceptions. Micro-titer plates were coated with unlabeled goat anti-mouse IgG+IgA+IgM (KPL). Individual serum samples were added to triplicate wells. Plates were incubated overnight at 4 °C, washed and incubated with HRP-labeled goat anti-mouse IgG+IgA+IgM (KPL) for 1 h at 37 °C.

Determination of IgG1/IgG2a responses

To determine the effect of estrogen on IgG1/IgG2a production, ELISA was used according to methods similar to those described above, with the following exceptions. Briefly, plates were coated with purified TT antigen (Sanofi-Aventis). Individual serum samples were diluted at 1:100 and added to triplicate wells. Plates were incubated overnight at 4 °C, washed and incubated with HRP-labeled goat anti-mouse IgG1 or HRP-labeled goat anti-mouse IgG2a (KPL) for 1 h at 37 °C.

Determination of natural autoantibody (NAA) production

ELISAs were done to assess NAA levels in mice according to methods described above. Briefly, plates were coated with 4 ug/ml calf thymus extract (CTE) (Immunovision). Individual and pooled serum samples were diluted at 1:200 and added to triplicate wells. Plates were incubated overnight at 4 °C. Wells were incubated with HRP-labeled goat anti-mouse IgG+A+M (KPL) for 1 h at 37 °C.

Statistical analysis

The SPSS program (SPSS Inc, Chicago, Illinois) version 12 was used for statistical analysis. Independent t-test was used to analyze group differences by immunization route. Analysis among multi-group data was carried out using ANOVA, followed by Turkey's significant difference test, or Tamhane's T2 test when the test of homogeneity of variances (Levene Statistic) indicated that the variance between the groups was not equal. Differences among groups were considered significant if $p < 0.05$.

RESULTS

As mentioned above, we used two different experimental protocols for this study. In the first protocol, mice received pellet-E2 replacement, whereas in the second protocol animals received oral-E2 replacement. Continuous release hormone pellets have been used and described extensively in the literature. In the present study, we used silastic pellets containing amounts of E2 previously shown to result in physiological levels of plasma E2 in balb/c mice (Wersinger, Haisenleder et al. 1999). However, we observed that within the third week of our study, mice receiving pellet-E2 delivery began to develop reddening and swelling of the vulva and perineum, eventually developing urinary retention and subsequent dribbling which resulted in scalding around the genital area. Therefore, OVEX-E2 mice were sacrificed at day 42. Continuous release of the E2 may not be suitable for chronic treatment. Indeed, urinary retention leading to subsequent death is documented in ovariectomized mice receiving chronic, subcutaneous estrogen replacement, which is not documented in hamsters or rats (Levin-Allerhand, Sokol et al. 2003).

In the second experimental protocol, we used oral administration through the drinking water as method of delivery. This method of E2 replacement was chosen for various reasons. First of all, it is safer because it results in uterotrophic responses, yet does not cause urine retention as seen with continuous release E2 capsules (Gordon, Osterburg et al. 1986; Levin-Allerhand, Sokol et al. 2003). Therefore, it is a more appropriate method for long-term delivery, as needed for measuring vaccine responses. Second, oral E2 delivery has been demonstrated to induce physiological responses in three different target tissues, including the vagina, uterus and pituitary

(Gordon, Osterburg et al. 1986). Finally, as a result of circadian patterns in drinking behavior, oral E2 administration does not produce chronically elevated levels of circulating E2. Rather, elevations occur during peak nocturnal drinking periods, and levels return to baseline approximately 15 hours later (Gordon, Osterburg et al. 1986). Therefore, this method results in cyclic estrogen levels that mimic natural E2 fluctuations during the estrus cycle.

Uterine weight

Uterine weights were measured and compared between the groups to determine the uterotrophic response to E2 delivery. As expected, in the pellet-E2 delivery group, OVEX-E2 mice had uterine weights approximately 3.1 and 1.3 times higher than OVEX and intact mice respectively. The uterine weights of OVEX mice were 2.4 times lower than those of the intact mice ($p < 0.001$) (Table 3.2). Similarly, in the oral E2-delivery group, OVEX-E2 mice had uterine weights approximately 2.5 and 1.4 times higher than the OVEX and intact mice, respectively ($p < 0.001$). The uterine weights of OVEX mice were 1.7 times lower than those of the intact mice ($p < 0.001$).

Pellet-E2 Experimental Protocol

Anti-TT antibody responses

To test the hypothesis that estrogen influences the antibody response to vaccines, groups of mice were ovariectomized, subjected to estrogen replacement and immunized with the TT vaccine. The IgM and IgG or IgG+A+M anti-TT responses produced by these groups of mice (OVEX-E2) were compared to the same response generated in OVEX mice and in control mice. OVEX-

E2 mice received a total of two immunizations (day 0 and 28), whereas OVEX and control mice received a total of three immunizations (day 0, 28 and 125). Serum samples obtained from all mice on day 42 were tested for IgM, IgG and IgG+A+M anti-TT responses by ELISA. Serum samples obtained from the OVEX and control groups on day 139 were tested for anti-TT responses. At day 42, the IgM anti-TT responses did not differ significantly between the OVEX-E2 and the other two groups of mice (data not shown), whereas OVEX-E2 mice produced significantly higher IgG and IgG+A+M TT-specific antibody levels than OVEX and control mice ($p > 0.05$) (Figure 3.2 and Figure 3.3). Antibody responses did not differ significantly between the OVEX and control groups. Even after receiving a third dose of TT vaccine on day 125, OVEX and control mice showed no significant differences in long-term specific IgG antibody response ($p > 0.05$) (Figure 3.4). Sera from OVEX-E2 mice were not available for comparison due to termination of this group after the second immunization dose. All samples were also tested against herpes B virus glycoprotein I as a negative control, which resulted in IgG OD values that were below 0.1. These were used to generate endpoint titers for the same data.

Anti-TT IgG1 antibody responses

To assess whether or not E2 treatment influenced expression of the IgG1 subclass, which is known to be the predominant subclass produced by mice in response to TT vaccination (Gupta and Siber 1994), TT-specific IgG1 and IgG2a responses were determined. Figure 3.5 shows that, according to what observed for overall IgG responses, OVEX-E2 mice produced the highest levels of TT-specific IgG1 following vaccination ($p < 0.01$), thus confirming that E2 replacement

Table 3.3. Uterotrophic responses in OVEX-E2, OVEX and intact mice.

Mice were sacrificed at day 227 and uteri were removed and weighed. Weights represent mean values of 32 mice \pm SD (OVEX-E2 and OVEX) or 30 mice \pm SD (intact). ¹significant difference in weight from intact and OVEX-E2 mice ($p < 0.001$). ²Significant difference in weight from OVEX and OVEX-E2 mice ($p < 0.001$).

Hormone group	Uterine weight (g)	
	Pellet E2 Delivery	Oral E2 Delivery
OVEX	0.044 \pm 0.03 ¹	0.055 \pm 0.02 ¹
INTACT	0.105 \pm 0.08 ²	0.096 \pm 0.06 ²
OVEX-E2	0.138 \pm 0.02	0.136 \pm 0.04

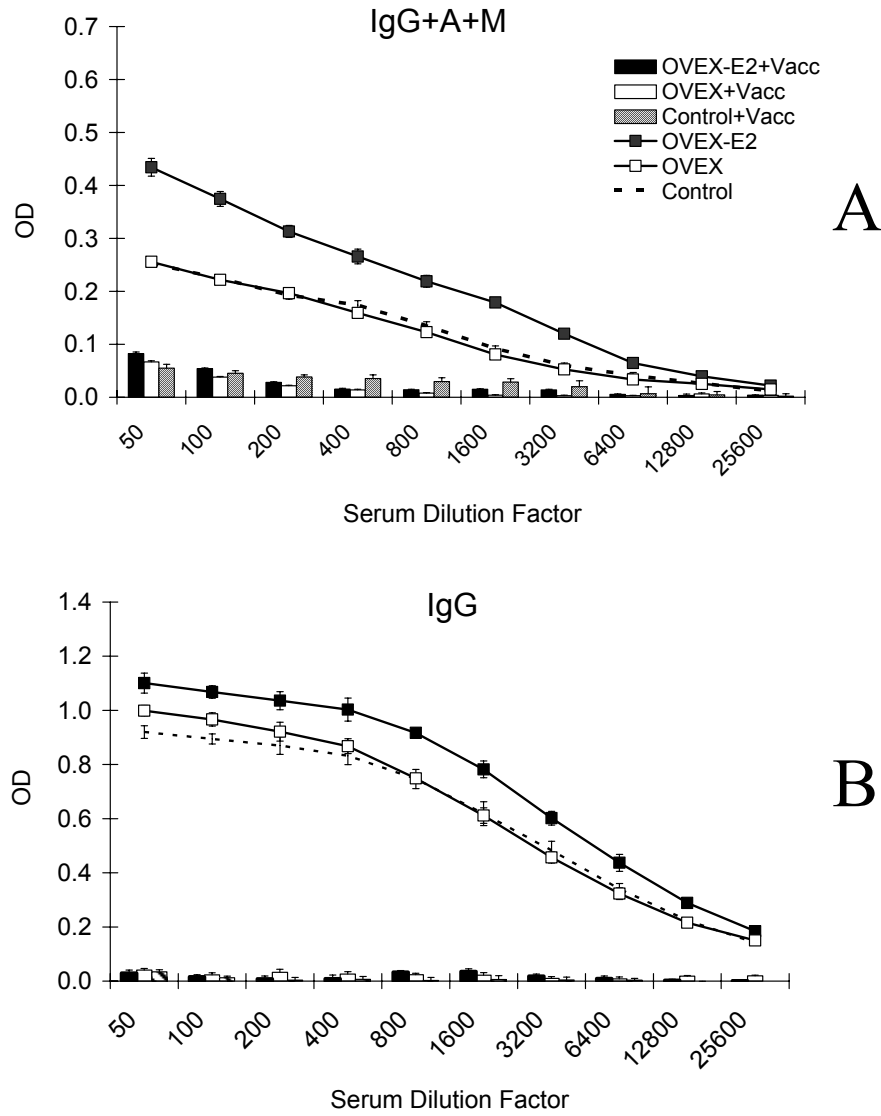


Figure 3.2. The effect of estrogen on TT-specific IgG and IgG+A+M antibody responses.

A, IgG+A+M and **B**, IgG (γ) responses against tetanus toxoid vaccination in mice 14 days after the second immunization (day 42). Binding curves represent 2-fold serial dilutions of sera. Curves represent mean values obtained from 11 mice per group. Curves represent data from the vaccinated groups, bars represent data from the unvaccinated groups to show background binding.

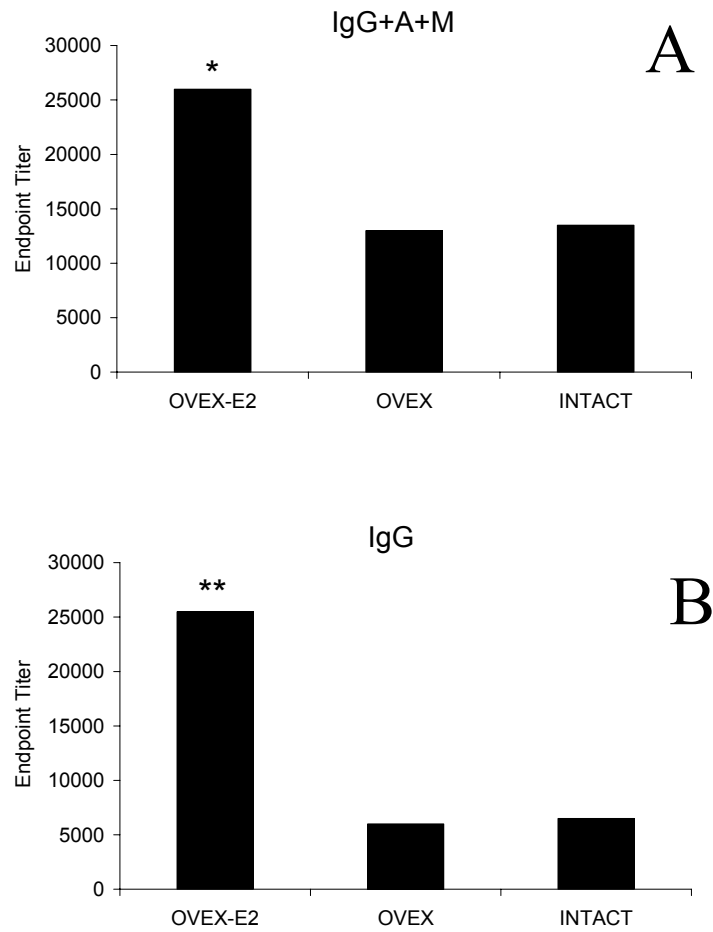


Figure 3.3. Anti-TT IgG and IgG+A+M titers in OVEX-E2, OVEX and intact mice.

A, IgG+A+M and **B**, IgG (γ) responses against tetanus toxoid vaccination in mice 14 days after the second immunization (day 42). Endpoint titers were calculated as 3 times the mean of the average OD of the negative control at a 1:50 dilution. Data shown represents the mean titers of 11 mice per group. * $p < 0.05$, ** $p < 0.01$.

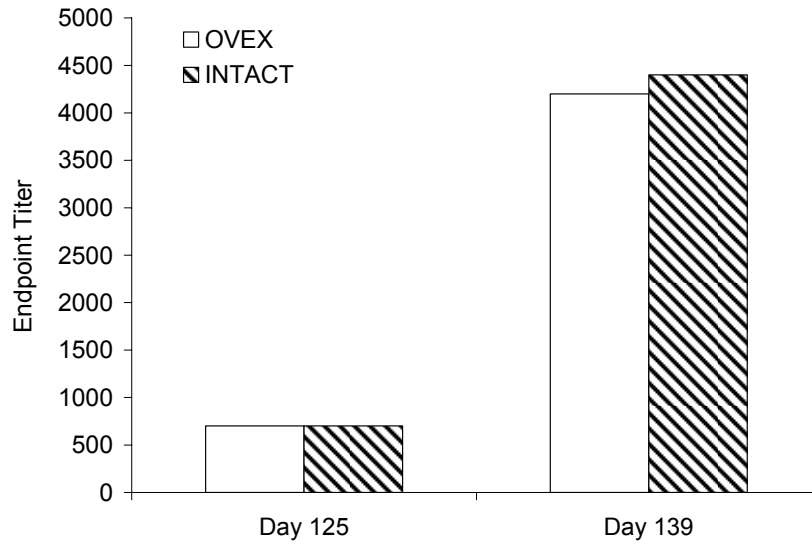


Figure 3.4. Longterm antibody responses.

Sera from OVEX and intact mice were tested for anti-TT IgG (γ) the day of (day 125) and 14 days after (day 139) the third immunization was administered to determine longterm responses. Endpoint titers were calculated as 3 times the mean of the average OD of the negative control at a 1:50 dilution. Data shown represents the mean titers of 11 mice per group \pm SEM.

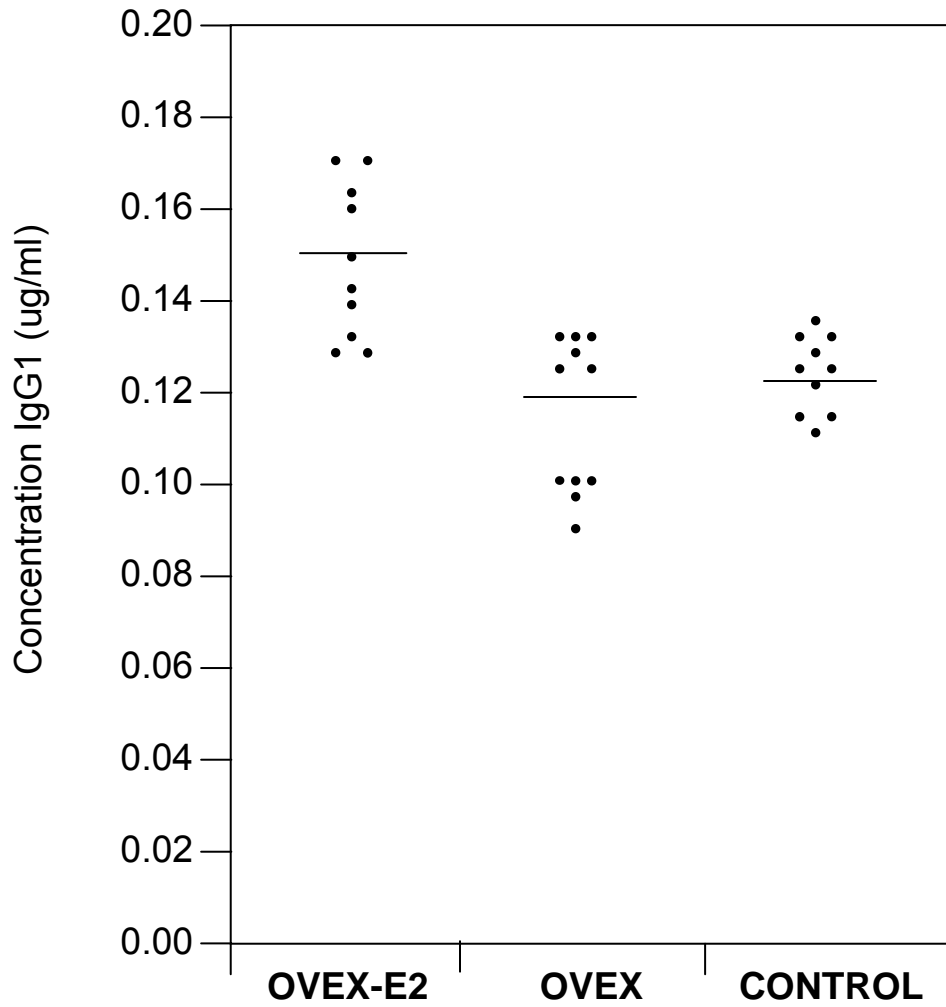


Figure 3.5. The effect of estrogen on TT-specific IgG1 production.

Individual mouse samples at day 42 were tested at 1:200 dilution. The concentration of IgG1 in serum was determined against a standard curve using purified mouse myeloma IgG1. OVEX-E2 mice have higher levels of anti-TT IgG1 compared to OVEX and intact mice ($p < 0.01$).

leads to production of higher TT-specific responses. TT-specific IgG1 levels did not differ between OVEX and intact mice, indicating the presence of a stronger Th2 response in the OVEX-E2 mice. TT-specific IgG2a was not detected in any of the mice (data not shown).

Oral-E2 Experimental Protocol

Anti-TT IgG responses

To test the hypothesis that estrogen influences the antibody response to vaccines, groups of mice were ovariectomized, subjected to estrogen replacement and immunized with the TT vaccine. The anti-TT response produced by this group of mice (OVEX-E2) was compared to the same response generated in OVEX and in intact mice. All mice received a total of three immunizations (day 0, 21 and 84). Serum samples obtained from all mice at day 0 as well as 14 days after the second and third immunizations (days 35 and 98, respectively) were tested for anti-TT IgG responses by ELISA. At day 35, OVEX-E2 and intact mice produced significantly higher anti-TT IgG levels than OVEX mice ($p < 0.05$) when vaccinated SC (Figure 3.6 and Figure 3.7). OVEX-E2 mice produced significantly higher anti-TT IgG levels than OVEX and intact mice when vaccinated IM ($p < 0.01$) (Figure 3.6 A). At day 98, OVEX-E2 produced significantly higher anti-TT IgG levels than the OVEX and intact mice when vaccinated either IM or SC ($p < 0.05$) (Figures 3.6 B and Figure 3.7). For both day 35 and 98, mice vaccinated IM produced significantly higher anti-TT IgG levels than mice vaccinated SC ($p < 0.05$). All samples were also tested against herpes B virus glycoprotein I as a negative control, which resulted in IgG OD values that were below 0.1. These were used to generate endpoint titer values for the same data,

shown in Figure 3.7. Unvaccinated animals showed similar low OD values compared with vaccinated animals (Figure 3.6).

Total Ig and IgG1/IgG2a responses

As already mentioned, it is known that estrogen may increase polyclonal B cell activation (Kanda and Tamaki 1999). To determine whether or not the increased anti-TT antibody levels detected in the OVEX-E2 mice as compared to the OVEX and intact mice were due to specific anti-TT responses and not just to increased total Ig production, total Ig levels were tested in serum samples from all mice. These levels are not significantly different between groups ($p > 0.05$) (Figure 3.8). These results confirm that OVEX-E2 mice produce higher levels of specific anti-TT antibody responses. To assess whether or not E2 treatment influenced expression of the IgG1 subclass, TT-specific IgG1 and IgG2a responses were determined. Figure 3.9 shows that, according to what observed for overall IgG responses, OVEX-E2 mice produced the highest levels of TT-specific IgG1 following vaccination ($p < 0.05$), thus confirming that E2 replacement leads to production of higher TT-specific responses. This effect was consistent whether mice were vaccinated SC or IM. TT-specific IgG1 Levels did not differ between OVEX and intact mice, indicating the presence of a stronger Th2 response in the OVEX-E2 mice. TT-specific IgG2a was not detected in any of the mice (data not shown).

Natural autoantibody (NAA) levels

NAA are found in the serum of healthy individuals in the absence of infection or immunization. Unlike specific, they do not undergo somatic hypermutation. As a result, they possess germline specificity and are polyreactive to conserved structures. Although their function is not clear,

Figure 3.6. The effect of estrogen and route of administration on TT-specific IgG responses (next page).

Sera obtained 14 days after the second immunization (day 35) and 14 days after the third immunization (day 98) were tested for anti-TT IgG. Binding curves and bars represent 2-fold serial dilutions of sera from TT-vaccinated and unvaccinated mice, respectively. Data shown represents the mean \pm SEM of 11 mice per group. At day 35, OVEX-E2 and intact mice had significantly higher anti-TT IgG levels than OVEX mice when vaccinated SC ($p < 0.05$) (A). OVEX-E2 mice have significantly higher anti-TT IgG levels than the OVEX and intact mice when vaccinated IM ($p < 0.01$) (C). At day 98, OVEX-E2 mice have significantly higher anti-TT IgG levels than the OVEX and intact mice when vaccinated either IM or SC ($p < 0.05$). Mice vaccinated IM had significantly higher anti-TT IgG levels than mice vaccinated SC ($p < 0.05$).

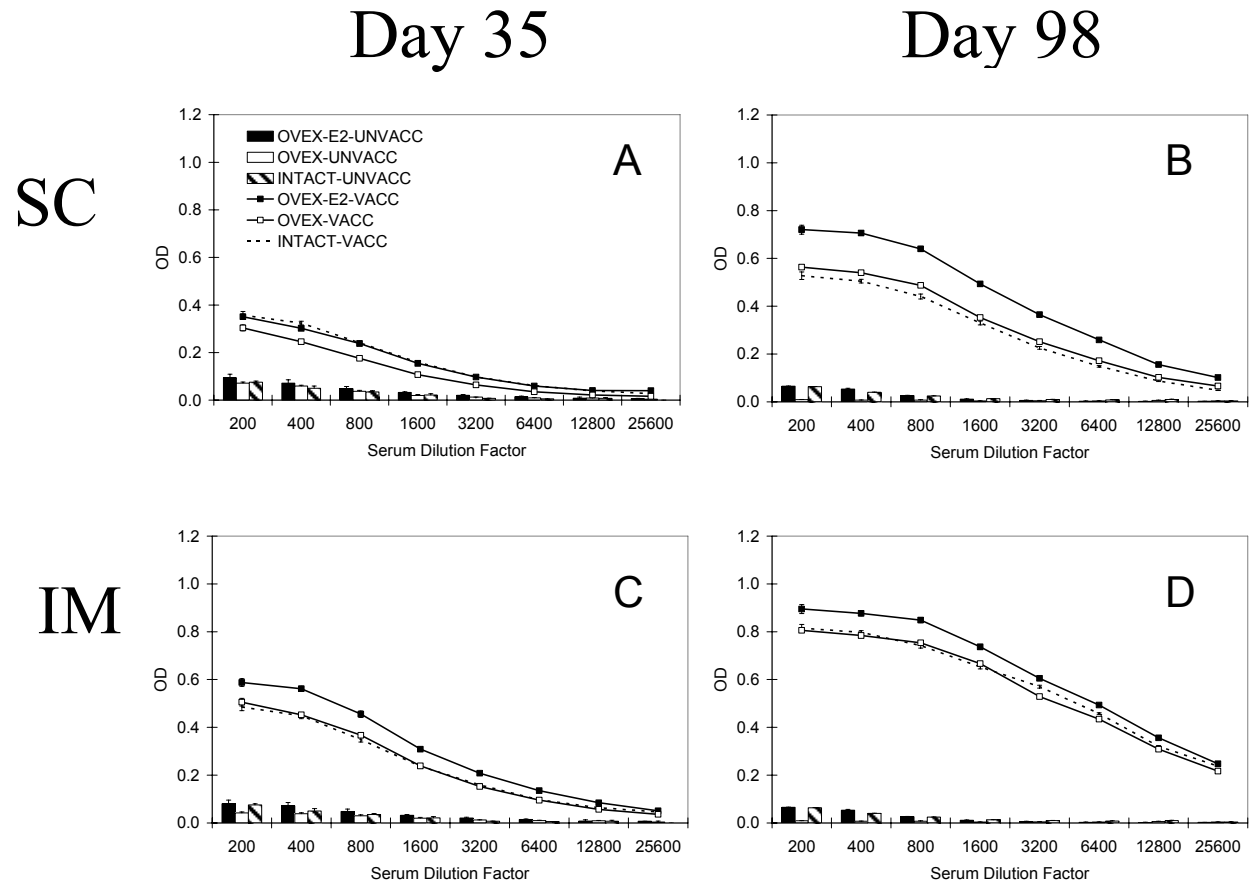


Figure 3.6

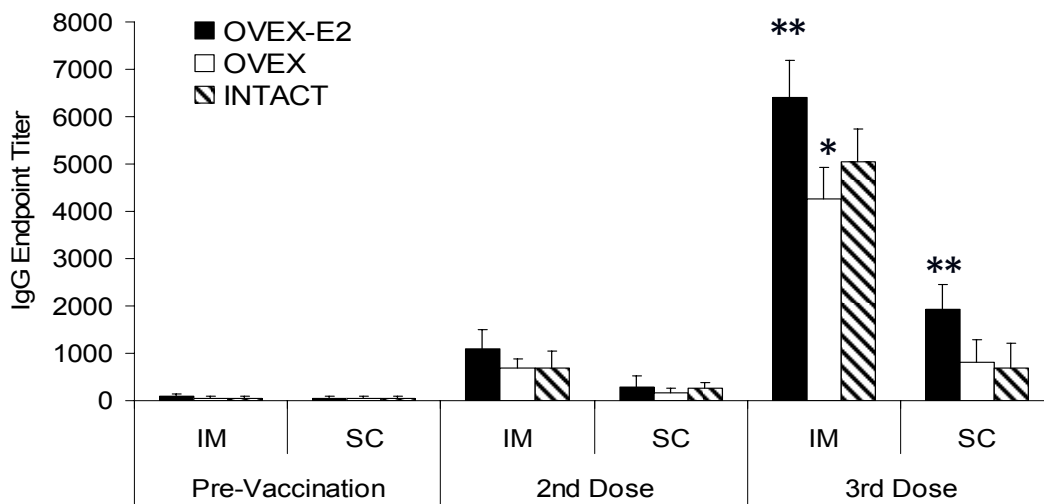


Figure 3.7. Anti-TT IgG titers in OVEX-E2, OVEX and intact mice.

Mice were vaccinated with TT either IM or SC and sera from days 0, 35 (14 days after the second dose) and 98 (14 days after the third dose) were tested for anti-TT IgG (γ). Endpoint titer was defined as 3 times the mean of the average OD of the negative control at 1:50 dilution. Data shown represents the mean \pm SD of 11 mice per group. * $p < 0.05$, ** $p < 0.01$ compared to intact mice.

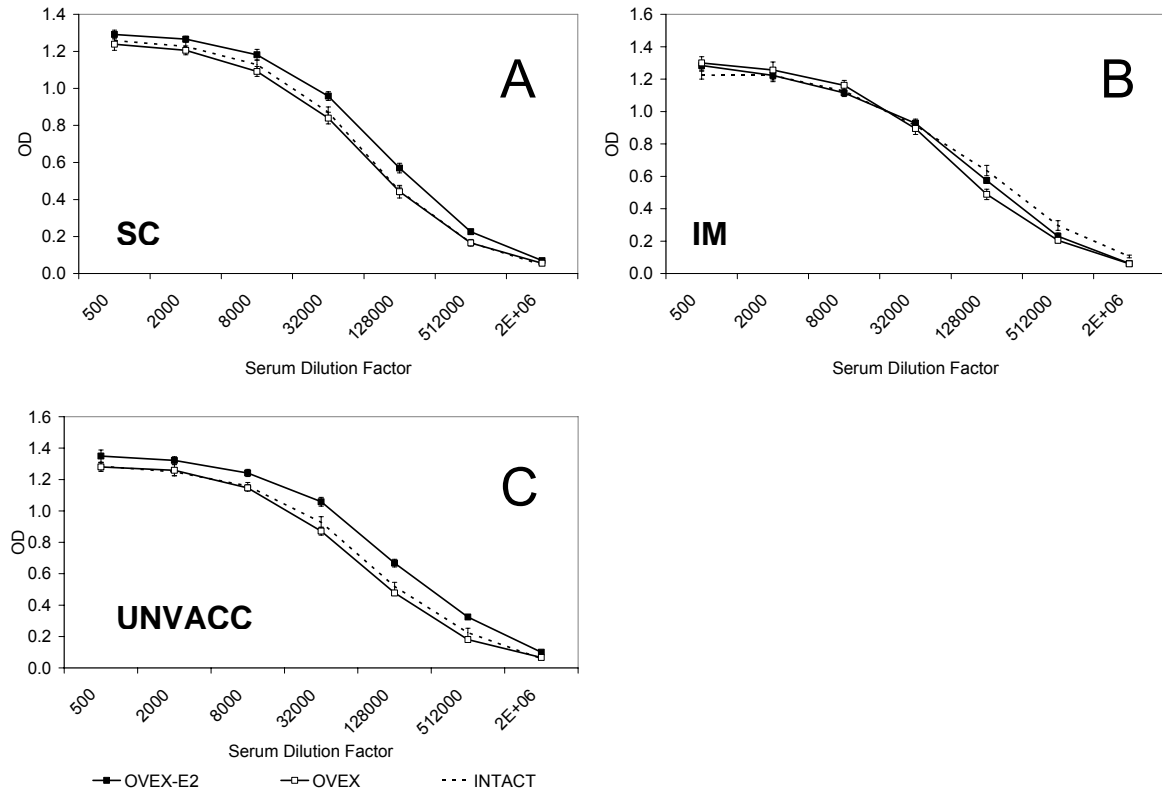


Figure 3.8. The effect of estrogen on total immunoglobulin levels.

Mice were vaccinated with TT by **A**, SC, **B**, IM routes or were **C**, unvaccinated. Individual mouse samples from day 98 were 2-fold serially-diluted to generate titration curves, and tested for levels of serum IgG+A+M by ELISA. Data shown represents the mean \pm SEM of 11 mice per group. There are no significant differences between the 3 groups ($p > 0.05$).

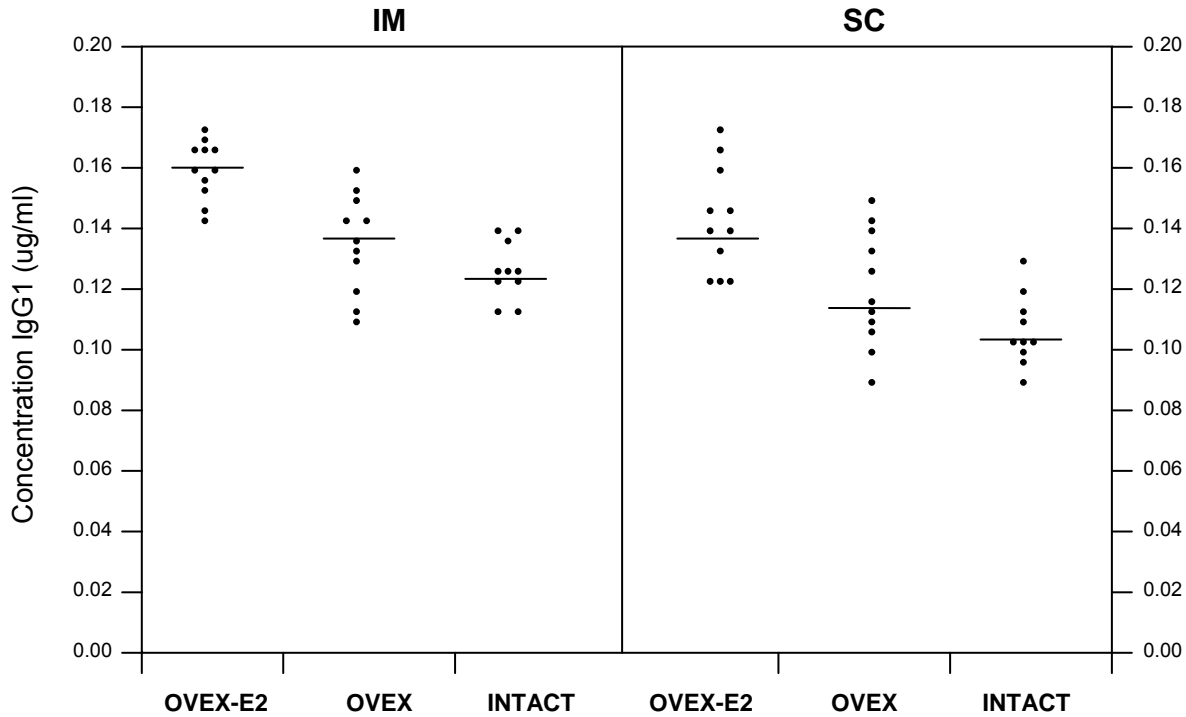


Figure 3.9. *The effect of estrogen on TT-specific IgG1 production.*

Individual mouse samples obtained at day 98 were diluted at 1:200 and tested for production of anti-TT IgG1 by ELISA. The concentration of IgG1 in serum was determined against a standard curve using purified mouse myeloma IgG1. OVEX-E2 mice have higher levels of anti-TT IgG1 compared to OVEX and intact mice when vaccinated either IM or SC ($p < 0.01$).

NAA constitute a large fraction of serum immunoglobulin. Immuno-adsorption studies show that up to 66% of IgG from healthy individual sera are auto-reactive. One theory is that NAA may play a role in the initial response to infection. To test whether vaccination or estrogen influences NAA levels, sera from day 98 were tested for reactivity against CTE. CTE was used to its high content of phylogenetically conserved structures and because of the nonspecific binding property of the NAAs. E2-treated mice had significantly higher NAA levels as compared to the OVEX and control mice.

To test whether vaccination or estrogen influences NAA levels, sera from day 98 were tested for reactivity against CTE. CTE was used to its high content of phylogenetically conserved structures and because of the nonspecific binding property of the NAAs. E2-treated mice had significantly higher NAA levels as compared to the OVEX and control groups ($p < 0.01$), whereas the OVEX and control groups did not differ (Fig. 3.10). This was consistent irrespective of vaccination. When sera taken prior to OVEX and E2-delivery were tested, there were no significant differences between any of the 3 groups (data not shown). Vaccination alone did not influence NAA levels in any of the three groups.

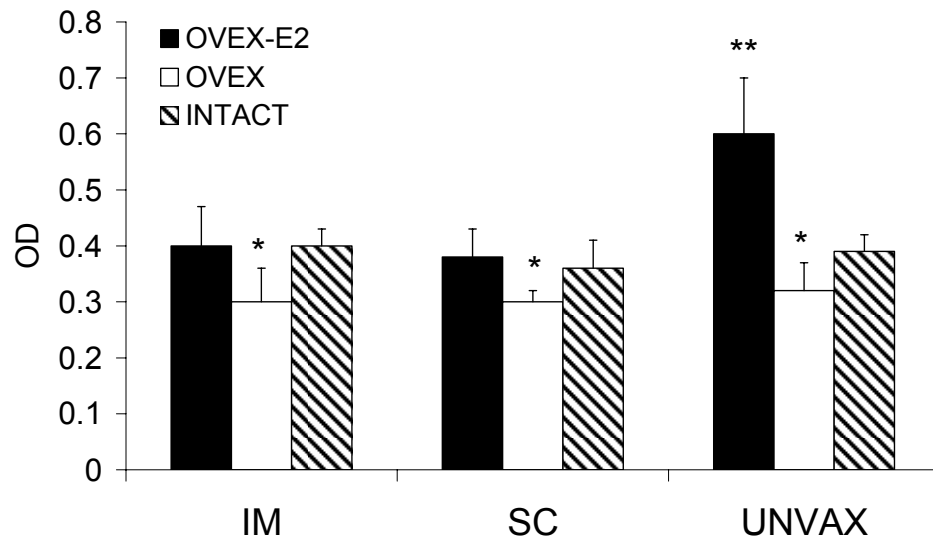


Figure 3.10. *The effect of estrogen and vaccination on natural autoantibody levels.*

Sera from TT-vaccinated and unvaccinated OVEX-E2, OVEX and intact mice at day 98 were diluted at 1:200 and tested for levels of IgG+A+M against 4 ug/ml CTE. Bars represent mean \pm SD of 11 mice per group. * $p < 0.05$, ** $p < 0.01$ compared to intact mice.

DISCUSSION

Results from several studies indicate that estrogen influences the immune response generated against a variety of immunogens. However, no information is available on the effects of estrogens on the immune response to specific vaccine preparations currently used in human populations. Therefore, the present study was carried out to assess whether or not estrogen may affect the antibody response to one of the currently available vaccines, i.e., the TT vaccine. TT represents one component of combination vaccines that include other components, i.e., tetanus toxoid, reduced diphtheria toxoid, acellular pertussis vaccine (Tdap); tetanus toxoid and diphtheria toxoids vaccine (Td); and diphtheria toxoid, tetanus toxoid and acellular pertussis vaccine (DTaP). These vaccines are recommended and routinely administered to humans for either childhood or adolescent and adult immunization. Our results show that E2 replacement results in the generation of higher anti-TT IgG responses for both routes of administration, SC and IM. However, for all groups, IM-vaccinated mice produced overall higher anti-TT IgG levels than did SC-vaccinated mice. In humans, these two routes of vaccine administration do not appear to result in different immunogenicity profiles (Mark, Carlsson et al. 1999; Ruben, Froeschle et al. 2001).

We used a uterine weight bioassay to confirm the efficacy of hormone treatment due to its well-described high sensitivity to low levels of estrogen treatment (Mobbs, Cheyney et al. 1985). Results obtained by measurement of uterine weight confirmed the efficacy of hormone replacement. The finding that no significant differences were detected in anti-TT antibody responses between OVEX and intact mice after a third immunization dose, indicates that

endogenously cycling estrogen levels may not ultimately be sufficient to enhance humoral responses to TT vaccination.

Consistent with the finding of increased anti-TT IgG production in the OVEX-E2 mice, significantly increased levels of anti-TT IgG1 were observed in the OVEX-E2 mice as compared to OVEX and intact mice. IgG2a and IgG1 are often used as indicators for the induction of murine Th1 and Th2 responses, respectively, and the IgG1/IgG2a ratio can help to define the T-cell phenotype induced by vaccination (Coffman, Seymour et al. 1988). Indeed, bacterial protein antigens administered in alum adjuvant, such as TT, are known to induce mostly IgG1 responses (van der Giessen and Groenboer-Kempers 1976; Stevens, Dichek et al. 1983; Seppala and Makela 1984; Rubin, Tang et al. 1986; Watt, Zardis et al. 1986; Grun and Maurer 1989; Comoy, Capron et al. 1997; Cox 1997). Production of Th1 cytokines is augmented in women after menopause. HRT prevents this increase, thereby restoring the Th1/Th2 balance that is implicated in inadequate immune responses and pathological conditions (Kamada, Irahara et al. 2001). However, in mice, E2 treatment results in increased IFN-gamma and decreased IL-4 production from *in vitro* antigen stimulated CD4⁺ T cells (Maret, Coudert et al. 2003). These differences might be explained on the basis of a biphasic effect of E2, resulting in enhanced IFN-gamma production at low E2 concentrations, but enhanced IL-10 production at higher E2 concentrations (Gilmore, Weiner et al. 1997; Correale, Arias et al. 1998; Beagley and Gockel 2003). Indeed, the dose of E2 replacement used in our study results in E2 levels that are significantly higher than in the OVEX and intact mice, as demonstrated by significantly increased uterine weights.

Several recent studies have examined immune responses in different compartments of female reproductive tract in response to immunization. Results from these studies show that E2 either alone or in combination with progesterone not only influence immune responses in the female reproductive tract, but also regulate susceptibility to infections (Crowley, Horner et al. 1997; Martin, Nyange et al. 1998; Sonnex 1998; Kaushic, Zhou et al. 2000; Gillgrass, Fernandez et al. 2005; Gillgrass, Tang et al. 2005).

Little information exists regarding the role that estrogen plays in the systemic immune responses. Results from earlier studies indicates that immunization with various T-dependent and T-independent antigens, such as horse or goat erythrocytes and polyvinylpyrrolidone, respectively, results in enhanced IgG and IgM titers in female mice as compared to male mice (Eidinger and Garrett 1972). Others have demonstrated that females have stronger and longer lasting humoral responses to bovine serum albumin compared to males (Terres, Morrison et al. 1968). Given the hormonal differences between males and females, these observations are consistent with the hypothesis that physiological levels of E2 enhance humoral immune responses to antigens. Indeed, results from additional earlier studies show that female rats produce higher anti-fluorescein antibody titers than male rats and that, in OVEX rats, primary and secondary anti-fluorescein antibody responses are significantly reduced compared to sham OVEX controls (Trawick and Bahr 1986; Erbach and Bahr 1991). Results from our study are in agreement with the majority of these earlier findings, with the possible exception of the latter mentioned study. Indeed, our results show no significant longterm differences between OVEX and intact mice at day 98, with increased responses present only in the OVEX-E2 mice. Differences in animal models (rats versus mice) and immunogen used may explain this discrepancy.

Clearly, additional studies are required to assess the influence of type, dose of antigen, adjuvant, and additional routes of administration on the immune response elicited in presence or absence of estrogen. Similarly, additional studies should include different doses of estrogens and different times of estrogen treatment. In conclusion, the results from our preliminary study show that estrogen may enhance the antibody response to a commonly used vaccine and suggests intriguing possibilities regarding the potential use of sex hormones as immunomodulators of the immune response to specific vaccines. In addition, our results emphasize the importance of taking into account the effects of estrogens in evaluating vaccine efficacy, especially in menopausal women (subjected or not to hormone replacement therapy) which represent a major subset of elderly individuals, a population known to exhibit a decreased response to vaccine administration. The future understanding of the specific mechanisms at the basis of the estrogen-induced modulation of the immune response might lead to the design of novel prevention strategies based on the use of vaccines.

CHAPTER 4

Estrogen-Induced Modulation of Murine Macrophage Genes Involved in Innate and Adaptive Immune Responses

SUMMARY

Macrophages play important roles in the immune response, as they are capable of not only phagocytosis and destruction of foreign cells and debris but also acting as antigen presenting cells. Thus they stand at the crossroads of innate and adaptive immunity. The key mechanisms by which macrophages exert their effects depend on the expression and function of different types of receptors. Toll-like receptors (TLRs) detect and bind directly to pathogenic ligands in the microenvironment and are critical in the first line of defense against infection. Antibody-Fc gamma receptors bind to antibodies-antigen complexes and are therefore involved in activation of the adaptive immune response. We have examined the effects of estrogen on TLR2 and Fc γ R gene expression from primary splenic and thioglycollate-elicited macrophages isolated from female mice either ovariectomized only or ovariectomized and given estrogen (E2) replacement and intact mice. We found that TLR2, Fc γ RI and Fc γ RIII but not Fc γ RIIB gene expression is greatly down-regulated in estrogen-replaced mice. When we compared intact female mice to male mice, we found a clear gender dimorphism in expression of these genes. When immunized with *M. tuberculosis* culture filtrate proteins containing TLR-inducing LAM, E2-replacement enhanced specific antibody responses. On the other hand, E2-replacement down-regulated gene expression while ovariectomy up-regulated expression. TLR and Fc γ R are co-expressed on macrophages, where co-ligation leads to increased pro-inflammatory cytokine release. Since

FcγRIIB gene expression was not affected by estrogen–replacement, this indicates that estrogen enhances macrophage genes encoding receptors involved in activation of the immune system, but not receptors involved in inhibition of the immune system. Altogether, our results suggest that gender differences as well as varying levels of estrogen in females may differentially influence immune function at the level of B cells and macrophages and that these differences should be considered when designing vaccine strategies for different target populations subject to endogenous or exogenous estrogen fluctuations.

INTRODUCTION

Macrophages play a significant role in host defense against infection. In innate immunity, macrophages are phagocytic cells involved in the activation of the inflammatory response. In adaptive immunity, macrophages are professional antigen-presenting cells which allow antigen recognition by T lymphocytes resulting in the activation and specific effector functions that lead to maximum antigen clearance. The critical functions of macrophages are mediated by various types of receptors, whose expression and function contribute to pathogen clearance as well as activation of adaptive responses necessary for long-term immunity. Macrophages express toll like receptors (TLRs), which are considered to be the principal membrane signaling molecules through which mammals sense infection. The innate immune response to bacterial infection is triggered when TLRs located on the cell surface recognize bacterial components, as for example lipopolysaccharide (LPS) and peptidoglycan (PGN), the major constituents of gram-negative and gram-positive bacterial cell walls, respectively. Ligation of TLRs leads to signaling mediated by the adaptor protein MyD88 and subsequent NF-κB activation, ultimately resulting in expression

of co-stimulatory molecules as well as pro-inflammatory cytokines including IL-6, IL-12 and TNF- α , which are important in activation of B and T cells and differentiation of T helper subsets.

Macrophages also express opsonic phagocytic receptors, including receptors for the Fc portion of immunoglobulin G (IgG), the Fc γ receptors (Fc γ R), which allow effector cells to recognize and bind immune IgG complexes rapidly and efficiently. Fc γ R cross-linking results in internalization of immune complexes by phagocytosis/endocytosis, antigen presentation, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators. Thus, the functions of TLRs and Fc γ R are the most critical for macrophages, as they are necessary for not only for activation during the initial response to infection but also for antigen presentation and signaling to activate the adaptive immune response.

Macrophage functions may be regulated to a large extent by sex hormones. Estrogen, a known immunomodulator, can act directly on monocytes and macrophages, decreasing the production of proinflammatory cytokines such as IL-1, IL-6 and TNF- α . Evidence for estrogen modulation of TLRs has been documented within the female reproductive tract (FRT). TLRs and estrogen receptors (ER) are co-expressed abundantly in uterine and vaginal mouse epithelial cells (Soboll, Shen et al. 2006). In endometrial epithelial and stromal cells, TLR2, 3, 4, and 9 exhibit differential spatio-temporal expression patterns according to menstrual cycle changes, where there is a general increase in expression of all four TLRs around menstruation and a decrease in expression around ovulation (Hirata, Osuga et al. 2007). Estrogen suppresses TLR3-dependent proinflammatory and anti-viral cytokine and chemokine production in endometrial epithelial cell

lines (Schaefer, Fahey et al. 2005; Lesmeister, Bothwell et al. 2006). Female peripheral blood lymphocytes (PBLs) produce significantly higher IFN- α levels in response to TLR7 stimulation compared with male PBLs (Berghofer, Frommer et al. 2006).

Estrogen also modulates Fc γ R expression. Pregnancy levels of estrogen enhance clearance of IgG-sensitized erythrocytes by increasing splenic Fc γ R1 and Fc γ R2 expression in guinea pigs (Friedman, Netti et al. 1985; Gomez, Ruiz et al. 2001) while androgens, including testosterone, have the opposite effect (Gomez, Ruiz et al. 2000). In human PBLs and monocytic cell lines, removal of estrogen results in increased Fc γ R transcripts as well as increased TNF- α , IL-1 β and IL-6 production upon receptor cross-linking (Kramer, Kramer et al. 2004; Kramer, Winger et al. 2007).

Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular pathogen capable of surviving and persisting within host mononuclear cells, such as macrophages (Quesniaux, Fremont et al. 2004). The mediators that are essential for control of *M. tuberculosis* infection are secreted by macrophages and activated lymphocytes and include IFN- γ , IL-12, TNF, lymphotoxins, CD40 and nitric oxide (Garcia, Guler et al. 2000; Jacobs, Marino et al. 2000; Holscher, Atkinson et al. 2001; Lazarevic, Myers et al. 2003). One way that mycobacteria have evolved to resist clearance by macrophages is by inhibition of phagosome-lysosome fusion. Another way is by neutralization of some of these mediators (TNF and IFN- γ), inhibition of iNOS and T cell depletion (Flynn and Chan 2003). Therefore, continuous activation of macrophages in concert with activated T cells is necessary to control infection.

When studying resistance against *M. tuberculosis* infection and pathogenesis, much of the focus has been on the proteins actively secreted from the bacteria during their growth in culture. These secreted proteins, termed culture filtrate proteins (CFP) are released into the medium of mycobacterial cultures and are the primary targets for the immune response in mice and humans with active tuberculosis. Furthermore, they are the major proteins involved in *M. tuberculosis* pathogenesis. CFP have been extensively characterized and employed in experimental vaccine studies using mice. They have been studied as single proteins, or in pools of several proteins. To date, there have not been any studies that have specifically addressed the influence of sex hormones on *M. tuberculosis* vaccine responses. Because CFP is well-studied and safe to administer to animals, we used *M. tuberculosis* CFP as a model vaccine to assess how estrogen modulates the immune response to immunization. We have previously shown that estrogen enhances humoral immune responses to vaccination. The enhancement mechanism is not fully understood. We carried out this study to determine whether estrogen influences macrophage function, which is necessary for response to pathogens and for activation of downstream adaptive responses. To activate the immune system, female mice that were intact, ovariectomized (OVEX), or ovariectomized and received estrogen replacement (OVEX-E2) were subjected to immunization with *M. tuberculosis* CFP. We then measured TLR2 as well as Fc γ RI, Fc γ RIIB and Fc γ RIIIA gene expression in primary splenic and thioglycollate-elicited macrophages to determine if these genes are hormonally regulated in the immune response to vaccination. In addition, TLR2, Fc γ RI, Fc γ RIIB and Fc γ RIIIA gene expression was measured in a group of non-immunized male mice.

TLR2 and FcγR are critical for proper activation of the immune response. Indeed, vaccination relies on such functions in order to effectively trigger immunity during secondary infection. Therefore, hormonal modulation of these receptors could be an important pathway which directly influences not only autoimmune disease states in females, but also by which immune responses to vaccination can be manipulated.

MATERIALS & METHODS

Animals

Eight week-old female Balb/c mice (Charles River Laboratories, Inc., Wilmington, MA) were divided into 3 experimental groups. The first group, designated OVEX-E2 group, was ovariectomized and subjected to estrogen replacement. The second group, designated OVEX group, received no estrogen replacement (Table 4.1). The third group remained intact. All animals were housed and subjected to experimental procedures in accordance to protocols approved by the appropriate institutional review committee.

Ovariectomy, E2 replacement, and uterotrophic responses

Mice were anesthetized with 5% isoflurane gas in 100% oxygen, and their ovaries, oviducts and tips of the uterine horns were bilaterally removed via two dorsal incisions. Immediately following surgery, mice in the OVEX-E2 group started E2 replacement. E2 was administered in the drinking water. E2-treated water was prepared as previously described by diluting stock solutions of E2 (Sigma- Aldrich Corp., St. Louis, MO) in 100% ethanol to solubilize (Levin-Allerhand, Sokol et al. 2003). Solubilized estrogen was added to drinking water to produce a final concentration of 1000 nM, as previously described

Table 4.1. Experimental design for E2-CFP study.

Mice were divided into 3 groups, where the first group was ovariectomized (OVEX), the second group was ovariectomized and was given 1000 nM 17 β -estradiol (E2) replacement in the drinking water (OVEX-E2), and the third group underwent sham ovariectomy (intact). Mice were then vaccinated with *M. tuberculosis* CFP. Within each of these groups, one third of mice were vaccinated with 10 ug CFP (CFP-10), one third received 50 ug CFP (CFP-50) and the final third received saline (unvaccinated).

Hormone group	Vaccine group	Group Designation	No. of mice
OVEX	10 ug CFP	OVEX, CFP-10	11
	50 ug CFP	OVEX, CFP-50	11
	Unvaccinated	OVEX, UNVAX	10
OVEX-E2	10 ug CFP	OVEX-E2, CFP-10	11
	50 ug CFP	OVEX-E2, CFP-50	11
	Unvaccinated	OVEX-E2, UNVAX	10
Sham OVEX	10 ug CFP	Intact, CFP-10	11
	50 ug CFP	Intact, CFP-50	11
	Unvaccinated	Intact, UNVAX	10
Untreated males	Unvaccinated	Male	6

(Levin-Allerhand, Sokol et al. 2003; Fernandez and Frick 2004). The final concentration of ethanol was 0.1%. No adverse effects of this concentration of ethanol or estrogen were reported (Levin-Allerhand, Sokol et al. 2003). The selected concentration of E2 results in an ingested dose of approximately 50-70 µg/kg per day, corresponding to about 15 µg/ml (Stone, Rozovsky et al. 1998; Levin-Allerhand, Sokol et al. 2003). Estrogen levels during the estrus cycle in mice typically range from 10-40 µg/ml (Nelson, Karelus et al. 1995). A 1000 nM dose in the drinking water was previously shown to result in uterine weights that were significantly higher than control mice, yet not significantly different from those of mice given either 1500 nM or 2500 nM doses, implying a uterotrophic response that plateaus with increasing E2 doses (Levin-Allerhand, Sokol et al. 2003). To test for uterotrophic responses, uteri were removed and weighed at termination.

Immunization

Mice were immunized with a primary (day 0), secondary (day 21) and tertiary (day 49) dose of *M. tuberculosis* CFP (Provided by J. T. Belisle, Colorado State University, Fort Collins, CO, under the National Institutes of Health, National Institute of Allergy and Infectious Diseases Contract NO1-AI-75320, titled Tuberculosis Research Materials and Vaccine Testing) (Figure 4.1). Mice received subcutaneous injections of either 10 µg or 50 µg of CFP, mixed with Imject Alum (Pierce, Rockford, IL) at a 2 to 1 ratio of CFP to Alum. Formulations were made in a final volume of 200µl, yielding a final dose of either 50 µg/ml or 250 µg/ml.

Isolation of splenic and thioglycollate-elicited macrophages

Splenic macrophages were isolated by passing cells through a 70µm nylon mesh (BD-Falcon, Bedford, MA), and blood cells were removed using Red Blood Cell Lysis Buffer (Sigma Aldrich Corp., St. Louis, MO). Cells were adjusted to 3×10^6 cells/ml in complete medium (DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM glutamine and 5% FBS) in 6-well plates in a final volume of 2.5 ml/well for 60 min at 37°C with 5% CO₂. Wells were rinsed with media to remove non-adherent cells. Remaining cells were used for RNA extraction. To induce activated peritoneal macrophages, 1 ml of 3% thioglycollate was injected i.p. into mice. Five days later, peritoneal exudates cells were collected in 10 ml of cold PBS. Cells were washed and incubated at 1×10^6 cells/ml in 2.5ml complete medium for 60 min and non-adherent cells were removed.

Macrophage stimulation

Thioglycollate-elicited and splenic macrophages (1×10^6 cells/ml) were stimulated in 12-well plates in a final volume of 2.5 ml/well with 1µg/ml LAM (CSU), 5µg/ml LAM, 10 µg/ml LAM, 10 µg/ml peptidoglycan (PGN, Sigma), 100 µg/ml PGN or media alone. Cultures were incubated for 4 hr and 48 hr at 37°C with 5% CO₂. Media was then removed and RNA was extracted from cells and used to determine TLR2 and FcγR expression by real-time PCR.

TLR2 expression detected by real-time PCR

Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA samples were quantified and used for cDNA synthesis using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics Corp., Indianapolis,

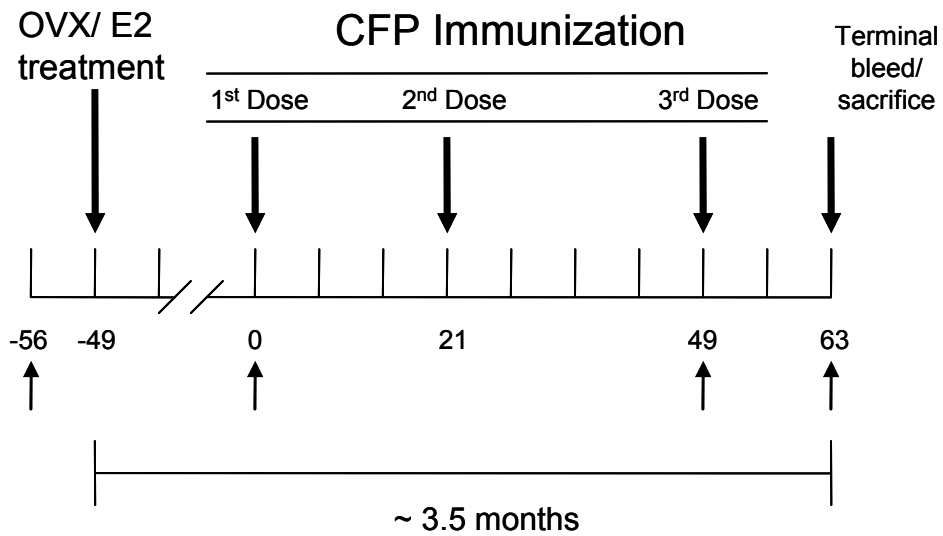


Figure 4.1. E2-CFP Study: Hormone treatment and vaccination schedule.

Mice were ovariectomized and mice designated to receive E2 replacement were immediately given E2 (1000 nM) orally in their drinking water. Mice were vaccinated on Day 0 with *M. tuberculosis* CFP, and then received subsequent booster doses on Days 21 and 49. All mice were sacrificed on day 63 for uterine weight measurements as well as macrophage isolation from the peritoneal cavity and the spleen. The large arrows indicate dates of vaccine administration. The small arrows indicate days of blood collection for determination of CFP-specific antibody responses.

IN) using approximately 1ug of total RNA. Real-time PCR was performed using TaqMan Gene Expression Assays pre-developed primers and probes for TLR2 at a 1 X concentration, 10ul of cDNA and 1 X TaqMan Gene Expression Master Mix (all supplied by Applied Biosystems, Foster City, CA). Relative quantification was determined by normalizing TLR2 expression to endogenous β -actin expression. $\Delta\Delta C_t$ values were calculated by calibrating average ΔC_t values of the OVEX-E2 and OVEX mice to those of the intact mice.

Detection of CFP-specific antibody responses

Anti-CFP antibody responses were assessed by ELISA. Micro-titer wells were coated with CFP at 4 ug/ml diluted in coating buffer (KPL, Inc., Gaithersburg, MD). To block nonspecific binding sites, wells were incubated for 30 min at 37°C with PBS containing 5% fetal calf serum (FCS-PBS) (Atlanta Biologicals, Lawrenceville, GA). Two-fold serial dilutions were performed on serum samples which were then added to triplicate wells and incubated overnight at 4° C. After washing with PBS containing 0.05% Tween-20, wells were incubated with HRP-labeled goat anti-mouse IgG (γ) (KPL, Gaithersberg, MD) for 1 hr and 15 min at 37° C. Wells were washed and then incubated with ABTS/ H₂O₂ (KPL) substrate for 10 min at room temperature, followed by addition of stop solution (KPL). Optical density (OD) values were assessed at 405 nm using a PowerWave HT plate reader (BioTek Instruments, Inc., Winooski, VT).

Statistical analysis

The SPSS program (SPSS Inc, Chicago, Illinois) version 12 was used for statistical analysis. Analysis among multi-group data was carried out using ANOVA, followed by Turkey's

significant difference test, or Tamhane's T2 test when the test of homogeneity of variances (Levene Statistic) indicated that the variance between the groups was not equal. Differences among groups were considered significant if $p < 0.05$.

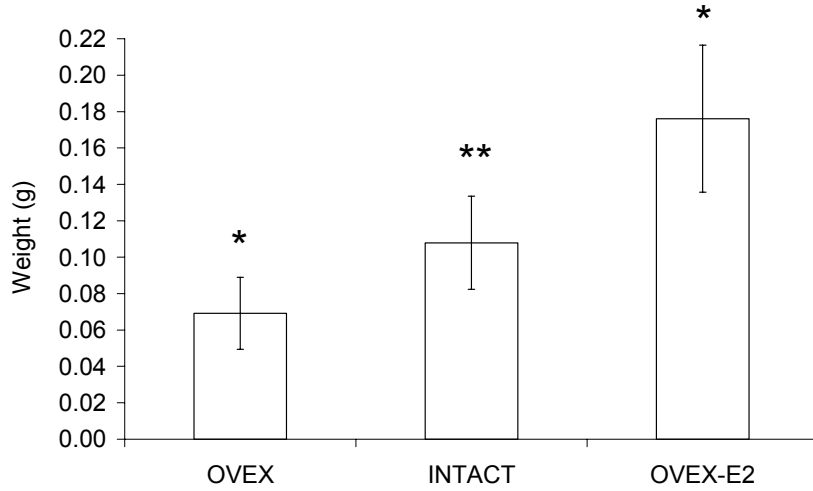
RESULTS

Uterotrophic responses

Mice were sacrificed after being subjected to approximately 3.5 months of ovariectomy with or without E2 replacement. At termination, uteri from each mouse were collected and weighed in order to test for uterotrophic responses to E2 delivery. This bioassay was chosen because of its high sensitivity to low amounts of E2 treatment (Mobbs, Cheyney et al. 1985). The mean uterine weights were compared between the groups. As expected, OVEX-E2 mice had uterine weights significantly higher than the OVEX and intact mice ($p < 0.001$). The uterine weights of OVEX mice were significantly lower than those of the intact mice ($p < 0.001$) (Figure 4.2). This confirms the desired effect of a supra-physiological level of E2 as a result of our method of delivering 1000 nM of E2 in the drinking water of OVEX mice.

Estrogen manipulation modulates TLR2 and FcγIIIA mRNA expression in macrophages isolated from non-vaccinated mice

To determine the effect of estrogen manipulation alone on macrophage gene expression, we measured and compared TLR2 and FcγR mRNA in unvaccinated OVEX-E2, OVEX and intact mice. Our results show that E2-replacement correlated with a reduction in TLR2 mRNA expression. Compared with intact mice, TLR2 expression in OVEX-E2 mice was 42% reduced in peritoneal macrophages and 60% reduced in splenic macrophages (Figure 4.3). On the other



TREATMENT	UTERINE WEIGHT (mg)
OVEX	0.069 ± 0.020 *
INTACT	0.108 ± 0.026 **
OVEX-E2	0.176 ± 0.040

Figure 4.2. Uterotrophic responses to hormone treatment.

Approximately 3.5 months after hormone manipulation, female mice were sacrificed and uteri were removed and weighed to compare uterotrophic responses between groups. * $p < 0.001$ compared to intact and OVEX-E2, ** $p < 0.001$ compared to OVEX and OVEX-E2.

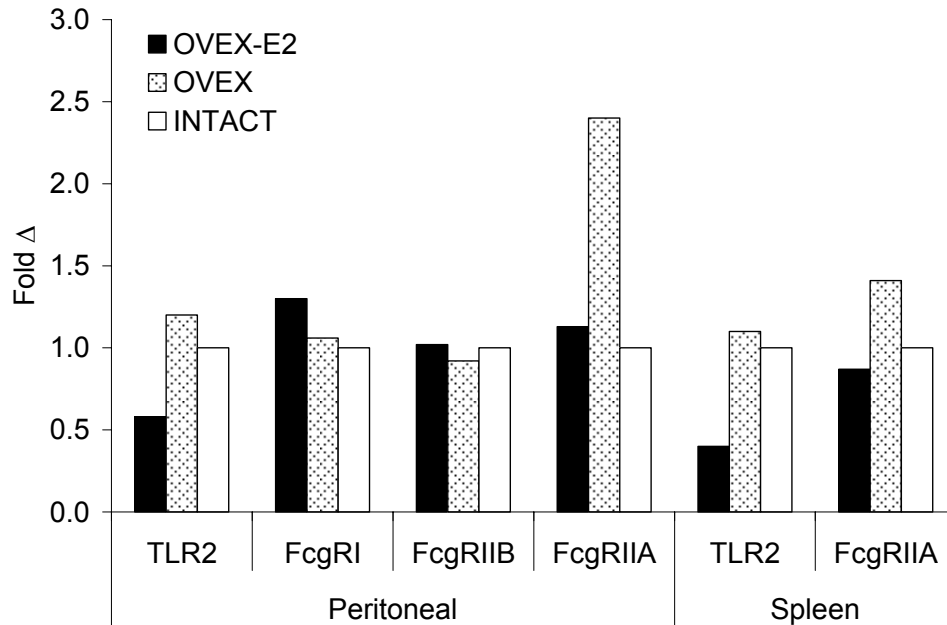


Figure 4.3. The effect of estrogen manipulation on macrophage gene expression.

Splenic and thioglycollate-elicited macrophages from OVEX-E2, OVEX and intact mice were isolated and total RNA was extracted and used for cDNA generation and real time PCR to measure levels of TLR2, FcγRI, FcγRIIB and FcγRIIA mRNA expression. Data are expressed as fold difference ($2^{-\Delta\Delta C_t}$) of OVEX-E2 and OVEX mice compared with intact mice (set to 1).

hand, OVEX mice showed a 17% increase in TLR2 mRNA expression compared to intact mice in peritoneal macrophages. In splenic macrophages, a lesser increase in TLR2 mRNA expression of only 6% in the OVEX mice was seen. OVEX mice expressed 139% more Fc γ RIIIA mRNA in peritoneal macrophages compared to intact mice. These patterns of E2-induced down-regulation were not observed when we measured Fc γ RI and Fc γ RIIB.

M. tuberculosis CFP-specific antibody responses

To test the effect of hormone treatment on specific response to vaccination, we measured serum levels of anti-CFP IgG in OVEX-E2, OVEX and intact mice. Mice were vaccinated with a total of three doses of 10 ug or 50 ug CFP, designated CFP-10 and CFP-50, respectively. Blood was collected according to the schedule indicated above (Figure 4.1) and serum was diluted and used for determination of specific antibody responses. After the third dose, OVEX mice in the CFP-10 group had significantly lower CFP-specific IgG levels compared to OVEX-E2 mice and reduced, although not significantly, lower anti-CFP IgG levels compared with the intact mice (Figure 4.4 A). These differences were more dramatic in the CFP-10 vaccination group than the CFP-50 group. Overall, CFP-50 mice showed higher responses than did the CFP-10 mice, likely due to the higher dose administered. These mice showed differences at higher serum dilutions of 1:800, where OVEX mice had reduced anti-CFP antibody responses compared to the OVEX-E2 and intact mice ($p < 0.05$ compared to intact mice) (Figure 4.4 B).

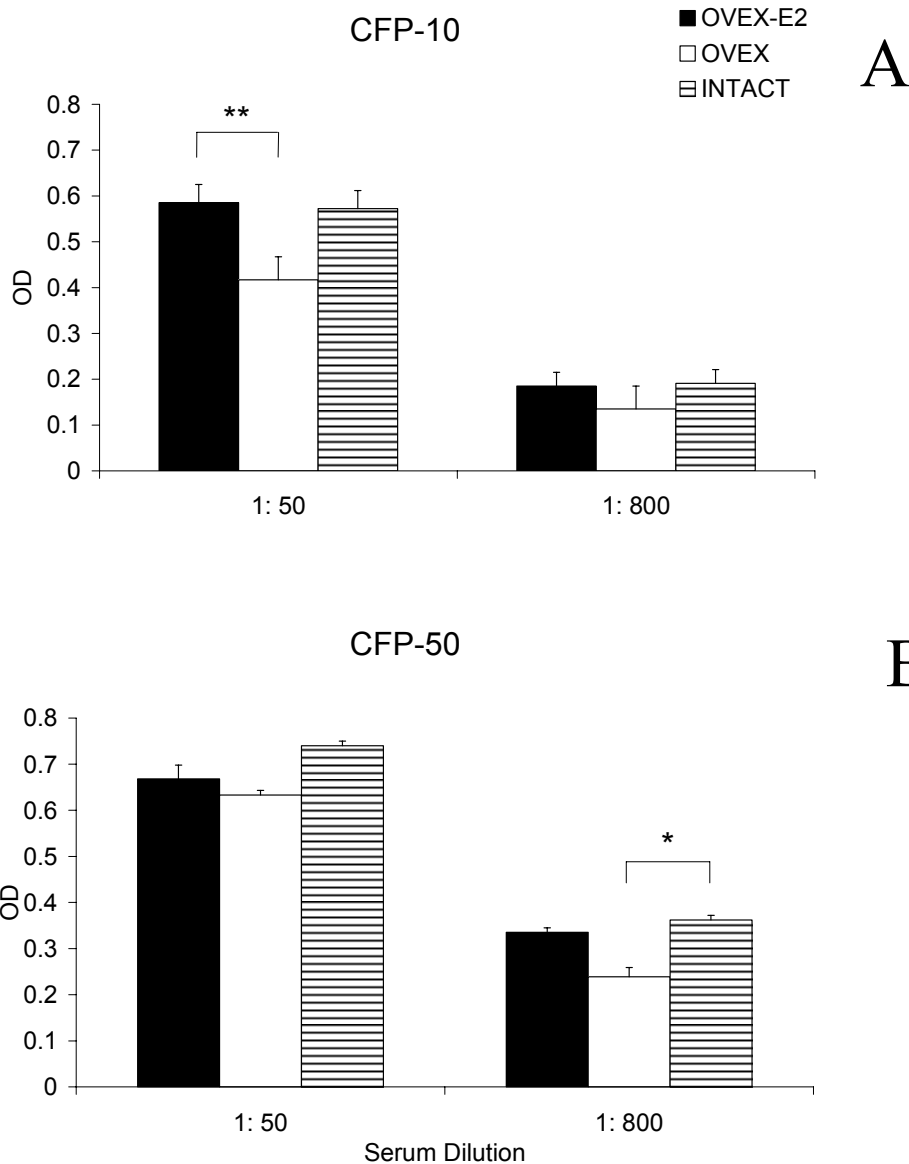


Figure 4.4. CFP-specific antibody responses.

Mice were vaccinated with a total of three doses of either **A**, 10 ug or **B**, 50 ug *M. tuberculosis* CFP. Blood was collected two weeks after the third dose was administered and serum was used for determination of anti-CFP IgG (γ) levels. Data expressed as OD values of the average of 10 or 11 mice per group \pm SEM. * $p < 0.05$, ** $p < 0.01$.

Macrophage TLR2 expression is enhanced in OVEX mice and reduced in E2-treated mice following vaccination

It is well-known that LAM from *M. tuberculosis*, also present in CFP, activates macrophages through TLR2. Our data shows that in normal intact female mice, vaccination with *M. tuberculosis* CFP results in up-regulation of TLR2 expression in thioglycollate-elicited macrophages. In both CFP-10 and CFP-50-vaccinated OVEX mice, this increase was more pronounced after vaccination when compared with the increase seen in the intact mice (Figure 4.5). In OVEX-E2 mice, vaccination caused reduced TLR2 mRNA expression compared to the response seen in the OVEX and intact mice. Interestingly, in CFP-10-vaccinated OVEX-E2 mice, TLR2 expression was down-regulated when compared with the unvaccinated OVEX-E2 mice. These data suggest that in the absence of E2, TLR2 mRNA expression in response to CFP is enhanced, whereas high levels of E2 cause a reduction of TLR2 mRNA expression in response to CFP. Such a pattern was most pronounced in the in CFP-10 vaccinated mice, thus indicating a possible dose-response effect. Similar vaccine-induced changes in TLR2 mRNA expression were not seen with the Fc γ R mRNA expression in the same mice (data not shown).

Vaccination and/or estrogen manipulation differentially effect TLR2 and Fc γ R gene expression in mice

Thioglycollate-elicited macrophages were isolated from OVEX-E2, OVEX and intact mice that were either unvaccinated or vaccinated with CFP-10 or CFP-50. To determine the effects of these treatments, alone or in combination, on macrophage gene expression, RNA from these cells was used for cDNA synthesis and real time PCR determination of TLR2 and Fc γ R mRNA

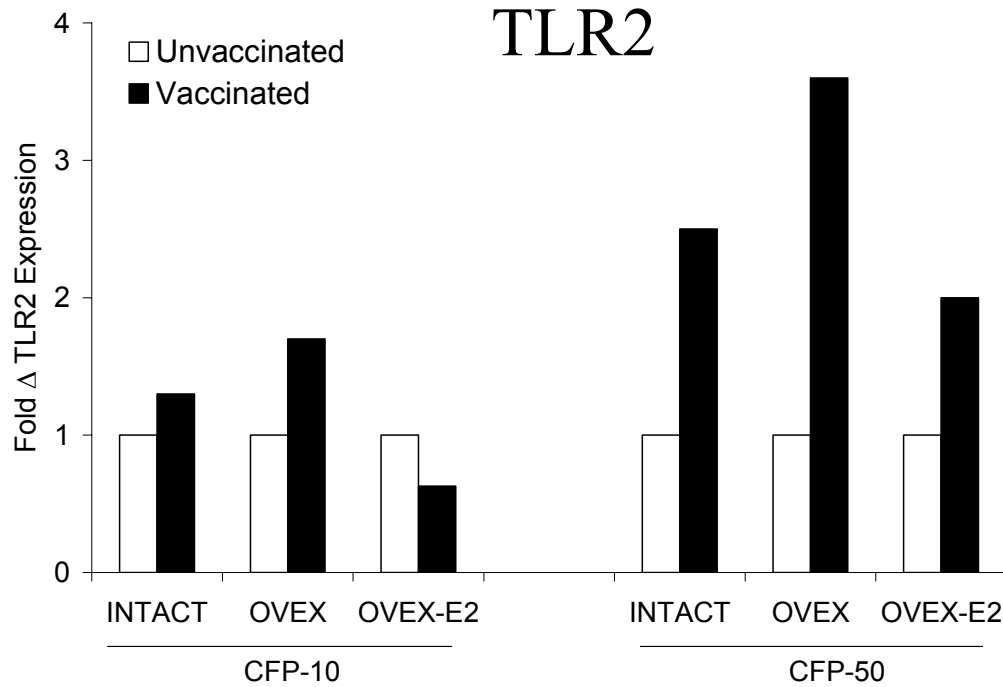


Figure 4.5. The effect of vaccination on TLR2 gene expression.

Real time PCR was used to determine TLR2 mRNA expression in thioglycollate-elicited peritoneal macrophages from unvaccinated mice and mice vaccinated with 10 ug (CFP-10) or 50 ug (CFP-50) *M. tuberculosis* CFP. Data are expressed as fold difference ($2^{-\Delta\Delta C_t}$) of vaccinated mice compared with unvaccinated mice (set to 1).

Figure 4.6. The effect of vaccination and/or estrogen on TLR2 and FcγR gene expression (next page).

Thioglycollate-elicited macrophages were isolated from mice and RNA was extracted and cDNA used for real time PCR determination of levels of **A**, TLR2, **B**, FcγRI, **C**, FcγRIIB and **D**, FcγRIIIA mRNA expression. Bars represent the average values of individual mice per group (n= 10 or 11). Bars depict mRNA levels of the indicated gene for each group in response to the indicated treatment. Data are expressed as fold difference ($2^{-\Delta\Delta C_t}$) of treated mice (filled bars) compared to those of the untreated intact mice (empty bars, set to 1) for each gene indicated.

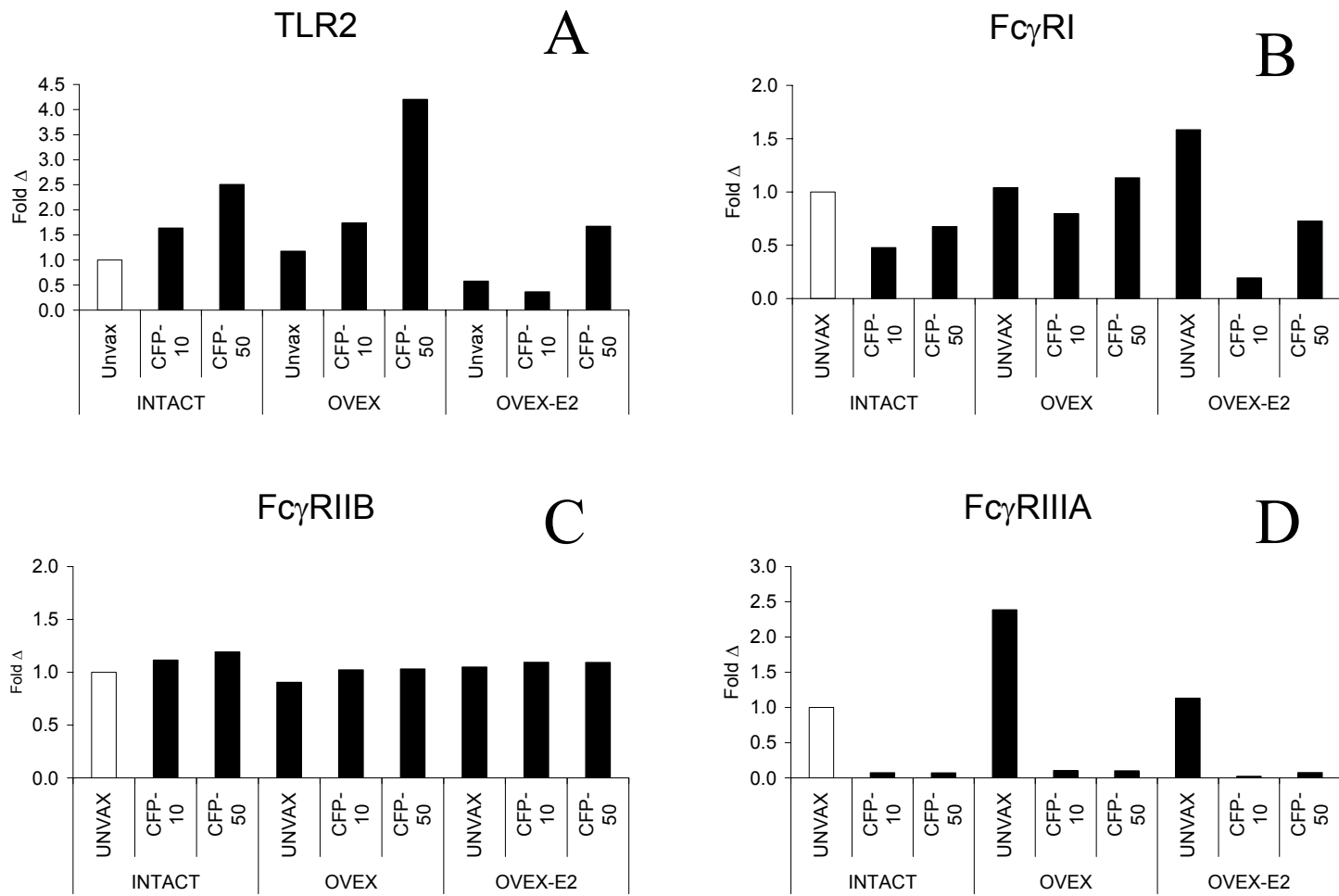


Figure 4.6.

expression. Figure 4.6 shows the expression patterns of each receptor according to the indicated treatment. These results indicate that gene expression patterns may vary with vaccination and/or estrogen manipulation (OVEX or OVEX-E2) (Figure 4.6 A, B). The most dramatic difference in gene expression related to estrogen manipulation but not vaccination was seen with Fc γ RIIIA (Figure 4.6 D). No difference in gene expression caused by such treatments was seen with Fc γ RIIB (Figure 4.6 C). Comparison of treated mice with untreated mice (unvax, intact mice) shows that macrophage gene expression levels are responsive and differentially affected depending on the receptor and type of treatment.

To determine the effect of estrogen on TLR2 and Fc γ R expression, within independent vaccine treatment, the data generated from the same mice was reanalyzed to compare gene expression levels of OVEX-E2 and OVEX mice with those of intact mice within each vaccine group. Our data show that TLR2 mRNA expression is significantly reduced in OVEX-E2 mice, irrespective of vaccination (Figure 4.7 A). Furthermore, there was a slight up-regulation of TLR2 mRNA expression in OVEX mice compared with intact mice. Fc γ R mRNA expression patterns varied greatly between the three types. Fc γ RI mRNA expression was significantly enhanced in OVEX and reduced in OVEX-E2 mice compared with intact mice in the CFP-10 vaccine group, whereas there were no differences in the CFP-50 vaccine group (Figure 4.7 B). Fc γ RIIIA mRNA expression was up-regulated in OVEX mice compared with intact mice irrespective of vaccination. However OVEX-E2 mice showed reduced Fc γ RIIIA mRNA levels only in the CFP-10 vaccine group (Figure 4.7 D).

Figure 4.7. The effect of estrogen on TLR2 and FcγR gene expression (next page).

Thioglycollate-elicited peritoneal macrophages were isolated from mice, RNA was extracted and cDNA was used for real time PCR determination of levels of **A**, TLR2, **B**, FcγRI, **C**, FcγRIIB and **D**, FcγRIIIA mRNA expression. Bars represent the average values of individual mice per group (n= 10 or 11). Bars depict mRNA levels of the indicated gene for each group in response to the indicated treatment. Data are expressed as fold differences ($2^{-\Delta\Delta C_t}$) of OVEX-E2, OVEX mice compared to intact mice (set to 1 for each gene indicated). * $p < 0.05$, ** $p < 0.01$.

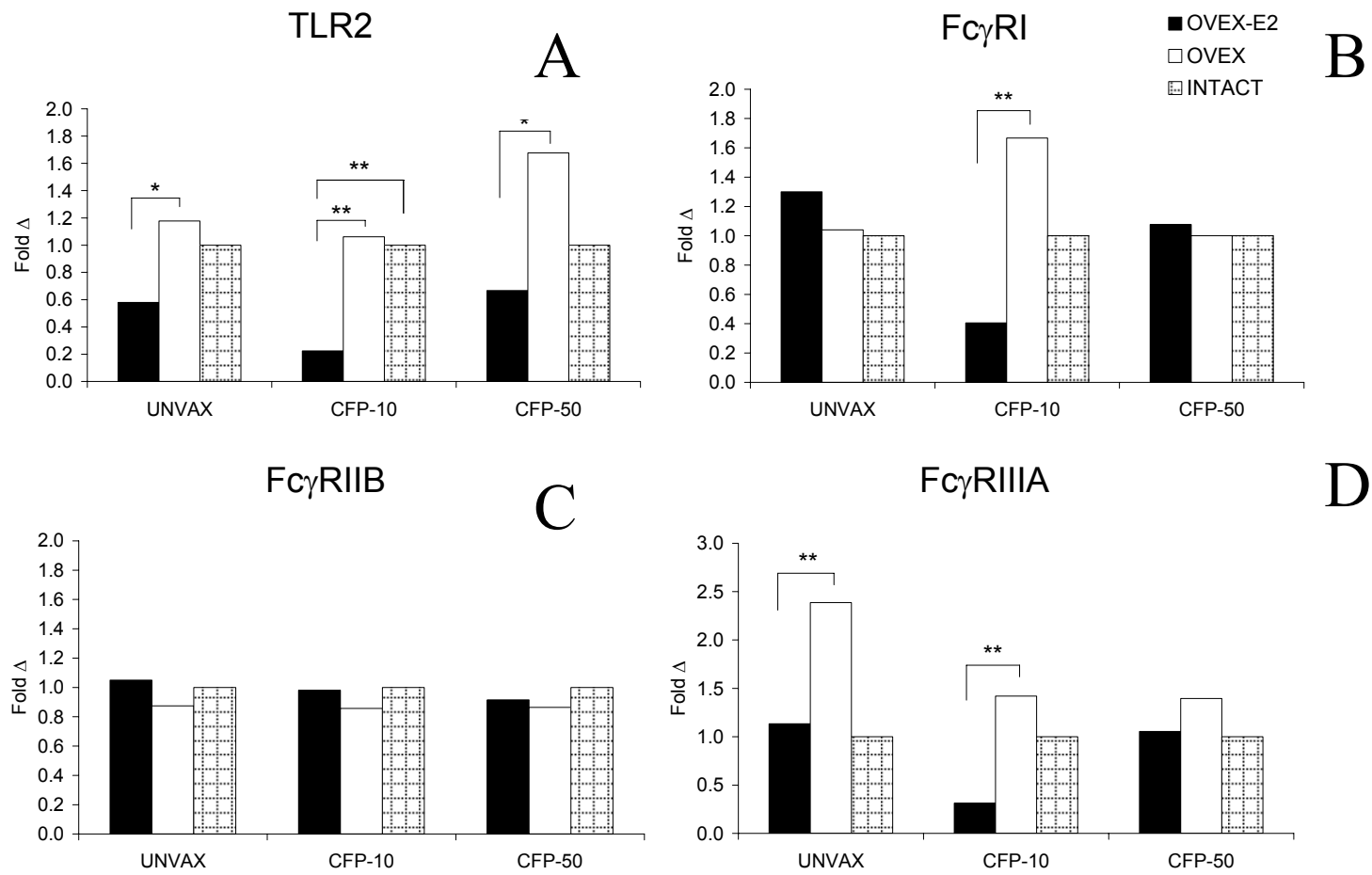


Figure 4.7

In vitro stimulation of macrophages with TLR2 ligands differentially up-regulates macrophage TLR2 mRNA expression in vaccinated and/or estrogen-manipulated mice

Our data so far show similar estrogen-modulated patterns of TLR2 and Fc γ R mRNA expression, where E2 generally reduces and OVEX enhances mRNA expression, with the exception of Fc γ RIIB, where no changes are seen. Common TLR2 ligands include gram-positive bacterial products such as peptidoglycan (PGN). Lipoarabinomannan (LAM) is a mycobacterial cell wall component that is also known to activate and unregulate TLR2 (Tapping and Tobias 2003; Quesniaux, Nicolle et al. 2004) and is present in mycobacterial culture filtrate preparations. Mice that are vaccinated with CFP have an advantage when encountering mycobacterial proteins because they have been primed to respond to secondary encounter. Therefore, we next determined whether estrogen manipulation affects macrophage responsiveness to *in vitro* stimulation with known TLR2 ligands in CFP-vaccinated and in unvaccinated mice.

Thioglycollate-elicited peritoneal macrophages were cultured for 48 hr in the presence of TLR2 ligands LAM (1 ug/ml and 10 ug/ml) and PGN (100 ug/ml). Total RNA was extracted and used for cDNA generation followed by real time PCR for detection of TLR2 transcripts. In unvaccinated mice, stimulation with PGN resulted in increased expression compared to the unstimulated controls, and these levels varied with respect to estrogen manipulation, where OVEX mice had up-regulated, and OVEX-E2 had down-regulated TLR2 expression compared with the intact mice (Figure 4.8 A). Very little differences in TLR2 mRNA expression were seen in response to stimulation with LAM when compared with the unstimulated control. This was not surprising since these mice were unvaccinated and therefore were not primed with CFP to respond as in a secondary encounter to LAM.

When mice were vaccinated with CFP, macrophage stimulation with LAM resulted in increased TLR2 expression in a dose-dependent manner. Stimulation with PGN in these groups served as a positive control for TLR2 expression based on the results from the unvaccinated group. In the CFP-10 vaccine group, there was a significant reduction in TLR2 mRNA expression in the OVEX-E2 mice compared to both the OVEX and intact, indicating a strong down-regulatory effect of estrogen (Figure 4.8 B). In the CFP-50 group, this reduction was apparent yet more subtle (Figure 4.8 C). Overall, TLR2 mRNA expression in stimulated macrophages was down-regulated in OVEX-E2 mice when compared with intact mice. OVEX mice showed equal or slightly up-regulated expression when compared to intact mice (Figure 4.8 B and C). Though both groups responded in a similar pattern to stimulation, mice vaccinated with CFP-10 showed higher TLR2 levels as compared with CFP-50. Furthermore, differences between OVEX-E2 mice and OVEX/intact mice were more pronounced in the CFP-10 group, indicating a CFP-vaccine dose response. This pattern of TLR2 mRNA expression was identical in splenic macrophages (data not shown).

The data discussed above focuses on TLR2 expression patterns of stimulated cells compared with un-stimulated control cells and show how treatment (vaccination and/or estrogen manipulation) affects TLR2 expression. Additional analysis was performed to compare TLR2 mRNA expression in the three hormone groups for each independent *in vitro* stimulation conditions. These data show consistent up-regulation of TLR2 mRNA expression in OVEX mice compared with intact mice, irrespective of vaccine dose and *in vitro* stimulation conditions (Figure 4.9). TLR2 mRNA expression was up-regulated from 36% up to 200% depending on the

Figure 4.8. The effects of estrogen on TLR2 gene expression in response to in vitro stimulation with TLR2 ligands (next page).

Thioglycollate-elicited peritoneal macrophages from **A**, unvaccinated mice or mice vaccinated with **B**, 10 ug CFP (CFP-50) or **C**, 50 ug CFP (CFP-10) were pooled according to group (n = 10 or 11 mice per group) and cultured for 48 hrs in the presence of 1 ug/ml and 10 ug/ml mycobacterial lipoarabinomannan (LAM) and 100 ug/ml peptidoglycan (PGN). RNA extracted from these cells was used for real time PCR determination of levels of TLR2 transcripts. Bars depict TLR2 mRNA levels of each group in response to the indicated stimulator. Data are expressed as fold difference ($2^{-\Delta\Delta C_t}$) of the stimulated cells (filled bars) compared to those of the unstimulated controls (empty bars, set to 1) for each hormone and vaccine treatment.

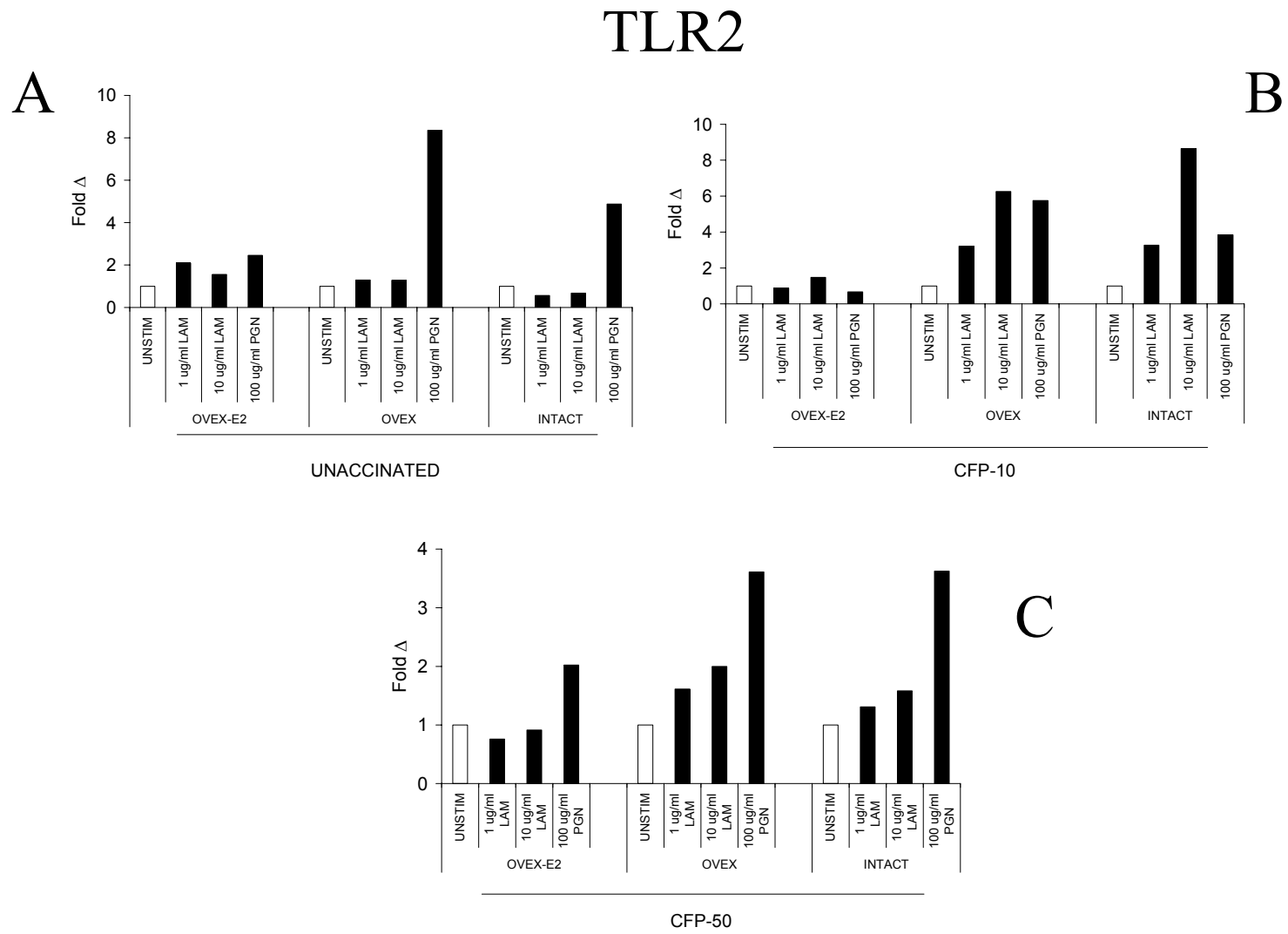


Figure 4.8.

TLR2

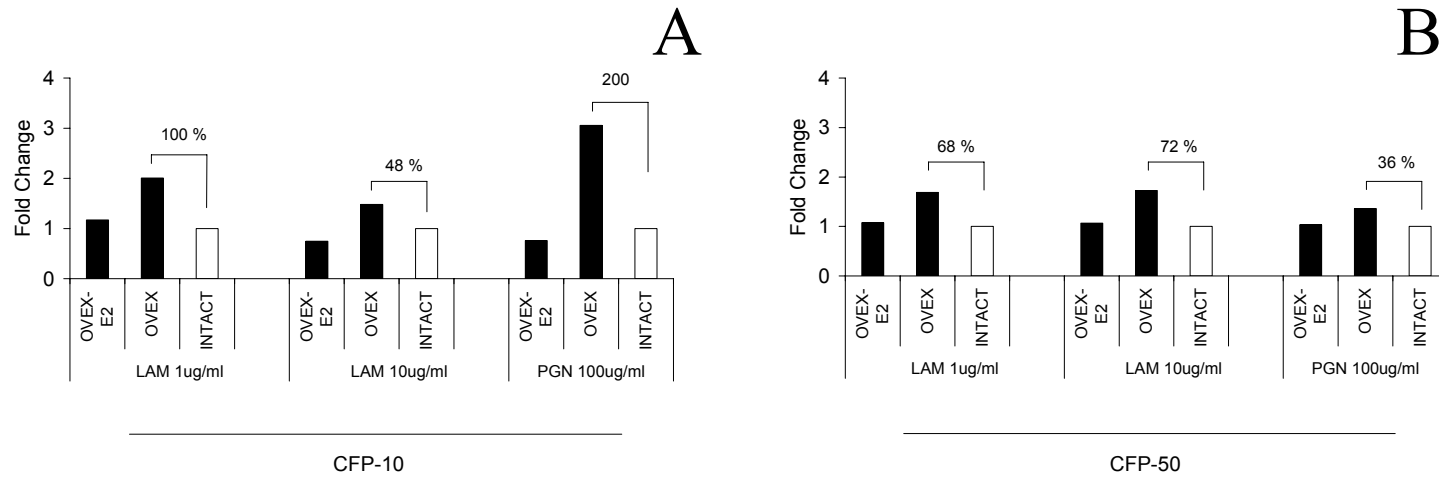


Figure 4.9. OVEX up-regulates TLR2 mRNA expression after stimulation with TLR2 ligands.

Thioglycollate-elicited peritoneal macrophages from mice vaccinated with A, 10 ug CFP (CFP-50) or B, 50 ug CFP (CFP-10) were pooled (n= 11) and stimulated for 48 hrs with LAM or PGN and RNA was used for real time PCR detection of TLR2 expression. Data expressed as fold difference ($2^{-\Delta\Delta Ct}$) of OVEX-E2 and OVEX mice (filled bars) compared to intact mice (empty bars, set to 1). Percent increase of TLR2 expression in the OVEX compared with the intact mice is indicated for each group.

type of stimulation and vaccine dose group. These data further indicate that in the absence of estrogen, CFP-primed macrophages express increased TLR2 transcripts in response to LAM and PGN compared to OVEX-E2 and intact control mice. TLR2 mRNA expression did not differ between OVEX-E2 and intact mice in response to *in vitro* stimulation with LAM or PGN.

Macrophage TLR2 and FcγR mRNA expression differs between male and female mice

Based on the observed differences between OVEX-E2, OVEX and intact mice, it is thus far clear that absence of estrogen results in up-regulated expression of TLR2, FcγRI and FcγRIIIA, whereas in presence of high levels of estrogen (OVEX-E2 mice), gene expression levels are down-regulated. Since estrogen levels differ greatly between males and females, we next compared baseline TLR2 and FcγR mRNA expression of intact female mice with those of male mice. For this comparison, we used thioglycollate-elicited peritoneal macrophages (all receptors) as well as splenic macrophages (TLR2 and FcγRIIIA only) to determine if expression differs within different tissues and compartments. Macrophages were obtained from individual female and male mice and total RNA was extracted and used for cDNA generation and realtime PCR to detect levels of TLR2 mRNA expression. In splenic macrophages, males expressed significantly higher levels of TLR2 and FcγRIIIA mRNA compared to females ($p < 0.01$) (Figure 4.10). In thioglycollate-elicited peritoneal macrophages, levels of TLR2, FcγRI and FcγRIIIA were significantly higher in the male mice compared with the female mice ($p < 0.05$ for TLR2 and $p < 0.01$ for FcγRI and FcγRIIIA). No gender-related differences in FcγRIIB mRNA expression were observed.

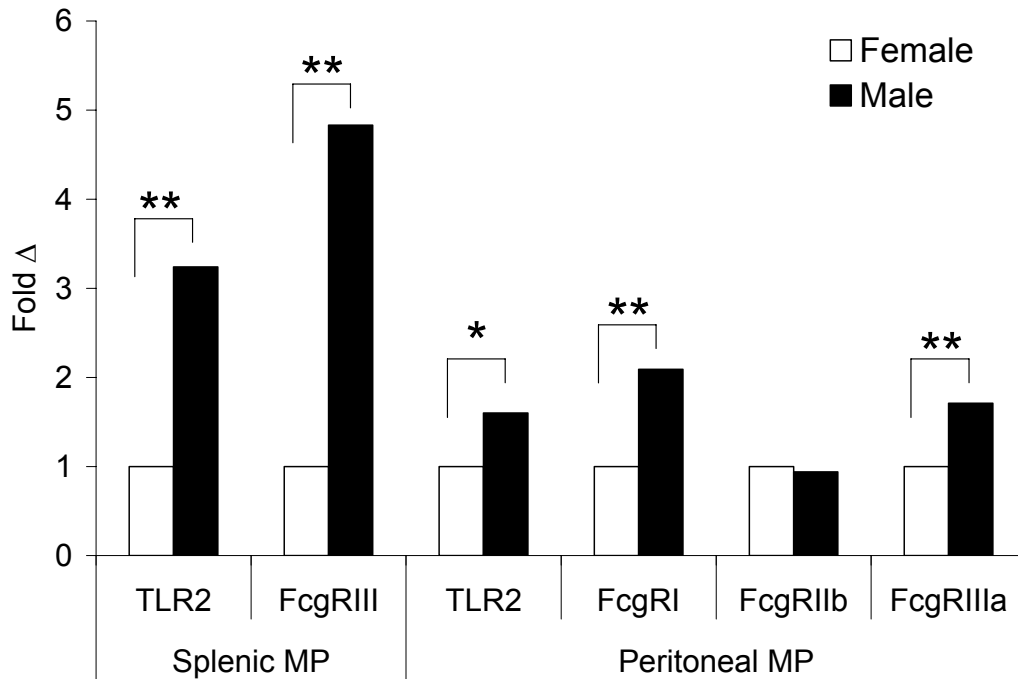


Figure 4.10. Sex differences in TLR2 and FcγR gene expression.

Thioglycollate-elicited peritoneal macrophages and splenic macrophages were isolated from female and male mice. RNA was extracted and reverse-transcribed, and cDNA was used for real time PCR determination of transcript levels of TLR2, FcγRI, FcγRIIB, FcγRIIIA. Bars represent the average of individual mice (n=10 females, n=6 males). Data are expressed as fold difference ($2^{-\Delta\Delta C_t}$) of transcript levels in males compared to females (set to 1). * $p < 0.05$, ** $p < 0.01$.

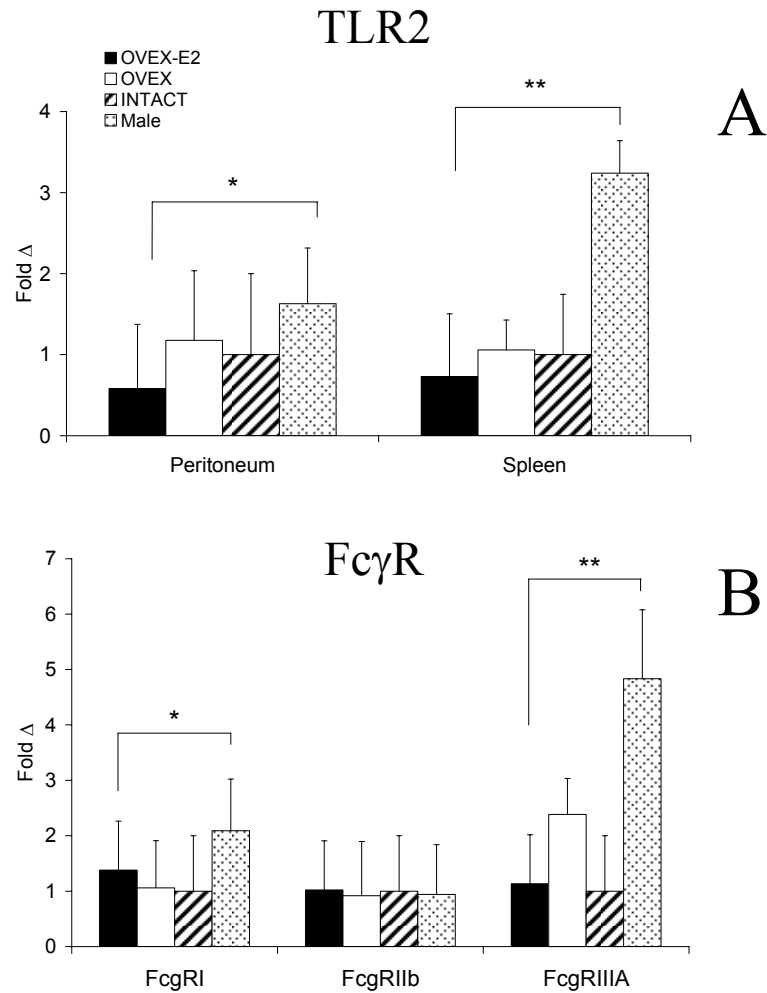


Figure 4.11. The effects of estrogen and sex on TLR2 and FcγR gene expression.

A, TLR2 mRNA expression in thioglycollate-elicited peritoneal and splenic macrophages and **B**, FcγRI, FcγRIIB and FcγRIIIA mRNA expression in thioglycollate-elicited peritoneal macrophages from individual non-vaccinated male (n=5) and female (n=10) mice. Data are expressed as fold differences ($2^{-\Delta\Delta Ct}$) of OVEX-E2, OVEX and male mice compared to intact female mice (set to 1). Error bars represent the SD converted to $2^{-\Delta\Delta Ct}$. * $p < 0.05$, ** $p < 0.01$.

To compare differences in expression under all hormone conditions TLR2 and Fc γ R, data were re-analyzed to include the unvaccinated female OVEX-E2, OVEX and intact mice along with male mice. In peritoneal macrophages, TLR2 mRNA expression in male mice was up regulated compared to OVEX-E2, OVEX and intact female mice, although statistical significance was only observed when compared with the OVEX-E2 mice ($p < 0.05$). In splenic macrophages, male mice expressed significantly higher levels of TLR2 mRNA compared with all groups of females ($p < 0.01$) (Figure 4.11 A). Fc γ R mRNA levels were determined in peritoneal macrophages. Fc γ RI and Fc γ RIIIA mRNA expression levels were higher in males compared with females ($p < 0.01$) (Figure 4.11 B). No gender differences were observed in Fc γ RIIB mRNA expression.

DISCUSSION

To study the effects of estrogen on macrophage gene expression, female Balb/c mice were ovariectomized to remove endogenously-produced E2. A group of these mice was then given E2 replacement in supra-physiological levels. E2 was diluted in the drinking water of designated mice. This administration method is a common and safer alternative to other methods of hormone delivery based on osmotic pumps, silastic implants or injections. This method resulted in significantly different uterotrophic responses when compared to OVEX and intact mice, confirming sufficient E2 delivery.

In studying the effects of estrogen on macrophage function, we focused on TLR2 and Fc γ R gene expression, as these receptors are involved in innate and adaptive immune responses, respectively. Therefore, estrogen-induced changes in receptor expression are expected to result

in significant alterations in immune function. Our data suggest a general up-regulation of macrophage gene expression in the absence of estrogen. OVEX mice expressed higher levels, whereas OVEX-E2 mice expressed lower levels, of TLR2, Fc γ RI and Fc γ RIIA. These results indicate that E2 down-regulates expression of these genes, as even intact mice, which produce physiological levels E2 showed slightly lower expression than did OVEX mice. When these groups were compared with male mice of identical age and subjected to the same housing and diet conditions, male mice expressed significantly higher levels of mRNA of these genes. Although males do endogenously produce estrogen through conversion of testosterone and other biosynthetic pathways, it is implied that this effect involves a more complex regulation of macrophage gene expression by sex hormones, as even differences between males and OVEX mice were drastically different. Fc γ RIIB consistently showed no change in mRNA expression between the groups, nor between males and females.

Fc γ RI and Fc γ RIIA are both expressed on myeloid cells (monocytes and macrophages) and both require the presence of associated subunits (γ or ζ) to be expressed on the cell surface and to transduce cell-activating signals (Nimmerjahn and Ravetch 2006; Kuby 2007). These two receptors are considered activating Fc γ R because they induce ADCC, endocytosis of immune complexes followed by antigen presentation, phagocytosis and release of cytokines or pro-inflammatory mediators. Fc γ RIIB, on the other hand, transduces inhibitory signals that down-regulate immune functions triggered by activating receptors, and is therefore considered an inhibitory Fc γ R. Differential patterns of Fc γ R expression induced by estrogen, therefore, is not surprising given the distinct functions of these receptors.

Fc γ RIIB is the only classic Fc receptor present on B cells where it regulates activating signals resulting from immune complex binding to the B cell receptor (BCR) (Phillips and Parker 1983). Normally, B cell signaling results in proliferation, class-switching and maturation into antibody-secreting plasma cells. Fc γ RIIB-deficient mice who display elevated levels of Ig. Fc γ RIIB also plays a role in peripheral tolerance, where deficient mice spontaneously develop lupus-like disease with high autoantibody production (Bolland and Ravetch 2000). Additionally, polymorphisms in the Fc γ RIIB gene leading to decreased transcription and surface expression on B cells of lupus patients have been identified (Blank, Stefanescu et al. 2005). Interestingly, when studied in equivalent animal models, the monocytic compartment still expressed Fc γ RIIB, while it was absent from peripheral B cells (Bolland and Ravetch 2000). Therefore, the present study, where estrogen treatment up-regulated specific antibody responses to vaccination, is consistent with the finding that although the activating Fc receptors were downregulated by estrogen, the inhibiting receptor which acts mostly on B cells, was not similarly affected by estrogen.

It is important to consider that hormonally-influenced immune modulation often times may not be apparent until activation of the immune system occurs, such as of immune cells during a vaccine response or during infection. During immune activation, the response to estrogen may be different than in an absence of activation. To test this hypothesis, we used a vaccine model consisting of *M. tuberculosis* CFP administered to mice in one of two dose concentrations (CFP-10 and CFP-50). In order to determine macrophage gene expression changes in response to antigenic stimulation under such hormonal conditions, OVEX-E2, OVEX and intact mice were vaccinated with *M. tuberculosis* CFP to induce immune activation. In the unvaccinated groups, we observed significantly increased TLR2 mRNA expression in peritoneal macrophages from

OVEX mice compared with intact and OVEX-E2 female mice, with the fold difference in expression most pronounced in OVEX mice. On the other hand, in the vaccinated groups, OVEX-E2 mice, there was a vaccine-induced decrease in TLR2 mRNA expression when compared with unvaccinated mice. These results support the hypothesis of an estrogen-related difference in macrophage activation and gene regulation in response to vaccination. Indeed, TLR2 gene expression post-vaccination was significantly higher in OVEX mice than prior to vaccination and was also significantly higher than both the intact and OVEX-E2 mice.

We have previously shown that estrogen enhances vaccine-specific antibody responses in mice (Chapter 3). We tested anti-CFP antibody responses to confirm the presence of a specific immune response, and therefore, immune activation. Patterns of antibody responses were negatively correlated with patterns observed for TLR2 and Fc γ R expression. CFP-vaccinated OVEX mice showed reductions anti-CFP IgG levels when compared with OVEX-E2 and intact mice. Unlike macrophage TLR2 and Fc γ R mRNA expression, we did not observe a difference in antibody responses between OVEX-E2 and intact mice, indicating that in the absence of estrogen, the immune response is more sensitive to humoral CFP vaccine responses than in presence of supra-physiological estrogen levels. This reduced antibody response negatively correlates with the observed increases in TLR2 and Fc γ RI and Fc γ RIIIA gene expression in OVEX mice compared to the intact mice. CFP-10 and CFP-50-vaccinated OVEX-E2 mice showed decreased TLR2 and I and Fc γ RIIIA expression, whereas OVEX mice showed slight up-regulation of expression. Therefore, in an activated immune system, in this case during a vaccine response, estrogen manipulation may not significantly alter the observed patterns of estrogen-induced modulation of macrophage gene expression, as seen in the unvaccinated mice.

M. tuberculosis is a gram-positive bacillus that, when sequestered, releases mycobacterial products (glycolipids, lipoproteins, secreted factors, etc.) that contribute to continued macrophage activation through TLR and MyD88-mediated signaling (Quesniaux, Fremont et al. 2004). An identified virulence factor of *M. tuberculosis* is the cell wall component lipoarabinomannan (LAM). Present in CFP, this has been shown to modulate a variety of immune activities including inhibition of phagosome maturation, apoptosis and IFN- γ signaling in macrophages and IL-12 secretion from dendritic cells (DC) (Pathak, Basu et al. 2007). It has been shown that that mycobacterial glycolipids such as LAM and its biosynthetic precursor lipomannan (LM) activate cells through TLR2 signaling (Rock, Hardiman et al. 1998). Therefore, it was not surprising that vaccination of mice with CFP up-regulated TLR2. The observed differences in TLR2 expression between the OVEX-E2 and OVEX mice compared to the intact mice implies that estrogen modulates CFP-induced TLR2 mRNA expression. The general pattern of E2-mediated down-regulation of TLR2 and Fc γ RIII appeared to be more pronounced in mice vaccinated with low-dose CFP, as a high-dose CFP appears to mask this effect.

Macrophages are critical components of innate immunity. Variability in TLR expression patterns may influence initial pathogen recognition and the subsequent response elicited. Indeed, results from our stimulation experiments show that splenic and thioglycollate-elicited peritoneal macrophages isolated from OVEX-E2, OVEX and intact mice respond differently to *in vitro* stimulation with TLR2 ligands, LAM and PGN. Although there are variations depending on the cell type and the dose of stimulator used, the general trend supports that OVEX mice appear to

have up-regulated levels of TLR2, and OVEX-E2 mice showed down-regulated levels of TLR2. In general, the differences seen between the groups were greater in CFP-10-vaccinated compared with those vaccinated with CFP-50-vaccinated mice. Splenic macrophages from CFP-50-vaccinated OVEX-E2, OVEX and intact mice did not show differences in TLR2 expression in response to stimulation. Comparison of the three hormone groups at each stimulator highlights a pattern in which OVEX mice appear to up-regulate TLR2 mRNA compared to OVEX-E2 and intact mice, although OVEX-E2 and intact mice do appear to differ in this respect.

Our results are consistent with studies which have shown that Fc γ RIIIA transcript increases in the human monocytic cell line THP-1 and primary macrophages in the absence of E2 (Kramer, Kramer et al. 2004). The finding that Fc γ RIIIA cross-linking stimulates production of TNF- α , IL-1 β and IL-6 in the absence of E2 indicates that the modulation of proinflammatory cytokine release from activated monocytes/macrophages occurs in part through modulation of Fc γ RIIA gene expression. Other studies using a guinea pig model show results contrary to ours. In these studies, estrogens enhanced, and androgens impaired, clearance of IgG-sensitized erythrocytes by increasing Fc γ RIIIA expression on splenic macrophages (Gomez, Ruiz et al. 2000; Gomez, Ruiz et al. 2001). While guinea pigs express two forms of this receptor, known as Fc γ P1 and Fc γ R2, they have no form of Fc γ RIIIA as do humans and mice. Therefore, expression patterns may vary with the type of receptor and thus these two species cannot be compared.

Besides differences in gene expression with respect to estrogen levels, our data show sex-related differences in levels of TLR2, Fc γ RI and Fc γ RIIIA in splenic and thioglycollate-elicited macrophages, with male mice expressing higher levels compared to normal intact female mice.

This finding is in contrast with results from other studies showing that TLR2 and CD16 surface expression on PBMCs is significantly higher in women than in men, as is LPS-stimulated TNF- α and IL-10 cytokine secretion (Ono, Tsujimoto et al. 2005). In the same study, the population of monocytes bearing Fc γ RIIIA was significantly higher in females than in males, which may have contributed to the observed differences in the levels of pro-inflammatory cytokine production.

Fc γ RIIIA expression on granulocytes appears to differ from the sex-related pattern seen on monocytes/macrophages. Indeed, gender seems to have no impact on Fc γ RIIIA expression on granulocytes (Moxey-Mims, Frank et al. 1993). Yet, pregnancy has shown to be associated with a lower intensity of granulocyte expression of Fc γ RIIIA (Naccasha, Gervasi et al. 2001). Absence of Fc γ RIIIA has been reported in some patients with higher prevalence of autoimmune diseases, although this absence was not associated with an increased risk of infection (de Haas, Kleijer et al. 1995).

The overall functional state of monocytes is reflected in the expression of surface antigens such as MHC class II, TLR and Fc γ R (Ono, Tsujimoto et al. 2005). Fc γ RIIIA-bearing monocytes express higher levels of class II MHC molecules, adhesion molecules and chemokine receptors and are able to more efficiently produce proinflammatory cytokines as compared with monocytes not expressing the receptor (Ziegler-Heitbrock, Fingerle et al. 1993). It has been found that Fc γ RIIIA-bearing monocytes more strongly express TLR2 levels and TNF- α in response to the TLR ligand lipopeptide (Iwahashi, Yamamura et al. 2004). This indicates that TLR2 is relevant to Fc γ RIIIA-bearing monocyte activation. Moreover, these two receptors appear to interact and regulate each other during inflammation. IL-12 is a pro-inflammatory

cytokine secreted by macrophages in response to TLR2 stimulation and is MyD88-dependent. It is important for both cell-mediated immune responses and to bias T_H cells to a T_H1 phenotype (Trinchieri 1998; Underhill 2003). Down-regulation of IL-12 by macrophages is attributed to the anti-inflammatory cytokines IL-4, IL-10 and TGF- β from T cells. Stimulation of macrophages with bacterial products such as LPS (TLR2 agonist) induces high IL-12 production and low IL-10 production (D'Andrea, Aste-Amezaga et al. 1993; Seki, Tsutsui et al. 2001). Co-ligation of TLR2 and Fc γ RIII abrogates IL-12 production and increases IL-10 production (D'Andrea, Ma et al. 1995; Gerber and Mosser 2001). Fc γ RIII ligation on macrophages thus seems to activate an anti-inflammatory cell program that has a dampening effect on TLR-mediated pro-inflammatory signals, possibly mediating inflammation.

Based on what we know of estrogen's immunomodulatory role in the FRT, it is possible to speculate that E2 plays a similar immunomodulatory role in helping to shape innate immunity during systemic infection as well as to shape adaptive immune activation in response to vaccination. Our results so far imply that ovariectomy consistently up-regulates TLR2, Fc γ RI and Fc γ RIIIA gene expression, and that E2 treatment results in down-regulation of these genes in macrophages. Furthermore, our data shows that the extent of modulation of gene expression in response to CFP may be vaccine dose-dependent. The data obtained from this study will provide some insight on how hormones, particularly estrogen, modulate macrophage gene expression which may influence both the innate and adaptive immune response to *M. tuberculosis*. Studies which address the interactions of the immune-endocrine systems in animal models are scarce. These studies are important in order to gain a better understanding of the complex interactions that determine the immune system's response to stressors (e.g. bacterial aggression, vaccination,

etc.) and how certain response may be modulated to either enhance or suppress a given response for clinical purposes such as disease treatment or vaccine development.

CHAPTER 6

Conclusions

There are many hormonal factors that collectively result in indirect modulation of the immune response. These factors include those related to the production of estrogen, progesterone, testosterone and their metabolites. Most of the research results currently available have implied an immunosuppressive effect of estrogen. Pregnancy, a state of elevated estrogen and progesterone, is associated with immunosuppression (Thong, Steele et al. 1973; Kovacs, Messingham et al. 2002). However, it must be considered that in *in vivo* models, these results may reflect a net effect of more complicated pathways. The influence of sex hormones on immune responses has been studied by the administration or blockade of various hormones and their metabolites. Results have uncovered important pro- and anti- inflammatory actions for sex hormones. For example, estradiol is metabolized to several hydroxyestrogens (Mueck, Seeger et al. 2002), and 4- and 16 α -hydroxyestrogens are considered to be pro-inflammatory, while 2-hydroxyestrogens are considered to be anti-inflammatory (Janele, Lang et al. 2006). Progesterone is also considered to be anti-inflammatory under some circumstances, such as during pregnancy, where along with estrogen, contributes to fetal tolerance. Within the context of this complicated system, we have studied the effects of estrogen on the immune response using an *in vivo* mouse model.

We have shown that estrogen acts indirectly and differentially on various components of the immune system. Administration of proestrus levels of estrogen to ovariectomized mice results in maximal vaccine-specific antibody responses *in vivo*, implying a net activating effect on B cell

function. A similar effect was seen in intact mice producing physiological levels of estrogen, where their antibody responses were higher than those of ovariectomized mice, although this may or may not be statistically significant, depending on the route of vaccine administration and the type of vaccine formulation administered. Similar patterns of responses were seen when examining the predominant T helper type response, where estrogen-replaced ovariectomized mice produced maximal levels of vaccine-specific IgG1, indicating that estrogen imposes signals that favor a predominant T_H2 response, further confirming estrogen's activating effect on B cell function.

While specific antibody responses are important indicators of induction of immunity by vaccination, there are several other cell types that are critically involved in the development of this response. Macrophages lie at the crossroads of innate and adaptive immunity by virtue of their many functions and signaling abilities. Thus, alterations in macrophage function can greatly influence immune responses. We have shown that macrophage genes involved in innate and adaptive immune function are differentially expressed depending on estrogen levels. Toll-like receptor 2 (TLR2) is important in recognition and signaling in response to gram-positive and gram-negative pathogen-derived molecules in the microenvironment as a first line of defense. Our results show that, in the absence of estrogen, macrophage TLR2 gene expression is greatly enhanced, whereas in the presence of proestrus levels of estrogen, TLR2 gene expression is greatly reduced. Furthermore, we found a strong sex-based correlation, where macrophages isolated from male mice expressed significantly higher levels of TLR2.

Another critical function of macrophages is to bind IgG molecules through surface receptors known as Fc γ R. Upon binding, immune complex internalization results in signaling and antigen presentation of antigenic epitopes to naïve T cells and therefore activation of the cellular immune response. Fc γ R knockout mice display compromised cellular and humoral immune responses. We have shown that in the absence of estrogen, there is an increase in Fc γ RI and Fc γ RIIIA gene expression, whereas there is a reduction in expression of these genes in the presence of proestrus estrogen levels. These genes are involved in signaling events that activate the immune system and are therefore referred to as activating Fc receptors. Interestingly, we saw different patterns of expression of Fc γ RIIB, which is involved in immune regulation by inhibiting overactivation of the immune system, and is thus referred to as the inhibiting Fc receptor. We showed that Fc γ RIIB gene expression is not influenced by estrogen. Similar to TLR2, we identified a strong correlation between sex and Fc γ RI and Fc γ RIIIA gene expression where male mice expressed higher levels compared to females. Consistent with the effects of estrogen, Fc γ RIIB gene expression was not different with respect to sex.

It is important to note that Fc γ RIIB is the only Fc γ R expressed on B cells and therefore exerts inhibitory signals when co-ligated with the BCR. Fc γ RIIB knockout mice display a phenotype consistent with increased polyclonal Ig production and also spontaneously develop autoantibodies in susceptible mouse strains. The finding that estrogen enhances B cell function by increasing vaccine-specific antibody responses and promotes a strong T_H2 response is consistent with the modulation of Fc γ R gene expression in macrophages. Estrogen decreased activating Fc γ R gene expression but did not interfere with Fc γ RIIB gene expression. Therefore, estrogen may enhance the humoral immune response not only at the level of B cells, but also

perhaps indirectly at the level of macrophages. Further studies should be carried out to assess the effects of estrogen on surface expression of these receptors.

Public Health Implications

As we have discussed, estrogen regulates a plethora of biological activities. The implications of this are important, particularly from a clinical standpoint. The ability to acutely change the outcome of autoimmunity and/or improve responses to vaccination has eluded investigators for over a century. Understanding the mechanisms that allow estrogen to accomplish rapid and dramatic effects will allow the development of refined therapeutics that may or may not require the broad-ranging effects of a sex steroid such as estrogen. This goal of this section is to discuss some of the physiological factors that influence the hormonal balance in humans and animals with an emphasis on the application of estrogen as an immunomodulator as well the need to consider these factors within the context of immunological research.

Infectious diseases remain an important cause of morbidity and mortality in the elderly population, who are more susceptible to severe infections and frequently respond poorly to vaccination. This is partially a consequence of immunosenescence, or the functional deterioration of the immune system with age (Grubeck-Loebenstien and Wick 2002). Some of the effects of immunosenescence on immune function as it relates to vaccine responses have been identified. These age-related alterations may partially result from hormonal changes in both males and females.

During menopause in females, ovarian function declines, resulting in serum estradiol levels lower than in younger females (Chahal and Drake 2007). In males, there is a conversion of dihydrotestosterone to either testosterone or estradiol. The pathway resulting in testosterone declines is potent in young males, but declines with age, whereas the pathway that results in estradiol is maintained with age. Thus, males are not excluded from the potential age-related changes of sex hormones, particularly estrogen.

A large percentage of post-menopausal females is under hormone replacement therapy (HRT) in the United States. The importance of understanding the effects of estrogen and other reproductive hormones on the body is exemplified in the results from the Women's Health Initiative (WHI) study. This study consisted of two parallel randomized, double-blind, placebo controlled clinical trials of hormone therapy to determine whether conjugated equine estrogen alone or in combination with progestin would reduce cardiovascular events in healthy post-menopausal women. The combined component of the WHI study was prematurely halted, as women taking this treatment were determined to have increased risk of breast cancer as well as heart disease and stroke (Rossouw, Anderson et al. 2002). The unopposed estrogen treatment was continued to find that similar results were discovered in terms of heart disease and stroke, but a striking decrease was seen with the incidence of breast cancer (Anderson, Limacher et al. 2004). Thus, there is a balance between the beneficial and harmful effects of estrogen on health.

Pregnancy is another reproductive phenomenon in which hormonal changes dictate immune status for both the mother and fetus. The maintenance of pregnancy requires suppression of the maternal immune system which would naturally recognize the developing fetus as a foreign

allograft and mount a T_H1 regulated cytotoxic immune response against it (Buyon 1998). During pregnancy, many soluble factors are produced by the placenta which collectively skew maternal immune regulation towards a protective T_H2 phenotype. Therefore, based on the relative contributions of sex hormones, vaccine responses during pregnancy may be greatly influenced.

The maternal immune status resulting from hormonal regulation also influences the developing fetal immune system. Indeed, all newborns initially have an immunological milieu based on a T_H2 phenotype. Vaccination during the neonatal period must therefore overcome the dual challenge of the inhibitory effect of maternally derived antibody and the natural T_H2 regulatory environment. Thus, it could be possible to redirect the neonatal immune response towards an appropriate T_H1 regulated reaction that confers protection. Understanding the contribution of maternal and fetal hormones to immune status can aid in strategies to induce immune responses with balanced T_H1 and T_H2 regulation in neonates (Morein, Blomqvist et al. 2007).

As mentioned previously, females are considerably more susceptible to developing autoimmune diseases than males, and their symptoms are more severe than in males. Physiological changes in estrogen contribute to this phenomenon. Animal models of autoimmune diseases have taught us that low estrogen concentrations lead to exacerbation of symptoms in certain models (rheumatoid arthritis and multiple sclerosis), while low estrogen ameliorates symptoms in other models (systemic lupus erythematosus) (Da Silva 1999). Epidemiological evidence in humans confirms this effect, where during pregnancy (high levels of estrogen), and post-partum and at menopause (low levels of estrogen), onset and symptoms follow the same patterns. It is thought that estrogen-dependent alteration of macrophage gene expression is one factor that contributes to

autoimmune disease states, possibly through modulation of receptor gene expression and function. These events are expected to have significant manifestations in immune responses in these patients.

From our studies and others, there is currently a substantial amount of evidence implicating estrogen as an immunomodulator, and based on such evidence, several applications should be considered. The use of laboratory animals in immunological research is an invaluable tool for human disease models. However, much of the studies that are conducted do not account for possible gender and hormonal influences on the experimental immunological endpoints. Besides just the direct effects of estrogen or sex hormones on immune parameters, it must also be considered that conditions of stress may also influence immunity in humans and animal models of research. As previously mentioned, the HPA axis, when activated, results in glucocorticoid release. It is found that there is a gender dimorphism in the HPA axis itself, where females produce higher levels of glucocorticoids in response to activation by immune aggression (Da Silva 1999). Removal of testosterone in male mice results in higher production of glucocorticoids, further confirming this effect. An imbalance in this pathway due to any number of factors including that of sex hormones can have important consequences. On one hand, excessive glucocorticoid production may lead to immunosuppression resulting in increased infection or cancer. On the other hand, insufficient glucocorticoid production may lead to increased vulnerability to autoimmune, inflammatory, or allergic disorders. Therefore, it is important to consider environmental and stress factors that may strongly influence immunological outcomes when conducting animal studies.

One of the primary aims of vaccine research is to provide scientific evidence leading to development of an agent that is capable of sufficiently stimulating the immune response in an effort to protect against infectious disease. Therefore, in vaccine research it is imperative to consider that the vaccine being tested may affect certain target populations and subpopulations differently. To this end, biomedical research involving human subjects should be carried out in a manner sufficient to elicit data from individuals from diverse groups and, in the case of clinical trials, to examine differential effects on such groups. In March of 1994, NIH issued a policy on the inclusion of women and minorities as subjects in clinical research (<http://grants.nih.gov/grants/guide/notice-files/not94-100.html>). Given the hormonal fluctuations in women and their resulting variations in immune status, it may be necessary to give increased attention to the division of subpopulations of women, especially at the Phase III clinical trial stage. Based on what we know of the effects of estrogen in particular, appropriate groups of women subjects should be included based on hormonal status.

Hormonal effects on classical endocrine target organs such as the female reproductive tract, mammary gland, ovary, and neuroendocrine system have been thoroughly studied, with significant advancements in our understanding of estrogen actions and disease conditions. Knowledge of the effects of estrogen in nonclassical endocrine organ systems, particularly the immune system, arising from epidemiological and clinical data, is only now becoming available based on the development and use of knock-out or transgenic animal models for the study of both estrogen and ER activities. By expanding this field of research, it is reasonable to expect that several important advancements will be made in the areas of vaccination against infectious disease as well as treatment of immune disorders.

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