AOP ID and Title:

SNAPSHOT

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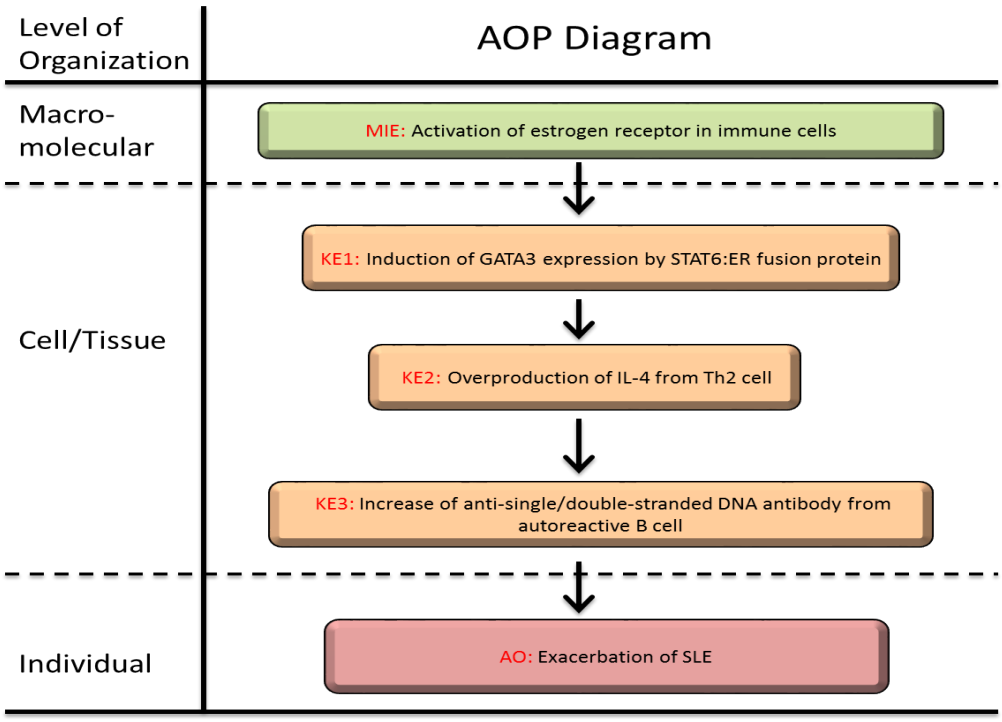
AOP 314: Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus

Short Title: Exacerbation of SLE by activation of estrogen receptor

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.73	Included in OECD Work Plan

Abstract

This AOP describes the linkage between the activation of estrogen receptor (ER)α and the exacerbation of the autoimmune disease systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition.

Estrogen Receptors (ERs), ERα and ERβ, are a group of proteins that are activated by the steroid hormone estrogen and are widely expressed in most tissue types, including most immune cells. ERs can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A is an example of a xenoestrogen that is considered an endocrine disrupting compound (EDC).

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Binding of ER in immune cells by a xenoestrogen or endogenous ER marks the molecular initiating event (MIE), which results in induction of GATA3 expression (KE1).

One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper2 (Th2) activity. Hyperactivation of ERα skew the immune system from a T helper 1 (Th1) to a Th2 profile and exacerbates autoimmune diseases and allergic diseases.

Complexes formed by the binding of ERα to stressors such as estrogen or EDC transport into cell nuclei, where they activate the transcription of specific genes. Excessive ERα-activation promotes the differentiation of naive CD4+ T cells into mature Th2 cells. This pathway leads to the overproduction of the cytokine interleukin-4 (IL-4) from Th2 cells and anti-single/double-stranded DNA antibody from autoreactive B cell are increased, which results in the adverse outcome of exacerbated SLE.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for activation of ERα in immune cells leading to exacerbated SLE.

Background

It has long been appreciated that most autoimmune disorders are characterized by increased prevalence in females, suggesting a potential role for sex hormones (estrogen) in the etiology of autoimmunity. ERs are involved in a wide range of physiological function. Women generally exhibit a stronger response to a variety of antigens including ER ligands than men, which is perhaps one reason that they are more prone to develop autoimmune and allergic diseases such as SLE in greater severity than men. This AOP could be helpful to assess the type of Th2 dominant autoimmune disorders

Humans and mammals have two ligand-activated transcription factors that bind estrogen, encoded by separate genes, estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ) (Maria, B. 2015). The estrogen receptors are composed of several domains important for hormone binding, DNA binding, dimer formation, and activation of transcription (Green S. 1986, Kumar V. 1986, Warnmark A. 2003). The ERs' expression patterns and functions vary in a receptor subtype, cell- and tissue-specific manner. In the adult human, large-scale sequencing approaches show that ERα mRNA is detected in numerous human tissues, with the highest levels in the uterus, liver, ovary, muscle, mammary gland, pituitary gland, adrenal gland, spleen and heart, and at lower levels in the prostate, testis, adipose tissue, thyroid gland, lymph nodes and spleen (Fagerberg L. 2014, Sayers EW. 2012) (www.ncbi.nlm.nih.gov/UniGene). In the same data sets, human ERβ mRNA is primarily detected in the lung and testis. There is increased ERα and decreased ERβ mRNA expression in PBMCs of SLE patients (Inui A. 2007). Although ERs are widely expressed in most tissue types, including most immune cells, this AOP mainly addresses hyperactivation of ERα in immune cells.

The effects of ERα signaling on T cells appear to be estrogen-dose dependent, i.e., low doses of estrogen stimulate a Th1 response, but higher doses promote a Th2 response (Priyanka HP. 2013). This AOP describes events occurring when high levels of estrogen shift the Th1/Th2 balance toward increased Th2 activity

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1710	Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)	Activation of estrogen receptor
	KE	1711	Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)	Induction of GATA3 expression
	KE	1712	Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)	Overproduction of IL-4
	KE	1713	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)	Increase of autoantibody production

Sequence	Type	Event ID	Title	Short name
	AO	1714	Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)	Exacerbation of SLE

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells (https://aopwiki.org/relationships/2020)	adjacent	Induction of GATA3 expression by STAT6:ER fusion protein	Moderate	Moderate
Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/relationships/2021)	adjacent	Overproduction of IL-4 from Th2 cell	Moderate	Moderate
Overproduction of IL-4 from Th2 cell (https://aopwiki.org/relationships/2022)	adjacent	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell	Moderate	Moderate
Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/relationships/2023)	adjacent	Exacerbation of systemic lupus erythematosus	Moderate	Moderate

Stressors

Name	Evidence
Estrogen	High
Bisphenol A	Moderate

Overall Assessment of the AOP

The immune system is the most complex and sophisticated in the body's defense mechanisms . Estrogen plays a role in controlling the immune balance. Hyperactivation of ERα can skew the immune system from a Th1 to a Th2 profile. This Th1/Th2 shift is one of the most important immunologic changes during gestation and occurs due to a progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress Th1-mediated responses and stimulate Th2-mediated responses (Doria A. 2006). Incidence of flare in patients with SLE is increased during pregnancy and within the 3-months postpartum (Amanda E. 2018). Thus, ERα activation can potentially induce immunoactivation-derived adverse outcomes, one effect of which could be exacerbation of SLE. The present AOP focused on ERα activation-induced exacerbation of SLE.

In general, ERα is activated when bound to a stressor, which subsequently binds to estrogen response elements (EREs) to transactivate or to suppress specific target genes. In naive CD4+ T cells, T cell expansion shifts toward a Th2 phenotype that produces Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, thereby increasing antibody production from autoantibody-producing B cells. We have identified a number of key events (KE) along this pathway and used these key event relationships (KER) to create an AOP that describes the activation of ERα leading to exacerbation of SLE.

Ordinary estrogen levels in women are 20-30 pg/mL during diestrus, 100-200 pg/mL during estrus, and 5000-10000 pg/mL during pregnancy (Offner H. 2000). While BPA binds in some assays with less than 2000-fold affinity compared to the binding of estradiol to estrogen receptors, it still has dramatic effects (Krishnan AV. 1993). Since each KE is quantifiable and shows similar dose responses with the stressors in vitro, the activation of ER leading to exacerbation of SLE comprise a suitable AOP. Additionally, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose response of adjacent KE.

Since ERα expresses in the cells of a vast variety of (vertebrate) species (Maria B. 2015) and there is common functionality in the immune systems of at least humans and mice, this AOP might be applicable to many mammal species, including humans and rodents.

Essentiality of KEs – what would be good is to have a table listing references that have demonstrated occurrence of individual KEs and their relationship with the AO.

Evidence assessment – here listing knockout or overexpression studies that intervene with a KE to show its essentiality to the AO

Quantitative assessment – if you have this informaiton

[Otsubo2]We will reconsider it and revise later.

[SH3]It seems like KE1 is not needed as it is not described much.

[Otsubo4]We want to discuss about it in WebEX meeting.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP describes the activation of ERα leading to exacerbation of SLE is dependent on estrogen level, which means it varies with life stage, sex, and age. SLE frequently develops or progresses when sympathoadrenomedullary and gonadal hormone levels are altered during pregnancy, the postpartum period, or menopause as well as during exposure to estrogen and includes the risk of preeclampsia or premature birth (Wilder RL. 1999, Whitelaw DA. 2008). Women using oral contraceptives that contain estrogen or undergoing hormone replacement therapy are susceptible to major flare ups and exacerbation of the disease (Whitelaw DA. 2007).

Since stressor-induced outcomes in humans are mimicked by similar responses in rodents, Th2 dominant conditions induced by activation of ERα is considered likely to occur in a variety of mammalian species.

Essentiality of the Key Events

Stressor , MIE and later events: ER knock out (KO) mice

It has been determined in a murine model of SLE that ERα is required for disease progression and that ERα deficiency impedes the course of the disease (Bynote KK. 2008).

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. Both NZB and NZW display limited autoimmunity, while NZB/W F1 hybrids develop severe lupus-like phenotypes comparable to that of lupus patients. SLE in the NZB/W F1 strain is strongly biased toward females, and this is at least in part due to estrogen levels. Indeed, ovariectomy of NZB/W F1 mice not only delayed onset of the disease but also decreased autoantibody titer. Meanwhile, restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cellsDNA-specific B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011).

In females of the lupus-prone NZB/NZW F1 strain, disruption of estrogen receptor-α (ERα or Esr1) both attenuated glomerulonephritis and increased survival. ERα deficiency also retarded development of anti-histone/DNA antibodies, suggesting that ERα promotes loss of immunologic tolerance. The presence of many autoantibodies is a hallmark of SLE. In particular, autoantibodies directed to double-stranded DNA (dsDNA) are characteristic (Isenberg DA. 2007). ERα deficiency in NZB/NZW F1 males increased survival and reduced anti-dsDNA antibodies, suggesting that ERα also modulates lupus in males (Bynote KK. 2008).

KE1 and later events: Stat6 KO mice

CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under null Th cell (ThN) conditions with added with IL-4 (Zhu J. 2001)

KE1 and later events: GATA3 KO mice

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells. Th2 cells from both knockout animals showed reduction in IL-4, IL-5, IL-13, and IL-10 production. Conversely, IFN-γ production was increased even under Th2 conditions (Zhu J. 2004, Pai SY. 2004).

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Appendix 1

List of MIEs in this AOP

Event: 1710: Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)

Short Name: Activation of estrogen receptor

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	MolecularInitiatingEvent

Stressors

Name
Estrogen
Bisphenol A

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

ERα is mainly expressed in uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue (Dahlman-Wright K. 2006). ERs is widely expressed in most tissue types including most immune cells (Couse JF. 1997). ERα and ERβ show a high degree of similarity when compered at the amino acid level (Dahlman-Wright K. 2006). Interspecies sequence identities for the entire ERα receptor are 88.5% (human-mouse), 87.5% (human-rat), and 97.5% (mouse-rat). For the ligand binding domain (ERα-LBD) alone, the interspecies sequence identities are 95.5% (human-mouse), 95.1% (human-rat), and 99.2% (mouse-rat) (White R. 1987). ERα is found in female reproductive organs, yet is robustly expressed in kidney, liver, heart, and lungs in males and females, as well as on most immune cells (Chelsea C. 2017).

Key Event Description

Estrogen receptor alpha (ERα) was discovered in the late 1960s and was cloned and characterized in 1985 (Melissa C. 2011). ERα and ERβ show a high degree of similarity when compared at the amino acid level (Dahlman-Wright K. 2006). 17β-estradiol (E2) activates ERα and ERβ with the same affinity although they share only 56% similarity in their ligand binding domains (Monroe DG. 2005, Papoutsi Z. 2009). The hormone binding domain of the estrogen receptor is required not only for binding estradiol but also to form stable homodimers of the protein and mediate transcriptional activation by the receptor. A direct genomic interaction occurs between the estrogen receptor (ER) ligand complex and specific sequences of DNA known as estrogen response elements (ERE). (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997). Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1 domain, located in the N-terminal domain of the receptor protein, and the ligand-dependent AF-2 domain, located in the C-terminal domain of the receptor protein (Delaunay F. 2000). In addition to above classical mechanism, ERα is also able to play roles both in ER binding and transcriptional activation; phosphorylation of ER and other proteins involved in transcriptional activation with cellular amounts of coactivators and adaptor proteins (Carolyn MK. 2001).

ERs are expressed in a variety of immunocompetent cells, including CD4+ (Th1, Th2, Th17, and Tregs) and CD8+ cells and macrophages (Salem ML. 2004, Robinson DP. 2014). One recent study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in CD4+ T cells than B cells (Melissa C. 2011).

How it is Measured or Detected

Recombinant human estrogen receptor hormone-binding domain (HBD) fragment is isolated from Escherichia coli. Purified HBD peptide is assayed for their ability to bind estradiol, [3H] estradiol binding using low concentrations (0.15 nM), by Radioreceptor Assay. Moreover HBD dimer dissociation is measured using size exclusion chromatography (Brandt ME. 1997).

On the other hand, a conditionally active form of STAT (the signal transducers and activator of transcription) 6 by fusing the HBD of a modified form of the mouse estrogen receptor (ER) gene is prepared as STAT6-ER fusion protein (STAT6:ER). 4-Hydroxytamoxifen (4-HT), estrogen analogue, (Research Biochemicals Institute, Natick, MA) was used to activate STAT6 fusion protein. M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, are used nuclear extracts and electrophoretic mobility shift assay (EMSA) with 1 μM 4HT. STAT6:ER DNA-binding activity is strongly and rapidly (within 1 hr) induced after addition of 4HT to these cells. BA/F3 cells prepared as the same manner are stimulated with 1 μM 4HT for 24 h at 37°C. The cells were harvested and assayed for luciferase activities using a Luciferase Assay Kit (Promega, Madison, WI). (Kamogawa et al. 1998).

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List of Key Events in the AOP

Event: 1711: Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)

Short Name: Induction of GATA3 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Involvement of GATA3 and STAT6 in Th2 cell development through ER is common in humans, rodents, and other mammalian species (Ho IC. 2009). A constitutively activated form of Stat6 introduced into CD4 T cells resulted in both Th2 differentiation and enhanced cell expansion. Stat6 is not only necessary but also sufficient to drive IL-4-mediated Th2 differentiation and cell expansion in naive CD4 T cells (Zhu J. 2001). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under ThN conditions with added with IL-4 (Zhu J. 2001).

Key Event Description

Transcription factors are critical for Th cell differentiation and cytokine production. Cell fate determination in each lineage requires at least two types of transcription factors: the master regulators as well as the signal transducers and activator of transcription (STAT) proteins (Zhu J. 2010). The ability of STAT6: ER to induce a Th2 phenotype correlates with the induction of GATA-3 mRNA expression. GATA3 is the Th2 master regulator (Zhu J 2010, Sung-Yun. 2004, Zhu J. 2004, Zheng W. 1997, Zhang DH. 1997), but it also plays important roles in multiple steps of CD4 T cell development (Ho IC. 2009).

How it is Measured or Detected

Purified naive T cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21. GATA-3 mRNAs can be measured using RNase protection assay in developing Th1 cells. RNase protection assay was performed with RiboQuant multiprobe kit (PharMingen) following the manufacturer's method using GATA-3. Stat6:ER Th1 cells expressed significant amounts of both GATA-3 mRNAs in a 4-HT-dependent manner. (Kurata H. 1999, Zhu J. 2001).

Constitutively activated Stat6 (Stat6VT) is primed under null Th cell (ThN) conditions in the absence of human (h)IL-4. The expression level of Gata3 in this primed cells are checked by RT-PCR (Zhu J. 2001).

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Event: 1712: Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)

Short Name: Overproduction of IL-4

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T-helper 2 cell

Organ term

Organ term
immune system

Key Event Description

Th2 cells produce IL-4, which stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL4 also plays an important role in the development of certain immune disorders, particularly allergies and some autoimmune diseases and especially when there is Th2 polarization.

How it is Measured or Detected

Purified naive T cells were activated and infected with RV-Stat6:ER. The cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 and the cells were analyzed for cytokine (IL-4) expression by flow cytometer analysis of intracellular cytokine production or cytokine ELISA (Kurata H. 1999, Zhu J. 2001).

Single-cell suspensions of lymph nodes removed from BALB/c mice 7 days after priming with KLH absorbed to aluminium hydroxide adjuvant in the footpads, were prepared and cultured in vitro with KLH in the absence or presence of either BPA (0.1, 1, 10, 50 and/or 100 μM) or NP. After 4 days, the levels of IL-4 and IFN-γ in the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) and mRNA levels of IL-4, IL-6 and IL-10 in the cells were assayed by reverse transcription–polymerase chain reaction (RT–PCR) (Lee MH. 2003). To evaluate the effects of exposure to BPA in adulthood, male Leishmania major– susceptible BALB/c and –resistant C57BL/6 mice were subcutaneously injected with BPA (0.625, 1.25, 2.5 and 5 μmol) dissolved in corn oil 1 week before being infected with L. major. A single cell suspension containing splenocytes from each mouse was incubated in 24-well tissue-culture plates in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were stimulated with L. major antigen (3 μg/mL) during the cultivation. Culture supernatants were collected 48 hr later. Concentrations of IL-4, IL-10, IL-13, and IFN-γ in culture supernatants were determined using CBA kits (Huimin Y. 2008).

Th2 cell-related cytokine (IL-4 and -10) in BPA (50 μM)-stimulated primary cultured mouse lymphocytes were evaluated using immunoblot analysis and reverse-transcription polymerase chain reaction (RT-PCR) (Lee et al. 2010).

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Event: 1713: Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)

Short Name: Increase of autoantibody production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
B cell

Organ term

Organ term
immune system

Key Event Description

In the development of T-cell dependent antibody producing cells, the interaction between IL-4 and its receptor delivers the first signal for switching to IgE. IL-4 produced by Th2 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The engagement of CD40 on B cells by CD154 (CD40L) expressed on T cells and DC provides the second signal required for switching to IgE.

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei et al. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei et al. 2009).

Bisphenol-A (BPA) as well as E2 and DES enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of EDs was more prominent on aged BWF1 mice developing lupus nephritis. B1 cells from aged mice exhibited increased expression of ERα mRNA compared to young mice (Yurino H. 2004).

How it is Measured or Detected

For the detection of anti-DNA antibodies in serum of female NZB/W F1 mice administrated of the estrogen antagonist tamoxifen, enzyme-linked immunosorbent assay (ELISA) was carried out. For the quantitated of total B cells and CD5+B cells expression in spleen and in peritoneal exudates were analyzed with fluorescence activated cell sorting (FACScan) (Wu et al. 2000). For the B cell subset analysis (including immature (transitional T1 and T2) and mature (MZ and follicular)) in BALB/c R4Ag-gamma 2b transgenic mice administrated the tamoxifen were performed with FACScan (Peeva et al. 2005).

In another study, used ERα deficiency in NZB/W F1 mice, autoantibody (anti-dsDNA antibodies) development and concentration was assessed by ELISA using serum isolated from blood collected monthlyvia (Bynote et al. 2008).

Using female NZB/WF1 mice, silastic implants containing the powdered form of endocrine disruptors were placed subcutaneously on the back of ovariectomized mice, and 3 to 4 months blood samples were collected peritoneal. 4 months after implantation, peritoneal lavage cells and splenic cells were obtained from mice. Anti-DNA antibody was measured in ELISA using ssDNA for the culture supernatant of and dsDNA for the serum. To examine the effect of EDs on autoantibody production by B1 cells, a PFC assay using autologous bromelain-treated erythrocytes (Br-RBC) was conducted. To evaluate autoantibody (IgG) production including plaque forming cell (PFC) assay for anti-RBC Ab. It has been reported

that B1 cells produce autoantibody against phosphatidylcholine expressed on bromelain-treated red blood cells (Br-RBC) using PFC assay (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E2: 100 nM, DES: 100 nM, BPA: 1 μM) for 4 days. The amount of total IgM and IgM anti-DNA Ab in the culture supernatant was measured by ELISA. Expression level of ERα and ERβ genes in B

cells was examined by RT-PCR and quantitative real-time PCR analysis (Yurino H. 2004).

For the investigate the in vitro effects of 17β-estradiol (E2) on spontaneous immunoglobulin production by human PBMCs, PBMCs from healthy human volunteers were cultured with E2. Levels of IgG and IgM and cytokine activity were measured by ELISA. Proliferation was determined by [3H]-thymidine uptake. The cell viability was assessed by a trypan blue exclusion test (Kanda et al. 1999).

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List of Adverse Outcomes in this AOP

Event: 1714: Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)

Short Name: Exacerbation of SLE

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Exacerbation of SLE is common in humans and rodents, and is considered likely to occur in other animal species, as well. SLE is an autoimmune disease that occurs primarily in women (9:1 compared to men) (Rider et al., 2001). SLE is an autoimmune disease that affects predominantly women during reproductive years, and its evolution is altered by hormonal events such as menses, menopause, and especially pregnancy (Luis et al., 2014). The incidence of SLE is markedly increased in females of child-bearing age (Grainne et al., 2013). Th1/Th2 shift is one of the most important immunologic changes during gestation. It is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses. For this reason, Th1-mediated diseases, such as rheumatoid arthritis, tend to improve, while Th2-mediated diseases, such as systemic lupus erythematosus (SLE) tend to worsen during pregnancy (Doria et al., 2006).

Key Event Description

SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition. Epstein– Barr virus (EBV) has been identified as a possible factor in the development of lupus. Over 100 drugs have been reported to cause drug-induced lupus (DIL), including a number of the newer biologics and antiviral agents. Although the pathogenesis of DIL is not well understood, these drugs may alter gene expression in CD4+ T cells by inhibiting DNA methylation and induce over-expression of lymphocyte function-associated antigen 1, thus promoting autoreactivity. Generally, sunlight is the most obvious environmental factor that may exacerbate SLE. High estrogen levels and BPA-induced ER activation skewed T cells toward a Th2 phenotype, thereby inducing hyperactivity by B-cells, which leads to exacerbation of SLE. T cell dysfunction is a characteristic of SLE, which is also associated with high levels of autoantibodies (Crispin et al. 2010).

How it is Measured or Detected

Most of the mouse models of lupus produce autoantibodies and develop immune complex glomerulonephritis. For the disease onset, mice can monitor by proteinuria levels, body weights, blood urea nitrogen and appearance over time. Additionally, serum levels of anti-dsDNA, anti-glomerular antigens (GA), total IgG can measure by ELISA. (Gabriela et al., 2019, Yurino et. al.,2004, John et. al.,2008, Wang et. al.1996).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2020: Activation of estrogen receptor leads to Induction of GATA3 expression (https://aopwiki.org/relationships/2020)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

Stressors bind to the ERs in immune cells, a ligand-activated transcription factor that regulates transcription of target genes in the nucleus or located in or adjacent to the plasma membrane (Deroo BJ. 2006). ERα is a nuclear hormone transcription factor that classically binds ligand stressors estrogen or EDC, further stabilizing dimers that subsequently bind estrogen response elements to transactivate or suppress specific target genes.

Evidence Supporting this KER

Biological Plausibility

The GATA3 expression induced by TNF-α was enhanced in the presence of BPA. However, the T-bet expression did not change when tested at various culture conditions (Guo H. 2010, Uemura Y. 2008). Naive Th cells primed by BPA/TNF-α-matured DCs differentiated into Th2 cells with characteristically high IL-5/IFN-γ, IL-10/IFN-γ, and IL-13/IFN-γ ratios. However, the IFN-γ production was not affected at all, thus indicating that

Th2 bias was induced by enhanced Th2 cytokine production (Guo H. 2010, Uemura Y. 2008). Also, dendritic cells exposed to BPA (100 nM) and TNF-α produced high levels of IL-10 relative to IL-12, and this induced Th2 deviation (Liu Y. 2009).

Uncertainties and Inconsistencies
Dendritic cells exposed to human exposure-relevant concentrations of BPA (10-100 nM) preferentially skewed T cells toward a Th2 phenotype. Th cells were primed by BPA/TNF-α-DCs. The administration of 17β-estradiol enhanced the differentiation of dendritic cells and increased IFN-γ production by dendritic cells in C57BL/6 mice.

Quantitative Understanding of the Linkage

Response-response relationship
When estrogen levels are low, T cell expansion shifts toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and experimental autoimmune encephalomyelitis (EAE) rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Melissa, and Gary 2011). Low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol have been shown to increase Th1 T-cell development in vitro through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high doses (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE model (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Known modulating factors
The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney et al. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2021: Induction of GATA3 expression leads to Overproduction of IL-4 (https://aopwiki.org/relationships/2021)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

Th2 cells produce IL-4, IL-5, IL-10, and IL-13, meanwhile Th1 cells produce IL-12, TNF-α, and IFN-γ. During Th2 polarization, IL-4 produced by Th2 cell. IL-12 plays a central role in promoting the differentiation of naive CD4+ T cells into mature Th1 effector cells. Secretion of IL-10 from Th2 has been suggested to downregulate the DC-derived IL-12 production and lead to a Th2 differentiation (Aste-Amezaga M. 1998).

Evidence Supporting this KER

Biological Plausibility

IFN-γ is noticeably reduced in pregnant women compared with non-pregnant women or in response to high levels of estrogen (Kruse et al. 2000). Thus, pharmacological or pregnancy levels of estrogen may skew the immune system from a Th1 to a Th2 profile (Ebru et al. 2011). Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells from GATA-3-deficient (FF and FF cre) mice (Sung-Yun. 2004, Zhu J. 2004). Antigen-specific immune response is evaluated with lymphocyte from FF and FF cre mice injected with KLH, and cytokine production was measured by sandwich ELISA (Sung-Yun. 2004). Mouse lymphocytes stimulated with a massive amount of BPA (50 μM) were Th2 polarized, with prominent elevation of IL-4 as well as IL-10 (Lee MH. 2010). Similarly, BPA enhanced IL-4 production in antigen-activated T cells by ELISA or RT-PCR, although the concentrations of BPA that they utilized (10–50 μM) were high (Lee MH. 2003). In this experiment, IL-4 level is confirmed baseline when treated with anti-CD4 mAb. Exposure to BPA in adulthood mice promoted antigen-stimulated levels of IL-4, IL-10, and IL-13, but not IFN-γ (Huimin et al. 2008).

Empirical Evidence

The proliferation of Stat6:ER Th1 cells was enhanced in a dose-dependent manner on days 10 and 31 after polarization by [^{3}H]thymidine incorporation (the effective concentration of 4-HT was between 0.08 and 2 μM, and the toxic concentration was greater than 5 μM) (Kurata H. 1999, Zhu J. 2001).

Uncertainties and Inconsistencies

The essential transcription factors of Th2 are GATA-3 and STAT5. Activation of GATA-3 and STAT5 induce IL-4 production in naïve CD4 T cells. IL-4-mediated STAT6 activation promotes Th2 differentiation (Kaplan MH. 1996, Shimoda K. 1996, Takeda K. 1996).

Quantitative Understanding of the Linkage

When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

IL-4 may serve multiple roles in the development of lupus: it may enhance autoantibody production via its direct B-cell effects, protect against autoimmunity via its T-cell suppressor effect, or perpetuate tissue damage via its direct effects on target organs (Ram Raj Singh 2003).

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2022: Overproduction of IL-4 leads to Increase of autoantibody production (https://aopwiki.org/relationships/2022)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

The receptor for IL-4 is IL-4Rα, which expresses in B cells. Th2 cells secrete cytokines IL-4 that upregulate antibody formation via B cells. Naive B cells that have not yet encountered antigen express immunoglobulin M and immunoglobulin D on their surface. During an immune response, B cells can express different immunoglobulin heavy chain isotypes sharing the same variable–diversity–joining (VDJ) region. This isotype-switching recombination allows a B-cell clone to produce antibodies with the same specificity for antigens but with different effector functions. To switch to a particular isotype, a B cell needs two signals, one cytokine-dependent and the other CD40-dependent. In B cells, estrogen-mediated events could occur through the CD40/CD40L costimulatory pathway. Estrogen can also enhance differentiation of immature DCs into mature functional DCs and regulate the expression of cytokines and chemokines such as IL-6, IL-10, CXCL8, and CCL2 (Liu Y. 2009, Guo H. 2010). This increase the number of B cells producing autoantibodies.

Evidence Supporting this KER

Biological Plausibility
Lack of ERα, in either male or female mice, did not increase B cell precursors (Smithson G. 1998).

Estrogen upregulates CD40L on B and T cells from SLE patients (Desai-Mehta A. 1996, Li X. 2006), and CD40L expression on B cells is increased two-fold in SLE patients (Díaz-Alderete A. 2004). Whereas anti-CD40L antibodies downregulate CD86 expression on normal and SLE B lymphocytes, blockade of CD86 only diminishes anti-DNA antibody production by SLE B cells (Nagafuchi H. 2003). Moreover, mice overexpressing CD40L develop a lupus-like disease with high levels of antibodies to nuclear antigens, DNA, and histones, as well as glomerulonephritis (Higuchi T. 2002). It is possible that this estrogen modulated elevation in CD40/CD40L crosstalk as well as stimulation via CD86 synergizes in the exacerbation of SLE by promoting autoantibody secretion as well as activation of T cells (Karpuzoglu E. 2011). In a murine model of SLE, BPA increased the number of B cells producing autoantibodies, and IgM antibody secretion by B1 cells was augmented (Yurino et al. 2004).

Direct exposure of PBMCs from SLE patients to 17β-estradiol induces secretion of anti-dsDNA antibodies and enhances the secretion of Igs, in particular IgG (Kanda et al. 1999).

Empirical Evidence
CD23 on M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, is up-regulated with treatment1 μM 4HT for 16 hr (Kamogawa et al. 1998).

The production of IgA and IgG2a was increased in B cells from mice fed BPA (Goto et al. 2007). Similarly, in mice exposed prenatally to BPA and then immunized in adulthood with hen egg lysozyme (HEL), the anti-HEL IgG2a measured three weeks later was elevated (Yoshino et al. 2004). These Ig can be measured by ELISA. The administration of the estrogen antagonist tamoxifen diminishes anti-DNA antibody levels by ELISA as well as decreases percentages of total B cells and CD5+ B cells by FCM (Wu et al. 2000). Tamoxifen Blocks Estrogen-Induced B Cell Maturation but not survival (Peeva et al. 2005). ERα deficiency in (NZB×NZW) F1 female mice downregulated levels of anti-dsDNA IgG antibodies, and the absence of ERα In (NZB×NZW) F1 males resulted in decreased anti-dsDNA antibodies (Bynote et al. 2008).

Quantitative Understanding of the Linkage

Response-response relationship
When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Time-scale
The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

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Relationship: 2023: Increase of autoantibody production leads to Exacerbation of SLE (https://aopwiki.org/relationships/2023)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

SLE patients appear to produce significant amounts of the anti-double-stranded DNA (anti-dsDNA) autoantibodies that cause the disease. Activation of autoantibody-producing B cells only serves to exacerbate that condition.

Evidence Supporting this KER

Biological Plausibility

SLE has been seen to flare up during pregnancy (Petri et al., 1991). Female MRL/lpr mice that developed lymphadenopathy and a lupus-like disease also exhibited a 50% higher mortality rate than males at 5 months of age (Carlsten H. 1992).

In (NZB×NZW) F1 mice too, females develop signs of SLE several months before males, with severe autoimmune hemolytic anemia, glomerulonephritis, and autoantibodies to single-stranded DNA, doublestranded DNA, and histones. In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Murine lupus models such as NZB×NZW F1 (NZB/W F1), NZB.H-2bm12, NZB×SWR F1 (SNF1), MRL.lpr/lpr, and BXSB mice have led to a better understanding of the pathogenic mechanisms of lupus (Zhang DH. 1997, Pai SY. 2004). All of these species of mice develop immunoglobulin G (IgG) anti-dsDNA antibody, which is a characteristic of lupus, and die of uremia in early life. Among these murine lupus models, the natural course of NZB/W F1 mice is closer to human lupus than MRL.lpr/lpr and BXSB mice. The administration of the estrogen antagonist tamoxifen diminishes immune complex deposition in the kidneys and increases survival. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney (Wu et al. 2000).

In NZM female mice, ERα inactivation markedly prolonged life-span, lowered proteinuria, and ameliorated glomerulonephritis but resulted in higher serum anti-dsDNA antibody levels (Svenson JL. 2008).

Empirical Evidence

Estrogen enhances anti-double-stranded DNA antibody and IgG, IgM production by PBMCs. PBMCs or B cells were cultured for 7 days with E2 (10^{-8} mol/L). Theamounts of total IgG and IgM in the supernatants were measured by ELISA. Proliferative responses PBMCs or B cells were measured by [3H]-thymidine (Kanda N. 1999).

Quantitative Understanding of the Linkage

Response-response relationship

When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). Treatment with low serum levels (60–100 pg/mL or 0.26–0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney et al. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria, A., et al. 2006).

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