SEX HORMONES AND GENDER DIFFERENCES IN IMMUNE RESPONSES

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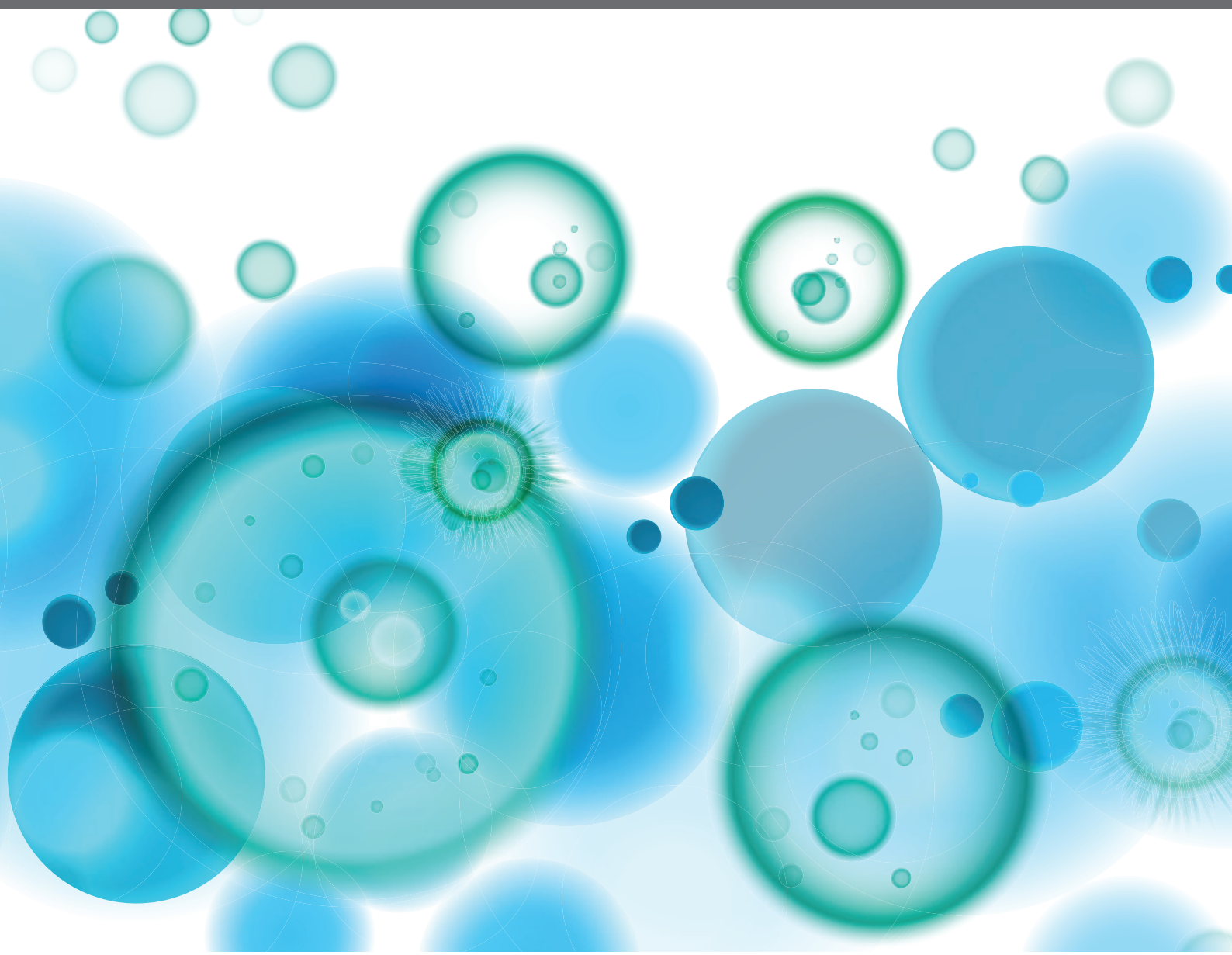

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SEX HORMONES AND GENDER DIFFERENCES IN IMMUNE RESPONSES

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Increasingly clear evidence points to the need to consider gender differences in human health. In this collection of papers, recent research that supports gender differences in the immune system are discussed.

We have loosely divided the eBook into two sections. The first section focuses on the role of steroid hormone interactions within the immune system, and their impact on autoimmune diseases, infection and allergy. This section contains comprehensive reviews and an opinion article about this topic. In the following section, original research articles revolve around the effects of the sex hormones on immune response. Two original manuscripts deal with the role of estrogen receptors in autoimmune diseases. Other two research articles discuss the role of the immune system during pregnancy. Finally, differences between males and females in infections are the topic of further two research articles.

We are confident this collection of papers will be important for exploring and developing a greater understanding of gender differences in human health and disease.

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EDITORIAL
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Editorial: Sex Hormones and Gender Differences in Immune Responses

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Editorial on the Research Topic

Sex Hormones and Gender Differences in Immune Responses

In general, females have stronger innate and adaptive (humoral and cellular) immune responses in comparison to males. The factors responsible for the stronger immune response in females than males may be due to biologic factors (i.e., sex differences, such as genetic and epigenetic factors, sex hormones) and to psychosocial factors (i.e., gender differences). Our aim in assembling this Research Topic was to highlight the current understanding of the role played by sex hormones (i.e., androgens, progesterone, prolactin, and estrogens) and their receptors in modulating the immune response. In addition, we wanted to highlight the possibility that sex differences could alter the susceptibility and/or the severity of autoimmune and infectious diseases. A better comprehension of sex hormone-immune response interactions could lead to innovative and readily available therapeutic interventions, such as hormone antagonists or agonists, as new approaches to manage immune-mediated diseases.

The collection is comprised of a series of reviews and original research papers underlining the role of sex hormones in immune response modulation as well as hormone influence on autoimmune diseases, infections and allergy. Borba et al., discuss the role of prolactin in immune system modulation and the involvement of prolactin in the pathogenesis and activity of several autoimmune disorders. The Authors describe the evidence for dopamine as an effective inhibitor of prolactin secretion and suggest that dopamine agonists could represent a promising novel therapy for autoimmune patients. Moulton summarizes a large body of evidence for estrogenic effects in the adaptive immune response in health and autoimmunity with an emphasis on systemic lupus erythematosus (SLE). Gubbels Bupp and Jorgensen provide a comprehensive review on the action of androgens, working through their receptors to dampen or alter immune responses. Androgens affect the onset of autoimmune diseases as well as disease progression. Gubbels Bupp et al. provide a timely and interesting review describing the age- and sex hormone-related changes to innate and adaptive immunity. Their review highlights the importance of age- and sex-associated changes in the immune system and the subsequent impact on the onset of autoimmunity, cancers, and the efficacy of vaccination and cancer immunotherapy. In an opinion article by Taneja, interactions among environmental factors (diet, infections, cigarette smoke) and sex hormones are postulated to influence immune responses. Bereshchenko et al. point to the importance of possible interactions between glucocorticoid and sex steroid receptors that could underpin the sexual disparity of autoimmune diseases. Additional research is necessary to investigate possible "cross talk" among steroid receptors to identify interacting signaling pathways that may be crucial in fully understanding the onset of autoimmune diseases and gender differences. Two reviews in the collection focus on sex hormones and viral infections.

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Kadel and Kovats discuss evidence that sex differences exist in both respiratory homeostasis and viral infections owing to differential regulation by sex hormones in innate immune cells in the lungs. Additional complications of sex differences in the respiratory system occur because of disparate influence of sex hormones on the proinflammatory/effector phase and/or the resolution/tissue repair phase in innate cells. These differences ultimately contribute to the host's ability to respond to respiratory viral infections. Ruggieri et al. discuss the effects of sex hormones on the immune system response to Hepatitis B and C virus infections. Included in their review is evidence for direct sex hormone influence on viral activity. Shah and Newcomb discuss data supporting differences in allergic responses between males and females. Fluctuations in sex hormones during puberty, menstruation, pregnancy, and menopause, may alter the symptoms and severity of asthma.

The second part of the collection includes original research articles that explore novel aspects of sex hormone action in immune responses.

Two research articles focus on estrogen receptors in immune modulation and their impact on autoimmune diseases. Rider et al. investigated cell signaling changes in human SLE T cells treated with estradiol and the estrogen receptor α antagonist, Fulvestrant, comparing the effects of blockading the action of estrogen receptor α in order to identify signaling pathways that could contribute to improved disease activity in women with SLE. The Authors identified alterations in several pathways including T helper cell differentiation, steroid receptor signaling, ubiquitination, and sumoylation. In their research article, Dupuis et al. provide new insight regarding the anti-inflammatory effects of the phytoestrogen silibinin. Silibin binds to estrogen receptor β in T lymphocytes from both female and male healthy subjects and patients with rheumatoid arthritis. Silibinin induces apoptosis, inhibits proliferation, and reduces expression of the pro-inflammatory cytokines IL-17 and TNF-α, suggesting a potential role for this phytoestrogen in rheumatoid arthritis management.

Adverse pregnancy outcome related to autoimmunity represents a hot topic in translational research. In this regard, Fredi et al. evaluated the risk factors for adverse pregnancy outcomes in patients with antiphospholipid antibodies positivity. The Authors observed maternal and fetal complications in some antiphospholipid antibodies—positive patients and a higher risk of adverse pregnancy outcome in patients with a previous thrombosis. Research on a similar topic by Truglia et al. focused at utilizing new and sensitive approaches to identify antiphospholipid antibodies in patients with obstetrical antiphospholipid syndrome who are negative when tested using conventional laboratory markers.

Sex differences in infection was the topic of two original research articles. Scalerandi et al., using a bacterial model of prostate inflammation, showed an intriguing effect of testosterone in promoting inefficient, anti-inflammatory neutrophils that prolonged bacterial inflammation, generating a pathogenic environment for several conditions. Celestino et al. observed that female mice are less susceptible than males to mouse-adapted influenza virus (A/PR8/H1N1). Their analysis of the underlying mechanism that contributes to the sex disparities suggested that the female mice generate higher total antioxidant power in their sera and lungs when compared with male mice.

Understanding how sex influences immunity is still in its infancy. However, recent evidence (1), including the papers of this collection, indicate that components of both innate and adaptive immunity are differently regulated in females and males. Sex differences contribute to differences in susceptibility and severity of immune-mediated and infectious diseases, and malignancies (1, 2). Sex hormones can affect different steps in immune processes. Thus, the complexity of endocrine-immune interaction represents a recurrent theme in the papers comprising this collection. Age-specific responses may also influence immune-hormone interactions. An integrated approach focused at analyzing the relationships among sex hormones, sex chromosomes and immune related genes is needed to better understand gender differences in immune response (3, 4).

We hope that this collection of primary research papers and review articles will prove useful to investigators interested in the current state-of-the-art research into sex hormones and immune responses.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol.* (2016) 16:626–38. doi: 10.1038/nri.2016.90
2. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. *J Immunol.* (2017) 198:1782–90. doi: 10.4049/jimmunol.1601166
3. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita.* (2016) 52:205–12. doi: 10.4415/ANN_16_02_12
4. Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. *Front Neuroendocrinol.* (2014) 35:347–69. doi: 10.1016/j.yfrne.2014.04.004

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Prolactin and Autoimmunity

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The great asymmetry of autoimmune diseases between genders represents one of the most enigmatic observations among the mosaic of autoimmunity. Sex hormones are believed to play a crucial role on this dimorphism. The higher prevalence of autoimmunity among women at childbearing ages, disease onset/relapses during pregnancy, and post-partum are some of the arguments that support this hypothesis. Certainly, motherhood represents one of the most remarkable challenges for the immune system, which not only has to allow for the conceptus, but also has to deal with complex endocrine alterations. Hormonal homeostasis is known to exert a crucial influence in achieving a competent and healthy immune system. Prolactin (PRL) has a bioactive function acting as a hormone and a cytokine. It interferes with immune system modulation, mainly inhibiting the negative selection of autoreactive B lymphocytes. Likewise, hyperprolactinemia has been described in relation to the pathogenesis and activity of several autoimmune disorders. Dopamine is an effective inhibitor of PRL secretion due to either a direct influence on the hypophysis or stimulation of postsynaptic dopamine receptors in the hypothalamus, arousing the release of the PRL inhibitory factor. Hence, dopamine agonists have proven to offer clinical benefits among autoimmune patients and represent a promising therapy to be explored. In this review, we attempt to provide a critical overview of the link between PRL, autoimmune diseases, and motherhood.

Keywords: sex hormones, prolactin, autoimmunity, systemic lupus erythematosus, multiple sclerosis, systemic sclerosis

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INTRODUCTION

Currently, more than 80 autoimmune disorders are recognized, in which aberrant immune responses against self-different organs and tissues play a crucial role (1). Gender dimorphism represents one of the most enigmatic observations among the mosaic of autoimmunity. Susceptibility genes, epigenetic modifications, gender-related composition of gut microbiota, and sex hormones are believed to be a mainstay of this asymmetry (2, 3). The greater prevalence of autoimmunity among childbearing age women, disease relapses during pregnancy, and post-partum are some of the arguments that support this hypothesis (4). Indeed, women have enhanced immune reactivity, larger antigen-presenting capability and mitogenic responses, increased antibody production, higher immunoglobulin (Ig) levels, and the ability to reject allografts more rapidly (5). The immune and neuroendocrine system

Abbreviations: IFN, interferon; Ig, immunoglobulin; IL, interleukin; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; PRL, prolactin; PRLR, prolactin receptor; Th, T helper cells; TNF, Tumor necrosis factor; Treg, T regulatory cells; STAT1, Signal transducer and activator of transcription 1.

are intimately connected, partaking of dynamic bidirectional communication. Prolactin (PRL) has a recognized immune-stimulatory effect, specially inhibiting the negative selection of autoreactive B lymphocytes, promoting autoimmunity. In accordance, hyperprolactinemia has been associated with several autoimmune diseases, influencing its pathogenesis (6). Although the mechanisms involving this interaction are not completely understood, it has been documented that PRL can influence the communication and regulation of immune cells (7).

PRL, THE HORMONE, AND THE CYTOKINE

Prolactin is a 23-kD peptide hormone secreted in the pituitary gland, through the hypothalamic–pituitary–adrenal axis, under tonic inhibition of dopamine. Interestingly, this hormone can also be produced in extra-pituitary locations, such as decidua, ovary, prostate, mammary gland, adipose tissue, brain, and immune cells. When produced in extra-pituitary sites, PRL has different molecular weight and bioactivity. Hyperprolactinemia is usually defined as fasting levels of above 20 ng/ml in men and above 25 ng/ml in women (8). The expected rate among healthy population is up to 3%. Levels physiologically increase during lactation, but also as result of several diseases, including prolactinoma, hypothyroidism, and adrenal insufficiency (9). Besides, PRL secretion is regulated by cytokines such as interleukin (IL)-1, IL-2, and IL-6, which are stimulators, while endothelin-3 and interferon (IFN)-γ play an inhibitory role. This hormone can be found adopting several isoforms due to variations in post-translational modifications (10). The three main isoforms are the monomeric (free little PRL), big PRL, and macroprolactin (big big). The most biologically potent isoform is the monomeric free (little) PRL, which consists of 199 amino acids and has a molecular weight of 23 kDa (11). The PRL receptor is a member of the type 1 cytokine/hematopoietic receptor superfamily and is widely expressed through the immune system, including monocytes, lymphocytes, macrophages, natural killer cells, granulocytes, and thymic epithelial cells (12). Hence, the binding of PRL to its receptor activates downstream signaling pathways that will manipulate immune cells proliferation, differentiation, secretion, and survival (13, 14). This molecule is an integral member of the immune-neuroendocrinology network and has been largely associated with autoimmune diseases (15).

PRL and Immune Modulation

Prolactin strongly persuades the innate and adaptive immune responses, managing the maturation of CD4− CD8− thymocytes to CD4+ CD8+ T cells, through IL-2 receptor expression (16, 17). A direct correlation between PRL levels and the number of B and CD4+ T lymphocytes has been reported (18). Indeed, hyperprolactinemia can impair B-cell clonal deletion, deregulate receptor editing and diminish the threshold for activation of B cells, promoting auto-reactivity (19–21). It is capable of changing Th1 and Th2 type cytokine production, promoting IL-6 and INF-γ secretion, and playing a regulatory role on IL-2 levels (22, 23). Furthermore, PRL increases Ig production, stimulates the development of antigen-presenting cells expressing major histocompatibility complex class II, and upholds the co-stimulatory molecules CD86, CD80, and CD40 (24). Interestingly, assorted autoantibodies, including anti-cardiolipin, anti-PRL, anti-La, anti-Ro, among others, were detected in patients with hyperprolactinemia (25–27). Finally, PRL has been shown to influence dendritic cells to skew antigen presentation to pro-inflammatory function phenotype, enhancing IFN-α production (28). During pregnancy, one of the most decisive immunologic adaptations is the shift from a Th1/Th17 pro-inflammatory response toward a Th2/T regulatory cell (Treg) response, which promotes tolerance and inhibits natural killer cells cytotoxicity (2, 29). In accordance, differences in the activity of assorted autoimmune diseases have been reported during pregnancy and post-partum. For a better comprehension, the effects of PRL on the immune system cells were summarized in **Table 1**.

PRL during Pregnancy and Breastfeeding

Sex hormones can influence different functions on the immune system network. Typically, PRL and estrogens act as immune stimulants, while progesterone and testosterone exert a suppressive role (51, 52). Pregnancy inspires unique changes in endocrine and immune signaling, in order to tolerate and support the development and survival of the placenta and fetus in the hostile maternal immune system environment. PRL levels increase during pregnancy and reach peak values during delivery (53). Suckling stimulates the nerve endings in the nipple-areolar complex and strongly promotes hormone production. A large study performed by Stuebe et al. (54) evaluated PRL levels in women who exclusively breastfed their infants. The authors successfully reported wide changing baseline values (from 9 ng/dl before to 74 ng/dl 10 min after breastfeeding), depending on the frequency of feedings (54). In accordance, during the pregnancy and lactation period, several patients experience disease onset or relapse, suggesting an active influence of PRL. Indeed, a significant association between PRL levels and disease activity was found in systemic lupus erythematosus (55), rheumatoid arthritis (50, 56), and peripartum cardiomyopathy patients (57, 58), therefore breastfeeding should not be encouraged among those patients.

PRL and the Role of Dopamine Agonists

Dopamine is an effective inhibitor of PRL secretion due either a direct influence on the hypophysis or stimulation of postsynaptic dopamine receptors in the hypothalamus, arousing the release of the PRL inhibitory factor. Bromocriptine is an ergot alkaloid that binds to the dopamine receptor and inhibits the central synthesis of PRL. In addition, this drug can also influence T and B lymphocytes through the dopamine receptor (59, 60). Bromocriptine has been shown to decrease autoantibodies production, influence lymphocyte function and modulate the expression of surface molecules. By contrast, it exerts no clear effect on extra-pituitary PRL production. In conclusion, the beneficial therapeutic effects in murine and human trials, and the low toxicity of the drug outline a solid rationale for its attempt in future therapeutic proposals (61).

TABLE 1 | Effects of prolactin (PRL) on the immune system cells.

Immune cells	PRL secretion	Prolactin receptor (PRLR) expression	Immunological effects of PRL	Reference
Thymocytes	✓	✓	• Promote the differentiation of CD4− CD8− thymocytes into CD4+ CD8+ cells • Regulate the maintenance of thymocyte viability during differentiation	(30, 31)
Dendritic cells	?	✓	• Enhance the production of cytokines (IL-12, TNF-α, IL-1β) • Increase the responsiveness in allogeneic mixed leukocyte reactions (upregulation of MHC surface expression and the co-stimulatory molecule CD80)	(32, 33)
T cells	✓	✓	• Exert an immunomodulatory role at early stages of T-cell activation • Increase secretion of TNF-α, IFN-γ, and IL-2 • Trigger the IL-2-stimulated proliferation • Promote dysfunction of regulatory T cells • Enhance adhesion to endothelial cells	(34–37)
B cells	✓	✓	• Influence B-cell maturation process, promoting the survival of self-reactive clones • Increase the viability of immature B cells by rescuing them from apoptosis	(38, 39)
Natural killer cells	?	✓	• Induce natural killer cells differentiation to PRL-activated killer cells (PAK cells) in a dose-dependent way • Interfere with proliferation and cytotoxic activity • Promote the release of IFN-γ	(40–42)
Monocytes	✓	✓	• Increase TNF expression	(43–45)
Granulocytes	?	✓	• Activate the STAT1 and MAPK pathways • Contribute for the transcription of IRF-1 and iNOS	(46, 47)
Macrophages	✓	✓	• Cooperate with other pro-inflammatory stimuli to activate macrophages *via* engagement with the PRLR • Promote the secretion of chemokines and cytokines (IL-1β, IL-12β, IFN-γ, and TNF)	(7, 48–50)

iNOS: inducible nitric oxide synthase; IFN, interferon; IL, interleukin; IRF-1, interferon regulatory factor 1; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; STAT1, signal transducer and activator of transcription 1; TNF, tumor necrosis factor.

HYPERPROLACTINEMIA AND AUTOIMMUNE DISEASES

Hyperprolactinemia has been reported in patients with several autoimmune diseases, commonly manipulating disease development and perpetuation (62). The link between PRL and autoimmunity has been proposed to have a genetic background (63, 64). The PRL gene is located on the short arm of chromosome 6, near the HLA-DRB1 region, which is known for its association with assorted immune-mediated disorders (65).

PRL and Systemic Lupus Erythematosus

Systemic lupus erythematosus is an autoimmune disease, typically affecting young women at reproductive age (66). Hyperprolactinemia has been reported in a wide range of lupus patients from both genders (15–33%). In accordance, PRL levels have shown direct correlation with clinical and serological disease activity (16, 67–69). Results from several trials report also an association with neurological, renal and hematological involvement, serositis, enhanced anti-double-stranded DNA antibodies, and diminished complement (70, 71). Furthermore, PRL bolsters the development of lupus-like phenotype in non-prone mice and exacerbated the disease in a lupus murine experimental study (72). During pregnancy, hyperprolactinemia has been associated with lupus anticoagulant, disease activity, and poor outcomes for mother and fetus (73). In accordance, the presence of anti-PRL antibodies was correlated with lower disease activity and better outcomes in pregnant patients (74, 75). The treatment of pregnant women with bromocriptine was shown to prevent disease relapses, improve outcomes, and reduce the doses of concomitant steroidal therapy (76, 77). In conclusion, the evidence strongly supports the role of PRL in the pathogenesis and activity of systemic lupus erythematosus.

PRL and Anti-Phospholipid Syndrome

Anti-phospholipid syndrome is a systemic autoimmune condition, characterized by thrombotic events and/or pregnancy morbidity in the presence of anti-phospholipid antibodies. Hyperprolactinemia was detected in 12% of patients with anti-phospholipid syndrome, with no differences among genders or disease subtypes. Likewise, hormone levels were shown to be correlated with the presence of lupus anticoagulants, intrauterine growth retardation, and miscarriages among pregnant patients (78). By contrast, no significant correlation was found with thrombotic events, although PRL was recently proposed as a novel risk factor for thrombotic disease, since it acts as a potent platelet aggregation co-activator (79–82). Previously, bromocriptine was tested in animal models with anti-phospholipid syndrome and lupus, showing a suppressive effect on both diseases, probably through induction of natural non-specific CD8 suppressor cells (59).

PRL and Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune disease that if untreated leads to progressive and irreversible destruction of cartilage and bone. The relationship between PRL and rheumatoid arthritis emerged from the adjacent location of the human PRL gene and HLA region (16). Recent studies reported higher levels of PRL in serum and synovial fluid of patients with rheumatoid arthritis. This suggests increased production, either systemic or locally secreted by immune cells, in putative relation with disease activity (45, 56). Pregnant women with

rheumatoid arthritis, due to a transient period of hypercortisolism, experience disease improvement. After delivery, flares are frequently reported (83). Women who breastfeed after the first pregnancy have a higher risk of developing rheumatoid arthritis, suggesting an active influence from PRL (84, 85). In addition, nearly 90% of these women will relapse within the first 3 months of postpartum and almost all patients will flair within the next 9 months. Indeed, severe disease was associated with longer breastfeeding periods and higher number of breast fed children. In animal models, bromocriptine was able to suppress postpartum exacerbation of collagen-induced arthritis (86). In humans, the treatment with bromocriptine revealed controversial findings (87, 88), probably because bromocriptine does not influence lymphocyte-derived PRL production (89). Hence, systemic and locally produced PRL may offer distinct contributions to inflammatory arthritis.

PRL and Systemic Sclerosis

Systemic sclerosis is a connective tissue disease characterized by alterations of the microvasculature, disturbances of the immune system, and massive deposition of collagen and other matrix substances in the skin and internal organs (90). High levels of PRL have been reported in 13–59% of patients with systemic sclerosis (91). Likewise, a significant correlation between hormone levels and the severity of skin sclerosis, lung, and cardiovascular involvement was found (92–94). The sources of PRL in this disease are believed to reside on enhanced lymphocytic secretion, increased dopaminergic central tone, and drug-induction, mainly by antidepressants and prokinetics (95). Pregnancy *per se* does not exacerbate the disease, even though cases have been reported of women with organ insufficiency mainly pulmonary hypertension and severe skin fibrosis (96, 97). Patients with disease duration of less than 4 years, with diffuse cutaneous subtype, presence of anti-RNA polymerase III or anti-topoisomerase I antibodies are at higher risk for obstetric complications and should delay pregnancy until the disease is quiescent (98). In conclusion, PRL was found to be correlated with disease severity and activity.

PRL and Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disorder involving the central nervous system (99, 100). In animal models, it is represented by experimental autoimmune encephalomyelitis, believed to be an inflammatory response against oligodendrocytes that form myelin sheaths surrounding neuronal axon driven by myelin-reactive CD4+ Th1/Th17 cells (101). Several studies reported a positive correlation between hyperprolactinemia and disease onset, relapse, and number of anti-myelin oligodendrocyte glycoprotein antibody secreting cells (102, 103). Indeed, the source of high PRL levels among those patients is unclear, albeit observations suggest that it may be part of a non-specific hypothalamic–pituitary–adrenal axis dysregulation due to neurodegeneration and/or demyelination (104). Currently, PRL is believed to have a dual impact in the central nervous system. On the one hand, PRL might support system repair by providing regenerative signals for neurons, oligodendrocytes, and adult neural stem/progenitor cells. On the other hand, its stimulation of peripheral immune cells might promote aberrant immune responses and negatively impact the disease (105, 106). Typically, pregnancies were believed to have a negative impact in women with multiple sclerosis, provoking postpartum exacerbations and increasing permanent disability (107). Nowadays, it is known that the risk of relapse significantly declines during the third trimester of pregnancy and increases three-fold in the first 3–4 months after delivery, with no references about medication consumption or breastfeeding options (108). Recently, studies revealed that an earlier return of menses was associated with a higher risk of disease relapse in the first 6 months after delivery, which suggests a natural protection from exclusive breastfeeding (109). Likewise, prolonged lactational amenorrhea was correlated with a lower risk of postpartum relapses (110). In conclusion, evidence supports a plausible protection from exclusive breastfeeding, although no studies have examined the long-term effects of breastfeeding, particularly in exclusive patterns.

PRL and Celiac Disease

Celiac disease is a gluten-sensitive autoimmune enteropathy where both adaptive immunity and innate immunity are involved in its development (111). Serum PRL levels were positively correlated with disease activity, degree of mucosal atrophy, and with the serum concentration of anti-endomysial antibodies. Recently, a longitudinal study revealed diminished levels of PRL after 6 months following a gluten-free diet. The evidence of decreasing PRL simultaneously with the decline of anti-transglutaminase antibodies suggests a direct connection with a gluten-free diet and hormone levels (112).

PRL and Autoimmune Thyroid Disease

Autoimmune thyroid diseases comprise mainly two disorders, Grave's disease and Hashimoto thyroiditis (113). Hyperprolactinemia was found in 20% of patients with autoimmune thyroid disease and had double the frequency among hypothyroidism patients. Around 90% of Hashimoto's thyroiditis patients presented significantly higher PRL levels in association with decreased cortisol titers (114). The role of dopamine agonists in the treatment of autoimmune thyroid disease is yet to be determined.

PRL and Peripartum Cardiomyopathy

Peripartum cardiomyopathy is a congestive heart failure occurring in the last month of pregnancy or 5 months after delivery, in the absence of preexisting heart disease (115). The etiology of this disease remains unclear, although plausible causes have been proposed, such as nutritional deficiency, viral infections, stress-activated cytokines, pathological response to hemodynamic stress, inflammation, and autoimmune reactions (116). Evidence supports an active role of PRL in the pathophysiology of this disease. Increased oxidative stress leads to subsequent 16-kDa PRL production, impairing the cardiac vasculature and its metabolism, culminating in systolic heart failure (117, 118). Interestingly, the presence of autoantibodies against sarcomeric myosin and troponin I were detected among women with peripartum myocardiopathy, suggesting the presence of an underlying autoimmune disorder. In addition, these antibodies were associated with the severity of left ventricle dysfunction and

lower rate of full cardiac recovery on follow-up (119). Interestingly, patients demonstrated an abnormal cytokine profile (increased levels of TNF, IL-6 and soluble Fas receptors), decreased levels of CD4+ CD25lo Tregs, a heightened level of fetal microchimerism, and a significant reduction in the plasma levels of progesterone, estradiol, and relaxin, contributing to abnormal immune responses and inflammatory processes (120, 121). Recently, dopamine agonists have shown promising results in the treatment of this disease, dramatically improving outcomes (58, 122–124). The 2010 European position statement does not encourage breastfeeding based on concerns regarding the perpetuation of PRL pathways (125).

CONCLUSION

The dimorphism between genders in autoimmune diseases is believed to rely on sex hormones. PRL exerts a great influence in immune system modulation, mainly inhibiting the negative selection of autoreactive B lymphocytes and has been associated with the pathogenesis of several autoimmune disorders. During pregnancy and the lactation period, assorted autoimmune patients experience disease relapse, suggesting an active influence of PRL. Immunological studies of pregnant and postpartum women with autoimmune diseases offer a biologically rich opportunity to improve our understanding of the hormonal impact on disease relapse pathophysiology. Although the interest on the relationship between PRL, immune modulation, and autoimmune diseases has emerged in the past few years, more studies are required to further delineate the influence of PRL in autoimmune disease. Eventually, gut microbiome, immune cells transcriptome, and proteome might be the answers to those questions being unsolved to date.

Highlights

- Susceptibility genes, epigenetic modifications, microbiome, and sex hormones are believed to be a mainstay of the gender asymmetry in autoimmune diseases.
- PRL influences the negative selection of autoreactive B cells, promoting their proliferation, survival, and antibody production.
- Hyperprolactinemia has been associated with several autoimmune diseases and is believed to play a crucial role in their pathogenesis.
- A significant association between PRL and disease flairs was found in systemic lupus erythematosus and rheumatoid arthritis.
- Dopamine agonists have been used in the treatment of many autoimmune diseases with great benefits.

AUTHOR CONTRIBUTIONS

VB, ZG, and YS contributed equally to the construction of this review.

REFERENCES

1. Perricone R, Perricone C, Shoenfeld Y. Autoimmunity: when the immune system becomes the self-ish giant. *Autoimmun Rev* (2011) 10(10):575–6. doi:10.1016/j.autrev.2011.05.003
2. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita* (2016) 52(2):205–12. doi:10.4415/ann_16_02_12
3. Krasselt M, Baerwald C. Sex, symptom severity, and quality of life in rheumatology. *Clin Rev Allergy Immunol* (2017) 11:1–16. doi:10.1007/s12016-017-8631-6
4. Orbach H, Shoenfeld Y. Hyperprolactinemia and autoimmune diseases. *Autoimmun Rev* (2007) 6(8):537–42. doi:10.1016/j.autrev.2006.10.005
5. Zandman-Goddard G, Peeva E, Shoenfeld Y. Gender and autoimmunity. *Autoimmun Rev* (2007) 6(6):366–72. doi:10.1016/j.autrev.2006.10.001
6. Buskila D, Sukenik S, Shoenfeld Y. The possible role of prolactin in autoimmunity. *Am J Reprod Immunol* (1991) 26(3):118–23. doi:10.1111/j.1600-0897.1991.tb00708.x
7. Tang MW, Garcia S, Gerlag DM, Tak PP, Reedquist KA. Insight into the endocrine system and the immune system: a review of the inflammatory role of prolactin in rheumatoid arthritis and psoriatic arthritis. *Front Immunol* (2017) 8:720. doi:10.3389/fimmu.2017.00720
8. Majumdar A, Mangal NS. Hyperprolactinemia. *J Hum Reprod Sci* (2013) 6(3):168–75. doi:10.4103/0974-1208.121400
9. Savino W. Prolactin: an immunomodulator in health and disease. *Front Horm Res* (2017) 48:69–75. doi:10.1159/000452906
10. Devi YS, Halperin J. Reproductive actions of prolactin mediated through short and long receptor isoforms. *Mol Cell Endocrinol* (2014) 382(1):400–10. doi:10.1016/j.mce.2013.09.016
11. Marcotegui AR, Garcia-Calvo A. [Biochemical diagnosis of monomeric hyperprolactinemia]. *An Sist Sanit Navar* (2011) 34(2):145–52. doi:10.4321/S1137-66272011000200002
12. Orbach H, Zandman-Goddard G, Amital H, Barak V, Szekanecz Z, Szucs G, et al. Novel biomarkers in autoimmune diseases: prolactin, ferritin, vitamin D, and TPA levels in autoimmune diseases. *Ann N Y Acad Sci* (2007) 1109:385–400. doi:10.1196/annals.1398.044
13. Thoreau E, Petridou B, Kelly PA, Djiane J, Mornon JP. Structural symmetry of the extracellular domain of the cytokine/growth hormone/prolactin receptor family and interferon receptors revealed by hydrophobic cluster analysis. *FEBS Lett* (1991) 282(1):26–31. doi:10.1016/0014-5793(91)80437-8
14. Jeganathan V, Peeva E, Diamond B. Hormonal milieu at time of B cell activation controls duration of autoantibody response. *J Autoimmun* (2014) 53:46–54. doi:10.1016/j.jaut.2014.02.007
15. Anaya JM, Shoenfeld Y. Multiple autoimmune disease in a patient with hyperprolactinemia. *Isr Med Assoc J* (2005) 7(11):740–1.
16. Vera-Lastra O, Jara LJ, Espinoza LR. Prolactin and autoimmunity. *Autoimmun Rev* (2002) 1(6):360–4. doi:10.1016/S1568-9972(02)00081-2
17. Pereira Suarez AL, Lopez-Rincon G, Martinez Neri PA, Estrada-Chavez C. Prolactin in inflammatory response. *Adv Exp Med Biol* (2015) 846:243–64. doi:10.1007/978-3-319-12114-7_11
18. Brand JM, Frohn C, Cziupka K, Brockmann C, Kirchner H, Luhm J. Prolactin triggers pro-inflammatory immune responses in peripheral immune cells. *Eur Cytokine Netw* (2004) 15(2):99–104.
19. Buckley AR. Prolactin, a lymphocyte growth and survival factor. *Lupus* (2001) 10(10):684–90. doi:10.1191/096120301717164912
20. Kochendoerfer SK, Krishnan N, Buckley DJ, Buckley AR. Prolactin regulation of Bcl-2 family members: increased expression of bcl-xL but not mcl-1 or bad in Nb2-T cells. *J Endocrinol* (2003) 178(2):265–73. doi:10.1677/joe.0.1780265
21. Saha S, Gonzalez J, Rosenfeld G, Keiser H, Peeva E. Prolactin alters the mechanisms of B cell tolerance induction. *Arthritis Rheum* (2009) 60(6):1743–52. doi:10.1002/art.24500
22. Tomio A, Schust DJ, Kawana K, Yasugi T, Kawana Y, Mahalingaiah S, et al. Prolactin can modulate CD4+ T-cell response through receptor-mediated alterations in the expression of T-bet. *Immunol Cell Biol* (2008) 86(7):616–21. doi:10.1038/icb.2008.29
23. Mackern-Oberti JP, Jara EL, Riedel CA, Kalergis AM. Hormonal modulation of dendritic cells differentiation, maturation and function: implications for the initiation and progress of systemic autoimmunity. *Arch Immunol Ther Exp (Warsz)* (2017) 65(2):123–36. doi:10.1007/s00005-016-0418-6

24. Peeva E, Zouali M. Spotlight on the role of hormonal factors in the emergence of autoreactive B-lymphocytes. *Immunol Lett* (2005) 101(2):123–43. doi:10.1016/j.imlet.2005.05.014
25. Buskila D, Berezin M, Gur H, Lin HC, Alosachie I, Terryberry JW, et al. Autoantibody profile in the sera of women with hyperprolactinemia. *J Autoimmun* (1995) 8(3):415–24. doi:10.1006/jaut.1995.0033
26. Krause I, Blumenfeld Z, Malchinsky M, Cohen S, Blank M, Eldor A, et al. Anti-endothelial cell antibodies in the sera of hyperprolactinemic women. *Lupus* (1998) 7(6):377–82. doi:10.1191/096120398678920316
27. De Bellis A, Colao A, Pivonello R, Savoia A, Battaglia M, Ruocco G, et al. Antipituitary antibodies in idiopathic hyperprolactinemic patients. *Ann N Y Acad Sci* (2007) 1107:129–35. doi:10.1196/annals.1381.014
28. Matera L, Mori M, Galetto A. Effect of prolactin on the antigen presenting function of monocyte-derived dendritic cells. *Lupus* (2001) 10(10):728–34. doi:10.1191/096120301717164967
29. Borchers AT, Naguwa SM, Keen CL, Gershwin ME. The implications of autoimmunity and pregnancy. *J Autoimmun* (2010) 34(3):J287–99. doi:10.1016/j.jaut.2009.11.015
30. Gagnerault MC, Touraine P, Savino W, Kelly PA, Dardenne M. Expression of prolactin receptors in murine lymphoid cells in normal and autoimmune situations. *J Immunol* (1993) 150(12):5673–81.
31. Lepletier A, de Carvalho VF, Rodrigues e Silva PM, Villar S, Perez AR, Savino W, et al. *Trypanosoma cruzi* disrupts thymic homeostasis by altering intrathymic and systemic stress-related endocrine circuitries. *PLoS Negl Trop Dis* (2013) 7(11):e2470. doi:10.1371/journal.pntd.0002470
32. Carreno PC, Jimenez E, Sacedon R, Vicente A, Zapata AG. Prolactin stimulates maturation and function of rat thymic dendritic cells. *J Neuroimmunol* (2004) 153(1–2):83–90. doi:10.1016/j.jneuroim.2004.04.020
33. Jara LJ, Benitez G, Medina G. Prolactin, dendritic cells, and systemic lupus erythematosus. *Autoimmun Rev* (2008) 7(3):251–5. doi:10.1016/j.autrev.2007.11.018
34. Dimitrov S, Lange T, Fehm HL, Born J. A regulatory role of prolactin, growth hormone, and corticosteroids for human T-cell production of cytokines. *Brain Behav Immun* (2004) 18(4):368–74. doi:10.1016/j.bbi.2003.09.014
35. Carreno PC, Sacedon R, Jimenez E, Vicente A, Zapata AG. Prolactin affects both survival and differentiation of T-cell progenitors. *J Neuroimmunol* (2005) 160(1–2):135–45. doi:10.1016/j.jneuroim.2004.11.008
36. Xu D, Lin L, Lin X, Huang Z, Lei Z. Immunoregulation of autocrine prolactin: suppressing the expression of costimulatory molecules and cytokines in T lymphocytes by prolactin receptor knockdown. *Cell Immunol* (2010) 263(1):71–8. doi:10.1016/j.cellimm.2010.02.018
37. Wu W, Sun M, Zhang HP, Chen T, Wu R, Liu C, et al. Prolactin mediates psychological stress-induced dysfunction of regulatory T cells to facilitate intestinal inflammation. *Gut* (2014) 63(12):1883–92. doi:10.1136/gutjnl-2013-306083
38. Legorreta-Haquet MV, Flores-Fernandez R, Blanco-Favela F, Fuentes-Panana EM, Chavez-Sanchez L, Hernandez-Gonzalez R, et al. Prolactin levels correlate with abnormal B cell maturation in MRL and MRL/lpr mouse models of systemic lupus erythematosus-like disease. *Clin Dev Immunol* (2013) 2013:287469. doi:10.1155/2013/287469
39. Flores-Fernandez R, Blanco-Favela F, Fuentes-Panana EM, Chavez-Sanchez L, Gorocica-Rosete P, Pizana-Venegas A, et al. Prolactin rescues immature B-cells from apoptosis induced by B-cell receptor cross-linking. *J Immunol Res* (2016) 2016:3219017. doi:10.1155/2016/3219017
40. Jara LJ, Lavalle C, Fraga A, Gomez-Sanchez C, Silveira LH, Martinez-Osuna P, et al. Prolactin, immunoregulation, and autoimmune diseases. *Semin Arthritis Rheum* (1991) 20(5):273–84. doi:10.1016/0049-0172(91)90028-X
41. Matera L, Buttiglieri S, Moro F, Geuna M. Effect of prolactin on natural killer and MHC-restricted cytotoxic cells. In: Matera L, Rapaport R, editors. *NeuroImmune Biology*. (Vol. 2), London: Elsevier (2002). p. 205–18.
42. Mavoungou E, Bouyou-Akotet MK, Kremsner PG. Effects of prolactin and cortisol on natural killer (NK) cell surface expression and function of human natural cytotoxicity receptors (NKp46, NKp44 and NKp30). *Clin Exp Immunol* (2005) 139(2):287–96. doi:10.1111/j.1365-2249.2004.02686.x
43. Matera L. Endocrine, paracrine and autocrine actions of prolactin on immune cells. *Life Sci* (1996) 59(8):599–614. doi:10.1016/0024-3205(96)00225-1
44. Tang C, Li Y, Lin X, Ye J, Li W, He Z, et al. Prolactin increases tumor necrosis factor alpha expression in peripheral CD14 monocytes of patients with rheumatoid arthritis. *Cell Immunol* (2014) 290(1):164–8. doi:10.1016/j.cellimm.2014.06.005
45. Tang MW, Reedquist KA, Garcia S, Gerlag DM, Tak PP. 1.57 Prolactin is locally produced in the synovium of patients with inflammatory arthritic diseases and promotes macrophage activation. *Ann Rheum Dis* (2014) 73(Suppl 1):A24. doi:10.1136/annrheumdis-2013-205124.56
46. Matera L, Galetto A, Geuna M, Vekemans K, Ricotti E, Contarini M, et al. Individual and combined effect of granulocyte-macrophage colony-stimulating factor and prolactin on maturation of dendritic cells from blood monocytes under serum-free conditions. *Immunology* (2000) 100(1):29–36. doi:10.1046/j.1365-2567.2000.00996.x
47. Dogusan Z, Hooghe R, Verdood P, Hooghe-Peters EL. Cytokine-like effects of prolactin in human mononuclear and polymorphonuclear leukocytes. *J Neuroimmunol* (2001) 120(1):58–66. doi:10.1016/S0165-5728(01)00420-9
48. Tripathi A, Sodhi A. Prolactin-induced production of cytokines in macrophages in vitro involves JAK/STAT and JNK MAPK pathways. *Int Immunol* (2008) 20(3):327–36. doi:10.1093/intimm/dxm145
49. Carvalho-Freitas MI, Anselmo-Franci JA, Palermo-Neto J, Felicio LF. Prior reproductive experience alters prolactin-induced macrophage responses in pregnant rats. *J Reprod Immunol* (2013) 99(1–2):54–61. doi:10.1016/j.jri.2013.03.005
50. Tang MW, Garcia S, Malvar Fernandez B, Gerlag DM, Tak PP, Reedquist KA. Rheumatoid arthritis and psoriatic arthritis synovial fluids stimulate prolactin production by macrophages. *J Leukoc Biol* (2017) 102(3):897–904. doi:10.1189/jlb.2A0317-115RR
51. Carp HJ, Selmi C, Shoenfeld Y. The autoimmune bases of infertility and pregnancy loss. *J Autoimmun* (2012) 38(2–3):J266–74. doi:10.1016/j.jaut.2011.11.016
52. Tan IJ, Peeva E, Zandman-Goddard G. Hormonal modulation of the immune system – a spotlight on the role of progestogens. *Autoimmun Rev* (2015) 14(6):536–42. doi:10.1016/j.autrev.2015.02.004
53. Zhang F, Xia H, Shen M, Li X, Qin L, Gu H, et al. Are prolactin levels linked to suction pressure? *Breastfeed Med* (2016) 11:461–8. doi:10.1089/bfm.2015.0083
54. Stuebe AM, Meltzer-Brody S, Pearson B, Pedersen C, Grewen K. Maternal neuroendocrine serum levels in exclusively breastfeeding mothers. *Breastfeed Med* (2015) 10(4):197–202. doi:10.1089/bfm.2014.0164
55. Song GG, Lee YH. Circulating prolactin level in systemic lupus erythematosus and its correlation with disease activity: a meta-analysis. *Lupus* (2017) 26(12):1260–8. doi:10.1177/0961203317693094
56. Fojtikova M, Tomasova Studynkova J, Filkova M, Lacinova Z, Gatterova J, Pavelka K, et al. Elevated prolactin levels in patients with rheumatoid arthritis: association with disease activity and structural damage. *Clin Exp Rheumatol* (2010) 28(6):849–54.
57. Hilfiker-Kleiner D, Kaminski K, Podewski E, Bonda T, Schaefer A, Sliwa K, et al. A cathepsin D-cleaved 16 kDa form of prolactin mediates postpartum cardiomyopathy. *Cell* (2007) 128(3):589–600. doi:10.1016/j.cell.2006.12.036
58. Hilfiker-Kleiner D, Haghikia A, Berliner D, Vogel-Claussen J, Schwab J, Franke A, et al. Bromocriptine for the treatment of peripartum cardiomyopathy: a multicentre randomized study. *Eur Heart J* (2017) 38(35):2671–9. doi:10.1093/eurheartj/ehx355
59. Blank M, Krause I, Buskila D, Teitelbaum D, Kopolovic J, Afek A, et al. Bromocriptine immunomodulation of experimental SLE and primary antiphospholipid syndrome via induction of nonspecific T suppressor cells. *Cell Immunol* (1995) 162(1):114–22. doi:10.1006/cimm.1995.1058
60. McMurray RW. Bromocriptine in rheumatic and autoimmune diseases. *Semin Arthritis Rheum* (2001) 31(1):21–32. doi:10.1053/sarh.2001.25482
61. Buskila D, Shoenfeld Y. Prolactin, bromocriptine and autoimmune diseases. *Isr J Med Sci* (1996) 32(1):23–7.
62. Shelly S, Boaz M, Orbach H. Prolactin and autoimmunity. *Autoimmun Rev* (2012) 11(6–7):A465–70. doi:10.1016/j.autrev.2011.11.009
63. Parada-Turska J, Targonska-Stepniak B, Majdan M. [Prolactin in connective tissue diseases]. *Postepy Hig Med Dosw (Online)* (2006) 60:278–85.
64. Arango MT, Perricone C, Kivity S, Cipriano E, Ceccarelli F, Valesini G, et al. HLA-DRB1 the notorious gene in the mosaic of autoimmunity. *Immunol Res* (2017) 65(1):82–98. doi:10.1007/s12026-016-8817-7
65. Viatte S, Massey J, Bowes J, Duffus K, Eyre S, Barton A, et al. Replication of associations of genetic loci outside the HLA region with susceptibility

to anti-cyclic citrullinated peptide-negative rheumatoid arthritis. *Arthritis Rheumatol* (2016) 68(7):1603–13. doi:10.1002/art.39619
66. Anaya JM, Shoenfeld Y, Cervera R. Systemic lupus erythematosus 2014. *Autoimmune Dis* (2014) 2014:274323. doi:10.1155/2014/274323
67. Jacobi AM, Rohde W, Ventz M, Riemekasten G, Burmester GR, Hiepe F. Enhanced serum prolactin (PRL) in patients with systemic lupus erythematosus: PRL levels are related to the disease activity. *Lupus* (2001) 10(8):554–61. doi:10.1191/096120301701549688
68. Pacilio M, Migliaresi S, Meli R, Ambrosone L, Bigliardo B, Di Carlo R. Elevated bioactive prolactin levels in systemic lupus erythematosus – association with disease activity. *J Rheumatol* (2001) 28(10):2216–21.
69. Cardenas-Mondragon G, Ulloa-Aguirre A, Isordia-Salas I, Goffin V, Leanos-Miranda A. Elevated serum bioactive prolactin concentrations in patients with systemic lupus erythematosus are associated with disease activity as disclosed by homologous receptor bioassays. *J Rheumatol* (2007) 34(7):1514–21.
70. Leanos-Miranda A, Cardenas-Mondragon G. Serum free prolactin concentrations in patients with systemic lupus erythematosus are associated with lupus activity. *Rheumatology (Oxford)* (2006) 45(1):97–101. doi:10.1093/rheumatology/kei115
71. Orbach H, Zandman-Goddard G, Boaz M, Agmon-Levin N, Amital H, Szekanecz Z, et al. Prolactin and autoimmunity: hyperprolactinemia correlates with serositis and anemia in SLE patients. *Clin Rev Allergy Immunol* (2012) 42(2):189–98. doi:10.1007/s12016-011-8256-0
72. Saha S, Tieng A, Pepeljugoski KP, Zandamn-Goddard G, Peeva E. Prolactin, systemic lupus erythematosus, and autoreactive B cells: lessons learnt from murine models. *Clin Rev Allergy Immunol* (2011) 40(1):8–15. doi:10.1007/s12016-009-8182-6
73. Jara LJ, Pacheco-Reyes H, Medina G, Angeles U, Cruz-Cruz P, Saavedra MA. Prolactin levels are associated with lupus activity, lupus anticoagulant, and poor outcome in pregnancy. *Ann N Y Acad Sci* (2007) 1108:218–26. doi:10.1196/annals.1422.024
74. Leanos A, Pascoe D, Fraga A, Blanco-Favela F. Anti-prolactin autoantibodies in systemic lupus erythematosus patients with associated hyperprolactinemia. *Lupus* (1998) 7(6):398–403. doi:10.1191/096120398678920280
75. Leanos-Miranda A, Cardenas-Mondragon G, Ulloa-Aguirre A, Isordia-Salas I, Parra A, Ramirez-Peredo J. Anti-prolactin autoantibodies in pregnant women with systemic lupus erythematosus: maternal and fetal outcome. *Lupus* (2007) 16(5):342–9. doi:10.1177/0961203307078197
76. Yang XY, Liang LQ, Xu HS, He M, Yao SZ, Zhan ZP, et al. [Efficacy of oral bromocriptine in protecting the postpartum systemic lupus erythematosus patients from disease relapse]. *Zhonghua Nei Ke Za Zhi* (2003) 42(9):621–4.
77. Qian Q, Liuqin L, Hao L, Shiwen Y, Zhongping Z, Dongying C, et al. The effects of bromocriptine on preventing postpartum flare in systemic lupus erythematosus patients from South China. *J Immunol Res* (2015) 2015:316965. doi:10.1155/2015/316965
78. Praprotnik S, Agmon-Levin N, Porat-Katz BS, Blank M, Meroni PL, Cervera R, et al. Prolactin's role in the pathogenesis of the antiphospholipid syndrome. *Lupus* (2010) 19(13):1515–9. doi:10.1177/0961203310373781
79. Wallaschofski H, Kobsar A, Sokolova O, Eigenthaler M, Lohmann T. Co-activation of platelets by prolactin or leptin – pathophysiological findings and clinical implications. *Horm Metab Res* (2004) 36(1):1–6. doi:10.1055/s-2004-814200
80. Raaz D, Wallaschofski H, Stumpf C, Yilmaz A, Cicha I, Klinghammer L, et al. Increased prolactin in acute coronary syndromes as putative co-activator of ADP-stimulated P-selectin expression. *Horm Metab Res* (2006) 38(11):767–72. doi:10.1055/s-2006-955090
81. Wallaschofski H, Lohmann T, Hild E, Kobsar A, Siegemund A, Spilcke-Liss E, et al. Enhanced platelet activation by prolactin in patients with ischemic stroke. *Thromb Haemost* (2006) 96(1):38–44. doi:10.1160/th05-09-0634
82. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum* (2013) 65(1):1–11. doi:10.1002/art.37715
83. Barrett JH, Brennan P, Fiddler M, Silman A. Breast-feeding and postpartum relapse in women with rheumatoid and inflammatory arthritis. *Arthritis Rheum* (2000) 43(5):1010–5. doi:10.1002/1529-0131(200005)43:5<1010:aid-anr8>3.0.co;2-o
84. Olsen NJ, Kovacs WJ. Hormones, pregnancy, and rheumatoid arthritis. *J Gend Specif Med* (2002) 5(4):28–37.
85. Karlson EW, Mandl LA, Hankinson SE, Grodstein F. Do breast-feeding and other reproductive factors influence future risk of rheumatoid arthritis? Results from the Nurses' Health Study. *Arthritis Rheum* (2004) 50(11):3458–67. doi:10.1002/art.20621
86. Whyte A, Williams RO. Bromocriptine suppresses postpartum exacerbation of collagen-induced arthritis. *Arthritis Rheum* (1988) 31(7):927–8. doi:10.1002/art.1780310717
87. Figueroa F, Carrion F, Martinez ME, Rivero S, Mamani I, Gonzalez G. [Effects of bromocriptine in patients with active rheumatoid arthritis]. *Rev Med Chil* (1998) 126(1):33–41.
88. Salesi M, Sadeghihaddadzavareh S, Nasri P, Namdarigharaghani N, Farajzadegan Z, Hajalikhani M. The role of bromocriptine in the treatment of patients with active rheumatoid arthritis. *Int J Rheum Dis* (2013) 16(6):662–6. doi:10.1111/1756-185x.12015
89. McMurray R, Keisler D, Kanuckel K, Izui S, Walker SE. Prolactin influences autoimmune disease activity in the female B/W mouse. *J Immunol* (1991) 147(11):3780–7.
90. Elhai M, Avouac J, Kahan A, Allanore Y. Systemic sclerosis: recent insights. *Joint Bone Spine* (2015) 82(3):148–53. doi:10.1016/j.jbspin.2014.10.010
91. Jara LJ, Medina G, Saavedra MA, Vera-Lastra O, Navarro C. Prolactin and autoimmunity. *Clin Rev Allergy Immunol* (2011) 40(1):50–9. doi:10.1007/s12016-009-8185-3
92. Straub RH, Zeuner M, Lock G, Scholmerich J, Lang B. High prolactin and low dehydroepiandrosterone sulphate serum levels in patients with severe systemic sclerosis. *Br J Rheumatol* (1997) 36(4):426–32. doi:10.1093/rheumatology/36.4.426
93. La Montagna G, Baruffo A, Pasquali D, Bellastella A, Tirri G, Sinisi AA. Assessment of pituitary gonadotropin release to gonadotropin releasing hormone/thyroid-stimulating hormone stimulation in women with systemic sclerosis. *Rheumatology (Oxford)* (2001) 40(3):310–4. doi:10.1093/rheumatology/40.3.310
94. Shahin AA, Abdoh S, Abdelrazik M. Prolactin and thyroid hormones in patients with systemic sclerosis: correlations with disease manifestations and activity. *Z Rheumatol* (2002) 61(6):703–9. doi:10.1007/s00393-002-0413-7
95. Vera-Lastra O, Jara LJ, Medina G, Rojas JL, Velaquez F, Ariza R, et al. Functional hyperprolactinemia and hypophyseal microadenoma in systemic sclerosis. *J Rheumatol* (2006) 33(6):1108–12.
96. Rueda de Leon Aguirre A, Ramirez Calvo JA, Rodriguez Reyna TS. Comprehensive approach to systemic sclerosis patients during pregnancy. *Reumatol Clin* (2015) 11(2):99–107. doi:10.1016/j.reuma.2014.06.006
97. Tincani A, Dall'Ara F, Lazzaroni MG, Reggia R, Andreoli L. Pregnancy in patients with autoimmune disease: a reality in 2016. *Autoimmun Rev* (2016) 15(10):975–7. doi:10.1016/j.autrev.2016.07.017
98. Taraborelli M, Ramoni V, Brucato A, Airo P, Bajocchi G, Bellisai F, et al. Brief report: successful pregnancies but a higher risk of preterm births in patients with systemic sclerosis: an Italian multicenter study. *Arthritis Rheum* (2012) 64(6):1970–7. doi:10.1002/art.34350
99. de Carvalho JF, Pereira RM, Shoenfeld Y. Pearls in autoimmunity. *Auto Immun Highlights* (2011) 2(1):1–4. doi:10.1007/s13317-011-0016-x
100. Belbasis L, Bellou V, Evangelou E, Ioannidis JP, Tzoulaki I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol* (2015) 14(3):263–73. doi:10.1016/s1474-4422(14)70267-4
101. Steinman L. Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol* (2014) 32:257–81. doi:10.1146/annurev-immunol-032713-120227
102. Azar ST, Yamout B. Prolactin secretion is increased in patients with multiple sclerosis. *Endocr Res* (1999) 25(2):207–14. doi:10.1080/07435809909066142
103. Correale J, Farez MF, Ysrraelit MC. Role of prolactin in B cell regulation in multiple sclerosis. *J Neuroimmunol* (2014) 269(1–2):76–86. doi:10.1016/j.jneuroim.2014.02.007
104. Zhornitsky S, Yong VW, Weiss S, Metz LM. Prolactin in multiple sclerosis. *Mult Scler* (2013) 19(1):15–23. doi:10.1177/1352458512458555
105. Costanza M, Binart N, Steinman L, Pedotti R. Prolactin: a versatile regulator of inflammation and autoimmune pathology. *Autoimmun Rev* (2015) 14(3):223–30. doi:10.1016/j.autrev.2014.11.005
106. Costanza M, Pedotti R. Prolactin: friend or foe in central nervous system autoimmune inflammation? *Int J Mol Sci* (2016) 17(12):2026. doi:10.3390/ijms17122026

107. Hellwig K, Haghikia A, Rockhoff M, Gold R. Multiple sclerosis and pregnancy: experience from a nationwide database in Germany. *Ther Adv Neurol Disord* (2012) 5(5):247–53. doi:10.1177/1756285612453192
108. Langer-Gould A, Huang SM, Gupta R, Leimpeter AD, Greenwood E, Albers KB, et al. Exclusive breastfeeding and the risk of postpartum relapses in women with multiple sclerosis. *Arch Neurol* (2009) 66(8):958–63. doi:10.1001/archneurol.2009.132
109. Hellwig K, Rockhoff M, Herbstritt S, Borisow N, Haghikia A, Elias-Hamp B, et al. Exclusive breastfeeding and the effect on postpartum multiple sclerosis relapses. *JAMA Neurol* (2015) 72(10):1132–8. doi:10.1001/jamaneurol.2015.1806
110. Langer-Gould A, Gupta R, Huang S, Hagan A, Atkuri K, Leimpeter AD, et al. Interferon-gamma-producing T cells, pregnancy, and postpartum relapses of multiple sclerosis. *Arch Neurol* (2010) 67(1):51–7. doi:10.1001/archneurol.2009.304
111. Parra-Medina R, Molano-Gonzalez N, Rojas-Villarraga A, Agmon-Levin N, Arango MT, Shoenfeld Y, et al. Prevalence of celiac disease in latin america: a systematic review and meta-regression. *PLoS One* (2015) 10(5):e0124040. doi:10.1371/journal.pone.0124040
112. Delvecchio M, Faienza MF, Lonero A, Rutigliano V, Francavilla R, Cavallo L. Prolactin may be increased in newly diagnosed celiac children and adolescents and decreases after 6 months of gluten-free diet. *Horm Res Paediatr* (2014) 81(5):309–13. doi:10.1159/000357064
113. Dong YH, Fu DG. Autoimmune thyroid disease: mechanism, genetics and current knowledge. *Eur Rev Med Pharmacol Sci* (2014) 18(23):3611–8.
114. Yamamoto M, Iguchi G, Takeno R, Okimura Y, Sano T, Takahashi M, et al. Adult combined GH, prolactin, and TSH deficiency associated with circulating PIT-1 antibody in humans. *J Clin Invest* (2011) 121(1):113–9. doi:10.1172/jci44073
115. Arany Z. Understanding peripartum cardiomyopathy. *Annu Rev Med* (2017) 69:1.1–1.12. doi:10.1146/annurev-med-041316-090545
116. Hilfiker-Kleiner D, Sliwa K. Pathophysiology and epidemiology of peripartum cardiomyopathy. *Nat Rev Cardiol* (2014) 11(6):364–70. doi:10.1038/nrcardio.2014.37
117. Haghikia A, Podewski E, Libhaber E, Labidi S, Fischer D, Roentgen P, et al. Phenotyping and outcome on contemporary management in a German cohort of patients with peripartum cardiomyopathy. *Basic Res Cardiol* (2013) 108(4):366. doi:10.1007/s00395-013-0366-9
118. Karaye KM, Henein MY. Peripartum cardiomyopathy: a review article. *Int J Cardiol* (2013) 164(1):33–8. doi:10.1016/j.ijcard.2011.11.069
119. Haghikia A, Kaya Z, Schwab J, Westenfeld R, Ehlermann P, Bachelier K, et al. Evidence of autoantibodies against cardiac troponin I and sarcomeric myosin in peripartum cardiomyopathy. *Basic Res Cardiol* (2015) 110(6):60. doi:10.1007/s00395-015-0517-2
120. Ansari AA, Fett JD, Carraway RE, Mayne AE, Onlamoon N, Sundstrom JB. Autoimmune mechanisms as the basis for human peripartum cardiomyopathy. *Clin Rev Allergy Immunol* (2002) 23:301–24. doi:10.1385/CRIAI:23:3:301
121. Sundstrom JB, Fett JD, Carraway RD, Ansari AA. Is peripartum cardiomyopathy an organ-specific autoimmune disease? *Autoimmun Rev* (2002) 1(1–2):73–7. doi:10.1016/S1568-9972(01)00009-X
122. Melo MA, Carvalho JS, Feitosa FE, Araujo Junior E, Peixoto AB, Costa Carvalho FH, et al. Peripartum cardiomyopathy treatment with dopamine agonist and subsequent pregnancy with a satisfactory outcome. *Rev Bras Ginecol Obstet* (2016) 38(6):308–13. doi:10.1055/s-0036-1584567
123. Arrigo M, Blet A, Mebazaa A. Bromocriptine for the treatment of peripartum cardiomyopathy: welcome on BOARD. *Eur Heart J* (2017) 38(35):2680–2. doi:10.1093/eurheartj/ehx428
124. Horn P, Saeed D, Akhyari P, Hilfiker-Kleiner D, Kelm M, Westenfeld R. Complete recovery of fulminant peripartum cardiomyopathy on mechanical circulatory support combined with high-dose bromocriptine therapy. *ESC Heart Fail* (2017) 4:641–4. doi:10.1002/ehf2.12175
125. Sliwa K, Hilfiker-Kleiner D, Petrie MC, Mebazaa A, Pieske B, Buchmann E, et al. Current state of knowledge on aetiology, diagnosis, management, and therapy of peripartum cardiomyopathy: a position statement from the Heart Failure Association of the European Society of Cardiology Working Group on peripartum cardiomyopathy. *Eur J Heart Fail* (2010) 12(8):767–78. doi:10.1093/eurjhf/hfq120

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REVIEW
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Sex Hormones in Acquired Immunity and Autoimmune Disease

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Women have stronger immune responses to infections and vaccination than men. Paradoxically, the stronger immune response comes at a steep price, which is the high incidence of autoimmune diseases in women. The reasons why women have stronger immunity and higher incidence of autoimmunity are not clear. Besides gender, sex hormones contribute to the development and activity of the immune system, accounting for differences in gender-related immune responses. Both innate and adaptive immune systems bear receptors for sex hormones and respond to hormonal cues. This review focuses on the role of sex hormones particularly estrogen, in the adaptive immune response, in health, and autoimmune disease with an emphasis on systemic lupus erythematosus.

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INTRODUCTION

From an evolutionary point of view, the paramount goal of all living organisms is to survive, reproduce and propagate the species. In humans and most vertebrates, the mother has the responsibility to bear the most vulnerable of the species—the offspring, and protect it from danger, to accomplish this supreme mission. Additionally there is non-genetic passive transfer of immunity from mother to offspring called trans-generational immune priming. Therefore, having the parental role may account for stronger immunity in females to defend and "prepare" for this responsibility. Intriguingly, the same immune response shifts during pregnancy to "tolerate" the foreign fetus and prevent rejection. Interestingly, in most fish species the father bears the parental responsibility. The Syngnathidae group includes seadragons, pipefish and the iconic seahorse. In these species, while it is the mother who produces the eggs, the father carries, nurtures the eggs through gestation, and gives birth to the young thus fulfilling the parental and immune priming role. There is evidence that there are differences in the immune response in the male seahorse during the parental vs. mating phases with improved immunity during the parental stage (1, 2). These observations suggest that the parental role comes with great immune power and responsibility. A "side-effect" of the stronger immune response is the higher propensity for developing autoimmune disease. This may be a plausible perspective to understand the gender bias of autoimmune disease.

Sex hormones not only control the reproductive system, but also regulate the development, and function of the immune response. Innate and adaptive, humoral and cell-mediated immune responses are impacted by hormones, and dysregulation of these mechanisms contribute to immune-mediated diseases including autoimmune disease (3–9). While the exact molecular mechanisms of how female hormones regulate the immune system are yet incompletely elucidated, studies show that they control development, homeostasis, gene expression, and signaling processes in T and B lymphocytes to influence their function in health and disease. This review focuses on

the role of sex hormones on the adaptive immune system and in autoimmune diseases with a focus on the prototype systemic autoimmune disease SLE (10–12).

ESTROGEN MECHANISMS OF ACTION

Estrogen acts via classical receptor-mediated, non-classical, and non-ligand-mediated genomic (nuclear) and non-genomic (extranuclear) pathways to control mechanisms of gene expression, protein modifications and signaling to influence cellular functions (**Figure 1**) (13–15).

Genomic Pathways of Action

In the classical genomic pathway, Estrogen, or its most potent form 17-β-estradiol (E2) binds to its cognate intracellular steroid hormone receptor–estrogen receptor (ER). Two types of classical ER have been identified–ERα and ERβ encoded by the *Esr1* and *Esr2* genes respectively. The ER is a ligand-activated transcription factor, which bears ligand- and DNA-binding domains. Estrogen diffuses through the cell membrane, binds to cytoplasmic ER, which undergoes conformational change in the ER, and homo- or hetero-dimerizes. ER dimers then translocate into the nucleus and bind to promoters of target genes to regulate gene expression. In the non-classical genomic pathways, ER bound to DNA can interact with other transcription factors, or the ER may act in tether-mediated manner as co-factor with transcription factors including Specificity protein 1 (Sp1), activating protein 1 (AP-1), NF-κB and p300 proteins. ER/Sp1 and ER/AP-1 interactions activate a large number of genes and pathways and the ligand structure and specific ER-subtype dependent activation of either (16, 17). Activating functions (AF) 1 and 2 domains of the ERα bind to coregulators to regulate transcription and are both important in E2-mediated effects (18). When bound to the ligand, there is differential activation of the two ERs. Specifically ERα transactivates while ERβ inhibits transcription.

The ER binds specific motifs known as estrogen response elements (ERE) within the target DNA. The consensus ERE site is 5′-GGTCAnnnTGACC-3′ (19). While ERE sites within gene promoters are important in transcription, a chromatin Immunoprecipitation (ChIP)-paired end diTag cloning and sequencing whole genome cartography strategy identified ER binding sites in MCF-7 breast cancer cells and noted several interesting findings (20). Only 5% of mapped sites are in the proximal promoter regions of genes while a vast majority is in intronic or distal locations indicating transcriptional regulatory mechanisms over physical distances. Majority of the mapped sites were full ERE sites while 25% were half-sites and a small proportion (4%) had no recognizable ERE sequence (20). ERα and ERβ display dynamic interplay in their chromatin binding capacities and function. ERα and ERβ exhibit substantial overlap in the sites they can recognize, in cells that express either one of these receptors, whereas in cells that express both, fewer sites are shared. Cognate sites for both ERs are ERE-rich, however in cells that express both receptors ERα can competitively displace ERβ shifting it to new sites less enriched in ERE elements (21).

Besides being richly expressed in reproductive tissues, ERs are widely expressed in most cells in the immune system therefore influencing both innate and adaptive immune responses. There is age- and stage-dependent expression of ERs by lymphocyte precursors. Activated T cells express estrogen receptors (22) and both mRNA and protein levels of ER have been described for T cells, B cells, monocytes and dendritic cells. Differential expression of ER genes has been demonstrated in human peripheral blood mononuclear cells (PBMC) (23) and peripheral blood lymphocytes (PBL) (24). PBL CD4, CD8 T cells, B cells, and natural killer (NK) cells contain intracellular ER of which the ERα46 isoform is the most-expressed isoform. A cell surface ERα46 was detected in PBLs, and existence of a functional membrane (m) ERα was confirmed when a membrane-impermeant E2 mediated intracellular signaling activation and proliferation of T cells (24). CD4 T cells express high levels of ERα over ERβ while B cells express more ERβ than ERα mRNA. CD8 T cells and monocytes express low levels of both receptors (23).

ERα undergoes various posttranslational modifications including phosphorylation, acetylation, and ubiquitination, which modulate its stability and/or transcriptional activity. An interesting aspect of ER signaling and ER-mediated gene regulation is the continuous proteasome-mediated turnover of ERα. Estrogen can activate the Ubiquitin-Proteasome Pathway (UPP) to influence post-translational modifications and degradation of proteins. Ubiquitin is a small ∼8 kDa protein which binds a series of three enzymes E1 (Ub-activating), E2 (Ub-carrier or conjugating), and E3 (Ub-ligase), which ultimately link it to the substrate protein. Ubiquitin-tagged proteins are targeted to the proteasome for degradation. This pathway is an important mechanism for tight control of the expression of short-lived inflammatory molecules and transcription factors including nuclear factor kappa B (NFκB), signal transducer and activator of transcription (STAT) 1 and cfos/jun to appropriately control their activity. Steroid hormone receptors including the ERs bind to protein components of the UPP including Ubc9, an E2 conjugating enzyme and E6-associated protein (E6-AP) which is an E3 ligase (25). Kruppel-like factor 5 (KLF5) is an important transcription factor, which inhibits cell proliferation, differentiation and carcinogenesis, and its levels are decreased in cancers including breast cancer. Estrogen induces the expression of estrogen responsive finger protein (EFP), an E3 ubiquitin ligase which leads to degradation of KLF5 in breast cancer cells (26). Similarly estrogen induces EFP-mediated degradation of another transcription factor tumor suppressor AT motif-binding factor 1 (ATBF1) which has an auto regulatory feedback with ERα signaling (27). Estrogen itself mediates downregulation of the ERα through the UPP (25, 28), and subsequently, the ERα mediated transcriptional activity and proteasomal degradation are inter-dependent. ERα was also shown to be a target for small ubiquitin-like modifier (SUMO)-1 modification (29). SUMOylation of the ERα hinge region is hormone-dependent and controls its transcriptional activity thus linking the estrogen and SUMO pathways. E3 ligases protein inhibitor of STAT1 (PIAS)1 and PIAS3 were shown to be E3 ligases for ERα (29), and addition of either Ubc9 or PIAS1 increased ERE-luciferase activity in COS cells (30).

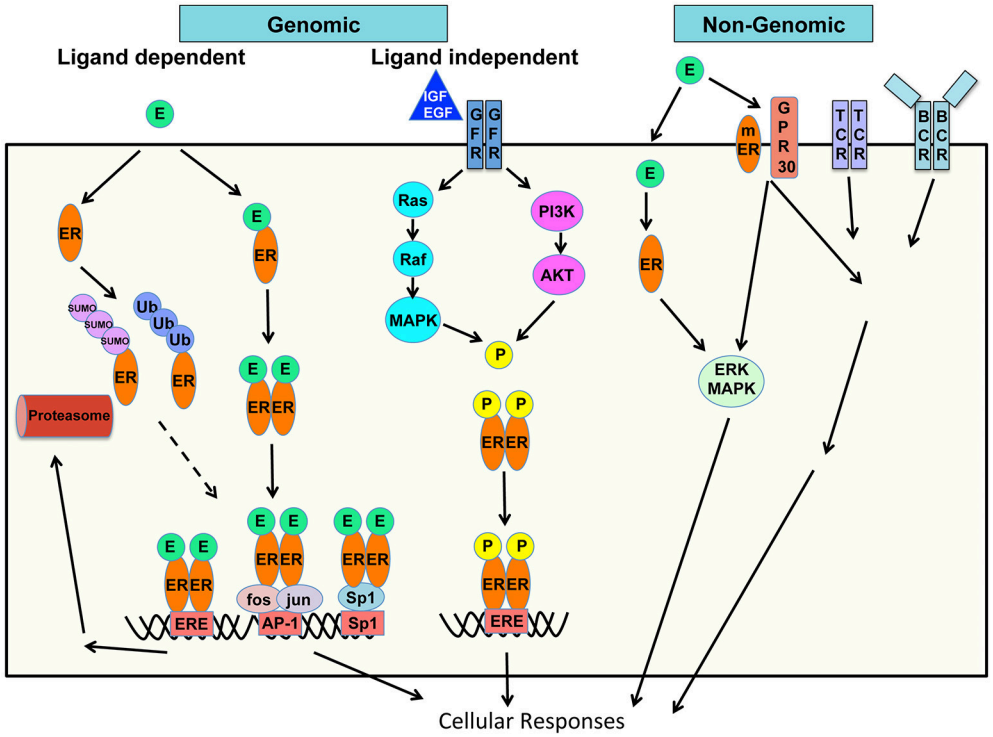

FIGURE 1 | Schematic of mechanisms of estrogen action on cellular responses. Genomic and non-genomic, ligand dependent and ligand-independent, classic and non-classic receptor mediated estrogen-estrogen receptor signaling pathways are shown.

Estrogen-independent functions of the ER include extensive phosphorylation, which control its transcriptional activity independently of its ligand. Environmental cues which activate the phosphoinositide 3-kinase/protein kinase B (PI3K)/Akt pathway and other kinases can phosphorylate the ER to regulate gene expression. ER independent functions of E2 were suggested in studies using ERα deficient wild-type (WT) or lupus-prone New Zealand Black × New Zealand White (NZB × NZW) founder 1 (F1) mice. A link between the ER and Toll-like receptor (TLR) signaling was shown as ERα deficiency led to reduced TLR9 signaling, reduced numbers of plasmacytoid dendritic cells (DC)s and impaired interferon (IFN)-α, interleukin (IL)-6, macrophage/monocyte chemoattractant protein (MCP)-1, IL-1β and IL-23 inflammatory cytokines (13).

Non-genomic Pathways of Action

Besides the genomic pathway of gene regulation, estrogen can mediate effects through non-genomic mechanisms, through cross-talk with signaling cascades. Besides the classical intracellular ERs, Estrogen can bind to membrane estrogen receptors (mER) and membrane-associated G-protein coupled receptors (GPCRs) and trigger signaling downstream in certain cell types. Estrogen binds the G protein-coupled estrogen receptor 1 (GPER1) originally identified as G protein-coupled receptor 30 (GPR30) (31). These are also called rapid effects of estrogen mediated through membrane receptors, receptor tyrosine kinases, and signaling pathways downstream (31, 32). There is also transcriptional activation of genes by the GPER-induced response which include a first tier of transcription factors serum response factor (SRF), cyclic AMP repressor element binding protein (CREB), Ets family, then followed by a second tier including Fos, Jun, connective tissue growth factor (CTGF), early growth response protein (EGR)1, cyclic AMP dependent transcription factor (ATF)3, CCAAT/enhancer binding protein delta (C/EBPγ), and nuclear receptor related (NR)4A2 (33). Ligand induced activation of the mER and GPER can also integrate into intracellular signaling of the immune cell receptor such as the B cell receptor (BCR) signaling and activation pathways. Thus, non-nuclear non-genomic cytoplasmic effects of estrogen are attributed to increased calcium, through phospholipase C beta (PLCβ) activation, Gα and Gβγ protein activation, and kinase pathway activation including the mitogen activated protein kinase (MAPK), (PI3K) and mammalian target of rapamycin (mTOR) pathways (34, 35).

Estrogen and MicroRNA in Post-transcriptional Gene Regulation

In the last decade, the role of microRNA (miR) in post-transcriptional gene regulation has been uncovered as a powerful mechanism of gene regulation in health and disease as evidenced by the dramatic rise in the number of studies and publications in this field (36). miRs are short 22-nucleotide non-coding RNA molecules which are transcribed from genomic DNA and bind complementary sequences within the 3′untranslated

region (UTR) of target genes to block translation or lead to degradation of the mRNA. miRs control genes involved in the immune response and aberrations in miR levels and activity can contribute to pathogenesis of autoimmune diseases. Therefore miRs are considered attractive biomarkers and targets for therapy. A large number (113) of miRs are encoded on the human X chromosome, second only to those on chromosome 1, which encodes 134 miRs, while the Y chromosome only encodes 2 miRs (37). Thus X-linked miRs likely contribute to the sex bias in autoimmunity. While the detailed functional characterization of all X-linked miRs in autoimmunity remains to be elucidated, a number of immune-suppressive genes are targeted by X-linked miRs including Forkhead box P3 (FoxP3), cytotoxic T lymphocyte associated protein 4 (CTLA4), Casitas B-lineage Lymphoma (CBL), CBL-B, suppressors of cytokine signaling (SOCS) genes, and programmed cell death 1 (PDCD1) as evidenced by putative predicted miR target sites within their 3′UTR (37). Besides the X-linked miR-mediated regulation, estrogen regulates microRNA expression to control genes of both innate and adaptive immune responses and therefore has implications for autoimmune disease (8, 36, 38, 39).

Estrogen upregulates miR-18a, miR-148a, miR-223, miR-451, miR-486, and miR-708, and downregulates SLE-linked miR-125, miR-145, and miR-146a. Microarray analysis showed that estrogen differentially regulates miRs in murine splenocytes *in vivo*. Treatment of mice with E decreases miR-146a and increases miR-223 which suppresses lipopolysaccharide (LPS)-induced IFN-γ and nitric oxide (NO) in splenic lymphocytes (40). miRs can also influence ER expression and modulate ER activity in disease (41). Estrogen activates STAT1-dependent transcriptional activation of TLR8 expression to promote inflammatory signaling via miR-21 in extracellular vesicles (42). A major role of estrogen is in bone remodeling and a protective role of estrogen is to suppress osteoclast mediated bone resorption. A novel mechanism by which estrogen preserves bone mass in bone marrow mesenchymal stem cells (BMMSC)s is to induce apoptosis of osteoclasts to protect from bone loss. Estrogen inhibits miR-181, which blocks FasL. Therefore estrogen promotes FasL protein expression by miR mediated posttranscriptional regulation in BMMSCs to maintain bone remodeling balance. In menopause, low estrogen levels, increased miR-181 and reduced FasL can promote survival of osteoclasts and increase bone loss (43).

ESTROGEN AND T LYMPHOCYTES

T Cell Development

It is well known that estrogen suppresses T and B cell lymphopoiesis and activates B cell function. ERs are present on thymocytes as well as thymic epithelial cells (44). Estrogen influences T cell development and lymphopoiesis, and its effects on the thymus are complex. High doses of exogenous estrogen reduce thymic cellularity and cause thymic atrophy. This reduction is attributed to reduced proliferation of thymocytes precursors, both in the thymus and in the bone marrow (45). Accordingly, ovariectomy to remove the endogenous source of estrogen increases thymic cellularity with a shift to increased double positive (DP) thymocytes with reduced double negative (DN) and single positive (SP) cells (46). Conversely, estrogen treatment leads to reduced thymic cellularity with decreased proportions of DP cells (45, 47, 48), increased proportions of single positive (SP) CD4 and CD8 expressing variable beta chain (Vβ) T cell receptor (TCR), and alters distribution and TCRVβ expression of DN thymocytes (49). Pregnancy or treatment with estrogen induces a dramatic involution of the thymus (50–53). Estrogen mediates the loss of cortical thymocytes as evidenced by the reduced size of the thymic cortex in histological studies in mice (54, 55). Estrogen activates extrathymic T cell differentiation in the liver while inactivating intrathymic T cell development (48). However endogenous E2/ERα signaling is necessary for normal thymic size and function, because male and female ERα knockout (ko) mice still had reduced thymi and it was shown that ERα in non-hematopoietic tissues is essential for a normal full-sized thymus. Other receptor pathways are likely involved in estrogen-mediated thymic atrophy (56, 57), possibly due to increased E2 mediated effects through the ERβ or through effects on thymic stromal cells.

Besides thymocytes, sex-hormones also have varied effects on thymic epithelial cells (TEC) as evidenced by transcriptomics studies of cortical (c) and medullary (m) TECs in male, female, and castrated male mice. Male mice accumulated more cTECs but exhibited lower proliferation rates and expressed lower levels of genes involved in thymocyte expansion (58). The autoimmune regulator (Aire) gene is a transcriptional regulator important for expression of tissue specific antigens in mTECs for the positive and negative selection of T lymphocytes in the thymus. Thus Aire is a key molecule in central tolerance. In both mice and humans, reduced levels of Aire were found in females compared to males after puberty (59, 60). Estrogen downregulated Aire in cultured TECs, in human thyme grafted into mice, and in murine fetal thymic organ cultures by epigenetic modifications within the *Aire* promoter (60). Therefore estrogen-mediated regulation of T cell development and repertoire selection are important for central tolerance and contribute to autoimmunity.

T Cell Homeostasis

The role of estrogen on cellular homeostasis is complex, depends on the cell/tissue type, concentrations of estrogen, and physiologic or pathological contexts (61). While physiologic concentrations of 17-β-estradiol stimulate survival and proliferation of cancer cells, and suppress apoptosis via Ras signaling in an ER dependent manner (62), pharmacological doses inhibit proliferation and induce apoptosis by ER independent pathways (63). Pharmacologic doses of 17-α-estradiol but not 17-β-estradiol induced G2/M cell cycle arrest in Jurkat cells which is exerted by ER independent mechanisms (64).

Estrogen stimulates growth and inhibits apoptosis a variety of cells but there is also evidence that estrogen induces apoptosis in breast cancer and other cells. Estrogen regulates apoptosis by both extrinsic Fas/FasL and intrinsic mitochondrial pathways (61). Culture of human PBMCs from healthy donors with 2-methoxyestradiol followed by pharmacologic phorbol myristic

acid (PMA)/Ionomycin or physiologic CD3/CD28 stimulation led to decreased apoptosis and decreased Caspase-9 activity and reduced T cell proliferation with modest decreases in tumor necrosis factor (TNF) and IFN-γ production (65).

Ovariectomy in female albino oxford (AO) inbred rats led to an increase in the CD8 T cell compartment in peripheral blood and spleen, reflected in increased thymic double positive and CD8 cells and recent thymic emigrants (RTE) in peripheral blood. It also increased CD4+FoxP3+ CD4 T cells generation in the peripheral lymphoid tissues (66).

T Cell Activation

Estrogen influences not only development but also various functions of T cells, in particular CD4 T cells including activation, cytokine production differentiation and regulatory functions with impact on physiology and autoimmune diseases (67, 68).

Estrogen and ERα are important in the activation, proliferation and pathogenic potential of T cells. T cell specific deletion of the ERα in mice led to transcriptomics changes with reduced expression of genes involved in T cell activation and reduced pathogenic potential in a T cell transfer model of colitis model (69). Estrogen downregulates DNA methyl transferase (DNMT) 1 expression and enhances global DNA hypomethylation in CD4 T cells from female SLE patients. While plasma β-estradiol levels were similar between patients and healthy controls, the mRNA expression of ERα but not ERβ was increased in SLE CD4 T cells (70). Aberrant extracellular regulated kinase (ERK)/MAPK signaling and resultant decrease in DNMT levels leading to DNA hypomethylation of a number of genes has been described and associated with autoimmune disease pathogenesis (71).

Estrogen controls immune cell activity through regulation of cellular metabolism via its receptors ERα, ERβ, membrane receptor mERα, mERβ, and GPER by direct and indirect mechanisms. The E2-ER-mediated control of transcription and signaling pathways stimulate mitochondrial function (72). The orphan nuclear receptor Estrogen related receptor (ERR) α controls transcription of a wide range of metabolic genes (73). ERRα was shown to control metabolic activity in T cells to influence T cell activation and is critical for Teffector (Teff) cell differentiation *in vivo* in ERRα-deficient mice. ERRα protein levels are low in resting T cells but increase upon activation. Glut1 upregulation, glucose uptake and mitochondrial processes were diminished in the absence of ERRα *in vivo* (74).

T Cell Differentiation and Cytokine Production

Estrogen regulates a number of cytokines that modulate the immune response. Pharmacologic doses of the synthetic estrogen diethylstilbestrol in mice led to reduced proliferation of splenic T cells, reduced IL-2 production and increased susceptibility to *Listeria monocytogenes* infection (75). Estrogen increases NFκB signaling activity and its ensuing cytokines including IL-1, IL-10, and IFN-γ in C57Bl/6 mouse splenocytes (76). To assess the role of estrogen on T cell immune responses, concentration dependent effects of 17-β-estradiol *in vitro* cultures of T cells and splenocytes from rats were studied to assess the effects on proliferation, cytokines (IL-2 and IFN-γ), and signaling molecules ERK1/2, CREB, and Akt (77). Lower concentrations of estrogen enhanced proliferation and IFN-γ production in an ER dependent manner. The ERα agonist propyl pyrazole triol (PPT) suppressed IL-2, but the ERβ agonist diarylpropionitrile (DPN) increased IL-2. These effects were associated with increased levels of phosphorylated (p)-ERK, p-Akt and p-CREB and increased activity of antioxidant enzymes and NO production (77).

The luteal phase of the menstrual cycle in healthy young women associates with reduced IL-2 levels as evidenced by bioassay activity of serum IL-2 measurements as well as intracellular IL-2 within peripheral blood lymphocytes stimulated *ex vivo* (78). This decreased IL-2 may account for the observed increase in pre-menstrual infections or may presumably be a facet of the immune suppression necessary for a potential pregnancy. In human studies, E2 suppressed IL-2 production in T cells from healthy women and increased the expression of Sp1 transcription factor and the cAMP response element modulator (CREM) transcriptional repressor (79, 80). These studies showed that estrogen has specific concentration- and receptor-subtype dependent effects on immune responses.

Estrogen increases T helper cell (Th) 1 differentiation, IFN-γ and the inflammatory effects mediated by IFN-γ including production of inflammatory mediators inducible nitric oxide synthetase (iNOS), NO, and (cyclooxygenase) Cox2. Estrogen increases IFN-γ mRNA levels in murine splenocytes by activating the *IFN-γ* promoter, which contains consensus ERE sites as shown by promoter reporter assays in Jurkat cells (81). Administration of estrogen to ovariectomized Bagg Albino (BALB)/c mice followed by immunization with exogenous antigens increased antigen-specific clonal expansion of CD4 T cells and selectively increased Th1 cells and IFN-γ production. ERα on hematopoietic cells was necessary for the Th1 responsiveness (82). Further, estrogen was shown to upregulate the Th1 driving transcription factor T-box protein expressed in T cells (T-bet) in murine splenocytes by IL-27 and partly by IFN-γ but not by IL-12 (83). IL-12 signaling activates two isoforms of signal transducer and activator of transcription (STAT) 4, a full length STAT4α and a short STAT4β isoform. Estrogen selectively activates the short isoform (84).

Estrogen increased levels of IL-17 and its driving transcription factor retinoic acid receptor (ROR)γt in activated splenocytes from male and female C57Bl/6 wild-type mice and in lupus-prone male NZB/W mice. IL-27 and IFN-γ suppressed the IL-17 induction (85).

Other studies have shown the opposite effect of estrogen and ER on Th1 and Th17 cytokines and disease. In the experimental autoimmune encephalomyelitis (EAE) murine model of the CNS autoimmune disease multiple sclerosis (MS), estrogen mediates a neuroprotective effect (86), and suppresses Th1, Th17 responses. The estrogen-mediated inhibition of Th17 responses in this system is specifically via ERα expression on T cells (87). Estrogen suppresses IL-17 and Th17 differentiation in mouse CD4 T cells by downregulating the RORγt transcription factor mRNA and protein expression. This effect was mediated by an E2-activated complex of ERα and repressor of ER activity (REA) binding to three ERE half sites within the RORγt promoter (88). These

studies indicate differential tissue-specific effects of estrogen on the immune response.

Estrogen is crucially important for its beneficial effects on bone metabolism, and postmenopausal estrogen decline is a critical factor in chronic inflammatory events including osteoporosis. IL-17 is implicated in the pathogenesis of inflammatory arthritis including RA and promotes bone loss in collagen-induced arthritis. In studies to assess the role of estrogen in IL-17 mediated regulation osteoclast and osteoblast differentiation, estrogen reversed the bone-destructing effects of IL-17. Therefore estrogen deficiency resulting in the de-repression of IL-17 may contribute to osteoporosis (89). Correspondingly, an evaluation of serum IL-17 levels in pre- and post-menopausal women showed a high prevalence of IL-17A levels in postmenopausal women, and inversely correlated with total lumbar T-scores, measures of bone loss (90). Estrogen also protects from bone loss through a transforming growth factor beta (TGF-β) signaling mediated pathway in T cells. TGF-β is an immunosuppressive cytokine and represses T cell activation, proliferation, and secretion of inflammatory cytokines. Accordingly, T cell specific TGF-β-signaling deficient mice had bone loss due to a de-repression of T cell activation and increased levels of osteoclastogenic cytokines TNF and receptor activator of NFκB ligand (RANKL) (91).

Peroxisome proliferator-activated receptor gamma (PPARγ) a nuclear receptor has recently been recognized as a critical regulator of adaptive immunity by negative regulation of T cell activation proliferation and differentiation. PPARγ mediated inhibition of Th1, Th2, and Th17 differentiation of naïve CD4 T cells from female C57Bl/6 mice whereas male cells only showed Th17 inhibition. Estradiol co-treatment of male cells inhibited Th1, Th2, and Th17 differentiation indicating that estrogen increases the sensitivity of male cells to the effects of PPARγ activation (92). Administration of the neurosteroid dehydroepiandrosterone (DHEA) inhibited Th17 responses and induced IL-10 producing regulatory cells in EAE and importantly reversed established paralysis and central nervous system (CNS) inflammation in mice. Further, DHEA-treated PBMCs from patients with relapsing remitting multiple sclerosis (RR-MS) exhibited decreased IFN-γ, IL-17, IL-4, and IL-2 responses but preserved IL-10. Thus such compounds, which suppress pro-inflammatory cells and expand regulatory subsets, could be useful as therapeutic agents (93).

Regulatory T Cells (Tregs)

Tregs are vitally important in the maintenance of self-tolerance and prevention of autoimmunity, and the X-linked master regulator transcription factor FoxP3 drives their generation, maintenance, and function (94, 95). Female gender and hormonal influences regulate FoxP3 expression and therefore are critical in the physiology of regulatory CD4 T cells and the gender bias of autoimmune disease (96). An imbalance between Teffs and Tregs is thought to contribute to dysregulated immune homeostasis and autoimmune disease.

In line with the observations that there is a maternal shift in the immune response to promote fetal tolerance, estrogen induced increased expression of CD25+ cells and increased FoxP3+ expression in naïve mice treated with Estrogen (97). Estrogen enhances Treg numbers and function, and induces FoxP3 expression both *in vitro* and *in vivo* (96). This effect is partially mediated through the checkpoint inhibitor programmed cell-death protein 1 (PD1). PD1 is a negative regulator of immune responses, is upregulated on activated T cells, considered a marker of dysfunctional T cells, is important for immune tolerance, and is an attractive target for autoimmune disease and cancer (98). Estrogen administration increased intracellular PD1 expression in CD4+FoxP3+ T cells, and PD1 expression was reduced in ER knockout mice (98).

Estrogen promoted the *ex vivo* proliferation of Tregs isolated from healthy human donors and also enhanced suppressive function in co-cultures with responder CD4+CD25- effector T cells (Teffs) (99). Increase in CD4+CD25+FoxP3+ T cells were observed in peripheral blood of fertile non-pregnant women in the late follicular phase of the menstrual cycle which correlated with β-estradiol levels, while there was a significant decline in Treg numbers in the luteal phase. Lower numbers of Tregs were found in follicular and luteal phases in women with recurrent spontaneous abortions (RSA) as well as from postmenopausal women. In addition Tregs from women with RSA also had reduced suppressive capacity compared to fertile women (100). Estrogen mediates its protective effect on bone metabolism through modulating Treg function on osteoclasts and bone resorption *in vitro* (101). E2 enhanced the suppressive capacity of Tregs on osteoclast differentiation from human embryonic bone marrow cells (BMC). Increased levels of both TGF-β1 and IL-10 suppressive cytokines were required for this effect because neutralizing both cytokines together but not individually, abolished the suppressive effect (101).

In Tregs derived from human cervical cancer tumor tissues, ERα blockade abolished FoxP3 expression and impaired suppressive function. ERE sites were found within the FoxP3 promoter ERα bound to the FoxP3 promoter in male blood-derived Tregs. Co-IP of E2 revealed E2-ERα complexes with FoxP3. Blocking with the anti-estrogen ICI 172 180 led to increase in IFN-γ & IL-4 production from Teffs derived from cervical-cancer suggesting that ER blockade could potentially restore certain Teff functions in tumors. These results showed that E2 and ERα are required for the FoxP3 expression and tumor-derived Treg and Teff function (102).

T Cell Trafficking

Estrogen contributes to immune cell trafficking and inflammation by regulating chemokines and chemokine receptors. T cells from female mice displayed increased mRNA and protein expression of CC chemokine receptor (CCR) 1-CCR5 and increased transmigration response to chemokines macrophage inflammatory protein (MIP)-1β and stromal cell-derived factor (SDF)-1β. Similar increases in CCR gene expression were found in T cells from mice treated with estrogen *in vivo* (103). Estrogen increased the secretion of MCP-1, MCP-5, eotaxin, and SDF from mitogen activated splenocytes from estrogen treated mice (104). Further, *in vivo* trafficking of T cells was shown to be gender and estrogen-dependent.

Ovariectomized DBA/1 mice treated with estrogen and subjected to collagen-induced arthritis had fewer Th17 cells in the joints and less severe arthritis. However increased numbers of Th17 cells were found in the lymph nodes in early phase of disease, followed by a decrease in Th17 cells in the joints during established arthritis. Increased expression of CCR6 on the Th17 cells and corresponding increase in the chemokine CCL20 was though to contribute to interference with the egress of Th17 cells from lymph nodes to the joints indicating that estrogen modulates Th17 migratory pathways in inflammatory arthritis (105).

T Follicular Helper (Tfh) Cell Function

Tfh cells provide cognate help to B cells to promote class switching and antibody production, and are implicated in autoantibody production in autoimmune diseases (106). Estrogen mediates gender-specific differences in regulation of Tfh cells responses via PPARγ. 4-hydroxy-3-nitrophenylacetyl hapten conjugated with ovalbumin (NP-Ova) immunization of female CD4/PPARγ deficient mice induced increased Tfh cells and germinal center (GC) B cells. Correspondingly treatment with a PPARγ agonist reduced responses in female and with E2 co-treatment in males (107). Estrogen increased Calcineurin and CD40 ligand (L) mRNA and protein expression in T cells from female SLE patients in an ER-dependent manner, therefore contributing to cognate B-cell help (108).

ESTROGEN AND B CELLS

Sex hormones play an important role in B cell development and function in physiology (109, 110) and contribute to their dysfunction in autoimmune disease (111). It has been known for a long time that estrogen enhances humoral responses, enhances B cell differentiation and immunoglobulin (Ig) production (112, 113).

B Cell Development

Similar to its effects on thymic T cell development, estrogen suppresses B cell lymphopoiesis. Estrogen controls lymphoid-restricted progenitors in the bone marrow. Early B cell precursors are estrogen-sensitive and are decreased in the bone marrow during pregnancy and following estrogen administration in mice and humans. Specifically estrogen blocks B cell development at the differentiation step from pro-B cell to the pre B-cell stage (114–118). The E2-mediated inhibition of B lymphopoiesis is both due to a direct effect on B cells as well as on the stromal cells partially due to reduced production of the homeostatic cytokine IL-7 and increased expression of soluble frizzled related protein 1 (sFRP1) (119, 120).

B Cell Homeostasis and Activation

Besides lymphopoiesis and differentiation, estrogen regulates peripheral B cell populations, and tolerance induction by promoting survival and activation of autoreactive B cells (121, 122). In splenic populations estrogen treatment leads to increased marginal zone (MZ) B cells, reduced transitional B cells and slightly increased follicular B cells (111, 123–125). In BALB/c R4a mice transgenic for an anti-DNA antibody, E2 treatment led to increased serum anti-dsDNA antibodies, peripheral lymphoid expansion of high-affinity antibody-positive B cells, and increased expression of anti-apoptotic protein Bcl-2 in the germinal center B cells (126). Estrogen increased expression of activation genes including CD22 and SHP-1 and overexpression of these genes led to reduced B cell receptor (BCR) signaling (124). These DNA-reactive B cells escape deletion and E2 mediates rescue of autoreactive cells at the immature and transitional B cell stages. Specifically it was the high-affinity DNA-reactive B cells competitively survived in E2 treated mice compared with the low-affinity B cells in control mice (125). While both ERα and ERβ mediated B cell maturation, and CD22 expression, ERα was involved in the E-mediated decrease in BCR signaling, indicating differential roles of ERα and ERβ in B cell maturation vs. selection (127). Thus autoreactive B cell differentiation depends on the hormonal milieu wherein estrogen promotes marginal zone B cells (123), their long-term persistence and autoantibody secretion (128).

B Cell Function

B lymphocyte stimulator (Blys) also called B cell activating factor (BAFF) is a vital cytokine for survival and maturation of B cells, and elevated serum levels have been found in SLE patients (129). Steady state mRNA and protein levels of BAFF were higher in immune cells from C57Bl/6 female mice and estrogen treatment increased BAFF expression which was mitigated in ERα, STAT1, or IRF5 deficient mice (130). Administration of β-estradiol by subcutaneous implants in NZB/W lupus-prone mice increased serum Blys levels, autoantibodies, and accelerated proteinuria and glomerulonephritis (131). In human studies, estrogen treatment led to increased BAFF mRNA levels in peripheral blood leukocytes from healthy men and women. Progesterone treatment increased BAFF mRNA in cells from women in a dose dependent manner, while lower concentrations increased but higher concentration decreased expression cells from men (132).

Besides the E2-mediated effect on B cell activation, which leads to increased immunoglobulin (Ig) antibody production from both bone marrow and splenic B cells, there is evidence of a direct effect of estrogen receptors on the Ig heavy chain locus. Specifically ERE were identified within the heavy chain switch (S) regions and an ERα antibody-mediated ChIP-sequencing (seq) analysis on genomic DNA from LPS-activated B cells revealed numerous ERα binding to key regulatory elements. These data support the idea that nuclear hormones and receptors can directly regulate class switch recombination and antibody expression (133).

In summary, estrogen mediates key effects on B cell physiology and function, which are vital in the pathogenesis of autoimmune diseases like SLE.

ESTROGEN AND AUTOIMMUNE DISEASES

The female predilection of autoimmune diseases ranging from 3:1 for MS to 15:1 for autoimmune thyroiditis clearly implicates the female gender and sex hormones in

autoimmunity (6, 8). While progesterone and androgens are considered immunosuppressive, therefore protective, estrogens in general are considered immune-stimulatory therefore pathogenic in autoimmune diseases. However, the role of estrogen is complicated and in some diseases, estrogens are immunostimulatory while in others they are inhibitory. There is an interesting dichotomy in the estrogen-mediated effects on different autoimmune diseases. While diseases like SLE worsen during pregnancy, others including MS, rheumatoid arthritis (RA), uveitis and thyroiditis improve, likely due to the maternal shift from a Th1 to Th2 immune response presumably as an attempt to avoid fetal rejection, and to enhance antibodies for passive transfer of immunity to the fetus. The diseases that are critically dependent on the T cell-dependent Th1 response, benefit from this diversion, while in SLE a shift to the Th2 propagates the autoantibody response to worsen disease.

SLE

SLE is a prototypical chronic systemic autoimmune disease afflicting women in the childbearing years and can affect any organ in the body (10, 11). Joints and skin are frequently involved, while complications in vital organs such as kidneys can lead to lupus nephritis and renal failure. Complex interaction of genetics, environmental factors, and hormones lead to the deregulation and aberrant activation of the innate and adaptive immune systems leading to circulating autoantibodies and inflammatory immune cells which eventually lead to destruction of target organs (134, 135). Historically, studies with gonadectomy/hormone deprivation and hormone supplementation in male and female lupus prone mice have shown a clear association of sex hormones with lupus, where estrogen accelerates or worsens disease and estrogen removal ameliorates disease in females. Male gonad removal increases susceptibility to disease in male mice and androgen supplementation improves disease in female mice (6).

The role of ERs has been studied in various murine models of lupus. Ovariectomized NZB/W mice treated with the potent ERα agonist PPT developed increased levels of autoantibodies and proteinuria earlier and succumbed to disease sooner than control counterparts. However, the ERβ agonist DPN reduced some anti-dsDNA autoantibodies but not total IgG, proteinuria or mortality. These studies indicate that ERα has a pro-inflammatory role while ERβ has mild immunosuppressive effects in this system (136). Correspondingly, ERα deficiency attenuated autoantibodies and glomerulonephritis and improved survival in female and male (NZBxNZW) F1 mice (137). Another study found amelioration of disease in ERα-deficient female but not male NZM2410 and MRL/lpr strains of lupus-prone mice (138). Monthly injections of estradiol into ERα deficient mice induced a serum Th2 cytokine profile, increased kidney damage and death while minimal changes were observed in similar experiments conducted in ERα deficient mice (139).

Estrogen and ER signaling contribute to the activation or repression of a number of immunomodulatory cytokines, which contribute to disease pathogenesis and organ pathology in lupus (68). The murine lupus susceptibility locus *Sle1c2* is a sublocus of the NZM2410-derived Sle1 major lupus susceptibility locus and contributes to CD4 T cell activation, increased IFNγ-expressing T cells, and increased susceptibility to chronic graft vs. host disease (cGVHD), When crossed into the NZB lupus-prone mice, *Sle1c2* enhanced B cell activation, autoantibodies, and renal pathology. This locus contains the estrogen related receptor γ (*Esrγ*), expressed in T cells, which encodes for an orphan nuclear receptor that controls mitochondrial function and oxidative metabolism. B6Sle1c2 CD4 T cells expressed reduced levels of Esrγ, which correlated inversely with CD4 activation compared to B6 CD4 T cells. Increased levels of mediators of glycolysis, with reduced mitochondrial mass and membrane potential, but increased reactive oxygen intermediates (ROI) indicating mitochondrial dysfunction (140, 141). While global deficiency of ERα in lupus-prone B6.Sle1 mice ameliorates disease (142), conditional deletion utilizing the Cre-lox technology has shown the effect of ERα in specific immune cells. B cell specific deletion of ERα by crossing ERα flox mice with CD19-Cre mice delayed autoantibody production and lupus nephritis in (NZBxNZW) F1 lupus-prone mice (143).

SLE T cells display numerous defects in homeostasis, phenotype, signaling, metabolism, and function (12, 135, 144) and estrogen influences T cell signaling and activation in T cells from SLE patients. While serum estrogen levels *per se* have not been found to be significantly different in women with SLE, increased estrogen metabolism is observed. Higher levels of more feminizing estrone metabolites are observed in SLE patients and their first degree relatives implying that more potent metabolites may induce more potently epigenetic changes via the ERs (6, 145). ERα and ERα transcripts are expressed in PBMCs (146), and T cells from SLE patients and exhibit biologically active ER proteins binding to ERE sites (22). Differential expression of the ER subtypes and antibodies against ERs impact disease activity. Some studies have found alterations in ER expression with increased ERα mRNA levels but decreased ERαβ transcripts in PBMC from SLE patients (147). Others examined of intracellular ERα and ERβ in T cells showed much greater variability of expression of the ERs in SLE patients compared to healthy controls. ERα is implicated in a pro-inflammatory pathogenic role while ERβ has some anti-inflammatory roles in SLE. Polymorphisms in the ERα (*Esr*) gene have been linked with SLE and found to be significantly associated with the development of disease or age at disease onset, with a higher frequency in childhood-onset vs. adult onset patients or with disease features and severity (148–152).

ERK pathway downregulation and DNA hypomethylation are well-known underlying epigenetic aberrations in SLE (71, 153, 154). Estrogen suppressed ERK phosphorylation in *ex vivo* stimulated SLE T cells from patients with inactive or mild disease (155). In (C57Bl/6xSJL) F1 mice transgenic for a dominant negative MEK (dnMEK) selectively in T cells, estrogen led to ERK inactivation, DNA hypomethylation of the X-linked gene *CD40L*, and increased autoantibodies in female but not male mice. Estrogen-induces miR148a (39) which targets and

suppresses DNMT1 expression in T cells leading to increased DNA hypomethylation (156). These results showed an effect of estrogen on epigenetic regulation of genes involved in disease pathophysiology (157).

The calcium-dependent phosphatase Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) to activate NFAT-mediated transcriptional activation of genes including the B-cell help molecule CD40L/CD154. Estrogen increases Calcineurin and CD154 expression levels in an ER dependent manner in T cells from women with SLE but not healthy controls (158, 159). Estradiol also increased the calcium-buffering protein Calreticulin in activated T cells from healthy donors but variably modulated it in activated T cells from SLE patients, suggesting a deregulated control in SLE T cells (160). Zinc finger acidic domain structure 3 (ZAS3) is a signaling and transcription factor, which regulates inflammatory responses. Increased ZAS3 mRNA and protein levels were found in PBMCs from SLE patients, and estradiol treatment increased ZAS3 expression levels in PBMCs and in mice injected with estradiol. ERα bound to ERE sites within the ZAS3 locus and was required for E2-mediated induction of ZAS3 (161).

Estrogen decreased activation induced cell death (AICD)-mediated apoptosis and downregulated FasL mRNA and protein expression in an ER-dependent manner in PMA-activated T cells *ex vivo* from SLE patients (162). Another study found that *in vitro* estradiol treatment of T cells from SLE patients led to increased expression of FasL and Caspase-8 but no change in Fas, Bcl-2, and Caspase-9 mRNA level (163). Thus the estrogen-mediated persistence of autoreactive cells may contribute to autoimmunity in SLE. Autoantibodies to ERα but not ERβ were identified in sera of about half of SLE patients tested, and ERα abs induced activation and apoptosis both in resting T cells and after CD3 activation. ERα autoantibody levels correlated with SLE disease activity index (SLEDAI) and arthritis clinical parameters (164) indicating that ERα autoantibodies disrupt T cell homeostasis in autoimmune disease.

Microarray gene profiles from activated T cells from female SLE patients and healthy controls showed alterations in a number of signaling pathways including Type I interferon, which has been clearly associated with disease initiation and progression. A Type I IFN gene altered was the vitamin D receptor interacting protein (DRIP150) suggesting that aberrant regulation of a cofactor may contribute to estradiol sensitivity in SLE T cells (165). Microarray analysis in PBMCs from SLE patients and healthy controls treated with estradiol revealed estrogen-mediated gene signatures. Many more genes were differentially regulated by estradiol in SLE T cells compared to healthy controls. Of note were pathways with genes involved in post-translational modification (161). A recent study utilized *in vitro* culture of T cells from female SLE patients or controls with the ER antagonist Fulvestrant/Faslodex (ICI 182, 780) to assess the global effects on estrogen-mediated genes signaling pathways by microarray gene profiling. Pathways of Th cell differentiation, steroid receptor (GR/ER) signaling, ubiquitination and sumoylation pathways were significantly altered. While the mRNA levels of both ERα and ERβ and protein levels of ERβ were similar, the protein expression of ERα in SLE T cells *ex vivo* was significantly lower in SLE compared to healthy controls suggesting an increased turnover (166). These studies suggest that increased turnover of ERα in SLE T cells may sensitize T cells to estradiol and contribute to their altered function.

In SLE, an imbalance between Th17 and Tregs is thought to contribute to and correlate with disease pathogenesis (167, 168). IL-6 is a crucial cytokine in this balance because IL-6 (with low dose TGFβ) drives naïve CD4 differentiation to Th17 cells, rather than Tregs (169), and inhibits TGFβ-induced Treg differentiation. High doses of TGFβ drive Treg differentiation. In addition, IL-6 in combination with IL-1β leads to degradation of FoxP3 (170). High serum and urine levels of IL-6 are found in SLE patients and correlate with disease activity (171–174). E2 stimulates IL-6 expression by biliary epithelial cells in mice and humans (175). IL-6 production is controlled genetically in an age- and gender dependent manner. In a human study (n.62, n.31 men and 31 women, aged 29 to 93 years), plasma IL-6 levels, IL-6 production by stimulated PBMC *ex vivo*, and a C to G transition at nucleotide−174 of the IL-6 gene promoter (−174 C/G locus) were assessed. Results showed that IL-6 production increases with age and is dominant in women (176). Accordingly, IL-6 knockout female C57BL/6 mice were resistant to syngeneic-activated lymphocyte-derived DNA (ALD-DNA)-induced SLE and IL-6 blockade increased FoxP3 expression, therefore showing that IL-6 suppresses Tregs to promote lupus (177). Thus IL-6 is a critical inflammatory cytokine, which shifts the balance from Tregs to Th17.

Type I as well as type II IFN cytokines are important in autoimmunity and inflammation (178, 179). Treatment of splenocytes from C57Bl/6 or lupus-prone NZB/W mice and murine cell lines with either IFN-α or IFN-γ led to increased expression of ERα mRNA and protein levels, via transcriptional activation of the *Esr1* promoter through STAT1. E2 and IFN signaling co-operatively activated ERα and IFN-responsive genes. These data bring to light a mutual positive regulatory feedback in which interferons activate ERα which activates IFN-γ and IFN-γ-mediated interferon regulatory factor (IRF) 9 to further amplify the inflammatory loop (180).

TNF-like weak inducer of apoptosis (TWEAK) is a TNF superfamily proinflammatory multifunctional cytokine, which can lead to increased inflammatory mediators including IL-6, MCP1 associated with renal damage in SLE (181). Higher urinary levels of soluble TWEAK were found in patients with renal damage compared to those without. Estrogen through ERα promotes expression of to accelerate the progression of lupus nephritis. E2 treatment of PBMCs from lupus nephritis (LN) patients led to increased mRNA levels of TWEAK, which were abolished in the presence of ERα inhibitor methyl-piperidino-pyrazole (MPP) and ER antagonist Fulvestrant (ICI 182 780). Similar results were obtained after ovariectomized MRL/lpr lupus-prone mice were treated with estrogen or antagonists. Severe renal pathology and high serum IL-6 levels in these mice were reversed by co-treatment *in vivo* with shRNA to inhibit TWEAK. (182). In C57BL/6 ERα knockout mice the nephrotoxic serum nephritis (NTN) model of immune-mediated

nephropathy was used to assess the role of ERα in lupus nephritis. Time-course microarrays on murine glomeruli from wt and ERα-ko NTN-induced mice showed increased PPAR-γ mediated lipid metabolism and decreased retinol metabolic pathways. In parallel, RNA-seq analysis of whole blood from SLE patients revealed similar expression profiles of these pathways (183). Thus ERα signaling impacts metabolic activity in the kidneys to promote immune-mediated nephropathy and has implications for lupus nephritis.

These studies indicate that female hormones particularly estrogen plays important roles in immune cell generation, homeostasis, and function which impact control of immune responses. Caution must be exercised while interpreting data due the differences in systems studied, heterogeneity in patient populations, numbers and disease state of patients examined, and most importantly, concentrations and durations of estrogen exposure. Importantly, depletion of ERα and estrogen supplementation studies must be very carefully interpreted because most studies have been carried out with ERα knockout mice which have a functional rather than genetic ERα deficiency because they carry an N-terminal truncated form which lacks the critical AF-1 domain required for most classic estrogen actions. However ovariectomized true ERα−/− mice with genetic deletion of ERα in the NZM2410 strain, were not protected from lupus-like disease suggesting that other hormones perhaps testosterone mediate protection rather than the loss of full-length ERα (184).

OTHER AUTOIMMUNE DISEASES

While estrogen and ERs contribute to SLE pathogenesis and worsen disease activity in mice and humans, immune-protective effects are observed in other autoimmune diseases such as Multiple Sclerosis (MS) and rheumatoid arthritis (RA) (185).

Multiple Sclerosis

In MS, autoreactive T cells attack myelin tissue in the central nervous system leading to axonal demyelination and CNS dysfunction. Disease follows a relapse-remitting or progressive type of course. In this disease, both in humans and in the EAE mouse model, estrogen is neuroprotective by shifting the immune response and suppressing immune activation (186–189). Serial brain magnetic resonance imaging (MRI) during follicular and luteal phases of the menstrual cycles in eight women with relapsing-remitting MS showed significant correlation between Progesterone/β-estradiol ratios with both the numbers and volumes of lesions (190). A major clinical observation was that during pregnancy, the relapse rate of MS declines in the third trimester, but increases in the 3 months post-partum period (186). A pilot trial treatment of non-pregnant women with the pregnancy hormone estriol showed improvement in disease lesions (187). These effects are presumed to be due to the shift from a proinflammatory Th1 to anti-inflammatory Th2 immune response environment. Estrogen ameliorates EAE, and E2-ERα leads to reduced pro-inflammatory Th1, Th17 cells, and cytokines IFN-γ, IL-17, TNF, and other molecules iNOS and MCP-1. In addition Estrogen induces anti-inflammatory cytokines IL-10 and TGF-β and promotes expansion of Tregs. Estrogen suppresses CD4 T cell expansion, increases T cell apoptosis. E was shown to protect from atrophy of gray matter in EAE. ERα is shown to be pathogenic while ERβ is protective in MS. Accordingly ERβ ligand estriol administration was neuroprotective in EAE in mice (191). A new ERβ ligand AC186 improved reduced neuropathology in chronic EAE (192). A placebo-controlled multi-center Phase2b trial with oral ERβ ligand estriol improved disease activity (193), and another clinical trial is currently ongoing (www.clinicaltrials.gov).

E2 is protective in the EAE model of autoimmune disease in both male and ovariectomized female mice and this effect is partially mediated by modulation of Tregs (194). Estrogen upregulated PD1 expression in CD4+FoxP3+ Tregs, and PD1 levels rather than the frequency of Tregs, correlated with the degree of E2-mediated EAE protection. E2 also dramatically reduced IL-17 production, and this effect and protection from EAE were partially abrogated in the PD1ko mice (195). While PD1ko mice had normal FoxP3 expression levels, Tregs were functionally defective in their suppressive capacity which was partially restored by pre-treatment of the mice with Estrogen without much increase in FoxP3 levels. These results imply that estrogen influences Treg function via both PD1-dependent and independent pathways (196). EAE was suppressed in pregnant mice and in ovariectomized mice that received pregnancy levels of estrogen. Estrogen suppressed proliferation of T cells and decreased proinflammatory Th1 (IFN-γ, TNF-α) and Th17 (IL-17, IL-6) cytokine protein and mRNA levels while elevated Th2 (IL-4) and Treg suppressive (IL-10, TGF-β) cytokines in MOG-restimulated splenocytes and lymph node cells *ex vivo* from immunized mice. Accordingly, the respective transcription factors T-bet and RORγt were decreased while GATA3 binding protein (GATA3) and FoxP3 expression were increased (197).

Rheumatoid Arthritis

RA is the most common systemic rheumatic autoimmune disease and has a female to male incidence of 4:1 before the age of 50 and about 2:1 after the age of 60 years with the peak incidence around the fifth decade. Therefore female hormones clearly play a role in disease (198–200). However the contribution and effects of hormones in RA disease development are complicated and still not fully understood. Serum hormone levels fluctuate throughout the lifespan in women and interact differentially with genetic and environmental factors to regulate immune responses and autoimmunity. A number of factors are associated with the risk vs. protective effects of hormones in RA. Different hormonal states including pregnancy, post-partum, breastfeeding, and exogenous hormones including oral contraceptives (OC), postmenopausal hormone replacement therapy (HRT), and hormone administration for infertility treatment alter the hormonal milieu and are associated differentially with RA. Low estrogen levels such as earlier age at menopause, multi-parity, longer breastfeeding (>17 months) are associated with increased risk for RA. Pregnancy is protective for RA development and disease activity and so have HRT and OCs. Synovial tissues from RA patients have higher expression of the ERβ over ERα, and inflammation induces its

expression to further induce proinflammatory cytokines TNF, IL-1β, and IL-6 by PBMCs (201). Diminished ovarian function and decreased circulating estrogen levels at menopause induces these cytokines and E2 inhibits them in PBMCs from postmenopausal women. However the E2-mediated effects on PBMC from pre-menopausal women are not consistent.

Therefore reduced estrogen bioavailability and decline in ovarian function contribute to development of RA. Hormones induce differential effects on immune system in pre-menopausal and post-menopausal women and therefore influence disease development differentially. The role of female hormones in the preclinical stages is still not fully understood (198).

HORMONES, RECEPTOR MODULATORS, AND RELATED THERAPIES

Because hormones play a critical role in physiology of reproductive tissue, bone, cardiovascular, lipid, and immune system, therefore contributing to disease pathogenesis, modulation of hormones or hormone receptors are considered therapies for cancer, bone diseases and autoimmune disease including SLE (202–204).

Effects of estrogen can be blocked by anti-estrogens or selective estrogen receptor modulators (SERM). Anti-estrogens include the pure ER antagonist Clomiphene citrate used for infertility treatment in anovulatory women and Fulvestrant (Faslodex) treatment of breast cancer. SERMs are synthetic estrogen-like ER-ligands, which have ER-agonistic or antagonistic effects depending on the target tissue without the adverse effects of steroid hormones. They have ER-agonistic effect on bone tissue, but minimal effects on reproductive tissues and are mainly used for their beneficial effects in postmenopausal vasomotor symptoms and osteoporosis. Tamoxifen is a first-generation SERM with competitive ER-antagonist effects on breast and agonist effects on bone, uterus and liver tissue. However, its uteroproliferative effects increase risk for endometrial cancer, negating its use for osteoporosis. Raloxifene, a second generation SERM is similar to Tamoxifen, but has anti-estrogen effects on breast and uterus but partial agonist in bone, lipids and cardiovascular system, and is approved for osteoporosis. Lasfoxifene and Bazedoxifene are third generation SERMs evaluated for their usefulness in osteoporosis (205).

A number of studies have assessed the effect of SERMs in bone loss in conjunction with effects on the immune system to assess their utility in postmenopausal osteoporosis. The effects of SERMs on the immune system are still being elucidated and some SERMs are shown to have immunoprotective effects. Continuous treatment with the selective estrogen receptor modulator (SERM) LY139478 ameliorated glomerulonephritis and improved survival in female MRL/lpr mice (206). MRL/lpr mice treated with the potent estrogen receptor antagonist Tamoxifen had reduced disease severity and decrease in numbers of double negative T cells and reduced IL-2 mediated DN cell proliferation *in vitro* (207). Oophorectomized normal mice treated with subcutaneous Raloxifene analog LY117018 had minimal changes on the thymus, T cell activity, and inflammation in DTH model indicating that Raloxifene does not exhibit similar effects as estrogen on T cell responsiveness and inflammation (208). Lasofoxifene and bazedoxifene are third generation SERMs with minimal estrogenic adverse effects used for treatment of postmenopausal osteoporosis. Similar to Raloxifene, Lasofoxifene, and Bazedoxifene did not increase peripheral B cell activity and only blocked B cell maturation at later stages of development therefore affecting fewer subpopulations, compared to estrogen treatment of ovariectomized female C57BL/6N mice indicating the safety of these drugs (209). A similar study assessed the effects of Raloxifene, Lasofoxifene, and Bazedoxifene on T cell development and T cell dependent inflammation (50). Raloxifene and Lasofoxifene but not Bazedoxifene reduced thymic weight but neither of these SERMS affected thymic T cell populations or delayed-type hypersensitivity (DTH) inflammation. Therefore Lasofoxifene and Bazedoxifene are safe to use because they do not impact T lymphopoiesis or T cell dependent inflammation (50).

Arctigenin is a plant-derived phytoestrogen SERM, considered a natural alternative to estrogen, and acts as a selective agonist of the immunosuppressive ERβ receptor. Arctigenin bound to and activated ERβ phosphorylation and nuclear translocation in the mouse EL4 T cell line, and inhibited mTORC1 activation and subsequent Th17 differentiation of naïve CD4 T cells from female C57BL/6 mice. This was associated with amelioration of dextran-sodium sulfate (DSS)-induced colitis in ovariectomized female C57BL/6 mice (210). A recent study showed that two novel SERMS (designated SERM2 and SERM7) and Raloxifene promoted anti-inflammatory signaling of CD14+ M2 type macrophages, diminished NFκB activity, induced the anti-inflammatory cytokine IL-10 and the IL-1R antagonist, and suppressed T cell proliferation (211).

Dehydroepiandrosterone (DHEA) is the natural steroid precursor of both androgens and estrogen in peripheral tissues. Increased metabolism of estrogen and reduced DHEA levels have been observed in SLE patients. Therefore treatment with DHEA is considered a therapeutic option for SLE (202). In a multicenter randomized double-blind placebo controlled clinical trial of adult women with SLE, Prasterone (generic DHEA) administration for 12 months was well-tolerated and improved or stabilized disease activity (212). Fulvestrant (Faslodex) a selective estrogen receptor downregulator and competitive inhibitor of estrogen was shown to improve SLE disease activity index (SLEDAI) scores and reduce T cell activation molecules CD154 and Calcineurin in a double-blind placebo-controlled trial in postmenopausal women with moderately active SLE (213).

Although female sex hormones are a culprit in the pathogenesis of autoimmune diseases such as SLE these hormones have vitally important beneficial effects on the reproductive system and bone metabolism. Therefore there are concerns about exogenous estrogen including the effects of hormone replacement therapy in post-menopausal women, oral contraceptives in pre-menopausal women, and hormone treatment for infertility, on disease activity in SLE (233, 234). A randomized, double-blind, placebo controlled trial evaluated the effect of combined estrogen-progesterone hormone replacement therapy in menopausal women inactive or stable-active SLE.

TABLE 1 | Effects of sex hormones on cells of the adaptive immune system.

Hormone	Cells	Process	Effects	References
Estrogen	T cells	Development	Suppresses thymopoiesis and thymic cellularity	(48–55)
			Activates extrathymic development in liver	(48)
			Downregulates Aire to impair negative selection of autoreactive T cells	(59, 60)
		Homeostasis (Physiologic conc)	Stimulates survival and proliferation and suppress apoptosis (cancer cells)	(61, 62)
		Homeostasis (Pharmacologic conc)	Reduces proliferation	(63–65)
		Activation	Increases T cell activation	(69)
			Increases NF-κB signaling	(76)
			Increases p-ERK, p-Akt, p-CREB signaling	(77)
			Stimulates mitochondrial function	(72, 74)
			Increases expression of Sp1 and CREM	(79)
			Impairs ERK/MAPK signaling, Decreases DNMT1, DNA hypomethylation	(71)
		Cytokine production	Reduces IL-2 (ERα), Increases IL-2 (ERβ)	(77–80)
			Increases IL-1, IL-10 IFN-γ	(81, 83, 84)
		Th Differentiation	Increases Th1 and Th17 differentiation Decreases Th2 differentiation	(82–85)
			Represses Th1, Th17, IFN-γ, IL-17 (Bone metabolism, CNS)	(87–90)
			Promotes TGF-β signaling (Bone metabolism)	(91)
		Tregs	Increases Treg numbers and FoxP3 expression	(96–99)
			Enhances Treg suppressive function	(99–102)
		T cell migration	Increases chemokine receptors CCR1-5	(103)
			Increases chemokines MCP1, MCP5, eotaxin and SDF1β	(104)
			Increases CCR6 on Th17 cells & chemokine CCL20; increases Th17 cell migration	(105)
		B cell help function (Tfh)	Increases Tfh cells	(107)
			Increases Calcineurin and CD40L expression	(108)
	B cells	Development	Suppresses B cell lymphopoiesis	(109, 110)
			Suppresses B cell differentiation from pro-B to pre-B cell stage	(114–120)
			Reduces threshold for negative selection; allows escape of autoreactive B cells	(126)
		Homeostasis/survival	Promotes survival of autoreactive B cells	(124, 125)
		Activation	Increases MZ and follicular B cells	(111, 123–125)
			Increases class switch and Ig antibody production	(128, 133)
		Cytokine production	Increases Blys (BAFF) levels	(129–131)
Progesterone	T cells	Homeostasis	Reduces T cell proliferation, Induces apoptosis	(214–216)
		Cytokine production	Increases IL-4, Decreases IFN-β, IL-17	
		Differentiation	Reduces Th1 Th17 differentiation	
		Function	Reduces T cell dependent antibody production	
			Inhibits cytotoxicity	
		Tregs	Increases Treg differentiation	
	B cells	Cytokine production	Promotes IL-10 production	
		Antibody production	Reduces class switch and T cell dependent antibody production	
Androgens	T cells	Development	Increase thymopoiesis	(217, 218)
			Increase Aire expression to promote deletion of autoreactive T cells	
		Differentiation	Inhibit Th1 and promotes Th2 and IL-10	
		Tregs	Increase FoxP3 and promotes Treg expansion	
	B cells	Development	Suppress B lymphopoiesis	
		Function	Reduce B cells and antibody responses	

(Continued)

TABLE 1 | Continued

Hormone	Cells	Process	Effects	References
Leptin	T cells	Activation and Differentiation	Promotes Th1 differentiation Increases RORγt, Promotes Th17	(219–225)
			Increases mTOR activation and proliferation of Teffs	(226, 227)
			Promotes Glycolysis to drive Teff differentiation	
			Increases availability of apoptotic cell-derived self-antigens, promotes autoimmunity	(228, 229)
		Tregs	Suppresses Treg proliferation and activity	(230)
	B cells	Homeostasis	Promotes survival by induction of Bcl-2 and Cyclin D1	(231)
		Activation	Increases JAK2/STAT3 and p38MAPK/ERK1/2	(232)
		Cytokine production	Increases TNF, IL-6, and IL-10	

Results from this trial showed increase in only mild to moderate but not severe flares compared to placebo and concluded that the benefits of HRT outweigh the small risk of flares in SLE (235). Similarly, combined oral contraceptives did not increase the risk of flares in women with stable disease activity in a double blind randomized noninferiority trial (236). A randomized placebo-controlled trial of another hormone replacement option Tibolone, a progestogen whose metabolites have affinity for the estrogen, progesterone and androgen receptors was conducted in postmenopausal women with inactive or controlled SLE. Tibolone was well tolerated and short-term use did not affect the frequency of flares (237). A pilot case-control prospective study investigated the immune-modulating effects of short-term controlled ovarian stimulation (COS) in infertile women to assess the effects of acute increase in E2 on serum BAFF levels, Immunoglobulins, anti-nuclear antibodies (ANA) and peripheral B cell phenotype and found no significant increases in these measures of immune activation suggesting the safety of COS in infertility treatment.

A modern HRT option is tissue-selective estrogen complex (TSEC) in which estrogen is combined with a SERM. In this therapy, the SERM competes for ER-binding in a tissue-specific manner to mediate protective effects on the tissue. An estrogen-Bazedoxifene combination was the first approved TSEC for prevention of postmenopausal vasomotor symptoms and osteoporosis and had better safety profiles and efficacy than conventional HRT, (238–241) and showed benefits by preventing bone loss in a collagen-induced arthritis (242). A study with E2 and Raloxifene showed suppressed E2-mediated autoreactive effects on B cells in NZB/NZW) F1 mice (243) However, the E2-Baze combination TSEC blocked uteroproliferation but did not affect the E2-mediated effects on thymus weight, or B lymphopoiesis or bone marrow B cell Ig secretion (244). Therefore, more studies of the role of TSECs in the immune system are needed to determine their usefulness.

ERβ is protective for bone loss and estrogen was shown to regulate bone marrow stromal cells senescence and stemness to prevent osteoporosis via ERβ and special AT-rich sequence binding protein 2 (SATB2) transcription factor. Estrogen induced ERβ-ERE binding to activate the promoter and upregulate SATB2. SATB2 ameliorated senescence, increased stemness and improved osteogenic differentiation of BMSCs from ovariectomized female SD rats (245). Therefore blocking estrogen or ERα are potential options, and targeting ERβ may be another potential avenue.

PROGESTERONE AND ANDROGENS

While estrogen in general has immunostimulatory roles, Progesterone, and androgens are immunosuppressive and counteract the pathways affected by estrogen (214, 217). Progesterone receptors are present in lymphoid organs and cells of the innate and adaptive immune systems and are intracellular (iPR) or membrane bound (mPR) (215). Progesterone is shown to impact CD4 Th differentiation and cytokine production with increased IL-4, and increased Treg differentiation, and reduced IFN-γ, Th17 responses, reduced T cell proliferation and T cell-dependent antibody responses, in human peripheral blood and cell line or mouse studies. In CD8 T cells, Progesterone reduced IFN-γ and cytotoxicity. Effects on B cells included reduced class switch recombination and reduced T cell dependent antibody production (216).

Androgens also have immunosuppressive effects on the immune response (217). Low testosterone levels are correlated with higher B cells and antibody responses. Studies of gonadectomy or androgen receptor (AR) deficiency in male mice showed increased B lymphopoiesis, which was reversed by administration of testosterone. Overall, androgens promote B lymphopoiesis through B cell intrinsic mechanisms or effects on bone marrow stromal cells. Gonadectomized or AR deficient male mice have thymic atrophy, which returns to normal size after testosterone supplementation. Testosterone reduces the numbers of DP and CD4 SP cell and promotes CD8+ thymocytes presumably by inhibiting proliferation and increasing apoptosis. Testosterone increases the negative selection of autoreactive thymocytes by upregulating Aire expression in MTECs, and increases thymic TGFβ production therefore promoting central self-tolerance. Androgens also limit the peripheral lymphoid compartments and androgen deficiency or gonadectomy leads to increased peripheral lymphoid populations. Testosterone can non-selectively cause death of peripheral T cells. Effects of T cell responses are also observed in response to androgens. Removal of androgens leads to increased T cell responses, and treating female mice with testosterone reduces antigen-specific responses.

Cytokine responses include a skewing toward the Th2 response with IL-4 and IL-10, and inhibiting Th1 differentiation, IL-12 and IFN-γ production. Testosterone promotes the expansion of Tregs and when ligand-bound, enhances FoxP3 expression in Tregs from rats or women in the ovulatory phase. Overall, androgens suppress the inflammatory responses of peripheral lymphoid cells through effects on T cells and indirect effects on B cells because peripheral B cells lack ARs (217). Although the incidence of SLE is far lower in men, disease is associated with poorer clinical outcomes in men. Indeed, testicular hypofunction was positively associated with SLE in a retrospective cohort study indicating that this requires consideration in patient management (218).

PROLACTIN AND LEPTIN

Prolactin and Leptin influence the immune system and contribute to autoimmune diseases and inflammation. Prolactin is a luteotrophic hormone, which in general has immunostimulatory roles in the immune system. The reader is directed to an excellent review on Prolactin and autoimmunity within this topic collection (246).

Leptin, an adipocytokine is produced by adipose tissue and has dual roles as a hormone and a cytokine (219–223). As a hormone it impacts energy homeostasis, endocrine functions, and bone metabolism. As a cytokine, Leptin has multiple roles in the innate and adaptive immune responses, and promotes autoimmune and non-autoimmune inflammation. Leptin is in general, a proinflammatory molecule, which affects survival, activation, differentiation, and function of both T and B lymphocytes. Leptin promotes T cell survival and activation. It promotes IL-2 and IFN-γ production, and drives Th1 over Th2 differentiation (224). Leptin promotes expression of RORγt to drive Th17 differentiation in human and mouse CD4 T cells *in vitro* and *in vivo* (225). In contrast, Leptin suppresses Treg proliferation and expansion (230). Leptin is shown to activate the mTOR pathway and promote T cell glycolytic metabolism to regulate both Teffs proliferation and Tregs responsiveness (226, 227). In B cells, Leptin promotes expression of anti-apoptotic proteins Bcl-2 and Cyclin D1 to promote survival (231). Leptin activates JAK2/STAT3 and p38/MAPK/ERK1/2 signaling pathways in human B cells, and activates TNF, IL-6, IL-10 production (232).

Leptin is elevated in a number of autoimmune diseases including SLE (247), in humans and in murine models of lupus, and exerts pathogenic effects through increased Th17 proinflammatory responses, increased autoantibody production, impaired Treg responses, and increased availability of apoptotic cell-derived self-antigens (228, 229). Accordingly genetic deletion of leptin in mice, and the neutralization of leptin are shown to benefit autoimmune disease by restoring immune cell functions (228). Based on these findings, Leptin blockade may be considered a useful therapeutic approach for inflammatory diseases. However, downregulating effector immune responses would be detrimental during infections. Therefore, caution must be exercised in this direction, and appropriate selective targeting of molecules in the Leptin pathway may be considered better options.

Better understanding of the role of these hormones in immune responses and autoimmunity will pave the path for development for clinically relevant therapeutics to treat autoimmune diseases.

CONCLUSIONS

The female gender-dependent bias in autoimmunity depends not only on the X chromosome but also the vast range of effects of sex hormones on the immune system and target organs. Sex hormones regulate molecular mechanisms in the innate and adaptive immune systems, and control immune responses in health. Complex interactions of hormones and environmental factors in genetically susceptible individuals lead to deregulation of the immune response, leading to immune-mediated diseases including autoimmune disease. While a large body of evidence exists for the role of estrogen in the immune response (**Table 1**), much remains to be learned. Complex roles of estrogen in different autoimmune diseases, with some protective roles in MS and RA, but pathogenic effects on others like SLE make it imperative to better understand the underlying basis for these dichotomies. Blocking estrogen receptors cautiously and in a targeted manner may yield better therapeutic outcomes than global treatment. Leptin is immunostimulatory, implicated in autoimmune disease, and targeting this hormone may be beneficial. Progesterone and androgens mediate immune-protective effects and therefore may be considered as potential therapeutic avenues.

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REFERENCES

1. Lin T, Zhang D, Liu X, Xiao D. Parental care improves immunity in the seahorse (Hippocampus erectus). *Fish Shellfish Immunol.* (2016) 58:554–62. doi: 10.1016/j.fsi.2016.09.065
2. Keightley MC, Wong BBM, Lieschke GJ. Immune priming: mothering males modulate immunity. *Curr Biol.* (2013) 23:R76–8. doi: 10.1016/j.cub.2012.11.050
3. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol.* (2016) 16:626–38. doi: 10.1038/nri.2016.90

4. Trombetta AC, Meroni M, Cutolo M. Steroids and autoimmunity. *Front Horm Res.* (2017) 48:121–32. doi: 10.1159/000452911
5. Edwards M, Dai R, Ahmed SA. Our environment shapes us: the importance of environment and sex differences in regulation of autoantibody production. *Front Immunol.* (2018) 9:478. doi: 10.3389/fimmu.2018.00478
6. Lahita RG. The immunoendocrinology of systemic lupus erythematosus. *Clin Immunol.* (2016) 172:98–100. doi: 10.1016/j.clim.2016.08.014
7. Hughes GC, Choubey D. Modulation of autoimmune rheumatic diseases by oestrogen and progesterone. *Nat Rev Rheumatol.* (2014) 10:740–51. doi: 10.1038/nrrheum.2014.144
8. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita* (2016) 52:205–12. doi: 10.4415/ANN_16_02_12
9. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol.* (2015) 294:63–9. doi: 10.1016/j.cellimm.2015.01.018
10. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med.* (2011) 365:2110–21. doi: 10.1056/NEJMra1100359
11. Moulton VR, Suarez-Fueyo A, Meidan E, Li H, Mizui M, Tsokos GC. Pathogenesis of human systemic lupus erythematosus: a cellular perspective. *Trends Mol Med.* (2017) 23:615–35. doi: 10.1016/j.molmed.2017.05.006
12. Katsuyama T, Tsokos GC, Moulton VR. Aberrant T cell signaling and subsets in systemic lupus erythematosus. *Front Immunol.* (2018) 9:1088. doi: 10.3389/fimmu.2018.01088
13. Cunningham M, Gilkeson G. Estrogen receptors in immunity and autoimmunity. *Clin Rev Allergy Immunol.* (2011) 40:66–73. doi: 10.1007/s12016-010-8203-5
14. Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. *Physiol Rev.* (2001) 81:1535–65. doi: 10.1152/physrev.2001.81.4.1535
15. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem.* (2001) 276:36869–72. doi: 10.1074/jbc.R100029200
16. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* (1997) 277:1508–10.
17. Safe S, Kim K, Kim K. Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. *J Mol Endocrinol.* (2008) 41:263–75. doi: 10.1677/JME-08-0103
18. Andersson A, Törnqvist AE, Moverare-Skrtic S, Bernardi AI, Farman HH, Chambon P, et al. Roles of activating functions 1 and 2 of estrogen receptor α in lymphopoiesis. *J Endocrinol.* (2018) 236:99–109. doi: 10.1530/JOE-17-0372
19. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* (2001) 29:2905–19.
20. Lin C-Y, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, et al. Whole-genome cartography of estrogen receptor α binding sites. *PLoS Genet.* (2007) 3:e87. doi: 10.1371/journal.pgen.0030087
21. Charn TH, Liu ET-B, Chang EC, Lee YK, Katzenellenbogen JA, Katzenellenbogen BS. Genome-wide dynamics of chromatin binding of estrogen receptors α and β: mutual restriction and competitive site selection. *Mol Endocrinol.* (2010) 24:47–59. doi: 10.1210/me.2009-0252
22. Suenaga R, Rider V, Evans MJ, Abdou NI. *In vitro*-activated human lupus T cells express normal estrogen receptor proteins which bind to the estrogen response element. *Lupus* (2001) 10:116–22. doi: 10.1191/096120301673870511
23. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunol Lett.* (2005) 97:107–13. doi: 10.1016/j.imlet.2004.10.007
24. Pierdominici M, Maselli A, Colasanti T, Giammarioli AM, Delunardo F, Vacirca D, et al. Estrogen receptor profiles in human peripheral blood lymphocytes. *Immunol Lett.* (2010) 132:79–85. doi: 10.1016/j.imlet.2010.06.003
25. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-α and coactivator turnover and for efficient estrogen receptor-α transactivation. *Mol Cell* (2000) 5:939–48. doi: 10.1016/S1097-2765(00)80259-2
26. Zhao K-W, Sikriwal D, Dong X, Guo P, Sun X, Dong J-T. Oestrogen causes degradation of KLF5 by inducing the E3 ubiquitin ligase EFP in ER-positive breast cancer cells. *Biochem J.* (2011) 437:323–33. doi: 10.1042/BJ20101388
27. Dong X-Y, Fu X, Fan S, Guo P, Su D, Dong J-T. Oestrogen causes ATBF1 protein degradation through the oestrogen-responsive E3 ubiquitin ligase EFP. *Biochem J.* (2012) 444:581–90. doi: 10.1042/BJ20111890
28. Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, et al. Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. *Mol Cell.* (2003) 11:695–707. doi: 10.1016/S1097-2765(03)00090-X
29. Sentis S, Le Romancer M, Bianchin C, Rostan M-C, Corbo L. Sumoylation of the estrogen receptor α hinge region regulates its transcriptional activity. *Mol Endocrinol.* (2005) 19:2671–84. doi: 10.1210/me.2005-0042
30. Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, Kurihara I, et al. FHL2, UBC9, and PIAS1 are novel estrogen receptor α-interacting proteins. *Endocr Res.* (2004) 30:617–21. doi: 10.1081/ERC-200043789
31. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (2005) 307:1625–30. doi: 10.1126/science.1106943
32. Barton M, Filardo EJ, Lolait SJ, Thomas P, Maggiolini M, Prossnitz ER. Twenty years of the G protein-coupled estrogen receptor GPER: historical and personal perspectives. *J Steroid Biochem Mol Biol.* (2018) 176:4–15. doi: 10.1016/j.jsbmb.2017.03.021
33. Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J Endocrinol.* (2010) 204:105–14. doi: 10.1677/JOE-09-0242
34. Seto K, Hoang M, Santos T, Bandyopadhyay M, Kindy MS, Dasgupta S. Non-genomic oestrogen receptor signal in B lymphocytes: an approach towards therapeutic interventions for infection, autoimmunity and cancer. *Int J Biochem Cell Biol.* (2016) 76:115–8. doi: 10.1016/j.biocel.2016.04.018
35. Stefkovich ML, Arao Y, Hamilton KJ, Korach KS. Experimental models for evaluating non-genomic estrogen signaling. *Steroids* (2018) 133:34–7. doi: 10.1016/j.steroids.2017.11.001
36. Klinge CM. Estrogen action: receptors, transcripts, cell signaling, and non-coding RNAs in normal physiology and disease. *Mol Cell Endocrinol.* (2015) 418(Pt 3):191–2. doi: 10.1016/j.mce.2015.11.028
37. Hewagama A. Role of X-Chromosome encoded miRNAs in Autoimmunity: suppressing the suppressor and female predisposition. *Rheumatol Curr Res.* (2013) 03:118. doi: 10.4172/2161-1149.1000118
38. Dai R, Ahmed SA. Sexual dimorphism of miRNA expression: a new perspective in understanding the sex bias of autoimmune diseases. *Ther Clin Risk Manag.* (2014) 10:151–63. doi: 10.2147/TCRM.S33517
39. Honarpisheh M, Köhler P, von Rauchhaupt E, Lech M. The involvement of MicroRNAs in modulation of innate and adaptive immunity in systemic lupus erythematosus and lupus nephritis. *J Immunol Res.* (2018) 2018:4126106. doi: 10.1155/2018/4126106
40. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA. Suppression of LPS-induced Interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. *Blood* (2008) 112:4591–7. doi: 10.1182/blood-2008-04-152488
41. Ma X, Liu Q. MicroRNAs in the pathogenesis of systemic lupus erythematosus. *Int J Rheum Dis.* (2013) 16:115–21. doi: 10.1111/1756-185X.12083
42. Young NA, Valiente GR, Hampton JM, Wu L-C, Burd CJ, Willis WL, et al. Estrogen-regulated STAT1 activation promotes TLR8 expression to facilitate signaling via microRNA-21 in systemic lupus erythematosus. *Clin Immunol.* (2017) 176:12–22. doi: 10.1016/j.clim.2016.12.005
43. Shao B, Liao L, Yu Y, Shuai Y, Su X, Jing H, et al. Estrogen preserves Fas ligand levels by inhibiting microRNA-181a in bone marrow-derived mesenchymal stem cells to maintain bone remodeling balance. *FASEB J.* (2015) 29:3935–44. doi: 10.1096/fj.15-272823
44. Savino W, Mendes-da-Cruz DA, Lepletier A, Dardenne M. Hormonal control of T-cell development in health and disease. *Nat Rev Endocrinol.* (2016) 12:77–89. doi: 10.1038/nrendo.2015.168

45. Zoller AL, Kersh GJ. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of β-selected thymocytes. *J Immunol.* (2006) 176:7371–8. doi: 10.4049/jimmunol.176.12.7371
46. Leposavić G, Karapetrović B, Obradović S, Vidiíc Dandović B, Kosec D. Differential effects of gonadectomy on the thymocyte phenotypic profile in male and female rats. *Pharmacol Biochem Behav.* (1996) 54:269–76.
47. Okasha SA, Ryu S, Do Y, McKallip RJ, Nagarkatti M, Nagarkatti PS. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. *Toxicology* (2001) 163:49–62. doi: 10.1016/S0300-483X(01)00374-2
48. Okuyama R, Abo T, Seki S, Ohteki T, Sugiura K, Kusumi A, et al. Estrogen administration activates extrathymic T cell differentiation in the liver. *J Exp Med.* (1992) 175:661–9.
49. Screpanti I, Meco D, Morrone S, Gulino A, Mathieson BJ, Frati L. *In vivo* modulation of the distribution of thymocyte subsets: effects of estrogen on the expression of different T cell receptor Vβ gene families in CD4-, CD8- thymocytes. *Cell Immunol.* (1991) 134:414–26.
50. Bernardi AI, Andersson A, Stubelius A, Grahnemo L, Carlsten H, Islander U. Selective estrogen receptor modulators in T cell development and T cell dependent inflammation. *Immunobiology* (2015) 220:1122–8. doi: 10.1016/j.imbio.2015.05.009
51. Clarke AG, Kendall MD. The thymus in pregnancy: the interplay of neural, endocrine and immune influences. *Immunol Today* (1994) 15:545–51. doi: 10.1016/0167-5699(94)90212-7
52. Hirahara H, Ogawa M, Kimura M, Iiai T, Tsuchida M, Hanawa H, et al. Glucocorticoid independence of acute thymic involution induced by lymphotoxin and estrogen. *Cell Immunol.* (1994) 153:401–11. doi: 10.1006/cimm.1994.1038
53. Marotti T, Sirotković M, Pavelić J, Gabrilovac J, Pavelić K. *In vivo* effect of progesteron and estrogen on thymus mass and T-cell functions in female mice. *Horm Metab Res.* (1984) 16:201–3. doi: 10.1055/s-2007-1014742
54. Kendall MD, Clarke AG. The thymus in the mouse changes its activity during pregnancy: a study of the microenvironment. *J Anat.* (2000) 197(Pt 3):393–411. doi: 10.1046/j.1469-7580.2000.19730393.x
55. Erlandsson MC, Ohlsson C, Gustafsson JA, Carlsten H. Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* (2001) 103:17–25. doi: 10.1046/j.1365-2567.2001.01212.x
56. Staples JE, Gasiewicz TA, Fiore NC, Lubahn DB, Korach KS, Silverstone AE. Estrogen receptor α is necessary in thymic development and estradiol-induced thymic alterations. *J Immunol.* (1999) 163:4168–74.
57. Yellayi S, Teuscher C, Woods JA, Welsh TH, Tung KS, Nakai M, et al. Normal development of thymus in male and female mice requires estrogen/estrogen receptor-α signaling pathway. *Endocrine* (2000) 12:207–13. doi: 10.1385/ENDO:12:3:207
58. Dumont-Lagacé M, St-Pierre C, Perreault C. Sex hormones have pervasive effects on thymic epithelial cells. *Sci Rep.* (2015) 5:12895. doi: 10.1038/srep12895
59. Bakhru P, Su MA. Estrogen turns down "the AIRE." *J Clin Invest.* (2016) 126:1239–41. doi: 10.1172/JCI86800
60. Dragin N, Bismuth J, Cizeron-Clairac G, Biferi MG, Berthault C, Serraf A, et al. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. *J Clin Invest.* (2016) 126:1525–37. doi: 10.1172/JCI81894
61. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res.* (2009) 11:206. doi: 10.1186/bcr2255
62. Fernando RI, Wimalasena J. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. *Mol Biol Cell.* (2004) 15:3266–84. doi: 10.1091/mbc.e03-11-0823
63. Jenkins JK, Suwannaroj S, Elbourne KB, Ndebele K, McMurray RW. 17-β-estradiol alters Jurkat lymphocyte cell cycling and induces apoptosis through suppression of Bcl-2 and cyclin A. *Int Immunopharmacol.* (2001) 1:1897–911.doi: 10.1016/S1567-5769(01)00114-X
64. Jun DY, Park HS, Kim JS, Kim JS, Park W, Song BH, et al. 17α-estradiol arrests cell cycle progression at G2/M and induces apoptotic cell death in human acute leukemia Jurkat T cells. *Toxicol Appl Pharmacol.* (2008) 231:401–12. doi: 10.1016/j.taap.2008.05.023
65. Luc JGY, Jackson K, Weinkauf JG, Freed DH, Nagendran J. Feasibility of lung transplantation from donation after circulatory death donors following portable *ex vivo* lung perfusion: a pilot study. *Transplant Proc.* (2017) 49:1885–92. doi: 10.1016/j.transproceed.2017.04.010
66. Arsenović-Ranin N, Kosec D, Nacka-Aleksić M, Pilipović I, Stojić-Vukanić Z, Djikić J, et al. Ovarian hormone level alterations during rat post-reproductive life-span influence $CD8^+$ T-cell homeostasis. *Exp Biol Med.* (2015) 240:1319–32. doi: 10.1177/1535370215570817
67. Pernis AB. Estrogen and $CD4^+$ T cells. *Curr Opin Rheumatol.* (2007) 19:414–20. doi: 10.1097/BOR.0b013e328277ef2a
68. Kassi E, Moutsatsou P. Estrogen receptor signaling and its relationship to cytokines in systemic lupus erythematosus. *J Biomed Biotechnol.* (2010) 2010:317452. doi: 10.1155/2010/317452
69. Mohammad I, Starskaia I, Nagy T, Guo J, Yatkin E, Väänänen K, et al. Estrogen receptor α contributes to T cell-mediated autoimmune inflammation by promoting T cell activation and proliferation. *Sci Signal.* (2018) 11:9415. doi: 10.1126/scisignal.aap9415
70. Wu Z, Sun Y, Mei X, Zhang C, Pan W, Shi W. 17β-oestradiol enhances global DNA hypomethylation in CD4-positive T cells from female patients with lupus, through overexpression of oestrogen receptor-α-mediated downregulation of DNMT1. *Clin Exp Dermatol.* (2014) 39:525–32. doi: 10.1111/ced.12346
71. Richardson B. The interaction between environmental triggers and epigenetics in autoimmunity. *Clin Immunol.* (2018) 192:1–5. doi: 10.1016/j.clim.2018.04.005
72. Klinge CM. Estrogens regulate life and death in mitochondria. *J Bioenerg Biomembr.* (2017) 49:307–24. doi: 10.1007/s10863-017-9704-1
73. Giguère V. Transcriptional control of energy homeostasis by the estrogen-related receptors. *Endocr Rev.* (2008) 29:677–96. doi: 10.1210/er.2008-0017
74. Michalek RD, Gerriets VA, Nichols AG, Inoue M, Kazmin D, Chang C-Y, et al. Estrogen-related receptor-α is a metabolic regulator of effector T-cell activation and differentiation. *Proc Natl Acad Sci USA.* (2011) 108:18348–53. doi: 10.1073/pnas.1108856108
75. Pung OJ, Tucker AN, Vore SJ, Luster MI. Influence of estrogen on host resistance: increased susceptibility of mice to Listeria monocytogenes correlates with depressed production of interleukin 2. *Infect Immun.* (1985) 50:91–6.
76. Dai R, Phillips RA, Ahmed SA. Despite inhibition of nuclear localization of NF-κ B p65, c-Rel, and RelB, 17-β estradiol up-regulates NF-κ B signaling in mouse splenocytes: the potential role of Bcl-3. *J Immunol.* (2007) 179:1776–83. doi: 10.4049/jimmunol.179.3.1776
77. Priyanka HP, Krishnan HC, Singh RV, Hima L, Thyagarajan S. Estrogen modulates *in vitro* T cell responses in a concentration- and receptor-dependent manner: effects on intracellular molecular targets and antioxidant enzymes. *Mol Immunol.* (2013) 56:328–39. doi: 10.1016/j.molimm.2013.05.226
78. Trzonkowski P, Myśliwska J, Tukaszuk K, Szmit E, Bryl E, Myśliwski A. Luteal phase of the menstrual cycle in young healthy women is associated with decline in interleukin 2 levels. *Horm Metab Res.* (2001) 33:348–53. doi: 10.1055/s-2001-15420
79. Moulton VR, Holcomb DR, Zajdel MC, Tsokos GC. Estrogen upregulates cyclic AMP response element modulator α expression and downregulates interleukin-2 production by human T lymphocytes. *Mol Med.* (2012) 18:370–8. doi: 10.2119/molmed.2011.00506
80. Moulton VR, Tsokos GC. Why do women get lupus? *Clin Immunol.* (2012) 144:53–6. doi: 10.1016/j.clim.2012.04.003
81. Fox HS, Bond BL, Parslow TG. Estrogen regulates the IFN-gamma promoter. *J Immunol.* (1991) 146:4362–7.
82. Maret A, Coudert JD, Garidou L, Foucras G, Gourdy P, Krust A, et al. Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development *in vivo*. essential role of estrogen receptor α expression in hematopoietic cells. *Eur J Immunol.* (2003) 33:512–21. doi: 10.1002/immu.200310027
83. Karpuzoglu E, Phillips RA, Gogal RM, Ansar Ahmed S. IFN-gamma-inducing transcription factor, T-bet is upregulated by estrogen in murine splenocytes: role of IL-27 but not IL-12. *Mol Immunol.* (2007) 44:1808–14. doi: 10.1016/j.molimm.2006.08.005

84. Karpuzoglu E, Phillips RA, Dai R, Graniello C, Gogal RM, Ahmed SA. Signal transducer and activation of transcription (STAT) 4β, a shorter isoform of interleukin-12-induced STAT4, is preferentially activated by estrogen. *Endocrinology* (2009) 150:1310–20. doi: 10.1210/en.2008-0832
85. Khan D, Dai R, Karpuzoglu E, Ahmed SA. Estrogen increases, whereas IL-27 and IFN-gamma decrease, splenocyte IL-17 production in WT mice. *Eur J Immunol.* (2010) 40:2549–56. doi: 10.1002/eji.201040303
86. Liu H, Loo KK, Palaszynski K, Ashouri J, Lubahn DB, Voskuhl RR. Estrogen receptor α mediates estrogen's immune protection in autoimmune disease. *J Immunol.* (2003) 171:6936–40. doi: 10.4049/jimmunol.171.12.6936
87. Lélu K, Laffont S, Delpy L, Paulet P-E, Périnat T, Tschanz SA, et al. Estrogen receptor α signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J Immunol.* (2011) 187:2386–93. doi: 10.4049/jimmunol.1101578
88. Chen R-Y, Fan Y-M, Zhang Q, Liu S, Li Q, Ke G-L, et al. Estradiol inhibits Th17 cell differentiation through inhibition of RORγT transcription by recruiting the ERα/REA complex to estrogen response elements of the RORγT promoter. *J Immunol.* (2015) 194:4019–28. doi: 10.4049/jimmunol.1400806
89. Tyagi AM, Srivastava K, Mansoori MN, Trivedi R, Chattopadhyay N, Singh D. Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis. *PLoS ONE* (2012) 7:e44552. doi: 10.1371/journal.pone.0044552
90. Molnár I, Bohaty I, Somogyiné-Vári É. High prevalence of increased interleukin-17A serum levels in postmenopausal estrogen deficiency. *Menopause* (2014) 21:749–52. doi: 10.1097/GME.0000000000000125
91. Gao Y, Qian W-P, Dark K, Toraldo G, Lin ASP, Guldberg RE, et al. Estrogen prevents bone loss through transforming growth factor β signaling in T cells. *Proc Natl Acad Sci USA.* (2004) 101:16618–23. doi: 10.1073/pnas.0404888101
92. Park H-J, Park H-S, Lee J-U, Bothwell ALM, Choi J-M. Sex-based selectivity of PPARγ regulation in Th1, Th2, and Th17 differentiation. *Int J Mol Sci.* (2016) 17:81347. doi: 10.3390/ijms17081347
93. Aggelakopoulou M, Kourepini E, Paschalidis N, Panoutsakopoulou V. ERβ in $CD4^+$ T cells is crucial for ligand-mediated suppression of central nervous system autoimmunity. *J Immunol.* (2016) 196:4947–56. doi: 10.4049/jimmunol.1600246
94. Kitagawa Y, Sakaguchi S. Molecular control of regulatory T cell development and function. *Curr Opin Immunol.* (2017) 49:64–70. doi: 10.1016/j.coi.2017.10.002
95. Li MO, Rudensky AY. T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat Rev Immunol.* (2016) 16:220–33. doi: 10.1038/nri.2016.26
96. Nie J, Li YY, Zheng SG, Tsun A, Li B. $FOXP3^+$ Treg cells and gender bias in autoimmune diseases. *Front Immunol.* (2015) 6:493. doi: 10.3389/fimmu.2015.00493
97. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF, et al. Cutting edge: estrogen drives expansion of the $CD4^+CD25^+$ regulatory T cell compartment. *J Immunol.* (2004) 173:2227–30. doi: 10.4049/jimmunol.173.4.2227
98. Dinesh RK, Hahn BH, Singh RP. PD-1, gender, and autoimmunity. *Autoimmun Rev.* (2010) 9:583–7. doi: 10.1016/j.autrev.2010.04.003
99. Prieto GA, Rosenstein Y. Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation. *Immunology* (2006) 118:58–65. doi: 10.1111/j.1365-2567.2006.02339.x
100. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of $CD4^+CD25^+$and $FOXP3^+$ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. *J Immunol.* (2007) 178:2572–8. doi: 10.4049/jimmunol.178.4.2572
101. Luo CY, Wang L, Sun C, Li DJ. Estrogen enhances the functions of $CD4^+CD25^+Foxp3^+$ regulatory T cells that suppress osteoclast differentiation and bone resorption *in vitro*. *Cell Mol Immunol.* (2011) 8:50–8. doi: 10.1038/cmi.2010.54
102. Adurthi S, Kumar MM, Vinodkumar HS, Mukherjee G, Krishnamurthy H, Acharya KK, et al. Oestrogen Receptor-α binds the FOXP3 promoter and modulates regulatory T-cell function in human cervical cancer. *Sci Rep.* (2017) 7:17289. doi: 10.1038/s41598-017-17102-w
103. Mo R, Chen J, Grolleau-Julius A, Murphy HS, Richardson BC, Yung RL. Estrogen regulates CCR gene expression and function in T lymphocytes. *J Immunol.* (2005) 174:6023–9. doi: 10.4049/jimmunol.174.10.6023
104. Lengi AJ, Phillips RA, Karpuzoglu E, Ahmed SA. Estrogen selectively regulates chemokines in murine splenocytes. *J Leukoc Biol.* (2007) 81:1065–74. doi: 10.1189/jlb.0606391
105. Andersson A, Stubelius A, Karlsson MN, Engdahl C, Erlandsson M, Grahnemo L, et al. Estrogen regulates T helper 17 phenotype and localization in experimental autoimmune arthritis. *Arthritis Res Ther.* (2015) 17:32. doi: 10.1186/s13075-015-0548-y
106. Blanco P, Ueno H, Schmitt N. T follicular helper (Tfh) cells in lupus: activation and involvement in SLE pathogenesis. *Eur J Immunol.* (2016) 46:281–90. doi: 10.1002/eji.201545760
107. Park H-J, Park H-S, Lee J-U, Bothwell ALM, Choi J-M. Gender-specific differences in PPARγ regulation of follicular helper T cell responses with estrogen. *Sci Rep.* (2016) 6:28495. doi: 10.1038/srep28495
108. Rider V, Jones S, Evans M, Bassiri H, Afsar Z, Abdou NI. Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. *J Rheumatol.* (2001) 28:2644–9.
109. Kincade PW, Medina KL, Payne KJ, Rossi MI, Tudor KS, Yamashita Y, et al. Early B-lymphocyte precursors and their regulation by sex steroids. *Immunol Rev.* (2000) 175:128–37. doi: 10.1111/j.1600-065X.2000.imr017502.x
110. Medina KL, Garrett KP, Thompson LF, Rossi MI, Payne KJ, Kincade PW. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat Immunol.* (2001) 2:718–24. doi: 10.1038/90659
111. Cohen-Solal JFG, Jeganathan V, Hill L, Kawabata D, Rodriguez-Pinto D, Grimaldi C, et al. Hormonal regulation of B-cell function and systemic lupus erythematosus. *Lupus* (2008) 17:528–32. doi: 10.1177/0961203308089402
112. Sthoeger ZM, Chiorazzi N, Lahita RG. Regulation of the immune response by sex hormones. I. *in vitro* effects of estradiol and testosterone on pokeweed mitogen-induced human B cell differentiation. *J Immunol.* (1988) 141:91–8.
113. Kanda N, Tamaki K. Estrogen enhances immunoglobulin production by human PBMCs. *J Allergy Clin Immunol.* (1999) 103:282–8.
114. Medina KL, Smithson G, Kincade PW. Suppression of B lymphopoiesis during normal pregnancy. *J Exp Med.* (1993) 178:1507–15.
115. Masuzawa T, Miyaura C, Onoe Y, Kusano K, Ohta H, Nozawa S, et al. Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. *J Clin Invest.* (1994) 94:1090–7. doi: 10.1172/JCI117424
116. Smithson G, Medina K, Ponting I, Kincade PW. Estrogen suppresses stromal cell-dependent lymphopoiesis in culture. *J Immunol.* (1995) 155:3409–17.
117. Smithson G, Couse JF, Lubahn DB, Korach KS, Kincade PW. The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis. *J Immunol.* (1998) 161:27–34.
118. Medina KL, Strasser A, Kincade PW. Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors. *Blood* (2000) 95:2059–67.
119. Miyaura C, Onoe Y, Inada M, Maki K, Ikuta K, Ito M, et al. Increased B-lymphopoiesis by interleukin 7 induces bone loss in mice with intact ovarian function: similarity to estrogen deficiency. *Proc Natl Acad Sci USA.* (1997) 94:9360–5.
120. Yokota T, Oritani K, Garrett KP, Kouro T, Nishida M, Takahashi I, et al. Soluble frizzled-related protein 1 is estrogen inducible in bone marrow stromal cells and suppresses the earliest events in lymphopoiesis. *J Immunol.* (2008) 181:6061–72. doi: 10.4049/jimmunol.181.9.6061
121. Suurmond J, Calise J, Malkiel S, Diamond B. DNA-reactive B cells in lupus. *Curr Opin Immunol.* (2016) 43:1–7. doi: 10.1016/j.coi.2016.07.002
122. Malkiel S, Barlev AN, Atisha-Fregoso Y, Suurmond J, Diamond B. Plasma cell differentiation pathways in systemic lupus erythematosus. *Front Immunol.* (2018) 9:427. doi: 10.3389/fimmu.2018.00427
123. Grimaldi CM, Michael DJ, Diamond B. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J Immunol.* (2001) 167:1886–90. doi: 10.4049/jimmunol.167.4.1886
124. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest.* (2002) 109:1625–33. doi: 10.1172/JCI14873

125. Grimaldi CM, Jeganathan V, Diamond B. Hormonal regulation of B cell development: 17 β-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *J Immunol.* (2006) 176:2703–10. doi: 10.4049/jimmunol.176.5.2703
126. Bynoe MS, Grimaldi CM, Diamond B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci USA.* (2000) 97:2703–8. doi: 10.1073/pnas.040577497
127. Hill L, Jeganathan V, Chinnasamy P, Grimaldi C, Diamond B. Differential roles of estrogen receptors α and β in control of B-cell maturation and selection. *Mol Med.* (2011) 17:211–20. doi: 10.2119/molmed.2010.00172
128. Jeganathan V, Peeva E, Diamond B. Hormonal milieu at time of B cell activation controls duration of autoantibody response. *J Autoimmun.* (2014) 53:46–54. doi: 10.1016/j.jaut.2014.02.007
129. Rawlings DJ, Metzler G, Wray-Dutra M, Jackson SW. Altered B cell signalling in autoimmunity. *Nat Rev Immunol.* (2017) 17:421–36. doi: 10.1038/nri.2017.24
130. Panchanathan R, Choubey D. Murine BAFF expression is up-regulated by estrogen and interferons: implications for sex bias in the development of autoimmunity. *Mol Immunol.* (2013) 53:15–23. doi: 10.1016/j.molimm.2012.06.013
131. Bassi N, Luisetto R, Ghirardello A, Gatto M, Valente M, Della Barbera M, et al. 17-β-estradiol affects BLyS serum levels and the nephritogenic autoantibody network accelerating glomerulonephritis in NZB/WF1 mice. *Lupus* (2015) 24:382–91. doi: 10.1177/0961203314559636
132. Drehmer MN, Suterio DG, Muniz YCN, de Souza IR, Löfgren SE. BAFF Expression is Modulated by Female Hormones in Human Immune Cells. *Biochem Genet.* (2016) 54:722–30. doi: 10.1007/s10528-016-9752-y
133. Jones BG, Penkert RR, Xu B, Fan Y, Neale G, Gearhart PJ, et al. Binding of estrogen receptors to switch sites and regulatory elements in the immunoglobulin heavy chain locus of activated B cells suggests a direct influence of estrogen on antibody expression. *Mol Immunol.* (2016) 77:97–102. doi: 10.1016/j.molimm.2016.07.015
134. Mohan C, Putterman C. Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis. *Nat Rev Nephrol.* (2015) 11:329–41. doi: 10.1038/nrneph.2015.33
135. Perl A. Review: metabolic control of immune system activation in rheumatic diseases. *Arthr Rheumatol.* (2017) 69:2259–70. doi: 10.1002/art.40223
136. Li J, McMurray RW. Effects of estrogen receptor subtype-selective agonists on autoimmune disease in lupus-prone NZB/NZW F1 mouse model. *Clin Immunol.* (2007) 123:219–26. doi: 10.1016/j.clim.2007.01.008
137. Bynoté KK, Hackenberg JM, Korach KS, Lubahn DB, Lane PH, Gould KA. Estrogen receptor-α deficiency attenuates autoimmune disease in (NZB x NZW)F1 mice. *Genes Immun.* (2008) 9:137–52. doi: 10.1038/sj.gene.6364458
138. Svenson JL, EuDaly J, Ruiz P, Korach KS, Gilkeson GS. Impact of estrogen receptor deficiency on disease expression in the NZM2410 lupus prone mouse. *Clin Immunol.* (2008) 128:259–68. doi: 10.1016/j.clim.2008.03.508
139. Feng F, Nyland J, Banyai M, Tatum A, Silverstone AE, Gavalchin J. The induction of the lupus phenotype by estrogen is via an estrogen receptor-α-dependent pathway. *Clin Immunol.* (2010) 134:226–36. doi: 10.1016/j.clim.2009.10.004
140. Chen Y, Cuda C, Morel L. Genetic determination of T cell help in loss of tolerance to nuclear antigens. *J Immunol.* (2005) 174:7692–702. doi: 10.4049/jimmunol.174.12.7692
141. Perry DJ, Yin Y, Telarico T, Baker HV, Dozmorov I, Perl A, et al. Murine lupus susceptibility locus Sle1c2 mediates $CD4^+$ T cell activation and maps to estrogen-related receptor γ. *J Immunol.* (2012) 189:793–803. doi: 10.4049/jimmunol.1200411
142. Yoachim SD, Nuxoll JS, Bynoté KK, Gould KA. Estrogen receptor α signaling promotes Sle1-induced loss of tolerance and immune cell activation and is responsible for sex bias in B6.Sle1 congenic mice. *Clin Immunol.* (2015) 158:153–66. doi: 10.1016/j.clim.2015.03.026
143. Tabor DE, Gould KA. Estrogen receptor α promotes lupus in (NZB×NZW)F1 mice in a B cell intrinsic manner. *Clin Immunol.* (2017) 174:41–52. doi: 10.1016/j.clim.2016.10.011
144. Moulton VR, Tsokos GC. T cell signaling abnormalities contribute to aberrant immune cell function and autoimmunity. *J Clin Invest.* (2015) 125:2220–7. doi: 10.1172/JCI78087
145. Cutolo M, Sulli A, Straub RH. Estrogen metabolism and autoimmunity. *Autoimmun Rev.* (2012) 11:A460–4. doi: 10.1016/j.autrev.2011.11.014
146. Kassi EN, Vlachoyiannopoulos PG, Moutsopoulos HM, Sekeris CE, Moutsatsou P. Molecular analysis of estrogen receptor α and β in lupus patients. *Eur J Clin Invest.* (2001) 31:86–93. doi: 10.1046/j.1365-2362.2001.00762.x
147. Inui A, Ogasawara H, Naito T, Sekigawa I, Takasaki Y, Hayashida Y, et al. Estrogen receptor expression by peripheral blood mononuclear cells of patients with systemic lupus erythematosus. *Clin Rheumatol.* (2007) 26:1675–8. doi: 10.1007/s10067-007-0568-3
148. Lee YJ, Shin KS, Kang SW, Lee CK, Yoo B, Cha HS, et al. Association of the oestrogen receptor α gene polymorphisms with disease onset in systemic lupus erythematosus. *Ann Rheum Dis.* (2004) 63:1244–9. doi: 10.1136/ard.2003.012583
149. Kassi E, Vlachoyiannopoulos PG, Kominakis A, Kiaris H, Moutsopoulos HM, Moutsatsou P. Estrogen receptor α gene polymorphism and systemic lupus erythematosus: a possible risk? *Lupus* (2005) 14:391–8. doi: 10.1191/0961203305lu2104oa
150. Johansson M, Arlestig L, Möller B, Smedby T, Rantapää-Dahlqvist S. Oestrogen receptor α gene polymorphisms in systemic lupus erythematosus. *Ann Rheum Dis.* (2005) 64:1611–7. doi: 10.1136/ard.2004.032425
151. Kisiel BM, Kosinska J, Wierzbowska M, Rutkowska-Sak L, Musiej-Nowakowska E, Wudarski M, et al. Differential association of juvenile and adult systemic lupus erythematosus with genetic variants of oestrogen receptors α and β. *Lupus* (2011) 20:85–9. doi: 10.1177/0961203310381514
152. Drehmer MN, Andrade D, Pereira IA, Marrero AR, Muniz YCN, de Souza IR, et al. Estrogen receptor α gene (ESR1) polymorphism can contribute to clinical findings in systemic lupus erythematosus patients. *Lupus* (2017) 26:294–8. doi: 10.1177/0961203316668041
153. Teruel M, Sawalha AH. Epigenetic variability in systemic lupus erythematosus: what we learned from genome-wide DNA methylation studies. *Curr Rheumatol Rep.* (2017) 19:32. doi: 10.1007/s11926-017-0657-5
154. Weeding E, Sawalha AH. Deoxyribonucleic acid methylation in systemic lupus erythematosus: implications for future clinical practice. *Front Immunol.* (2018) 9:875. doi: 10.3389/fimmu.2018.00875
155. Gorjestani S, Rider V, Kimler BF, Greenwell C, Abdou NI. Extracellular signal-regulated kinase 1/2 signalling in SLE T cells is influenced by oestrogen and disease activity. *Lupus* (2008) 17:548–54. doi: 10.1177/0961203307087982
156. Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus $CD4^+$ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol.* (2010) 184:6773–81. doi: 10.4049/jimmunol.0904060
157. Strickland FM, Hewagama A, Lu Q, Wu A, Hinderer R, Webb R, et al. Environmental exposure, estrogen and two X chromosomes are required for disease development in an epigenetic model of lupus. *J Autoimmun.* (2012) 38:J135–43. doi: 10.1016/j.jaut.2011.11.001
158. Rider V, Jones SR, Evans M, Abdou NI. Molecular mechanisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus T cells. *Clin Immunol.* (2000) 95:124–34. doi: 10.1006/clim.2000.4844
159. Rider V, Li X, Peterson G, Dawson J, Kimler BF, Abdou NI. Differential expression of estrogen receptors in women with systemic lupus erythematosus. *J Rheumatol.* (2006) 33:1093–101.
160. Ward JM, Rider V, Abdou NI, Kimler B. Estradiol differentially regulates calreticulin: a potential link with abnormal T cell function in systemic lupus erythematosus? *Lupus* (2013) 22:583–96. doi: 10.1177/0961203313482742
161. Young NA, Friedman AK, Kaffenberger B, Rajaram MVS, Birmingham DJ, Rovin BH, et al. Novel estrogen target gene ZAS3 is overexpressed in systemic lupus erythematosus. *Mol Immunol.* (2013) 54:23–31. doi: 10.1016/j.molimm.2012.10.026
162. Kim W-U, Min S-Y, Hwang S-H, Yoo S-A, Kim K-J, Cho C-S. Effect of oestrogen on T cell apoptosis in patients with systemic lupus erythematosus. *Clin Exp Immunol.* (2010) 161:453–8. doi: 10.1111/j.1365-2249.2010.04194.x
163. Rastin M, Hatef MR, Tabasi N, Mahmoudi M. The pathway of estradiol-induced apoptosis in patients with systemic lupus erythematosus. *Clin Rheumatol.* (2012) 31:417–24. doi: 10.1007/s10067-011-1821-3

164. Colasanti T, Maselli A, Conti F, Sanchez M, Alessandri C, Barbati C, et al. Autoantibodies to estrogen receptor α interfere with T lymphocyte homeostasis and are associated with disease activity in systemic lupus erythematosus. *Arthritis Rheum.* (2012) 64:778–87. doi: 10.1002/art.33400
165. Walters E, Rider V, Abdou NI, Greenwell C, Svojanovsky S, Smith P, et al. Estradiol targets T cell signaling pathways in human systemic lupus. *Clin Immunol.* (2009) 133:428–36. doi: 10.1016/j.clim.2009.09.002
166. Rider V, Abdou NI, Kimler BF, Lu N, Brown S, Fridley BL. Gender bias in human systemic lupus erythematosus: a problem of steroid receptor action? *Front Immunol.* (2018) 9:611. doi: 10.3389/fimmu.2018.00611
167. Giang S, La Cava A. Regulatory T cells in SLE: biology and use in treatment. *Curr Rheumatol Rep.* (2016) 18:67. doi: 10.1007/s11926-016-0616-6
168. Mizui M, Tsokos GC. Targeting regulatory T cells to treat patients with systemic lupus erythematosus. *Front Immunol* (2018) 9:786. doi: 10.3389/fimmu.2018.00786
169. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol.* (2010) 40:1830–5. doi: 10.1002/eji.201040391
170. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* (2008) 29:44–56. doi: 10.1016/j.immuni.2008.05.007
171. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. a putative role in pathogenesis. *J Immunol.* (1991) 147:117–23.
172. Ripley BJ, Goncalves B, Isenberg DA, Latchman DS, Rahman A. Raised levels of interleukin 6 in systemic lupus erythematosus correlate with anaemia. *Ann Rheum Dis.* (2005) 64:849–53. doi: 10.1136/ard.2004.022681
173. Sabry A, El-Agroudy A, Sheashaa H, Hawas S, El-Shahat FB, Barakat N. Co-administration of ketoconazole and tacrolimus therapy: a transplanted rat model. *Int Urol Nephrol.* (2006) 38:713–8. doi: 10.1007/s11255-006-0062-x
174. Li Y, Tucci M, Narain S, Barnes EV, Sobel ES, Segal MS, et al. Urinary biomarkers in lupus nephritis. *Autoimmun Rev.* (2006) 5:383–8. doi: 10.1016/j.autrev.2005.10.006
175. Isse K, Specht SM, Lunz JG, Kang L-I, Mizuguchi Y, Demetris AJ. Estrogen stimulates female biliary epithelial cell interleukin-6 expression in mice and humans. *Hepatology* (2010) 51:869–80. doi: 10.1002/hep.23386
176. Olivieri F, Bonafè M, Cavallone L, Giovagnetti S, Marchegiani F, Cardelli M, et al. The−174 C/G locus affects *in vitro/in vivo* IL-6 production during aging. *Exp Gerontol.* (2002) 37:309–14. doi: 10.1016/S0531-5565(01)00197-8
177. Mao X, Wu Y, Diao H, Hao J, Tian G, Jia Z, et al. Interleukin-6 promotes systemic lupus erythematosus progression with Treg suppression approach in a murine systemic lupus erythematosus model. *Clin Rheumatol.* (2014) 33:1585–93. doi: 10.1007/s10067-014-2717-9
178. Lu R, Munroe ME, Guthridge JM, Bean KM, Fife DA, Chen H, et al. Dysregulation of innate and adaptive serum mediators precedes systemic lupus erythematosus classification and improves prognostic accuracy of autoantibodies. *J Autoimmun.* (2016) 74:182–93. doi: 10.1016/j.jaut.2016.06.001
179. Munroe ME, Lu R, Zhao YD, Fife DA, Robertson JM, Guthridge JM, et al. Altered type II interferon precedes autoantibody accrual and elevated type I interferon activity prior to systemic lupus erythematosus classification. *Ann Rheum Dis.* (2016) 75:2014–21. doi: 10.1136/annrheumdis-2015-208140
180. Panchanathan R, Shen H, Zhang X, Ho S-M, Choubey D. Mutually positive regulatory feedback loop between interferons and estrogen receptor-α in mice: implications for sex bias in autoimmunity. *PLoS ONE* (2010) 5:e10868. doi: 10.1371/journal.pone.0010868
181. Michaelson JS, Wisniacki N, Burkly LC, Putterman C. Role of TWEAK in lupus nephritis: a bench-to-bedside review. *J Autoimmun.* (2012) 39:130–42. doi: 10.1016/j.jaut.2012.05.003
182. Xue L, Liu Z, Hu J, Huang J, Wen J, Liu Z. Estrogen-induced expression of tumor necrosis factor-like weak inducer of apoptosis through ERα accelerates the progression of lupus nephritis. *Rheumatology* (2016) 55:1880–8. doi: 10.1093/rheumatology/kew248
183. Corradetti C, Jog NR, Cesaroni M, Madaio M, Caricchio R. Estrogen receptor α signaling exacerbates immune-mediated nephropathies through alteration of metabolic activity. *J Immunol.* (2018) 200:512–22. doi: 10.4049/jimmunol.1700770
184. Scott JL, Wirth JR, Eudaly J, Ruiz P, Cunningham MA. Complete knockout of estrogen receptor α is not directly protective in murine lupus. *Clin Immunol.* (2017) 183:132–41. doi: 10.1016/j.clim.2017.08.010
185. Khan D, Ansar Ahmed S. The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol.* (2016) 6:635. doi: 10.3389/fimmu.2015.00635
186. Confavreux C, Hutchinson M, Hours MM, Cortinovis-Tourniaire P, Moreau T. Rate of pregnancy-related relapse in multiple sclerosis. pregnancy in multiple sclerosis group. *N Engl J Med.* (1998) 339:285–91. doi: 10.1056/NEJM199807303390501
187. Sicotte NL, Liva SM, Klutch R, Pfeiffer P, Bouvier S, Odesa S, et al. Treatment of multiple sclerosis with the pregnancy hormone estriol. *Ann Neurol.* (2002) 52:421–8. doi: 10.1002/ana.10301
188. Bebo BF, Fyfe-Johnson A, Adlard K, Beam AG, Vandenbark AA, Offner H. Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. *J Immunol.* (2001) 166:2080–9. doi: 10.4049/jimmunol.166.3.2080
189. Duncan GS, Brenner D, Tusche MW, Brüstle A, Knobbe CB, Elia AJ, et al. 2-Methoxyestradiol inhibits experimental autoimmune encephalomyelitis through suppression of immune cell activation. *Proc Natl Acad Sci USA.* (2012) 109:21034–9. doi: 10.1073/pnas.1215558110
190. Pozzilli C, Falaschi P, Mainero C, Martocchia A, D'Urso R, Proietti A, et al. MRI in multiple sclerosis during the menstrual cycle: relationship with sex hormone patterns. *Neurology* (1999) 53:622–4.
191. Spence RD, Voskuhl RR. Neuroprotective effects of estrogens and androgens in CNS inflammation and neurodegeneration. *Front Neuroendocrinol.* (2012) 33:105–15. doi: 10.1016/j.yfrne.2011.12.001
192. Itoh N, Kim R, Peng M, DiFilippo E, Johnsonbaugh H, MacKenzie-Graham A, et al. Bedside to bench to bedside research: estrogen receptor β ligand as a candidate neuroprotective treatment for multiple sclerosis. *J Neuroimmunol.* (2017) 304:63–71. doi: 10.1016/j.jneuroim.2016.09.017
193. Voskuhl RR, Wang HJ, Wu TCJ, Sicotte NL, Nakamura K, Kurth F, et al. Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2016) 15:35–46. doi: 10.1016/S1474-4422(15)00322-1
194. Seifert HA, Benedek G, Nguyen H, Kent G, Vandenbark AA, Offner H. Estrogen protects both sexes against EAE by promoting common regulatory cell subtypes independent of endogenous estrogen. *Metab Brain Dis.* (2017) 32:1747–54. doi: 10.1007/s11011-017-0063-8
195. Wang C, Dehghani B, Li Y, Kaler LJ, Vandenbark AA, Offner H. Oestrogen modulates experimental autoimmune encephalomyelitis and interleukin-17 production via programmed death 1. *Immunology* (2009) 126:329–35. doi: 10.1111/j.1365-2567.2008.03051.x
196. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *Int Immunol.* (2007) 19:337–43. doi: 10.1093/intimm/dxl151
197. Haghmorad D, Amini AA, Mahmoudi MB, Rastin M, Hosseini M, Mahmoudi M. Pregnancy level of estrogen attenuates experimental autoimmune encephalomyelitis in both ovariectomized and pregnant C57BL/6 mice through expansion of Treg and Th2 cells. *J Neuroimmunol.* (2014) 277:85–95. doi: 10.1016/j.jneuroim.2014.10.004
198. Alpízar-Rodríguez D, Pluchino N, Canny G, Gabay C, Finckh A. The role of female hormonal factors in the development of rheumatoid arthritis. *Rheumatology* (2017) 56:1254–63. doi: 10.1093/rheumatology/kew318
199. Islander U, Jochems C, Lagerquist MK, Forsblad-d'Elia H, Carlsten H. Estrogens in rheumatoid arthritis; the immune system and bone. *Mol Cell Endocrinol.* (2011) 335:14–29. doi: 10.1016/j.mce.2010.05.018
200. Alpízar-Rodríguez D, Finckh A. Environmental factors and hormones in the development of rheumatoid arthritis. *Semin Immunopathol.* (2017) 39:461–8. doi: 10.1007/s00281-017-0624-2
201. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev.* (2007) 28:521–74. doi: 10.1210/er.2007-0001
202. Furie R. Dehydroepiandrosterone and biologics in the treatment of systemic lupus erythematosus. *Curr Rheumatol Rep.* (2000) 2:44–50. doi: 10.1007/s11926-996-0068-5

203. Karpuzoglu E, Zouali M. The multi-faceted influences of estrogen on lymphocytes: toward novel immuno-interventions strategies for autoimmunity management. *Clin Rev Allergy Immunol.* (2011) 40:16–26. doi: 10.1007/s12016-009-8188-0
204. Nilsson S, Koehler KF, Gustafsson J-Å. Development of subtype-selective oestrogen receptor-based therapeutics. *Nat Rev Drug Discov.* (2011) 10:778–92. doi: 10.1038/nrd3551
205. An K-C. Selective estrogen receptor modulators. *Asian Spine J.* (2016) 10:787–91. doi: 10.4184/asj.2016.10.4.787
206. Apelgren LD, Bailey DL, Fouts RL, Short L, Bryan N, Evans GF, et al. The effect of a selective estrogen receptor modulator on the progression of spontaneous autoimmune disease in MRL lpr/lpr mice. *Cell Immunol.* (1996) 173:55–63. doi: 10.1006/cimm.1996.0251
207. Wu WM, Suen JL, Lin BF, Chiang BL. Tamoxifen alleviates disease severity and decreases double negative T cells in autoimmune MRL-lpr/lpr mice. *Immunology* (2000) 100:110–8.
208. Erlandsson MC, Gömöri E, Taube M, Carlsten H. Effects of raloxifene, a selective estrogen receptor modulator, on thymus, T cell reactivity, and inflammation in mice. *Cell Immunol.* (2000) 205:103–9. doi: 10.1006/cimm.2000.1719
209. Bernardi AI, Andersson A, Grahnemo L, Nurkkala-Karlsson M, Ohlsson C, Carlsten H, et al. Effects of lasofoxifene and bazedoxifene on B cell development and function. *Immun Inflamm Dis.* (2014) 2:214–25. doi: 10.1002/iid3.37
210. Wu X, Tong B, Yang Y, Luo J, Yuan X, Wei Z, et al. Arctigenin functions as a selective agonist of estrogen receptor β to restrict mTORC1 activation and consequent Th17 differentiation. *Oncotarget* (2016) 7:83893–906. doi: 10.18632/oncotarget.13338
211. Polari L, Wiklund A, Sousa S, Kangas L, Linnanen T, Härkönen P, et al. SERMs promote anti-inflammatory signaling and phenotype of $CD14^+$ cells. *Inflammation* (2018) 41, 1157–71. doi: 10.1007/s10753-018-0763-1
212. Petri MA, Mease PJ, Merrill JT, Lahita RG, Iannini MJ, Yocum DE, et al. Effects of prasterone on disease activity and symptoms in women with active systemic lupus erythematosus. *Arthritis Rheum.* (2004) 50:2858–68. doi: 10.1002/art.20427
213. Abdou NI, Rider V, Greenwell C, Li X, Kimler BF. Fulvestrant (Faslodex), an estrogen selective receptor downregulator, in therapy of women with systemic lupus erythematosus. clinical, serologic, bone density, and T cell activation marker studies: a double-blind placebo-controlled trial. *J Rheumatol.* (2008) 35:797.
214. Hughes GC. Progesterone and autoimmune disease. *Autoimmun Rev.* (2012) 11:A502–14. doi: 10.1016/j.autrev.2011.12.003
215. Greenstein B, Roa R, Daher Y, Nunn E, Greenstein A, Khamashta M, et al. Estrogen and progesterone receptors in murine models of systemic lupus erythematosus. *Int Immunopharmacol.* (2001) 1:1025–35. doi: 10.1016/S1567-5769(01)00034-0
216. Tan IJ, Peeva E, Zandman-Goddard G. Hormonal modulation of the immune system—a spotlight on the role of progestogens. *Autoimmun Rev.* (2015) 14:536–42. doi: 10.1016/j.autrev.2015.02.004
217. Gubbels Bupp MR, Jorgensen TN. Androgen-induced immunosuppression. *Front Immunol.* (2018) 9:794. doi: 10.3389/fimmu.2018.00794
218. Pakpoor J, Goldacre R, Goldacre MJ. Associations between clinically diagnosed testicular hypofunction and systemic lupus erythematosus: a record linkage study. *Clin Rheumatol.* (2018) 37:559–62. doi: 10.1007/s10067-017-3873-5
219. Procaccini C, Pucino V, Mantzoros CS, Matarese G. Leptin in autoimmune diseases. *Metab Clin Exp.* (2015) 64:92–104. doi: 10.1016/j.metabol.2014.10.014
220. Naylor C, Petri WA. Leptin regulation of immune responses. *Trends Mol Med.* (2016) 22:88–98. doi: 10.1016/j.molmed.2015.12.001
221. La Cava A. Leptin in inflammation and autoimmunity. *Cytokine* (2017) 98:51–8. doi: 10.1016/j.cyto.2016.10.011
222. Pérez-Pérez A, Vilariño-García T, Fernández-Riejos P, Martín-González J, Segura-Egea JJ, Sánchez-Margalet V. Role of leptin as a link between metabolism and the immune system. *Cytokine Growth Factor Rev.* (2017) 35:71–84. doi: 10.1016/j.cytogfr.2017.03.001
223. Francisco V, Pino J, Campos-Cabaleiro V, Ruiz-Fernández C, Mera A, Gonzalez-Gay MA, et al. Obesity, fat mass and immune system: role for leptin. *Front Physiol.* (2018) 9:640. doi: 10.3389/fphys.2018.00640
224. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* (1998) 394:897–901. doi: 10.1038/29795
225. Yu Y, Liu Y, Shi F-D, Zou H, Matarese G, La Cava A. Cutting edge: leptin-induced RORγt expression in $CD4^+$ T cells promotes Th17 responses in systemic lupus erythematosus. *J Immunol.* (2013) 190:3054–8. doi: 10.4049/jimmunol.1203275
226. Procaccini C, De Rosa V, Galgani M, Carbone F, Cassano S, Greco D, et al. Leptin-induced mTOR activation defines a specific molecular and transcriptional signature controlling $CD4^+$ effector T cell responses. *J Immunol.* (2012) 189:2941–53. doi: 10.4049/jimmunol.1200935
227. Gerriets VA, Danzaki K, Kishton RJ, Eisner W, Nichols AG, Saucillo DC, et al. Leptin directly promotes T-cell glycolytic metabolism to drive effector T-cell differentiation in a mouse model of autoimmunity. *Eur J Immunol.* (2016) 46:1970–83. doi: 10.1002/eji.201545861
228. Lourenço EV, Liu A, Matarese G, La Cava A. Leptin promotes systemic lupus erythematosus by increasing autoantibody production and inhibiting immune regulation. *Proc Natl Acad Sci USA.* (2016) 113:10637–42. doi: 10.1073/pnas.1607101113
229. Amarilyo G, Iikuni N, Liu A, Matarese G, La Cava A. Leptin enhances availability of apoptotic cell-derived self-antigen in systemic lupus erythematosus. *PLoS ONE* (2014) 9:e112826. doi: 10.1371/journal.pone.0112826
230. De Rosa V, Procaccini C, Calì G, Pirozzi G, Fontana S, Zappacosta S, et al. A key role of leptin in the control of regulatory T cell proliferation. *Immunity* (2007) 26:241–55. doi: 10.1016/j.immuni.2007.01.011
231. Lam QLK, Wang S, Ko OKH, Kincade PW, Lu L. Leptin signaling maintains B-cell homeostasis via induction of Bcl-2 and Cyclin D1. *Proc Natl Acad Sci USA.* (2010) 107:13812–7. doi: 10.1073/pnas.1004185107
232. Agrawal S, Gollapudi S, Su H, Gupta S. Leptin activates human B cells to secrete TNF-α, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway. *J Clin Immunol.* (2011) 31:472–8. doi: 10.1007/s10875-010-9507-1
233. Lateef A, Petri M. Hormone replacement and contraceptive therapy in autoimmune diseases. *J Autoimmun.* (2012) 38:J170–6. doi: 10.1016/j.jaut.2011.11.002
234. Williams WV. Hormonal contraception and the development of autoimmunity: a review of the literature. *Linacre Q.* (2017) 84:275–95. doi: 10.1080/00243639.2017.1360065
235. Buyon JP, Petri MA, Kim MY, Kalunian KC, Grossman J, Hahn BH, et al. The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial. *Ann Intern Med.* (2005) 142:953–62. doi: 10.7326/0003-4819-142-12_Part_1-200506210-00004
236. Petri M, Kim MY, Kalunian KC, Grossman J, Hahn BH, Sammaritano LR, et al. Combined oral contraceptives in women with systemic lupus erythematosus. *N Engl J Med.* (2005) 353:2550–8. doi: 10.1056/NEJMoa051135
237. Vieira CS, Pereira FV, de Sá MFS, Paulo LJ, Martins WP, Ferriani RA. Tibolone in postmenopausal women with systemic lupus erythematosus: a pilot study. *Maturitas* (2009) 62:311–6. doi: 10.1016/j.maturitas.2008.12.021
238. Panay N, Hamoda H, Arya R, Savvas M. The 2013 British Menopause Society & Women's Health Concern recommendations on hormone replacement therapy, The 2013 British Menopause Society & Women's Health Concern recommendations on hormone replacement therapy. *Menopause Int.* (2013) 19:59–68. doi: 10.1177/1754045313489645
239. Lindsay R, Gallagher JC, Kagan R, Pickar JH, Constantine G. Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. *Fertil Steril.* (2009) 92:1045–52. doi: 10.1016/j.fertnstert.2009.02.093
240. Lobo RA, Pinkerton JV, Gass MLS, Dorin MH, Ronkin S, Pickar JH, et al. Evaluation of bazedoxifene/conjugated estrogens for the treatment of menopausal symptoms and effects on metabolic

parameters and overall safety profile. *Fertil Steril.* (2009) 92:1025–38. doi: 10.1016/j.fertnstert.2009.03.113

241. Pickar JH, Komm BS. Selective estrogen receptor modulators and the combination therapy conjugated estrogens/bazedoxifene: a review of effects on the breast. *Post Reprod Health* (2015) 21:112–21. doi: 10.1177/2053369115599090
242. Andersson A, Bernardi AI, Nurkkala-Karlsson M, Stubelius A, Grahnemo L, Ohlsson C, et al. Suppression of experimental arthritis and associated bone loss by a tissue-selective estrogen complex. *Endocrinology* (2016) 157:1013–20. doi: 10.1210/en.2015-1820
243. Zhang Y, Saha S, Rosenfeld G, Gonzalez J, Pepeljugoski KP, Peeva E. Raloxifene modulates estrogen-mediated B cell autoreactivity in NZB/W F1 mice. *J Rheumatol.* (2010) 37:1646–57. doi: 10.3899/jrheum.090911
244. Nordqvist J, Bernardi A, Islander U, Carlsten H. Effects of a tissue-selective estrogen complex on B lymphopoiesis and B cell function. *Immunobiology* (2017) 222:918–23. doi: 10.1016/j.imbio.2017.05.013
245. Wu G, Xu R, Zhang P, Xiao T, Fu Y, Zhang Y, et al. Estrogen regulates stemness and senescence of bone marrow stromal cells to prevent osteoporosis via ERβ-SATB2 pathway. *J Cell Physiol.* (2017) 233:4194–204. doi: 10.1002/jcp.26233
246. Borba VV, Zandman-Goddard G, Shoenfeld Y. Prolactin and autoimmunity. *Front Immunol.* (2018) 9:73. doi: 10.3389/fimmu.2018.00073
247. McMahon M, Skaggs BJ, Sahakian L, Grossman J, FitzGerald J, Ragavendra N, et al. High plasma leptin levels confer increased risk of atherosclerosis in women with systemic lupus erythematosus, and are associated with inflammatory oxidised lipids. *Ann Rheum Dis.* (2011) 70:1619–24. doi: 10.1136/ard.2010.142737

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REVIEW
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Androgen-Induced Immunosuppression

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In addition to determining biological sex, sex hormones are known to influence health and disease *via* regulation of immune cell activities and modulation of target-organ susceptibility to immune-mediated damage. Systemic autoimmune disorders, such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis are more prevalent in females, while cancer shows the opposite pattern. Sex hormones have been repeatedly suggested to play a part in these biases. In this review, we will discuss how androgens and the expression of functional androgen receptor affect immune cells and how this may dampen or alter immune response(s) and affect autoimmune disease incidences and progression.

Keywords: androgen, testosterone, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, cancer, autoimmunity, sex hormones

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INTRODUCTION

Sex hormones exert their effects on many cellular targets, including cells of the immune system. Indeed, sex hormones directly influence immune cell function and development as well as the susceptibility of cells and tissues to damage from aberrant (autoimmune) processes. In this review, we will discuss how androgens and the androgen receptor (AR) affect immune cells and how this may dampen or alter immune response(s) to affect disease incidence and progression.

ANDROGEN AND ARs

Androgens

The four androgen hormones, dihydrotestosterone (DHT), testosterone, androstenedione, and dehydroepiandrosterone (DHEA), are all synthesized from cholesterol in the gonads and adrenal glands (1). DHT is more potent than testosterone, while androstenedione and DHEA exhibit only 10 and 5% of the potency of testosterone, respectively (1). However, testosterone is the most concentrated androgen in adult male serum, with DHT present at one-tenth the concentration of testosterone. Testosterone can be converted to androstenedione (and *vice versa*) and both can be aromatized to estrogens by aromatase (2). Aromatase is widely expressed and thus studies in which testosterone and androstenedione have been used for *in vivo* treatment can be difficult to interpret. DHEA binds several steroid hormone receptors, including AR and estrogen receptors α and β, albeit with lower affinity than their cognate ligands (3). Moreover, DHEA can be reversibly modified to form DHEA-S, which can be peripherally metabolized to testosterone (especially in premenopausal women) and estrogens (especially in postmenopausal women) (3), further complicating our understanding of DHEA-mediated effects. Of the four androgens, only DHT cannot be converted to estrogens and thus, studies utilizing DHT are most easily interpreted.

Androgen Receptors

Beyond its required role in the development and expression of male phenotypes, the AR regulates immune function *via* modulating the transcription of a number of genes by DNA-binding-dependent and -independent mechanisms (4). Encoded on the X chromosome, the AR is a signal transduction protein and transcription factor required for the development and expression of male phenotypes (4). The AR is bound by heat shock proteins and chaperones in the cytoplasm until bound by its ligands (5–10). Signal transduction through the classical AR is a multi-step process dependent upon receptor dimerization, the binding of ligand, interaction with cofactors, and DNA binding. Upon binding ligand, heat-shock proteins and chaperones are exchanged for cofactors, and the receptor:ligand complex translocates into the nucleus to bind specific DNA regulatory sequences [androgen response elements (AREs)] and regulate transcription (4). Due to the differences in binding affinities and dissociation constants, AR:DHT complexes remains bound to AREs longer than AR:testosterone complexes, further adding to the increased potency of DHT (11–13).

In addition to its well-characterized ability to function as a transcription factor as outlined above, the AR also signals through DNA-binding-independent mechanisms and can even signal in a ligand-independent fashion (14). Activation of non-classical (NC) AR rapidly affects the regulation of other nuclear receptors, transcription factors, and cytoplasmic signaling events including the release of intracellular calcium and the formation of inositol 1,4,5-triphosphate (15). NC receptors may be located in the plasma membrane, where they are associated with G-protein coupled receptors and subject to internalization, or in the cytoplasm (16, 17) [reviewed in Ref. (18, 19)]. NC ARs include receptors that bind androgen either directly or indirectly *via* the steroid hormone binding globulin (SHBG) (20, 21). Finally, in the context of cancer, AR may be activated by a variety of growth factors independently of androgens (14).

Polymorphisms in the AR gene, *NR3C5*, are known to influence androgen signaling strength. The most widely studied polymorphism affects the number of CAG repeat sequences in exon one of the AR gene. Specifically, AR's transactivational activity decreases with the presence of longer CAG repeats and *vice versa* (22, 23). Interestingly, women with shorter CAG repeats (i.e., those with more potent AR signaling) exhibit higher androgen levels, while men with shorter CAG repeats experience more dramatic reductions in testosterone as they age (24, 25), suggesting that CAG repeats differentially affect AR signaling in men and women.

The expression of AR in various immune organs and multiple immune cells provides some indication of the level at which androgens influence immunity (**Table 1**). For example, T cells are sensitive to androgens throughout development and beyond, while B cells are primarily sensitive during development. Indeed, thymocytes and thymic epithelial cells express intracellular AR (26–28) as do peripheral T cells, which also express NC, membrane associated receptors (17, 28). Bone marrow stromal cells and B cell precursors, but not peripheral B cells, express AR (29–31). Gene expression studies show that the AR is expressed by all myeloid progenitor cells as well as some terminally differentiated cells of myeloid lineage, including neutrophils, monocytes, and macrophages (16, 32–36). Thus, there is great potential for androgen modulation of the development and function of both the lymphoid and myeloid branches of the immune system.

TABLE 1 | Expression of androgen receptor (AR) in hematopoietic cells.

Cell type	AR expression (method)	Reference
Stem cells and progenitor cells		
Hematopoietic stem cell	Yes (RT-PCR, IF)	(33, 34)
Common myeloid progenitor	Yes (RT-PCR)	(33)
Common lymphoid progenitor	Yes (RT-PCR)	(33)
Granulocyte-macrophage progenitor	ND	
Common dendritic cell (DC) progenitor	ND	
Megakaryocyte-erythroid progenitor	ND	
Erythroblast	Yes (binding assay)	(32)
Early T cell progenitor	ND	
Myeloid-derived cells		
Megakaryocyte	Yes (IHC, RT-PCR, IF)	(37, 38)
Platelet	Yes (IF)	(38)
Erythroid cell (nucleated and enucleated)	*Not expressed* (IHC)	(37)
Monocyte	Yes (RT-PCR)	(37)
Macrophage	Yes (C+NC) (flow, IF, IHC, RT-PCR)	(16, 36, 37, 39)
Myeloid-derived DC	*Not expressed* (WB)	(40)
Myelocyte	Yes (IHC)	(37)
Metamyelocyte	Yes (IHC)	(37)
Neutrophil (band cell)	Yes (IHC)	(37)
Neutrophil (segmented)	Yes (IHC)	(37)
Mature eosinophil	*Not expressed* (IHC)	(37)
Basophil	ND	
Mature mast cell	Yes (ImmGold)	(35, 41)
Lymphoid-derived cells		
DN T cell	Yes (binding assay)	(42)
DP T cell	Yes (binding assay)	(42)
CD4+ T cell	Yes (C+NC) (flow, IF, binding assay)	(17, 28, 42)
CD8+ T cell	Yes (C+NC) (binding assay)	(17, 42)
Plasmacytoid DC	ND	
Pro-B cell	Yes (WB)	(43)
Pre-B cell	Yes (WB)	(43)
Immature B cell	ND	
Mature B cell	*Not expressed* (IHC, WB)	(37, 43)
Other		
Thymic epithelial cells	Yes (IF)	(44)
Bone marrow stromal cells	Yes (IHC, WB)	(37, 43)

ND, not determined; C, classical; NC, non-classical. ImmGold, ImmunoGold staining; RT-PCR, reverse-transcriptase-polymerase chain reaction; WB, western blotting; IF, immunofluorescence; IHC, immunohistochemical staining; flow, flow cytometry.

ANDROGENS AND IMMUNE CELL SUBSETS

There is ample evidence that androgens alter immune cell development and immune activation. In the following section, we review the effect of androgens on specific immune cell subsets and the potential effect this may have on immune responses and immune homeostasis in general.

Myeloid Cells

The innate immune system consists of a number of different cell subsets, predominantly of myeloid origin. Most myeloid cells initiate their track of differentiation from hematopoietic stem cells in the bone marrow only to undergo final differentiation at sites of infection or inflammation. As mentioned above, all myeloid progenitor cells express the classical AR (34, 35) and testosterone has been shown to affect early myelopoiesis (45–47). Myeloid cell-specific effects of androgens are further discussed below.

Neutrophils

Several lines of evidence suggest that androgens directly promote the differentiation of neutrophils from myeloid progenitors. For example, both genetically manipulated AR-deficient mice and androgen insensitive mice carrying the naturally occurring testicular feminization mutation (*tfm*) exhibit neutropenia (45, 46). Similarly, androgen-deficient prostate cancer patients and gonadectomized male mice also display neutropenia, prior to androgen-replacement therapy/DHT treatment (48, 49). Further support for androgen-induced granulopoiesis and neutrophil differentiation comes from studies of stanozolol, a testosterone analog, showing an increased prevalence of myelocytes and metamyelocytes as well as accelerated neutrophil maturation in treated female mice (50). Neutrophilia can also be observed in young women with hyperandrogenism due to polycystic ovarian syndrome (47). Interestingly, treatment with the anti-androgen, flutamide, and metformin (known to reduce circulating levels of androgens) decreases numbers of neutrophils in these patients (47). Together, these studies suggest that androgens drive neutrophil differentiation and/or survival in mice and humans.

Although, less well understood, there is growing evidence that androgens might also affect neutrophil function. For example, testosterone suppresses both the anti-microbial activity and the production of pro-inflammatory cytokines by human neutrophils, while promoting the production of the anti-inflammatory cytokine IL-10 (51, 52). It is interesting that there are no reports of elevated numbers of myeloid cells in athletes taking anabolic androgenic steroids (AAS) as performance enhancing drugs, although increased production of pro-inflammatory cytokines (IL-1β and TNFα) and greater oxidative stress responses in PBMCs from AAS users [reviewed in Ref. (53, 54)] do suggest an effect on myeloid cell activity. In summary, androgens appear to promote neutrophil differentiation in mice and humans and may also dampen the inflammatory potential of mature neutrophils.

Monocytes/Macrophages

Both monocytes and macrophages have been found to express classical as well as NC AR (16, 37, 39, 41). Testosterone treatment was shown to elevate levels of circulating monocytes in a population of type II diabetic men with partial androgen deficiency (55), however, whether this effect was due to augmented differentiation of monocytes in the BM or altered trafficking patterns remains unknown. Studies evaluating the importance of androgens and/or AR in macrophages during wound healing have shown that AR deficiency accelerates wound healing, while DHT treatment improves the quality of the wound by increasing collagen fibers (56, 57). It remains to be determined at which stages of wound healing testosterone/DHT binding to the AR is required, which may explain these seemingly contradictory results. Finally, gonadectomy has been found to drive increased TLR4 expression by male murine macrophages leading to elevated pro-inflammatory responses during infection (58), suggesting that one mechanism by which androgens are immunosuppressive is by limiting myeloid cell responsiveness to pathogens. This observation is further supported by data showing higher TLR4 expression, increased phagocytosis, and enhanced oxidative burst in female macrophages (59) and a specific downregulation of TLR4 expression by testosterone *in vitro* (58). Interestingly, male mice subjected to sepsis fare worse than females (60), although whether the outcome is dependent on testosterone-mediated suppression of myeloid cell activity remains unknown.

At the molecular level, studies have identified the presence of plasma membrane-located G-protein receptor coupled NC ARs on macrophages. These receptors are capable of binding testosterone either directly or bound to SHBG and elicit non-transcriptional stimulatory effects, such as rapid intracellular calcium mobilization and ERK phosphorylation (16, 20, 21). More research is needed to fully understand the impact of NC AR activation on macrophage function in males and females.

Other Myeloid-Derived Cell Subsets

Mast Cells

Skin residing mast cells have been found to express the AR, however, neither numbers nor distribution of these cells are affected by altering levels of androgens (35, 41). Instead, mast cell function, as determined by histamine release, may be regulated by androgens, as histamine levels at some peripheral sites are reduced after castration (61). More recently, it was shown that at least under some circumstances testosterone directly induces *Il33* expression by mast cells (62). Interestingly, IL-33 drives the generation of both innate lymphoid cells and basophils, known to produce Th2-associated cytokines and promote antibody class switching to IgE; an immunoglobulin found to be increased in young males over females in individuals suffering from allergic rhinitis (63, 64). Future studies will determine if androgens drive activation of other mast cell-specific proteins and processes.

Eosinophils

In contrast to neutrophils, numbers of eosinophils increased in the periphery and in the bone marrow of gonadectomized male mice (65). Castration did not, however, affect eosinophil numbers within sites of exposure, as eosinophil counts in nasal mucosa of unmanipulated and castrated male mice challenged with phospholipase A2 and *Schistosoma mansoni* egg antigen were comparable (66). Interestingly, testosterone directly reduced human eosinophil viability and adhesion properties *in vitro* (67), and DHEA suppressed eosinophil trafficking to the lung during infection (68), suggesting that androgens affect eosinophil numbers *via* control of tissue infiltration rather than *de novo* differentiation in the bone marrow. Additional studies evaluating the effect of the non-aromatizable DHT are needed to firmly address the role of AR and androgens on eosinophil maturation and function.

Basophils

Basophils are largely unaffected by testosterone treatment (69) and expression of AR by these cells has not been determined.

Dendritic Cells (DCs)

At the border of the innate and the acquired immune systems, reside DCs. These can originate from either myeloid or lymphoid progenitors. Only few studies have investigated the effect of androgens on DC differentiation and function but overall, testosterone has been assigned an immunoinhibitory function. It remains controversial, however, whether this effect is direct or indirect, as at least one study demonstrated a lack of AR expression in myeloid-derived DCs (40). Nevertheless, exogenous DHT treatment has been found to either downregulate surface levels of MHC/HLA and costimulatory molecules or inhibit cytokine production in animal models resulting in reduced T cell activation, proliferation, and differentiation. For example, after LCMV infection, infiltrating DCs isolated from the brains of male mice were less activated (reduced MHC-II and CD86 expression) than cells isolated from females and gonadectomized male mice (70). This observation was due to testosterone, as DHT treatment of gonadectomized male mice reversed the DC phenotype back to that of intact males (70). Similarly, gonadectomy studies have shown that removal of testicular testosterone production in male mice results in increased expression of MHC and costimulatory molecules on DC (71). A similar pattern is found in hypogonadal men, in whom DC activation markers are significantly elevated, but reversed in response to exogenous testosterone treatment (72). The *in vivo* nature of these experiments and observations, however, do not necessarily support a direct effect of androgens on DCs, as both MHC and costimulatory molecules are also regulated by cytokines secreted by other cells subject to androgenic regulation. Specifically addressing this concern, bone marrow-derived DCs, exposed briefly to DHT during antigen uptake, have been found to be less efficient T cell activators *in vitro* than BMDCs not exposed to DHT (73).

Lymphoid Cells

B Cells

It has been known for decades that the average female produces higher levels of antibodies in response to infections and vaccinations [reviewed in Ref. (74)]. A number of studies have found strong correlations between low testosterone and elevated numbers of B cells (75–79), and high testosterone levels in men correlates with poorer antibody responses to vaccination (80), suggesting that androgens inhibit B lymphopoiesis. Recent studies of B cell subsets in 3- to 8-year-old children identified different distributions in males and females (81, 82). Specifically, boys demonstrate elevated levels of immature CD5+ B cells, while girls exhibit more memory-type B cells. Lundell et al. further evaluated levels of DHT in these children and found a positive correlation between DHT levels at birth and the frequency of immature B cells. Given the minimal exposure to exogenous agents, these data suggest that males and females are subject to differential genetic- and/or hormonal-driven gestational regulation of B cell lymphopoiesis.

In mouse studies, gonadectomy of male mice has been found repeatedly to drive B cell lymphopoiesis in the bone marrow, with both testosterone and DHT treatment capable of reversing this effect (43, 83, 84). Similarly, global AR-deficient mice present with elevated B cell precursors in the bone marrow (pro-B stage and beyond) and studies of the B cell repertoire in B cell-specific AR knockout animals suggest that the lymphopoietic effect of testosterone is AR-dependent and intrinsic to the B cell—at least at the later stages of B cell development (pre-B cells and beyond) (85). However, other studies have suggested that the inhibitory effect of testosterone on B lymphopoiesis is dependent on bone marrow stromal cells (29, 30). Recently, AR expression by bone marrow osteoblasts was found to specifically inhibit early B lymphopoiesis resulting in an accumulation of pro-B cells (86). Thus, it is likely that the differentiation of pro-B cells from common lymphoid progenitors is inhibited by AR expression by osteoblasts, while further differentiation along the B cell lineage is negatively affected by AR expression by the B cell progenitors themselves. A possible mechanism of action has been suggested based on studies showing that testosterone upregulates TGFβ production by bone marrow stromal cells leading to inhibition of IL-7 production and suppression of B lymphopoiesis (29, 87). In summary, B lymphopoiesis is inhibited by androgens both directly and indirectly *via* effects on bone marrow stromal cells.

T Cells

Thymic size and the selection of developing thymocytes is significantly affected by androgens. Testosterone deficient or insensitive males, due to gonadectomy or AR deficiency, experience thymic enlargement (27, 88–93). Thymic size returns to normal when gonadectomized males are treated with DHT (91). Studies involving AR-deficient bone marrow chimeric mice demonstrated that androgen signaling through AR in thymic epithelial cells mediates androgen's effects in the thymus (27). A similar observation was made in thymic epithelial cell-specific AR−/− mice (94). In addition, androgens limit the numbers of CD4+ CD8+ and CD4+ CD8− in favor of CD4− CD8+ thymocytes, perhaps by suppressing proliferation and accelerating the apoptosis of immature thymocytes (88, 90, 95, 96). Finally, androgens enhance the negative selection of self-reactive T cells by upregulating the expression of *autoimmune regulator* (*Aire*) in medullary thymic epithelial cells (97). Androgens may also influence T cell development in tolerance-promoting ways by enhancing TGF-β production in the thymus (98).

Similar to its effect on thymic size, androgens also limit the total number of T cells residing in the periphery. Postpubescent gonadectomized male mice exhibit larger peripheral lymphoid organs housing a greater number of lymphocytes, including both CD4+ and CD8+ T cells (92, 99). The expansion of peripheral lymphoid organs after the removal of androgens may be related to increases in thymic output and/or lessened peripheral T cell death, as an *in vitro* study recently demonstrated that DHT can non-selectively induce cell death in peripheral T cells (100).

Androgens are likely responsible for some portion of the effect of sex on peripheral T cell responses. Thymocytes and lymphocytes isolated from non-autoimmune female mice respond more vigorously to exogenous and allogeneic antigens *in vitro* than cells

isolated from male mice (101, 102). Treating female mice with testosterone reduces the proliferative T cell response to OVA and KLH (101). Similarly, gonadectomized male mice, compared to intact male mice, proliferate more vigorously in response to TCR stimulation and OVA *in vitro* and *in vivo* (92, 99).

Cytokine production evoked by specific antigens are also often affected by sex, with male cells favoring Th2 type responses and female cells favoring Th1 type responses (92). For example, anti-CD3 stimulation provokes the secretion of Th2 cytokines, IL-10 and IL-4, from CD4+ T cells isolated from male experimental autoimmune encephalomyelitis (EAE)-prone SJL mice, but IL-12 from cells isolated from females (26). Interestingly, the addition of DHT to T cell cultures is sufficient to upregulate IL-10 expression (26). Beyond its ability to enhance the production of Th2 cytokines, androgens actively inhibit Th1 differentiation (103, 104), by inhibiting IL-12 and IFN-γ production downstream of antigen stimulation (105–109).

Androgens may influence the differentiation and function of regulatory T cells differently in males versus females. *In vivo* androgen supplementation of women with adrenal insufficiency and female rats with experimental autoimmune orchitis expands the number of regulatory T cells (104, 110). More specifically, when bound to ligand, the AR directly enhances the expression of *Foxp3* in T cells (regulatory or otherwise) isolated from rats or women during the ovulatory phase, but not men (111). Thus, androgens are capable of directly converting peripheral T cells into regulatory T cells in women. By contrast, androgens may interfere with regulatory T cell function in men, as occurs in a mouse model of Sjögren's syndrome that predominantly affects male mice (112).

Overall, androgens directly influence the development of lymphoid cells; and at least in mice, lymphoid cells that develop in the presence of androgens may retain differential characteristics even when later placed in an androgen-deficient environment. Moreover, androgens appear to suppress the inflammatory potential of peripheral lymphoid cells. In some cases, such effects may be direct, but the absence of AR in peripheral B cells, for example, suggests that differences are more likely due to prior exposure to androgens during development, or regulation by other androgen-sensitive peripheral cells.

ANDROGENS IN AUTOIMMUNITY

Many autoimmune disorders are more prevalent in females, including autoimmune thyroiditis, systemic lupus erythematosus (SLE), Sjögren's syndrome, multiple sclerosis (MS), and rheumatoid arthritis (RA) (113). Both sex hormones and genes expressed on the X or Y chromosomes have been proposed to drive this bias, as exemplified by the fact that Klinefelter's patients (XXY karyotype) express not only two X chromosomes but also reduced levels of androgens, and present with an increased risk for most of these disorders (114). Many patients of either sex with autoimmune disorders that predominantly affect women also demonstrated lower serum concentrations of androgens (76, 115, 116). Here, we will discuss the influence of androgens on the development and severity of RA, MS, and SLE.

Rheumatoid Arthritis

Rheumatoid arthritis is characterized by synovial inflammation and swelling, as well as cartilage and bone destruction. Some patients may develop one or more additional systemic sequelae, including cardiovascular disease, pulmonary disorders, lymphoma, lung cancer, psychological disorders, and osteoporosis (117). Like many other autoimmune diseases, RA is 2–4 times more frequent in women than in men (113). Most studies have concluded that in addition to increased susceptibility, female RA patients suffer from a more severe version of the disease, with higher disease activity scores, faster progression, more pain, and lower remission rates (118–122).

While estrogens likely contribute to the increased female risk of RA (123), it has been hypothesized that androgens may also offer some protection from the disease. Indeed, serum androgen levels tend to be lower in men with RA as compared to healthy controls. For example, the incidence of RA increases as androgen production declines in aging men and several groups have reported lower serum testosterone concentrations in male RA patients as compared to controls (76, 124–130). Furthermore, men who experience a dramatic loss of serum androgens with age may develop a more aggressive form of RA with earlier onset (131–133). In some cases, low serum androgens also correlate with increased risk of developing RA. Men with genetic hypogonadism (Klinefelter's syndrome) and prostate cancer patients treated with androgen-ablating therapy are at increased risk of developing RA (114, 134).

Interestingly, serum androgen levels are not lower prior to the onset of RA in all patients, suggesting that low androgens are not universally predisposing to the development of RA (127, 133). Instead, as a recent large study reported, men with lower serum testosterone levels prior to the onset of RA may be more likely to develop a specific subset of RA, characterized as rheumatoid factor negative (135). It is possible that the correlation of low serum androgen levels and RA in men can be explained by inflammatory cytokines, such as IL-6, which become elevated during the disease process and are known to suppress the secretion of adrenal androgens (136). The notion that low serum androgens are a consequence of RA, as opposed to a cause in some cases, is supported by an inverse correlation between low free testosterone and inflammatory markers and disease activity (129, 137), as well as the finding that successful treatment of RA correlates with the restoration of normal levels of free testosterone (130). To summarize, androgens may protect against the development of RA in men in some circumstances; and in others, the inhibition of androgen secretion by the RA-inflammatory response is secondary to RA, but may still influence the severity of disease in men.

The effect of androgens on RA susceptibility and severity in women is less well understood. Androgens may protect against RA in some women, but other studies suggest that androgens may actually worsen disease severity. As in men, low serum concentrations of androgens, particularly DHEA and/or DHEA-S, are linked with RA in women (136, 138–140). However, as was found in men, serum androgen levels are within the normal range 10 years prior to the onset of RA and levels of DHEA-S inversely correlate with disease duration and severity in women (127, 140).

Thus, for most female patients, the inhibitory effect of inflammation on the secretion of androgens may explain this correlation. Two notable exceptions to this include women who inherited particular polymorphisms resulting in higher or lower androgen levels correlating with protection from disease or exacerbation, respectively. First, women predisposed to produce androgens in greater concentrations due to inheritance of a polymorphism in the CYB5A gene are protected from developing RA characterized by RF and antibodies to citrullinated proteins (141); and, second, women with lower serum androgens are more likely to develop a type of RA that is not responsive to combination therapy consisting of nonsteroidal anti-inflammatory drugs, low-dose prednisolone, methotrexate, and more than one of several other disease modifying anti-rheumatic drugs (142).

In contrast to the above findings, high androgen concentrations or more potent AR signaling have been reported in some women with more severe RA. For example, one small study reported normal androgen concentrations in premenopausal RA patients, and higher testosterone and DHEA-S in postmenopausal women with RA (143). More strikingly, women with higher concentrations of serum androgens due to a low number of CAG repeats in the AR developed a more aggressive RA with earlier onset, though overall susceptibility to RA was not affected (131–133, 144).

Because of the correlation between low serum androgens and RA and the known immunosuppressive properties of androgens, androgens have been utilized to some extent as a treatment for RA. Overall, the administration of androgens to male and female patients had a positive effect for both sexes (145–148). However, it should be noted that such studies are few in number, with small patient populations, short-term treatments, modest improvements, and in some cases, no effect at all.

Animal Models of RA

Several animal models of RA also show increased susceptibility or more severe disease in females as compared to males (149–152). For example, the incidence of RA is greater or more severe for females in collagen-induced arthritis (CIA) in rats, SKG mice injected with zymosan, LEW/N rats injected with polysaccharide fragments from group A streptococcal bacteria, and BALB/c mice with cotton-pellet induced inflammation (149–152). Moreover, androgens have been shown to exert protective effects in RA, even in animal models with equal or more severe disease in males (150, 153). Gonadectomy of male animals worsens RA in CIA-rats and SKG mice; and, the addition of DHT to gonadectomized CIA-rats inhibits disease (149, 152). Male and female rats injected with complete Freund's adjuvant (adjuvant arthritis) do not demonstrate a sex bias; however, similar to human studies, arthritic males demonstrate lower testosterone levels after disease induction (153). Finally, the expression of the AR on B cell progenitors has been shown to have protective effects in male mice with CIA (150).

Cellular and Molecular Targets of Androgens in RA

Regardless of whether serum androgen levels or receptor activity is involved in systemic RA etiology, a separate case has been made for its involvement in disease pathogenesis within affected joints. The synovial fluid of RA patients exhibits elevated levels of free estrogens and reduced concentrations of free androgens, possibly due to increased local aromatization of androgens to estrogens (154). The conversion of androgens to estrogens heightens the local inflammatory response, since androgens have been shown to inhibit the synthesis and secretion of IL-1 and IL-6, two important inflammatory cytokines in RA (155–158). The relationship between androgens, inflammatory cytokines, and aromatase activity is reciprocal; IL1 and IL6 stimulate aromatase activity, while androgens inhibit it (157).

In summary, with some notable exceptions, the correlation between low androgen and RA likely exists because RA-associated inflammation dampens serum androgen levels. Improvements in our ability to group RA into less heterogenous disease subgroups may reveal particular subgroups that are more affected by androgen levels than others.

MS and Androgens

Multiple sclerosis is an autoimmune disorder in which neuronal axons are actively demyelinated leading to neuronal damage and eventual paralysis. The disease precipitates in patterns of relapsing-remitting or progressive-onset. Only the former of these shows a sex-bias; relapsing-remitting MS (RRMS) develops 3–4 times more frequently in females than in males and predominantly in individuals of childbearing age, suggesting a role for sex hormones [reviewed in Ref. (159)]. In addition to higher incidence rates among female RRMS patients, many studies have also shown that women exhibit higher relapse rates than men (160–165), further supporting a gender-bias in this disease. Several studies have evaluated levels of sex hormones in MS patients, and testosterone, DHEA, or DHEA-S levels have been found to be lower in both men and women with MS as compared to healthy age-matched controls (115, 165–168). Although it is generally thought that testosterone's protective effect is mediated *via* immune modulation, treatment with testosterone improved cognitive performance and slowed brain atrophy (169) and has been suggested to increase gray matter in a small cohort of men with RRMS, suggesting a direct neuroprotective function of testosterone (170).

Multiple sclerosis patients display a chronic inflammatory profile characterized by T cell-derived cytokines (IFNγ and IL-17) and circulating antibodies reactive to brain autoantigens (171–176). Both T cell and B cell differentiation and effector functions have been shown to be affected by androgens (as mentioned above), however, clinical trials with drugs specifically targeting IL-17A (secukinumab) or B cells (rituximab) were only somewhat effective (177, 178), and targeting IFNγ *via* blockade of IL-12 (ustekinumab) was not effective (179).

Animal Models of MS

Studies of EAE (animal model of MS) have largely confirmed a protective role for androgens (180–183). For example, gonadectomy of male SJL mice resulted in increased disease susceptibility, while treatment with exogenous testosterone or DHT reduced disease incidence in both females and males (180, 181). Although C57Bl/6 male and female mice develop EAE at a similar rate, C57Bl/6 male mice were also protected by treatment with DHT (181),

and similar results have been obtained in EAE-susceptible Dark Agouti rats (183). By contrast, Ziehn et al. showed that only testosterone, not DHT, exerted a direct neuoprotective effect (182), suggesting that testosterone and DHT may have independent effects on cells of the hippocampus and infiltrating immune cells. Further studies are needed to thoroughly investigate if AR binding is required for the protective effect of androgenic treatment.

Cellular and Molecular Targets of Androgens in MS/EAE

While most studies support an overall protective effect of testosterone in MS and EAE, specific immunological targets are less well explored. Androgens may affect T cells in at least two specific ways. As mentioned above, androgens stimulate the *Aire* promoter, driving increased Aire expression by medullary thymic epithelial cells and increased negative selection (97). As a result hereof, male mice or mice treated with DHT are relatively protected from EAE (97). Second, EAE is typically driven by T helper cells (Th1 and Th17) and is characterized by the presence of key signature cytokines such as IFNγ and IL-17 within the brain, secondary lymphoid organs, and circulation. A general Th1 propensity has been observed in female patients with MS and EAE animal models (184–187) and it has been suggested that low levels of testosterone drive this phenotype. In support hereof, *ex vivo* exposure of encephalomyelitic T cells to testosterone has been shown to significantly change the secreted cytokine profile (from IFNγ to IL-10) and the pathogenic potential of these T cells (180, 188). Furthermore, myelin-basic protein-primed female T cells and T cells from gonadectimized males express significantly higher levels of the VLA-4 integrin β1 subunit and secrete higher levels of pro-inflammatory cytokines, such as IL-1β, than male-derived cells (189), thereby promoting T cell infiltration into the brain and brain pathogenesis. Although the mechanism driving differential T cell activation in males and females is largely unknown, Dunn et al. recently described that PPARα was highly expressed in male T cells in a testosterone-dependent manner and that deficiency of PPARα specifically worsened EAE in male mice (109). Further studies are needed to establish the interrelationship between PPARα, Aire, and other DHT-dependent immune regulators.

In conclusion, low levels of androgens are observed in patients with MS and gonadectomy of male mice increases their susceptibility to induced EAE. T cells have been found to respond to androgens throughout development and recent studies have started to unravel molecular mechanisms associated with androgen-induced T cell suppression.

Androgens in SLE

Systemic lupus erythematosus, a chronic and potentially fatal disease with the potential to cause damage in multiple organ systems, is nine times more prevalent in women than men (190). Physicians commonly see patients with a wide range of clinical manifestations, which may spontaneously flare and remit. For example, patients with mild lupus may present with intermittent skin rash and joint pain and require little medication, while patients with severe glomerulonephritis may show progressive renal deterioration despite treatment with high doses of corticosteroids and cytotoxic drugs. Other significant health consequences can include central nervous system involvement, vasculitis, thrombosis, thrombocytopenia, anemia, fevers, fatigue, and heart and lung involvement (190, 191).

Antinuclear autoantibodies (ANAs) are generally considered to be a hallmark of lupus (190, 191). A portion of the tissue damage in SLE is related to autoantibodies that target cell surface antigens. In other cases, such as in the kidney, the deposition, or *in situ* formation of ANA immune complexes with subsequent complement activation and inflammatory cell recruitment are responsible for the damage. The damage generated by immune complexes is not trivial; SLE is a leading cause of kidney disease, stroke, and premature cardiovascular disease in young women (192, 193).

A number of reports have suggested that androgens are protective in SLE. Although many male lupus patients have normal levels of androgens (194), men with hypogonadism are at increased risk of developing SLE (114, 116, 195) and testosterone supplementation of male lupus patients with genetic hypogonadism (Klinefelter's syndrome) has, in two cases, been beneficial in treating lupus (196, 197). However, no large-scale studies involving testosterone supplementation in male lupus patients have been reported (198). Women with lupus generally have lower androgen levels, including testosterone, DHT, DHEA, and DHEA-S (199–201), and demonstrate an accelerated inactivation of testosterone *via* oxidation than healthy age-matched controls (202). In addition, it has been suggested that inflammatory cytokines in affected tissues modulate aromatase activity to locally dampen the effects of androgens in favor of estrogens in lupus patients (203). Finally, male lupus patients with reduced AR signaling produce higher quantities of IgG autoantibodies (204), while female lupus patients with the same polymorphism exhibited reduced disease (78). It is not clear why reduced androgen signaling is associated with opposite effects on lupus in male and female patients. However, recent studies in the BWF1 animal model (discussed below) have also identified instances whereby androgen-mediated mechanisms of disease suppression are not effective in females (48). Together these observations suggest that androgens affect lupus pathogenesis differently in males and females and that the mechanisms by which androgens limit disease may not be immediately applicable as therapies for female patients.

Although survival rates of male lupus patients are comparable to survival rates in female lupus patients (205–210), it has been reported that the severity of SLE is worse in males than in females, suggesting that genetic susceptibility must be more potent in men to overcome the protection afforded by androgens (211–215). This is supported by reports that male lupus patients have an increased frequency of renal involvement (208, 210, 216–218) and that women with an affected male relative are 3.5 times more likely to develop renal disease than women without an affected male relative (219).

Some effort has been made to treat lupus with androgenic compounds. The small numbers of patients included in many of the following clinical trials make definitive conclusions difficult. However, in general, these studies support an ameliorating role for androgens. Danazol, a weakly androgenic synthetic compound,

reduced total serum IgG as well as anti-dsDNA autoantibody levels in women (220, 221). However, all patients did not benefit from therapy and some experienced disease flares (220, 221). Likewise, treatment with a testosterone-like anabolic steroid, nandrolone, afforded some patients clinical improvement, but other patients saw no improvement and masculinizing side effects made this drug a somewhat untenable treatment option for most women (202, 222, 223).

Larger clinical trials have been conducted with DHEA, an adrenal steroid with mild androgenic activity, in the hopes of separating the disease-ameliorating properties of androgens from its masculinizing ones. However, as for other androgenic drugs, some patients treated with DHEA (also known as prasterone) experienced reduced disease activity, improved cognitive function, enhanced mental well-being, and a reduced need for corticosteroid treatment (224–228), while others experienced no improvement (229, 230). Overall, treatment with this agent did not meet primary objectives of these studies and it has not been approved by the Federal Drug Agency for treatment of lupus patients. It should be mentioned that in a recent study, female lupus patients with a particular polymorphism in the extra-pituitary prolactin gene associated with low serum DHEA levels experienced the most dramatic improvements after DHEA treatment (201). Thus, genetic differences of particular study populations may explain the varied results from different clinical trials with DHEA in lupus patients.

Androgens in Mouse Models of Lupus

Inbred mice that spontaneously develop a lupus-like disease have been extremely helpful toward elucidating the etiology and pathogenesis of lupus. Several spontaneous models of lupus exist [reviewed in Ref. (231)]. Here, we focus exclusively on studies conducted with the F1 offspring of New Zealand black (NZB) and New Zealand white (NZW) mice. The female F1 offspring of NZB and NZW mice (BWF1) develop a lupus-like disease characterized by high levels of IgG ANAs accompanied by a severe and progressive glomerulonephritis in the first year of life (232, 233). By contrast, less than half of BWF1 male mice develop severe proteinuria within the same time period (232, 233). As in humans, several studies have determined that androgens suppress lupus pathogenicity BWF1 mice. For example, prepubescent gonadectomy of BWF1 male mice increases the incidence of proteinuria and mortality and accelerates the appearance of ANAs as compared to intact males (233–235). In addition, administration of DHT to gonadectomized male mice is sufficient to reduce disease development comparable to that observed in intact male mice (233–235).

Chemical manipulation of androgens in lupus-prone BWF1 mice also generally supports the hypothesis that androgens are protective in lupus. For example, treatment of BWF1 females with nandrolone decanoate (236–238) and ethylestrenol (239), which are both testosterone-like anabolic steroids, ameliorated disease. Nandrolone decanoate also reduced IgG anti-dsDNA antibody, reduced the incidence of proteinuria, and improved survival (236, 238). Similarly, DHEA treatment significantly delayed disease onset, reduced IgG anti-dsDNA autoantibodies, and reduced mortality (240). By contrast, danazol did not protect BWF1 females from accelerated development of disease (241), and treatment of BWF1 females with the anti-androgenic drug, flutamide, resulted only in a slight decrease in survival, with no noticeable effect on autoantibody levels (242). Overall, BWF1 male and female mice recapitulate much of the sex bias observed in lupus patients and are a useful model for advancing our understanding of the role of androgens in lupus-like disease.

Cellular and Molecular Targets of Androgens in Lupus

Many autoimmune diseases that are more prevalent in females, including SLE, are characterized by increased numbers of B cells and circulating autoantibody levels [reviewed in Ref. (243)]. Some evidence suggests that androgens may indirectly regulate isotype switching from IgM to more pathogenic IgG autoantibodies in BWF1 mice. Serum testosterone levels dramatically drop in intact BWF1 males at 9 months of age (234), paralleling the time at which autoantibodies in intact males class switch to IgG (233, 244). Furthermore, treating 9-month-old BWF1 males with physiological levels of DHT greatly decreases mortality, prevents IgM anti-polyA antibodies from class switching to IgG, and reduces the levels of IgG anti-dsDNA antibodies (234). By contrast, DHT treatment in intact BWF1 female mice after autoantibody production (6 months) does not affect levels of IgG anti-dsDNA autoantibodies, although mortality is reduced (245). Though the mechanism by which androgens suppress disease in older mice remains unclear, some studies suggest that androgens enhance (and estrogens delay) immune complex clearance (246); a process frequently associated with the development of SLE and lupus in mouse models. For example, androgens have been shown to enhance serum levels of complement components C4, Slp, C5, C6, and Ss binding protein, which could underlie more efficient IC clearance (247–249). More current studies evaluating a relationship between androgens and complement has to our knowledge not been performed.

As indicated above, androgens have been shown to have discreet effects on B cell function and downstream kidney damage in males and females. Androgens also appear to modulate the development and function of neutrophils differently in lupus-prone males and female. We have previously shown that Gr1+ CD11b+ cells accumulate in male mice, inhibiting the function of T follicular helper cells, germinal center formation, and plasma cell differentiation (48, 49, 250). Interestingly, these myeloid cells suppress disease in male, but not female, mice. It is intriguing to speculate that perhaps the increased frequency of suppressive Gr1+ CD11b+ cells in males is a related to CD11b+ cell overexpression of the DHT-regulated gene, *colony-stimulating factor 3-receptor* (*Csf3-r*) (233). Together with its ligand, G-CSF, CSF3-R is involved in maintaining neutrophil homeostasis and also regulates several aspects of neutrophil function (251). Interestingly, high doses of G-CSF suppress lupus-like disease in at least one animal model (252) and polymorphisms, the *Csf3-r* gene influence the development of lupus and RA (253, 254). Thus, it is not unreasonable to hypothesize that sex-specific alterations in expression of CSF3-R could influence neutrophil phenotype and function and thereby differentially influence lupus pathogenesis in males and females. Overall, at least in mice, it seems as though

lupus pathogenesis may proceed in fundamentally different ways in male and female mice.

CANCER AND ANDROGENS

Immune cells must be capable of distinguishing modified-self from self, and yet, must not become unduly activated against unmodified self-antigens. Immune activity outside of these boundaries likely risks the development of cancer or autoimmunity and it follows that immune systems especially good at protecting against cancer may run the risk of triggering autoimmunity (and *vice versa*). Given that women are more susceptible to many autoimmune diseases, one might expect men to be more susceptible to cancer. Indeed, a number of epidemiologic studies and meta-analyses have recently confirmed that cancer develops more frequently in males than in females (255–257). In a meta-study examining mortality rates from 1977 to 2006, the male-to-female mortality-rate ratios were also found to be increased all malignant cancers, although predominantly for cancers affecting the upper gastro-intestinal tract and respiratory systems (lip, tongue, hypopharynx, esophagus, larynx, lung, etc.) as well as liver and bladder (255). Many factors have been proposed to explain this discrepancy including environmental exposures (smoking, obesity, infections) and sex hormone levels and signaling (255, 258).

As mentioned above, chronic inflammation can lead to suppression of testosterone. Cancer is known to induce a stage of chronic inflammation, and thus in order to study a potential causal relationship between testosterone and cancer development, retrospective cohort analyses investigating levels of testosterone *prior to* the diagnosis of cancer are necessary. Not many studies of that kind have been done. In one study, testosterone levels were found to positively correlate with the risk for a subset of epithelial ovarian cancers (259). Similarly, higher levels of testosterone correlated with an increased risk of breast cancer in women (260). It will be interesting to see if this pattern holds for other types of cancer.

Animal Models of Cancer

More direct evidence supporting a tumorigenic role for androgen in cancer development comes from animal studies, where susceptibility to cancer in response to chemicals and genetic manipulation often depend on sex hormone levels (261). For example, gonadectomy of male rats reduced both chemical-induced pancreatic tumor burden (262) and renal cell carcinoma (263), while re-administration of testosterone at least partly reversed this effect. Furthermore, injection of prostate cancer cells into unmanipulated and gonadectomized nude male mice showed reduced tumor growth in gonadectomized mice and increased tumor growth upon subsequent testosterone treatment (94). It should be noted that whether these effects of testosterone were due to inhibition of immune activation or *via* direct effects on tumor cells remains to be tested. In a separate study of induced thyroid cancer in male mice, gonadectomy led to an upregulation of tumor-suppressor genes (264). With tumor-suppressor proteins present, CCL5 chemokine expression by tumor cells increased driving infiltration by inflammatory macrophages and CD8+ cytotoxic T cells and subsequently reduced tumor growth. Thus, testosterone may be predictive of increased cancer risk, warranting further research into the immunosuppressive and potential cancer-promoting effects of testosterone during early establishment of cancer.

CONCLUDING REMARKS

To conclude, sex-specific biases in autoimmunity and cancer incidence are associated with many differences in immune cell development and function. A significant portion of these differences are the result of exposure to androgens. Androgen-mediated suppression of immune reactivity and inflammation increases the threshold for autoimmunity to develop, but likely lowers the threshold for cancer. Studies further uncovering immune-specific effects of androgens are needed and may lead to the identification of pathways that could be targeted therapeutically to inhibit the incidence and progression of autoimmunity and cancer.

AUTHOR CONTRIBUTIONS

MGB and TNJ contributed equally to this project.

REFERENCES

1. Marchetti PM, Barth JH. Clinical biochemistry of dihydrotestosterone. *Ann Clin Biochem* (2013) 50:95–107. doi:10.1258/acb.2012.012159
2. Griffin JE, Wilson JD. The syndromes of androgen resistance. *N Engl J Med* (1980) 302:198–209. doi:10.1056/NEJM198001243020404
3. Prough RA, Clark BJ, Klinge CM. Novel mechanisms for DHEA action. *J Mol Endocrinol* (2016) 56:R139–55. doi:10.1530/JME-16-0013
4. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem* (1991) 266:510–8.
5. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM. Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. *J Biol Chem* (1999) 274:37219–25. doi:10.1074/jbc.274.52.37219
6. He B, Kemppainen JA, Wilson EM. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. *J Biol Chem* (2000) 275:22986–94. doi:10.1074/jbc.M002807200
7. Loy CJ, Sim KS, Yong EL. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. *Proc Natl Acad Sci U S A* (2003) 100:4562–7. doi:10.1073/pnas.0736237100
8. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. *Mol Endocrinol* (2000) 14:1618–26. doi:10.1210/mend.14.10.0541
9. Cardozo CP, Michaud C, Ost MC, Fliss AE, Yang E, Patterson C, et al. C-terminal Hsp-interacting protein slows androgen receptor synthesis and reduces its rate of degradation. *Arch Biochem Biophys* (2003) 410:134–40. doi:10.1016/S0003-9861(02)00680-X
10. Shatkina L, Mink S, Rogatsch H, Klocker H, Langer G, Nestl A, et al. The cochaperone Bag-1L enhances androgen receptor action via interaction with the NH2-terminal region of the receptor. *Mol Cell Biol* (2003) 23:7189–97. doi:10.1128/MCB.23.20.7189-7197.2003
11. Gaughan L, Logan IR, Neal DE, Robson CN. Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. *Nucleic Acids Res* (2005) 33:13–26. doi:10.1093/nar/gki141
12. Haelens A, Tanner T, Denayer S, Callewaert L, Claessens F. The hinge region regulates DNA binding, nuclear translocation, and transactivation of the

androgen receptor. *Cancer Res* (2007) 67:4514–23. doi:10.1158/0008-5472.CAN-06-1701
13. Lee DK, Chang C. Endocrine mechanisms of disease: expression and degradation of androgen receptor: mechanism and clinical implication. *J Clin Endocrinol Metab* (2003) 88:4043–54. doi:10.1210/jc.2003-030261
14. Davey RA, Grossmann M. Androgen receptor structure, function and biology: from bench to bedside. *Clin Biochem Rev* (2016) 37:3–15.
15. Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC. Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol* (2010) 42:813–27. doi:10.1016/j.biocel.2009.11.013
16. Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F. Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. *Mol Biol Cell* (1999) 10:3113–23. doi:10.1091/mbc.10.10.3113
17. Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, et al. Functional testosterone receptors in plasma membranes of T cells. *FASEB J* (1999) 13:123–33. doi:10.1096/fasebj.13.1.123
18. Boonyaratanakornkit V, Edwards DP. Receptor mechanisms mediating non-genomic actions of sex steroids. *Semin Reprod Med* (2007) 25:139–53. doi:10.1055/s-2007-973427
19. Durdiakova J, Ostatnikova D, Celec P. Testosterone and its metabolites – modulators of brain functions. *Acta Neurobiol Exp (Wars)* (2011) 71:434–54.
20. Pi M, Parrill AL, Quarles LD. GPRC6A mediates the non-genomic effects of steroids. *J Biol Chem* (2010) 285:39953–64. doi:10.1074/jbc.M110.158063
21. De Toni L, Guidolin D, De Filippis V, Tescari S, Strapazzon G, Santa RM, et al. Osteocalcin and sex hormone binding globulin compete on a specific binding site of GPRC6A. *Endocrinology* (2016) 157:4473–86. doi:10.1210/en.2016-1312
22. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* (1994) 22:3181–6. doi:10.1093/nar/22.15.3181
23. Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL. Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab* (1997) 82:3777–82. doi:10.1210/jcem.82.11.4385
24. Krithivas K, Yurgalevitch SM, Mohr BA, Wilcox CJ, Batter SJ, Brown M, et al. Evidence that the CAG repeat in the androgen receptor gene is associated with the age-related decline in serum androgen levels in men. *J Endocrinol* (1999) 162:137–42. doi:10.1677/joe.0.1620137
25. Westberg L, Baghaei F, Rosmond R, Hellstrand M, Landen M, Jansson M, et al. Polymorphisms of the androgen receptor gene and the estrogen receptor beta gene are associated with androgen levels in women. *J Clin Endocrinol Metab* (2001) 86:2562–8. doi:10.1210/jcem.86.6.7614
26. Liva SM, Voskuhl RR. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. *J Immunol* (2001) 167:2060–7. doi:10.4049/jimmunol.167.4.2060
27. Olsen NJ, Olson G, Viselli SM, Gu X, Kovacs WJ. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* (2001) 142:1278–83. doi:10.1210/endo.142.3.8032
28. Benten WP, Becker A, Schmitt-Wrede HP, Wunderlich F. Developmental regulation of intracellular and surface androgen receptors in T cells. *Steroids* (2002) 67:925–31. doi:10.1016/S0039-128X(02)00055-7
29. Olsen NJ, Gu X, Kovacs WJ. Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. *J Clin Invest* (2001) 108:1697–704. doi:10.1172/JCI200113183
30. Smithson G, Couse JF, Lubahn DB, Korach KS, Kincade PW. The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis. *J Immunol* (1998) 161:27–34.
31. Benten WP, Stephan C, Wunderlich F. B cells express intracellular but not surface receptors for testosterone and estradiol. *Steroids* (2002) 67:647–54. doi:10.1016/S0039-128X(02)00013-2
32. Claustres M, Sultan C. Androgen and erythropoiesis: evidence for an androgen receptor in erythroblasts from human bone marrow cultures. *Horm Res* (1988) 29:17–22. doi:10.1159/000180959
33. Igarashi H, Kouro T, Yokota T, Comp PC, Kincade PW. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. *Proc Natl Acad Sci U S A* (2001) 98:15131–6. doi:10.1073/pnas.011513098
34. Mierzejewska K, Borkowska S, Suszynska E, Suszynska M, Poniewierska-Baran A, Maj M, et al. Hematopoietic stem/progenitor cells express several functional sex hormone receptors-novel evidence for a potential developmental link between hematopoiesis and primordial germ cells. *Stem Cells Dev* (2015) 24:927–37. doi:10.1089/scd.2014.0546
35. Sinha-Hikim I, Taylor WE, Gonzalez-Cadavid NF, Zheng W, Bhasin S. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. *J Clin Endocrinol Metab* (2004) 89:5245–55. doi:10.1210/jc.2004-0084
36. Wunderlich F, Benten WP, Lieberherr M, Guo Z, Stamm O, Wrehlke C, et al. Testosterone signaling in T cells and macrophages. *Steroids* (2002) 67:535–8. doi:10.1016/S0039-128X(01)00175-1
37. Mantalaris A, Panoskaltsis N, Sakai Y, Bourne P, Chang C, Messing EM, et al. Localization of androgen receptor expression in human bone marrow. *J Pathol* (2001) 193:361–6. doi:10.1002/1096-9896(0000)9999:9999<::AID-PATH803>3.0.CO;2-W
38. Khetawat G, Faraday N, Nealen ML, Vijayan KV, Bolton E, Noga SJ, et al. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood* (2000) 95:2289–96.
39. Sader MA, McGrath KC, Hill MD, Bradstock KF, Jimenez M, Handelsman DJ, et al. Androgen receptor gene expression in leucocytes is hormonally regulated: implications for gender differences in disease pathogenesis. *Clin Endocrinol (Oxf)* (2005) 62:56–63. doi:10.1111/j.1365-2265.2004.02173.x
40. Paharkova-Vatchkova V, Maldonado R, Kovats S. Estrogen preferentially promotes the differentiation of CD11c+ CD11b(intermediate) dendritic cells from bone marrow precursors. *J Immunol* (2004) 172:1426–36. doi:10.4049/jimmunol.172.3.1426
41. Chen W, Beck I, Schober W, Brockow K, Effner R, Buters JT, et al. Human mast cells express androgen receptors but treatment with testosterone exerts no influence on IgE-independent mast cell degranulation elicited by neuromuscular blocking agents. *Exp Dermatol* (2010) 19:302–4. doi:10.1111/j.1600-0625.2009.00969.x
42. Kovacs WJ, Olsen NJ. Androgen receptors in human thymocytes. *J Immunol* (1987) 139:490–3.
43. Viselli SM, Reese KR, Fan J, Kovacs WJ, Olsen NJ. Androgens alter B cell development in normal male mice. *Cell Immunol* (1997) 182:99–104. doi:10.1006/cimm.1997.1227
44. Lai KP, Lai JJ, Chang P, Altuwaijri S, Hsu JW, Chuang KH, et al. Targeting thymic epithelia AR enhances T-cell reconstitution and bone marrow transplant grafting efficacy. *Mol Endocrinol* (2013) 27:25–37. doi:10.1210/me.012-1244
45. Chuang KH, Altuwaijri S, Li G, Lai JJ, Chu CY, Lai KP, et al. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *J Exp Med* (2009) 206:1181–99. doi:10.1084/jem.20082521
46. McDonnell ND, Livingston RB. Severe reversible neutropenia following treatment of prostate cancer with flutamide. *J Urol* (1994) 151:1353–4. doi:10.1016/S0022-5347(17)35251-5
47. Ibanez L, Jaramillo AM, Ferrer A, de ZF. High neutrophil count in girls and women with hyperinsulinaemic hyperandrogenism: normalization with metformin and flutamide overcomes the aggravation by oral contraception. *Hum Reprod* (2005) 20:2457–62. doi:10.1093/humrep/dei072
48. Trigunaite A, Khan A, Der E, Song A, Varikuti S, Jorgensen TN. Gr1 CD11b cells suppress B cell differentiation and lupus-like disease in lupus-prone male mice. *Arthritis Rheum* (2013) 65:2392–402. doi:10.1002/art.38048
49. Bird AK, Chang M, Barnard J, Goldman BI, Meednu N, Rangel-Moreno J, et al. Neutrophils slow disease progression in murine lupus via modulation of autoreactive germinal centers. *J Immunol* (2017) 199:458–66. doi:10.4049/jimmunol.1700354
50. Inamdar Doddamani LS, Jayamma Y. Acceleration of neutrophil precursors' maturation and immunostimulation of CD3+, CD4+ lymphocytes by stanozolol in mice. *J Steroid Biochem Mol Biol* (2012) 129:172–8. doi:10.1016/j.jsbmb.2011.11.008
51. Marin DP, Bolin AP, dos Santos RC, Curi R, Otton R. Testosterone suppresses oxidative stress in human neutrophils. *Cell Biochem Funct* (2010) 28:394–402. doi:10.1002/cbf.1669
52. Malkin CJ, Pugh PJ, Jones RD, Kapoor D, Channer KS, Jones TH. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid

profiles in hypogonadal men. *J Clin Endocrinol Metab* (2004) 89:3313–8. doi:10.1210/jc.2003-031069
53. Hughes TK, Fulep E, Juelich T, Smith EM, Stanton GJ. Modulation of immune responses by anabolic androgenic steroids. *Int J Immunopharmacol* (1995) 17:857–63. doi:10.1016/0192-0561(95)00078-X
54. Arazi H, Mohammadjafari H, Asadi A. Use of anabolic androgenic steroids produces greater oxidative stress responses to resistance exercise in strength-trained men. *Toxicol Rep* (2017) 4:282–6. doi:10.1016/j.toxrep.2017.05.005
55. Corrales JJ, Almeida M, Miralles JM, Orfao A. Persistence of androgenic effects on the production of proinflammatory cytokines by circulating antigen-presenting cells after withdrawal of testosterone treatment in aging type 2 diabetic men with partial androgen deficiency. *Fertil Steril* (2009) 92:311–9. doi:10.1016/j.fertnstert.2008.05.040
56. Ashcroft GS, Mills SJ. Androgen receptor-mediated inhibition of cutaneous wound healing. *J Clin Invest* (2002) 110:615–24. doi:10.1172/JCI15704
57. Goncalves RV, Novaes RD, Sarandy MM, Damasceno EM, da Matta SL, de Gouveia NM, et al. 5alpha-dihydrotestosterone enhances wound healing in diabetic rats. *Life Sci* (2016) 152:67–75. doi:10.1016/j.lfs.2016.03.019
58. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod* (2008) 78:432–7. doi:10.1095/biolreprod.107.063545
59. Angele MK, Schwacha MG, Ayala A, Chaudry IH. Effect of gender and sex hormones on immune responses following shock. *Shock* (2000) 14:81–90. doi:10.1097/00024382-200014020-00001
60. Newsome CT, Flores E, Ayala A, Gregory S, Reichner JS. Improved antimicrobial host defense in mice following poly-(1,6)-beta-D-glucopyranosyl-(1,3)-beta-D-glucopyranose glucan treatment by a gender-dependent immune mechanism. *Clin Vaccine Immunol* (2011) 18:2043–9. doi:10.1128/CVI.05202-11
61. Lima AP, Lunardi LO, Rosa E Silva AA. Effects of castration and testosterone replacement on peritoneal histamine concentration and lung histamine concentration in pubertal male rats. *J Endocrinol* (2000) 167:71–5. doi:10.1677/joe.0.1670071
62. Russi AE, Ebel ME, Yang Y, Brown MA. Male-specific IL-33 expression regulates sex-dimorphic EAE susceptibility. *Proc Natl Acad Sci U S A* (2018) 115:E1520–9. doi:10.1073/pnas.1710401115
63. Kornizky Y, Topilsky M, Fireman E, Kivity S, Kivity S. Specific IgE antibodies to aeroallergens and food among Israelis. *Ann Allergy Asthma Immunol* (1999) 83:149–52. doi:10.1016/S1081-1206(10)62627-0
64. Paula Couto TA, Falsarella N, Mattos CC, Mattos LC. Total IgE plasma levels vary according to gender and age in Brazilian patients with allergic rhinitis. *Clinics (Sao Paulo)* (2014) 69:740–4. doi:10.6061/clinics/2014(11)06
65. Nakanishi H, Horii Y, Fujita K. Effect of testosterone on the eosinophil response of C57BL/6 mice to infection with *Brugia pahangi*. *Immunopharmacology* (1992) 23:75–9. doi:10.1016/0162-3109(92)90030-G
66. Yamatomo T, Okano M, Ono T, Nakayama E, Yoshino T, Satoskar AR, et al. Sex-related differences in the initiation of allergic rhinitis in mice. *Allergy* (2001) 56:525–31. doi:10.1034/j.1398-9995.2001.056006525.x
67. Hamano N, Terada N, Maesako K, Numata T, Konno A. Effect of sex hormones on eosinophilic inflammation in nasal mucosa. *Allergy Asthma Proc* (1998) 19:263–9. doi:10.2500/108854198778557773
68. Liou CJ, Huang WC. Dehydroepiandrosterone suppresses eosinophil infiltration and airway hyperresponsiveness via modulation of chemokines and Th2 cytokines in ovalbumin-sensitized mice. *J Clin Immunol* (2011) 31:656–65. doi:10.1007/s10875-011-9529-3
69. Kamis AB, Ibrahim JB. Effects of testosterone on blood leukocytes in plasmodium berghei-infected mice. *Parasitol Res* (1989) 75:611–3. doi:10.1007/BF00930957
70. Lin AA, Wojciechowski SE, Hildeman DA. Androgens suppress antigen-specific T cell responses and IFN-gamma production during intracranial LCMV infection. *J Neuroimmunol* (2010) 226:8–19. doi:10.1016/j.jneuroim.2010.05.026
71. Koh YT, Gray A, Higgins SA, Hubby B, Kast WM. Androgen ablation augments prostate cancer vaccine immunogenicity only when applied after immunization. *Prostate* (2009) 69:571–84. doi:10.1002/pros.20906
72. Corrales JJ, Almeida M, Cordero M, Martin-Martin L, Mendez C, Miralles JM, et al. Enhanced immunological response by dendritic cells in male hypogonadism. *Eur J Clin Invest* (2012) 42:1205–12. doi:10.1111/j.1365-2362.2012.02712.x
73. Hepworth MR, Hardman MJ, Grencis RK. The role of sex hormones in the development of Th2 immunity in a gender-biased model of *Trichuris muris* infection. *Eur J Immunol* (2010) 40:406–16. doi:10.1002/eji.200939589
74. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* (2016) 16:626–38. doi:10.1038/nri.2016.90
75. Cutolo M, Balleari E, Accardo S, Samanta E, Cimmino MA, Giusti M, et al. Preliminary results of serum androgen level testing in men with rheumatoid arthritis. *Arthritis Rheum* (1984) 27:958–9. doi:10.1002/art.1780270821
76. Spector TD, Perry LA, Tubb G, Silman AJ, Huskisson EC. Low free testosterone levels in rheumatoid arthritis. *Ann Rheum Dis* (1988) 47:65–8. doi:10.1136/ard.47.1.65
77. Olsen NJ, Kovacs WJ. Effects of androgens on T and B lymphocyte development. *Immunol Res* (2001) 23:281–8. doi:10.1385/IR:23:2-3:281
78. Olsen NJ, Benko AL, Kovacs WJ. Variation in the androgen receptor gene exon 1 CAG repeat correlates with manifestations of autoimmunity in women with lupus. *Endocr Connect* (2014) 3:99–109. doi:10.1530/EC-14-0039
79. Robeva R, Tanev D, Andonova S, Kirilov G, Savov A, Stoycheva M, et al. Androgen receptor (CAG)n polymorphism and androgen levels in women with systemic lupus erythematosus and healthy controls. *Rheumatol Int* (2013) 33:2031–8. doi:10.1007/s00296-013-2687-2
80. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci U S A* (2014) 111:869–74. doi:10.1073/pnas.1321060111
81. Lundell AC, Nordstrom I, Andersson K, Strombeck A, Ohlsson C, Tivesten A, et al. Dihydrotestosterone levels at birth associate positively with higher proportions of circulating immature/naive CD5(+) B cells in boys. *Sci Rep* (2017) 7:15503. doi:10.1038/s41598-017-15836-1
82. Lundell AC, Hesselmar B, Nordstrom I, Adlerberth I, Wold AE, Rudin A. Higher B-cell activating factor levels at birth are positively associated with maternal dairy farm exposure and negatively related to allergy development. *J Allergy Clin Immunol* (2015) 136:1074–82. doi:10.1016/j.jaci.2015.03.022
83. Smithson G, Beamer WG, Shultz KL, Christianson SW, Shultz LD, Kincade PW. Increased B lymphopoiesis in genetically sex steroid-deficient hypogonadal (hpg) mice. *J Exp Med* (1994) 180:717–20. doi:10.1084/jem.180.2.717
84. Ellis TM, Moser MT, Le PT, Flanigan RC, Kwon ED. Alterations in peripheral B cells and B cell progenitors following androgen ablation in mice. *Int Immunol* (2001) 13:553–8. doi:10.1093/intimm/13.4.553
85. Altuwaijri S, Chuang KH, Lai KP, Lai JJ, Lin HY, Young FM, et al. Susceptibility to autoimmunity and B cell resistance to apoptosis in mice lacking androgen receptor in B cells. *Mol Endocrinol* (2009) 23:444–53. doi:10.1210/me.2008-0106
86. Wilhelmson AS, Stubelius A, Borjesson AE, Wu J, Stern A, Malin S, et al. Androgens regulate bone marrow B lymphopoiesis in male mice by targeting osteoblast-lineage cells. *Endocrinology* (2015) 156:1228–36. doi:10.1210/en.2014-1822
87. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH. TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. *J Immunol* (1997) 159:117–25.
88. Olsen NJ, Viselli SM, Fan J, Kovacs WJ. Androgens accelerate thymocyte apoptosis. *Endocrinology* (1998) 139:748–52. doi:10.1210/endo.139.2.5729
89. Olsen NJ, Kovacs WJ. Increased thymic size and thymocyte interleukin 2 production in androgen-resistant mice. *Scand J Immunol* (1989) 29:733–8. doi:10.1111/j.1365-3083.1989.tb01178.x
90. Olsen NJ, Watson MB, Henderson GS, Kovacs WJ. Androgen deprivation induces phenotypic and functional changes in the thymus of adult male mice. *Endocrinology* (1991) 129:2471–6. doi:10.1210/endo-129-5-2471
91. Greenstein BD, Fitzpatrick FT, Adcock IM, Kendall MD, Wheeler MJ. Reappearance of the thymus in old rats after orchidectomy: inhibition of regeneration by testosterone. *J Endocrinol* (1986) 110:417–22. doi:10.1677/joe.0.1100417
92. Roden AC, Moser MT, Tri SD, Mercader M, Kuntz SM, Dong H, et al. Augmentation of T cell levels and responses induced by androgen deprivation. *J Immunol* (2004) 173:6098–108. doi:10.4049/jimmunol.173.10.6098
93. Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, Berzins SP, Heng TS, et al. Activation of thymic regeneration in mice and humans

following androgen blockade. *J Immunol* (2005) 175:2741–53. doi:10.4049/jimmunol.175.4.2741
94. Song W, Soni V, Soni S, Khera M. Testosterone inhibits the growth of prostate cancer xenografts in nude mice. *BMC Cancer* (2017) 17:635. doi:10.1186/s12885-017-3569-x
95. Olsen NJ, Viselli SM, Shults K, Stelzer G, Kovacs WJ. Induction of immature thymocyte proliferation after castration of normal male mice. *Endocrinology* (1994) 134:107–13. doi:10.1210/endo.134.1.8275924
96. Aboudkhil S, Bureau JP, Garrelly L, Vago P. Effects of castration, depo-testosterone and cyproterone acetate on lymphocyte T subsets in mouse thymus and spleen. *Scand J Immunol* (1991) 34:647–53. doi:10.1111/j.1365-3083.1991.tb01588.x
97. Zhu ML, Bakhru P, Conley B, Nelson JS, Free M, Martin A, et al. Sex bias in CNS autoimmune disease mediated by androgen control of autoimmune regulator. *Nat Commun* (2016) 7:11350. doi:10.1038/ncomms11350
98. Olsen NJ, Zhou P, Ong H, Kovacs WJ. Testosterone induces expression of transforming growth factor-beta 1 in the murine thymus. *J Steroid Biochem Mol Biol* (1993) 45:327–32. doi:10.1016/0960-0760(93)90001-D
99. Viselli SM, Stanziale S, Shults K, Kovacs WJ, Olsen NJ. Castration alters peripheral immune function in normal male mice. *Immunology* (1995) 84: 337–42.
100. Jia T, Anandhan A, Massilamany C, Rajasekaran RA, Franco R, Reddy J. Association of autophagy in the cell death mediated by dihydrotestosterone in autoreactive T cells independent of antigenic stimulation. *J Neuroimmune Pharmacol* (2015) 10:620–34. doi:10.1007/s11481-015-9633-x
101. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *J Immunol* (1984) 132:656–61.
102. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. *Endocr Rev* (1996) 17:369–84. doi:10.1210/edrv-17-4-369
103. Massa MG, David C, Jorg S, Berg J, Gisevius B, Hirschberg S, et al. Testosterone differentially affects T cells and neurons in murine and human models of neuroinflammation and neurodegeneration. *Am J Pathol* (2017) 187:1613–22. doi:10.1016/j.ajpath.2017.03.006
104. Fijak M, Schneider E, Klug J, Bhushan S, Hackstein H, Schuler G, et al. Testosterone replacement effectively inhibits the development of experimental autoimmune orchitis in rats: evidence for a direct role of testosterone on regulatory T cell expansion. *J Immunol* (2011) 186:5162–72. doi:10.4049/jimmunol.1001958
105. Kissick HT, Sanda MG, Dunn LK, Pellegrini KL, On ST, Noel JK, et al. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. *Proc Natl Acad Sci U S A* (2014) 111:9887–92. doi:10.1073/pnas.1402468111
106. Wilcoxen SC, Kirkman E, Dowdell KC, Stohlman SA. Gender-dependent IL-12 secretion by APC is regulated by IL-10. *J Immunol* (2000) 164:6237–43. doi:10.4049/jimmunol.164.12.6237
107. Bao M, Yang Y, Jun HS, Yoon JW. Molecular mechanisms for gender differences in susceptibility to T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol* (2002) 168:5369–75. doi:10.4049/jimmunol.168.10.5369
108. Zhang MA, Ahn JJ, Zhao FL, Selvanantham T, Mallevaey T, Stock N, et al. Antagonizing peroxisome proliferator-activated receptor alpha activity selectively enhances Th1 immunity in male mice. *J Immunol* (2015) 195: 5189–202. doi:10.4049/jimmunol.1500449
109. Dunn SE, Ousman SS, Sobel RA, Zuniga L, Baranzini SE, Youssef S, et al. Peroxisome proliferator-activated receptor (PPAR)alpha expression in T cells mediates gender differences in development of T cell-mediated autoimmunity. *J Exp Med* (2007) 204:321–30. doi:10.1084/jem.20061839
110. Rutkowski K, Sowa P, Rutkowska-Talipska J, Kuryliszyn-Moskal A, Rutkowski R. Dehydroepiandrosterone (DHEA): hypes and hopes. *Drugs* (2014) 74:1195–207. doi:10.1007/s40265-014-0259-8
111. Walecki M, Eisel F, Klug J, Baal N, Paradowska-Dogan A, Wahle E, et al. Androgen receptor modulates Foxp3 expression in CD4+CD25+Foxp3+ regulatory T-cells. *Mol Biol Cell* (2015) 26:2845–57. doi:10.1091/mbc.E14-08-1323
112. Lieberman SM, Kreiger PA, Koretzky GA. Reversible lacrimal gland-protective regulatory T-cell dysfunction underlies male-specific autoimmune dacryoadenitis in the non-obese diabetic mouse model of Sjogren syndrome. *Immunology* (2015) 145:232–41. doi:10.1111/imm.12439
113. Whitacre CC. Sex differences in autoimmune disease. *Nat Immunol* (2001) 2:777–80. doi:10.1038/ni0901-777
114. Seminog OO, Seminog AB, Yeates D, Goldacre MJ. Associations between Klinefelter's syndrome and autoimmune diseases: English national record linkage studies. *Autoimmunity* (2015) 48:125–8. doi:10.3109/08916934.2014.968918
115. Foster SC, Daniels C, Bourdette DN, Bebo BF Jr. Dysregulation of the hypothalamic-pituitary-gonadal axis in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Neuroimmunol* (2003) 140:78–87. doi:10.1016/S0165-5728(03)00177-2
116. Baillargeon J, Al SS, Raji MA, Urban RJ, Sharma G, Sheffield-Moore M, et al. Hypogonadism and the risk of rheumatic autoimmune disease. *Clin Rheumatol* (2016) 35:2983–7. doi:10.1007/s10067-016-3330-x
117. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med* (2011) 365:2205–19. doi:10.1056/NEJMra1004965
118. Kuiper S, van Gestel AM, Swinkels HL, de Boo TM, Da Silva JA, van Riel PL. Influence of sex, age, and menopausal state on the course of early rheumatoid arthritis. *J Rheumatol* (2001) 28:1809–16.
119. Tengstrand B, Ahlmen M, Hafstrom I. The influence of sex on rheumatoid arthritis: a prospective study of onset and outcome after 2 years. *J Rheumatol* (2004) 31:214–22.
120. Forslind K, Hafstrom I, Ahlmen M, Svensson B. Sex: a major predictor of remission in early rheumatoid arthritis? *Ann Rheum Dis* (2007) 66:46–52. doi:10.1136/ard.2006.056937
121. Iikuni N, Sato E, Hoshi M, Inoue E, Taniguchi A, Hara M, et al. The influence of sex on patients with rheumatoid arthritis in a large observational cohort. *J Rheumatol* (2009) 36:508–11. doi:10.3899/jrheum.080724
122. Weyand CM, Schmidt D, Wagner U, Goronzy JJ. The influence of sex on the phenotype of rheumatoid arthritis. *Arthritis Rheum* (1998) 41:817–22. doi:10.1002/1529-0131(199805)41:5<817:AID-ART7>3.0.CO;2-S
123. Islander U, Jochems C, Lagerquist MK, Forsblad-d'Elia H, Carlsten H. Estrogens in rheumatoid arthritis; the immune system and bone. *Mol Cell Endocrinol* (2011) 335:14–29. doi:10.1016/j.mce.2010.05.018
124. Davidson JM, Chen JJ, Crapo L, Gray GD, Greenleaf WJ, Catania JA. Hormonal changes and sexual function in aging men. *J Clin Endocrinol Metab* (1983) 57:71–7. doi:10.1210/jcem-57-1-71
125. Gordon D, Beastall GH, Thomson JA, Sturrock RD. Androgenic status and sexual function in males with rheumatoid arthritis and ankylosing spondylitis. *Q J Med* (1986) 60:671–9.
126. Cutolo M, Balleari E, Giusti M, Monachesi M, Accardo S. Sex hormone status of male patients with rheumatoid arthritis: evidence of low serum concentrations of testosterone at baseline and after human chorionic gonadotropin stimulation. *Arthritis Rheum* (1988) 31:1314–7. doi:10.1002/art.1780311015
127. Heikkila R, Aho K, Heliovaara M, Knekt P, Reunanen A, Aromaa A, et al. Serum androgen-anabolic hormones and the risk of rheumatoid arthritis. *Ann Rheum Dis* (1998) 57:281–5. doi:10.1136/ard.57.5.281
128. Khalkhali-Ellis Z, Moore TL, Hendrix MJ. Reduced levels of testosterone and dehydroepiandrosterone sulphate in the serum and synovial fluid of juvenile rheumatoid arthritis patients correlates with disease severity. *Clin Exp Rheumatol* (1998) 16:753–6.
129. Kanik KS, Chrousos GP, Schumacher HR, Crane ML, Yarboro CH, Wilder RL. Adrenocorticotropin, glucocorticoid, and androgen secretion in patients with new onset synovitis/rheumatoid arthritis: relations with indices of inflammation. *J Clin Endocrinol Metab* (2000) 85:1461–6. doi:10.1210/jcem.85.4.6534
130. Tengstrand B, Carlstrom K, Hafstrom I. Gonadal hormones in men with rheumatoid arthritis – from onset through 2 years. *J Rheumatol* (2009) 36:887–92. doi:10.3899/jrheum.080558
131. Kawasaki T, Ushiyama T, Ueyama H, Inoue K, Mori K, Ohkubo I, et al. Polymorphic CAG repeats of the androgen receptor gene and rheumatoid arthritis. *Ann Rheum Dis* (1999) 58:500–2. doi:10.1136/ard.58.8.500
132. Lo SF, Huang CM, Tsai CH, Chen WC, Lai CC, Tsai Y, et al. Androgen receptor gene polymorphism and rheumatoid arthritis in Taiwan. *Clin Exp Rheumatol* (2006) 24:209–10.
133. Karlson EW, Chibnik LB, McGrath M, Chang SC, Keenan BT, Costenbader KH, et al. A prospective study of androgen levels, hormone-related genes and risk of rheumatoid arthritis. *Arthritis Res Ther* (2009) 11:R97. doi:10.1186/ar2742

134. Pope JE, Joneja M, Hong P. Anti-androgen treatment of prostatic carcinoma may be a risk factor for development of rheumatoid arthritis. *J Rheumatol* (2002) 29:2459–62.
135. Pikwer M, Giwercman A, Bergstrom U, Nilsson JA, Jacobsson LT, Turesson C. Association between testosterone levels and risk of future rheumatoid arthritis in men: a population-based case-control study. *Ann Rheum Dis* (2014) 73:573–9. doi:10.1136/annrheumdis-2012-202781
136. Imrich R, Rovensky J, Malis F, Zlnay M, Killinger Z, Kvetnansky R, et al. Low levels of dehydroepiandrosterone sulphate in plasma, and reduced sympathoadrenal response to hypoglycaemia in premenopausal women with rheumatoid arthritis. *Ann Rheum Dis* (2005) 64:202–6. doi:10.1136/ard.2003.019844
137. Gordon D, Beastall GH, Thomson JA, Sturrock RD. Prolonged hypogonadism in male patients with rheumatoid arthritis during flares in disease activity. *Br J Rheumatol* (1988) 27:440–4. doi:10.1093/rheumatology/27.6.440
138. Masi AT, Chatterton RT, Aldag JC. Perturbations of hypothalamic-pituitary-gonadal axis and adrenal androgen functions in rheumatoid arthritis: an odyssey of hormonal relationships to the disease. *Ann N Y Acad Sci* (1999) 876:53–62. doi:10.1111/j.1749-6632.1999.tb07622.x
139. Cutolo M, Foppiani L, Prete C, Ballarino P, Sulli A, Villaggio B, et al. Hypothalamic-pituitary-adrenocortical axis function in premenopausal women with rheumatoid arthritis not treated with glucocorticoids. *J Rheumatol* (1999) 26:282–8.
140. Deighton CM, Watson MJ, Walker DJ. Sex hormones in postmenopausal HLA-identical rheumatoid arthritis discordant sibling pairs. *J Rheumatol* (1992) 19:1663–7.
141. Stark K, Straub RH, Rovensky J, Blazickova S, Eiselt G, Schmidt M. CYB5A polymorphism increases androgens and reduces risk of rheumatoid arthritis in women. *Arthritis Res Ther* (2015) 17:56. doi:10.1186/s13075-015-0574-9
142. Yu SF, Cheng TT, Hsu YH, Lai HM, Chen YC, Chiu CK, et al. Association of tri-nucleotide (CAG and GGC) repeat polymorphism of androgen receptor gene in Taiwanese women with refractory or remission rheumatoid arthritis. *Clin Rheumatol* (2007) 26:2051. doi:10.1007/s10067-007-0616-z
143. Cutolo M, Balleari E, Giusti M, Monachesi M, Accardo S. Sex hormone status in women suffering from rheumatoid arthritis. *J Rheumatol* (1986) 13:1019–23.
144. Dziedziejko V, Kurzawski M, Safranow K, Ossowski A, Piatek J, Parafiniuk M, et al. CAG repeat polymorphism in the androgen receptor gene in women with rheumatoid arthritis. *J Rheumatol* (2012) 39:10–7. doi:10.3899/jrheum.110894
145. Margolis HM, Caplan PS. The effect of some steroids (testosterone propionate, desoxycorticosterone acetate and ascorbic acid, and 21-acetoxy delta-5-pregnenolone, artisone acetate, Wyeth) in rheumatoid arthritis. *Ann Intern Med* (1951) 34:61–71. doi:10.7326/0003-4819-34-1-61
146. Cutolo M, Balleari E, Giusti M, Intra E, Accardo S. Androgen replacement therapy in male patients with rheumatoid arthritis. *Arthritis Rheum* (1991) 34:1–5. doi:10.1002/art.1780340102
147. Booji A, Biewenga-Booji CM, Huber-Bruning O, Cornelis C, Jacobs JW, Bijlsma JW. Androgens as adjuvant treatment in postmenopausal female patients with rheumatoid arthritis. *Ann Rheum Dis* (1996) 55:811–5. doi:10.1136/ard.55.11.811
148. Hall GM, Larbre JP, Spector TD, Perry LA, Da Silva JA. A randomized trial of testosterone therapy in males with rheumatoid arthritis. *Br J Rheumatol* (1996) 35:568–73. doi:10.1093/rheumatology/35.6.568
149. Ganesan K, Balachandran C, Manohar BM, Puvanakrishnan R. Comparative studies on the interplay of testosterone, estrogen and progesterone in collagen induced arthritis in rats. *Bone* (2008) 43:758–65. doi:10.1016/j.bone.2008.05.025
150. Homo-Delarche F, Fitzpatrick F, Christeff N, Nunez EA, Bach JF, Dardenne M. Sex steroids, glucocorticoids, stress and autoimmunity. *J Steroid Biochem Mol Biol* (1991) 40:619–37. doi:10.1016/0960-0760(91)90285-D
151. Da Silva JA, Larbre JP, Seed MP, Cutolo M, Villaggio B, Scott DL, et al. Sex differences in inflammation induced cartilage damage in rodents. The influence of sex steroids. *J Rheumatol* (1994) 21:330–7.
152. Keith RC, Sokolove J, Edelman BL, Lahey L, Redente EF, Holers VM, et al. Testosterone is protective in the sexually dimorphic development of arthritis and lung disease in SKG mice. *Arthritis Rheum* (2013) 65:1487–93. doi:10.1002/art.37943
153. Bruot BC, Clemens JW. Effect of adjuvant-induced arthritis on serum luteinizing hormone and testosterone concentrations in the male rat. *Life Sci* (1987) 41:1559–65. doi:10.1016/0024-3205(87)90722-3
154. Castagnetta LA, Carruba G, Granata OM, Stefano R, Miele M, Schmidt M, et al. Increased estrogen formation and estrogen to androgen ratio in the synovial fluid of patients with rheumatoid arthritis. *J Rheumatol* (2003) 30:2597–605.
155. Cutolo M, Accardo S, Villaggio B, Barone A, Sulli A, Balleari E, et al. Androgen metabolism and inhibition of interleukin-1 synthesis in primary cultured human synovial macrophages. *Mediators Inflamm* (1995) 4:138–43. doi:10.1155/S096293519500024X
156. Keller ET, Chang C, Ershler WB. Inhibition of NFkappaB activity through maintenance of IkappaBalpha levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. *J Biol Chem* (1996) 271:26267–75. doi:10.1074/jbc.271.42.26267
157. Capellino S, Straub RH, Cutolo M. Aromatase and regulation of the estrogen-to-androgen ratio in synovial tissue inflammation: common pathway in both sexes. *Ann N Y Acad Sci* (2014) 1317:24–31. doi:10.1111/nyas.12398
158. Li ZG, Danis VA, Brooks PM. Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clin Exp Rheumatol* (1993) 11:157–62.
159. Dunn SE, Lee H, Pavri FR, Zhang MA. Sex-based differences in multiple sclerosis (part I): biology of disease incidence. *Curr Top Behav Neurosci* (2015) 26:29–56. doi:10.1007/7854_2015_371
160. Kalincik T, Vivek V, Jokubaitis V, Lechner-Scott J, Trojano M, Izquierdo G, et al. Sex as a determinant of relapse incidence and progressive course of multiple sclerosis. *Brain* (2013) 136:3609–17. doi:10.1093/brain/awt281
161. Tremlett H, Zhao Y, Joseph J, Devonshire V. Relapses in multiple sclerosis are age- and time-dependent. *J Neurol Neurosurg Psychiatry* (2008) 79:1368–74. doi:10.1136/jnnp.2008.145805
162. Held U, Heigenhauser L, Shang C, Kappos L, Polman C. Predictors of relapse rate in MS clinical trials. *Neurology* (2005) 65:1769–73. doi:10.1212/01.wnl.0000187122.71735.1f
163. Weatherby SJ, Mann CL, Davies MB, Fryer AA, Haq N, Strange RC, et al. A pilot study of the relationship between gadolinium-enhancing lesions, gender effect and polymorphisms of antioxidant enzymes in multiple sclerosis. *J Neurol* (2000) 247:467–70. doi:10.1007/s004150070179
164. Pozzilli C, Tomassini V, Marinelli F, Paolillo A, Gasperini C, Bastianello S. 'Gender gap' in multiple sclerosis: magnetic resonance imaging evidence. *Eur J Neurol* (2003) 10:95–7. doi:10.1046/j.1468-1331.2003.00519.x
165. Tomassini V, Onesti E, Mainero C, Giugni E, Paolillo A, Salvetti M, et al. Sex hormones modulate brain damage in multiple sclerosis: MRI evidence. *J Neurol Neurosurg Psychiatry* (2005) 76:272–5. doi:10.1136/jnnp.2003.033324
166. Foroughipour A, Norbakhsh V, Najafabadi SH, Meamar R. Evaluating sex hormone levels in reproductive age women with multiple sclerosis and their relationship with disease severity. *J Res Med Sci* (2012) 17:882–5.
167. Bove R, Musallam A, Healy BC, Raghavan K, Glanz BI, Bakshi R, et al. Low testosterone is associated with disability in men with multiple sclerosis. *Mult Scler* (2014) 20:1584–92. doi:10.1177/1352458514527864
168. Tellez N, Comabella M, Julia E, Rio J, Tintore M, Brieva L, et al. Fatigue in progressive multiple sclerosis is associated with low levels of dehydroepiandrosterone. *Mult Scler* (2006) 12:487–94. doi:10.1191/135248505ms1322oa
169. Sicotte NL, Giesser BS, Tandon V, Klutch R, Steiner B, Drain AE, et al. Testosterone treatment in multiple sclerosis: a pilot study. *Arch Neurol* (2007) 64:683–8. doi:10.1001/archneur.64.5.683
170. Kurth F, Luders E, Sicotte NL, Gaser C, Giesser BS, Swerdloff RS, et al. Neuroprotective effects of testosterone treatment in men with multiple sclerosis. *Neuroimage Clin* (2014) 4:454–60. doi:10.1016/j.nicl.2014.03.001
171. Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* (1995) 37:424–35. doi:10.1002/ana.410370404
172. Balashov KE, Rottman JB, Weiner HL, Hancock WW. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci U S A* (1999) 96:6873–8. doi:10.1073/pnas.96.12.6873

173. Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, et al. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol* (2009) 66:390–402. doi:10.1002/ana.21748
174. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* (2008) 172:146–55. doi:10.2353/ajpath.2008.070690
175. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* (2002) 8:500–8. doi:10.1038/nm0502-500
176. Krumbholz M, Derfuss T, Hohlfeld R, Meinl E. B cells and antibodies in multiple sclerosis pathogenesis and therapy. *Nat Rev Neurol* (2012) 8:613–23. doi:10.1038/nrneurol.2012.203
177. Havrdova E, Belova A, Goloborodko A, Tisserant A, Wright A, Wallstroem E, et al. Activity of secukinumab, an anti-IL-17A antibody, on brain lesions in RRMS: results from a randomized, proof-of-concept study. *J Neurol* (2016) 263:1287–95. doi:10.1007/s00415-016-8128-x
178. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* (2008) 358:676–88. doi:10.1056/NEJMoa0706383
179. Segal BM, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol* (2008) 7:796–804. doi:10.1016/S1474-4422(08)70173-X
180. Dalal M, Kim S, Voskuhl RR. Testosterone therapy ameliorates experimental autoimmune encephalomyelitis and induces a T helper 2 bias in the autoantigen-specific T lymphocyte response. *J Immunol* (1997) 159:3–6.
181. Palaszynski KM, Loo KK, Ashouri JF, Liu HB, Voskuhl RR. Androgens are protective in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *J Neuroimmunol* (2004) 146:144–52. doi:10.1016/j.jneuroim.2003.11.004
182. Ziehn MO, Avedisian AA, Dervin SM, Umeda EA, O'Dell TJ, Voskuhl RR. Therapeutic testosterone administration preserves excitatory synaptic transmission in the hippocampus during autoimmune demyelinating disease. *J Neurosci* (2012) 32:12312–24. doi:10.1523/JNEUROSCI.2796-12.2012
183. Giatti S, Rigolio R, Romano S, Mitro N, Viviani B, Cavaletti G, et al. Dihydrotestosterone as a protective agent in chronic experimental autoimmune encephalomyelitis. *Neuroendocrinology* (2015) 101:296–308. doi:10.1159/000381064
184. Cua DJ, Hinton DR, Stohlman SA. Self-antigen-induced Th2 responses in experimental allergic encephalomyelitis (EAE)-resistant mice. Th2-mediated suppression of autoimmune disease. *J Immunol* (1995) 155:4052–9.
185. Bebo BF Jr, Zelinka-Vincent E, Adamus G, Amundson D, Vandenbark AA, Offner H. Gonadal hormones influence the immune response to PLP 139-151 and the clinical course of relapsing experimental autoimmune encephalomyelitis. *J Neuroimmunol* (1998) 84:122–30. doi:10.1016/S0165-5728(97)00214-2
186. Pelfrey CM, Cotleur AC, Lee JC, Rudick RA. Sex differences in cytokine responses to myelin peptides in multiple sclerosis. *J Neuroimmunol* (2002) 130:211–23. doi:10.1016/S0165-5728(02)00224-2
187. Moldovan IR, Cotleur AC, Zamor N, Butler RS, Pelfrey CM. Multiple sclerosis patients show sexual dimorphism in cytokine responses to myelin antigens. *J Neuroimmunol* (2008) 193:161–9. doi:10.1016/j.jneuroim.2007.10.010
188. Bebo BF Jr, Schuster JC, Vandenbark AA, Offner H. Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. *J Immunol* (1999) 162:35–40.
189. Brahmachari S, Pahan K. Gender-specific expression of beta1 integrin of VLA-4 in myelin basic protein-primed T cells: implications for gender bias in multiple sclerosis. *J Immunol* (2010) 184:6103–13. doi:10.4049/jimmunol.0804356
190. Kotzin BL. Systemic lupus erythematosus. *Cell* (1996) 85:303–6. doi:10.1016/S0092-8674(00)81108-3
191. Mills JA. Systemic lupus erythematosus. *N Engl J Med* (1994) 330:1871–9. doi:10.1056/NEJM199406303302608
192. Zeller CB, Appenzeller S. Cardiovascular disease in systemic lupus erythematosus: the role of traditional and lupus related risk factors. *Curr Cardiol Rev* (2008) 4:116–22. doi:10.2174/157340308784245775
193. Manzi S, Meilahn EN, Rairie JE, Conte CG, Medsger TA Jr, Jansen-McWilliams L, et al. Age-specific incidence rates of myocardial infarction and angina in women with systemic lupus erythematosus: comparison with the Framingham Study. *Am J Epidemiol* (1997) 145:408–15. doi:10.1093/oxfordjournals.aje.a009122
194. Stahl NI, Decker JL. Androgenic status of males with systemic lupus erythematosus. *Arthritis Rheum* (1978) 21:665–8. doi:10.1002/art.1780210609
195. Jimenez-Balderas FJ, Tapia-Serrano R, Fonseca ME, Arellano J, Beltran A, Yanez P, et al. High frequency of association of rheumatic/autoimmune diseases and untreated male hypogonadism with severe testicular dysfunction. *Arthritis Res* (2001) 3:362–7. doi:10.1186/ar328
196. Olsen NJ, Kovacs WJ. Case report: testosterone treatment of systemic lupus erythematosus in a patient with Klinefelter's syndrome. *Am J Med Sci* (1995) 310:158–60. doi:10.1097/00000441-199510000-00006
197. Sasaki N, Yamauchi K, Sato R, Masuda T, Sawai T, Inoue H. Klinefelter's syndrome associated with systemic lupus erythematosus and autoimmune hepatitis. *Mod Rheumatol* (2006) 16:305–8. doi:10.1007/s10165-006-0511-5
198. Bove R. Autoimmune diseases and reproductive aging. *Clin Immunol* (2013) 149:251–64. doi:10.1016/j.clim.2013.02.010
199. Jungers P, Nahoul K, Pelissier C, Dougados M, Tron F, Bach JF. Low plasma androgens in women with active or quiescent systemic lupus erythematosus. *Arthritis Rheum* (1982) 25(4):454–7. doi:10.1002/art.1780250415
200. Lahita RG, Bradlow HL, Ginzler E, Pang S, New M. Low plasma androgens in women with systemic lupus erythematosus. *Arthritis Rheum* (1987) 30:241–8. doi:10.1002/art.1780300301
201. Treadwell EL, Wiley K, Word B, Melchior W, Tolleson WH, Gopee N, et al. Prolactin and dehydroepiandrosterone levels in women with systemic lupus erythematosus: the role of the extrapituitary prolactin promoter polymorphism at -1149G/T. *J Immunol Res* (2015) 2015:435658. doi:10.1155/2015/435658
202. Lahita RG. Sex hormones as immunomodulators of disease. *Ann N Y Acad Sci* (1993) 685:278–87. doi:10.1111/j.1749-6632.1993.tb35876.x
203. Cutolo M, Sulli A, Capellino S, Villaggio B, Montagna P, Seriolo B, et al. Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. *Lupus* (2004) 13:635–8. doi:10.1191/0961203304lu1094oa
204. Tessnow AH, Olsen NJ, Kovacs WJ. Expression of humoral autoimmunity is related to androgen receptor CAG repeat length in men with systemic lupus erythematosus. *J Clin Immunol* (2011) 31:567–73. doi:10.1007/s10875-011-9519-5
205. Estes D, Christian CL. The natural history of systemic lupus erythematosus by prospective analysis. *Medicine (Baltimore)* (1971) 50:85–95. doi:10.1097/00005792-197103000-00001
206. Studenski S, Allen NB, Caldwell DS, Rice JR, Polisson RP. Survival in systemic lupus erythematosus. A multivariate analysis of demographic factors. *Arthritis Rheum* (1987) 30:1326–32. doi:10.1002/art.1780301202
207. Sthoeger ZM, Geltner D, Rider A, Bentwich Z. Systemic lupus erythematosus in 49 Israeli males: a retrospective study. *Clin Exp Rheumatol* (1987) 5:233–40.
208. Jonsson H, Nived O, Sturfelt G. Outcome in systemic lupus erythematosus: a prospective study of patients from a defined population. *Medicine (Baltimore)* (1989) 68:141–50. doi:10.1097/00005792-198905000-00002
209. Reveille JD, Bartolucci A, Alarcon GS. Prognosis in systemic lupus erythematosus. Negative impact of increasing age at onset, black race, and thrombocytopenia, as well as causes of death. *Arthritis Rheum* (1990) 33:37–48. doi:10.1002/art.1780330105
210. Pistiner M, Wallace DJ, Nessim S, Metzger AL, Klinenberg JR. Lupus erythematosus in the 1980s: a survey of 570 patients. *Semin Arthritis Rheum* (1991) 21:55–64. doi:10.1016/0049-0172(91)90057-7
211. Wallace DJ, Podell T, Weiner J, Klinenberg JR, Forouzesh S, Dubois EL. Systemic lupus erythematosus – survival patterns. Experience with 609 patients. *JAMA* (1981) 245:934–8. doi:10.1001/jama.245.9.934
212. Folomeev M, Alekberova Z. Survival pattern of 120 males with systemic lupus erythematosus. *J Rheumatol* (1990) 17:856–9.
213. Ward MM, Pyun E, Studenski S. Long-term survival in systemic lupus erythematosus. Patient characteristics associated with poorer outcomes. *Arthritis Rheum* (1995) 38:274–83. doi:10.1002/art.1780380218

214. Blanco FJ, Gomez-Reino JJ, de la Mata J, Corrales A, Rodriguez-Valverde V, Rosas JC, et al. Survival analysis of 306 European Spanish patients with systemic lupus erythematosus. *Lupus* (1998) 7:159–63. doi:10.1191/096120398678919930
215. Kiss E, Regeczy N, Szegedi G. Systemic lupus erythematosus survival in Hungary. Results from a single centre. *Clin Exp Rheumatol* (1999) 17:171–7.
216. Tareyeva IE, Janushkevitch TN, Tuganbekova SK. Lupus nephritis in males and females. *Proc Eur Dial Transplant Assoc Eur Ren Assoc* (1985) 21:712–6.
217. Blum A, Rubinow A, Galun E. Predominance of renal involvement in male patients with systemic lupus erythematosus. *Clin Exp Rheumatol* (1991) 9: 206–7.
218. Mok CC, Lau CS, Chan TM, Wong RW. Clinical characteristics and outcome of southern Chinese males with systemic lupus erythematosus. *Lupus* (1999) 8:188–96. doi:10.1191/096120399678847605
219. Stein CM, Olson JM, Gray-McGuire C, Bruner GR, Harley JB, Moser KL. Increased prevalence of renal disease in systemic lupus erythematosus families with affected male relatives. *Arthritis Rheum* (2002) 46:428–35. doi:10.1002/art.10105
220. Letchumanan P, Thumboo J. Danazol in the treatment of systemic lupus erythematosus: a qualitative systematic review. *Semin Arthritis Rheum* (2011) 40:298–306. doi:10.1016/j.semarthrit.2010.03.005
221. Agnello V, Pariser K, Gell J, Gelfand J, Turksoy RN. Preliminary observations on danazol therapy of systemic lupus erythematosus: effects on DNA antibodies, thrombocytopenia and complement. *J Rheumatol* (1983) 10:682–7.
222. Hazelton RA, McCruden AB, Sturrock RD, Stimson WH. Hormonal manipulation of the immune response in systemic lupus erythematosus: a drug trial of an anabolic steroid, 19-nortestosterone. *Ann Rheum Dis* (1983) 42: 155–7. doi:10.1136/ard.42.2.155
223. Lahita RG, Cheng CY, Monder C, Bardin CW. Experience with 19-nortestosterone in the therapy of systemic lupus erythematosus: worsened disease after treatment with 19-nortestosterone in men and lack of improvement in women. *J Rheumatol* (1992) 19:547–55.
224. Chang DM, Lan JL, Lin HY, Luo SF. Dehydroepiandrosterone treatment of women with mild-to-moderate systemic lupus erythematosus: a multicenter randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* (2002) 46:2924–7. doi:10.1002/art.10615
225. van Vollenhoven RF. Dehydroepiandrosterone for the treatment of systemic lupus erythematosus. *Expert Opin Pharmacother* (2002) 3:23–31. doi:10.1517/14656566.3.1.23
226. Petri MA, Mease PJ, Merrill JT, Lahita RG, Iannini MJ, Yocum DE, et al. Effects of prasterone on disease activity and symptoms in women with active systemic lupus erythematosus. *Arthritis Rheum* (2004) 50:2858–68. doi:10.1002/art.20427
227. Nordmark G, Bengtsson C, Larsson A, Karlsson FA, Sturfelt G, Ronnblom L. Effects of dehydroepiandrosterone supplement on health-related quality of life in glucocorticoid treated female patients with systemic lupus erythematosus. *Autoimmunity* (2005) 38:531–40. doi:10.1080/08916930500285550
228. Petri MA, Lahita RG, van Vollenhoven RF, Merrill JT, Schiff M, Ginzler EM, et al. Effects of prasterone on corticosteroid requirements of women with systemic lupus erythematosus: a double-blind, randomized, placebo-controlled trial. *Arthritis Rheum* (2002) 46:1820–9. doi:10.1002/art.10364
229. Marder W, Somers EC, Kaplan MJ, Anderson MR, Lewis EE, McCune WJ. Effects of prasterone (dehydroepiandrosterone) on markers of cardiovascular risk and bone turnover in premenopausal women with systemic lupus erythematosus: a pilot study. *Lupus* (2010) 19:1229–36. doi:10.1177/0961203310371156
230. Hartkamp A, Geenen R, Godaert GL, Bijl M, Bijlsma JW, Derksen RH. Effects of dehydroepiandrosterone on fatigue and well-being in women with quiescent systemic lupus erythematosus: a randomised controlled trial. *Ann Rheum Dis* (2010) 69:1144–7. doi:10.1136/ard.2009.117036
231. Celhar T, Fairhurst AM. Modelling clinical systemic lupus erythematosus: similarities, differences and success stories. *Rheumatology (Oxford)* (2017) 56:i88–99. doi:10.1093/rheumatology/kew400
232. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Delayed androgen treatment prolongs survival in murine lupus. *J Clin Invest* (1979) 63:902–11. doi:10.1172/JCI109390
233. Gubbels Bupp MR, Jorgensen TN, Kotzin BL. Identification of candidate genes that influence sex hormone-dependent disease phenotypes in mouse lupus. *Genes Immun* (2008) 9:47–56. doi:10.1038/sj.gene.6364447
234. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J Exp Med* (1978) 147:1568–83. doi:10.1084/jem.147.6.1568
235. Roubinian JR, Papoian R, Talal N. Androgenic hormones modulate autoantibody responses and improve survival in murine lupus. *J Clin Invest* (1977) 59:1066–70. doi:10.1172/JCI108729
236. Verheul HA, Deckers GH, Schuurs AH. Effects of nandrolone decanoate in NZB/W mice treated concomitantly with maintenance doses of dexamethasone sodium phosphate. *Int J Immunopharmacol* (1985) 7:249–54. doi:10.1016/0192-0561(85)90033-5
237. Verheul HA, Deckers GH, Schuurs AH. Effects of nandrolone decanoate or testosterone decanoate on murine lupus: further evidence for a dissociation of autoimmunosuppressive and endocrine effects. *Immunopharmacology* (1986) 11:93–9. doi:10.1016/0162-3109(86)90029-9
238. Verheul HA, Stimson WH, den Hollander FC, Schuurs AH. The effects of nandrolone, testosterone and their decanoate esters on murine lupus. *Clin Exp Immunol* (1981) 44:11–7.
239. Verheul HA, Schot LP, Deckers GH, Schuurs AH. Effects of tibolone, lynestrenol, ethylestrenol, and desogestrel on autoimmune disorders in NZB/W mice. *Clin Immunol Immunopathol* (1986) 38:198–208. doi:10.1016/0090-1229(86)90138-8
240. Lucas JA, Ahmed SA, Casey ML, MacDonald PC. Prevention of autoantibody formation and prolonged survival in New Zealand black/New Zealand white F1 mice fed dehydroisoandrosterone. *J Clin Invest* (1985) 75:2091–3. doi:10.1172/JCI111929
241. Steinberg AD, Melez KA, Raveche ES, Reeves JP, Boegel WA, Smathers PA, et al. Approach to the study of the role of sex hormones in autoimmunity. *Arthritis Rheum* (1979) 22:1170–6. doi:10.1002/art.1780221103
242. Walker SE, Besch-Williford CL, Keisler DH. Accelerated deaths from systemic lupus erythematosus in NZB x NZW F1 mice treated with the testosterone-blocking drug flutamide. *J Lab Clin Med* (1994) 124:401–7.
243. Jackson SW, Kolhatkar NS, Rawlings DJ. B cells take the front seat: dysregulated B cell signals orchestrate loss of tolerance and autoantibody production. *Curr Opin Immunol* (2015) 33:70–7. doi:10.1016/j.coi.2015.01.018
244. Papoian R, Pillarisetty R, Talal N. Immunological regulation of spontaneous antibodies to DNA and RNA. II. Sequential switch from IgM to IgG in NZB/NZW F1 mice. *Immunology* (1977) 32:75–9.
245. Roubinian J, Talal N, Siiteri PK, Sadakian JA. Sex hormone modulation of autoimmunity in NZB/NZW mice. *Arthritis Rheum* (1979) 22(11):1162–9. doi:10.1002/art.1780221102
246. Shear HL, Roubinian JR, Gil P, Talal N. Clearance of sensitized erythrocytes in NZB/NZW mice. Effects of castration and sex hormone treatment. *Eur J Immunol* (1981) 11:776–80. doi:10.1002/eji.1830111008
247. Churchill WH Jr, Weintraub RM, Borsos T, Rapp HJ. Mouse complement: the effect of sex hormones and castration on two of the late-acting components. *J Exp Med* (1967) 125:657–72. doi:10.1084/jem.125.4.657
248. Shreffler DC. The S region of the mouse major histocompatibility complex (H-2): genetic variation and functional role in complement system. *Transplant Rev* (1976) 32:140–67.
249. Ferreira A, Takahashi M, Nussenzweig V. Purificaiton and characterization of mouse serum protein with specific binding affinity for C4 (Ss protein). *J Exp Med* (1977) 146:1001–8. doi:10.1084/jem.146.4.1001
250. Der E, Dimo J, Trigunaite A, Jones J, Jorgensen TN. Gr1+ cells suppress T-dependent antibody responses in (NZB x NZW)F1 male mice through inhibition of T follicular helper cells and germinal center formation. *J Immunol* (2014) 192:1570–6. doi:10.4049/jimmunol.1302479
251. Touw IP, van de Geijn GJ. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. *Front Biosci* (2007) 12:800–15. doi:10.2741/2103
252. Zavala F, Masson A, Hadaya K, Ezine S, Schneider E, Babin O, et al. Granulocyte-colony stimulating factor treatment of lupus autoimmune disease in MRL-lpr/lpr mice. *J Immunol* (1999) 163:5125–32.
253. Eyles JL, Hickey MJ, Norman MU, Croker BA, Roberts AW, Drake SF, et al. A key role for G-CSF-induced neutrophil production and trafficking during inflammatory arthritis. *Blood* (2008) 112:5193–201. doi:10.1182/blood-2008-02-139535
254. Lantow M, Sivakumar R, Zeumer L, Wasserfall C, Zheng YY, Atkinson MA, et al. The granulocyte colony stimulating factor pathway regulates autoantibody

production in a murine induced model of systemic lupus erythematosus. *Arthritis Res Ther* (2013) 15:R49. doi:10.1186/ar4208
255. Cook MB, McGlynn KA, Devesa SS, Freedman ND, Anderson WF. Sex disparities in cancer mortality and survival. *Cancer Epidemiol Biomarkers Prev* (2011) 20:1629–37. doi:10.1158/1055-9965.EPI-11-0246
256. Cook MB. Epidemiology: excess cancer in men – a call for an increased research focus. *Nat Rev Clin Oncol* (2013) 10:186–8. doi:10.1038/nrclinonc.2013.37
257. Dorak MT, Karpuzoglu E. Gender differences in cancer susceptibility: an inadequately addressed issue. *Front Genet* (2012) 3:268. doi:10.3389/fgene.2012.00268
258. Dev R, Hui D, Del FE, Delgado-Guay MO, Sobti N, Dalal S, et al. Association between hypogonadism, symptom burden, and survival in male patients with advanced cancer. *Cancer* (2014) 120:1586–93. doi:10.1002/cncr.28619
259. Ose J, Poole EM, Schock H, Lehtinen M, Arslan AA, Zeleniuch-Jacquotte A, et al. Androgens are differentially associated with ovarian cancer subtypes in the ovarian cancer cohort consortium. *Cancer Res* (2017) 77:3951–60. doi:10.1158/0008-5472.CAN-16-3322
260. Kaaks R, Berrino F, Key T, Rinaldi S, Dossus L, Biessy C, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl Cancer Inst* (2005) 97:755–65. doi:10.1093/jnci/dji132
261. Peter HH, Chouroulinkov I, Guillon JC, Ventura A, Lenormand M. The influence of castration and oestradiol treatment on tumor transplantation immunity in mice. *Z Krebsforsch* (1970) 74:207–18. doi:10.1007/BF00525887
262. Lhoste EF, Roebuck BD, Brinck-Johnsen T, Longnecker DS. Effect of castration and hormone replacement on azaserine-induced pancreatic carcinogenesis in male and female Fischer rats. *Carcinogenesis* (1987) 8:699–703. doi:10.1093/carcin/8.5.699
263. Deguchi J, Miyamoto M, Okada S. Sex hormone-dependent renal cell carcinogenesis induced by ferric nitrilotriacetate in Wistar rats. *Jpn J Cancer Res* (1995) 86:1068–71. doi:10.1111/j.1349-7006.1995.tb03022.x
264. Zhang LJ, Xiong Y, Nilubol N, He M, Bommareddi S, Zhu X, et al. Testosterone regulates thyroid cancer progression by modifying tumor suppressorgenes and tumor immunity. *Carcinogenesis* (2015) 36:420–8. doi:10.1093/carcin/bgv001

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REVIEW
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The Confluence of Sex Hormones and Aging on Immunity

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The immune systems of post-pubescent males and females differ significantly with profound consequences to health and disease. In many cases, sex-specific differences in the immune responses of young adults are also apparent in aged men and women. Moreover, as in young adults, aged women develop several late-adult onset autoimmune conditions more frequently than do men, while aged men continue to develop many cancers to a greater extent than aged women. However, sex differences in the immune systems of aged individuals have not been extensively investigated and data addressing the effectiveness of vaccinations and immunotherapies in aged men and women are scarce. In this review, we evaluate age- and sex hormone-related changes to innate and adaptive immunity, with consideration about how this impacts age- and sex-associated changes in the incidence and pathogenesis of autoimmunity and cancer as well as the efficacy of vaccination and cancer immunotherapy. We conclude that future preclinical and clinical studies should consider age and sex to better understand the ways in which these characteristics intersect with immune function and the resulting consequences for autoimmunity, cancer, and therapeutic interventions.

Keywords: sex, sex hormones, immunity, autoimmunity, cancer, vaccines, immunotherapy, checkpoint blockade

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INTRODUCTION

In developed countries, the population is aging, with the number of people over the age of 65 doubling in size from 2012 to 2050 (1). In developed and even developing countries, lifespan is longer for women than men (2, 3). Both sex (i.e., biological differences between males and females) and gender (i.e., social or cultural norms that define masculine and feminine) contribute to male–female differences in mortality rates among individuals 65 years and older. Why and how the sexes differ in the incidence and progression of immune-related diseases that are either specific to advanced age or that worsen with age, such as particular infections, autoimmune disease, and cancer, has not been well studied.

Aging is associated with the development of chronic inflammation and a general reduction in immune function. The effect of sex on immune function during the aging process has not been well studied. But, some studies indicate that the innate immune system of aged females may be more inflammation-prone when compared with aged males. However, aging of the adaptive immune system may occur at a faster rate in men, when compared with women. Several diseases that are associated with age are also sensitive to changes in the immune system. Therefore, herein, we will discuss the effects of age and sex on the innate and adaptive immune systems and the contribution of sex hormones to these effects. We will also examine the functional consequences of age- and sex-related changes to immunity in the contexts of vaccination, autoimmunity, cancer, and cancer

immunotherapy. We conclude that sex and age should be considered in future clinical and preclinical studies to improve our understanding and treatment of age-associated diseases.

AGE-RELATED CHANGES IN IMMUNE FUNCTION

With age, there is a decline in the functioning of the immune system (4) that has, until recently, been assumed to occur equally in males and females. "Inflammaging," as defined by aberrant chronic low-grade inflammatory responses, is one of the most well-characterized attributes of an aging immune system (5). The activity of dendritic cell (DC) subsets, macrophages, and neutrophils, each of which are associated with inflammation, also become altered with age (6–9). Inflammatory responses are necessary to clear pathogens and repair tissues; chronicity of inflammatory responses, however, can contribute to tissue damage and disease, especially among aged individuals. Similarly, adaptive immunity becomes less functional with age (10, 11). Reductions in lymphopoeisis along with exposure to pathogens throughout the lifespan contribute to reduced numbers of naïve lymphocytes with increased proportions of memory and memory-like lymphocytes that are associated with less robust functional outcomes (12, 13). Overall, age-associated changes to the functions of innate and adaptive immune cells (summarized in **Figure 1**) likely contribute to increased risk of specific autoimmune diseases and cancer, as well as altered vaccine and cancer immunotherapy efficacy.

Age-Related Changes in Innate Immunity

Aging is associated with the secretion of pro-inflammatory cytokines, such as TNF, IL-6, and IL-1β, the cellular source of which has not yet been clearly identified (14). Innate immune cells, including DCs, neutrophils, and macrophages, become less functional and, paradoxically, more inflammatory with age. It has been difficult to determine whether systemic inflammation causes innate cell dysfunction or *vice versa*. Recent evidence discussed below suggests that inflammaging may alter the development and signaling potential of innate cells, contributing to inflammation in the absence of infection and, at the same time, a reduced ability to clear infections (15–17). Together, the elevated levels of inflammatory cytokines and diminished ability to resolve infections or local inflammation likely contribute to less functional innate responses to vaccination and increased risk of certain autoimmune diseases.

The number and proportion of plasmacytoid DCs declines during healthy aging, while frailty appears to be associated with reduced numbers of conventional DCs (8). Regardless of their number, conventional DCs from aged mice and humans migrate, phagocytose, express costimulatory molecules, secrete cytokines, and prime T cells poorly in response to exogenous antigens when compared with DCs from young conspecifics (6, 18–32). At least some of these defects appear to be cell intrinsic and related to the altered expression of toll-like receptors (TLRs) and dysregulated downstream signaling [reviewed in Ref. (33)].

Neutrophils from aged individuals have defects in accurately migrating to inflamed tissues, phagocytosing microbes, producing reactive oxygen species (ROS), and capturing microbes using neutrophil extracellular traps (9, 34–38). Like neutrophils, many functions of macrophages are negatively affected by aging including migration, phagocytosis, production of ROS and cytokines, and expression of major histocompatibility complex class II and costimulatory molecules (15, 39–43). Studies examining the cytokine response of monocytes isolated from older patients have yielded mixed results, likely due to differences in health status, isolation techniques, assay conditions, and stimuli between studies [reviewed in Ref. (33)]. Some studies have revealed a diminished ability of aged monocytes and macrophages to

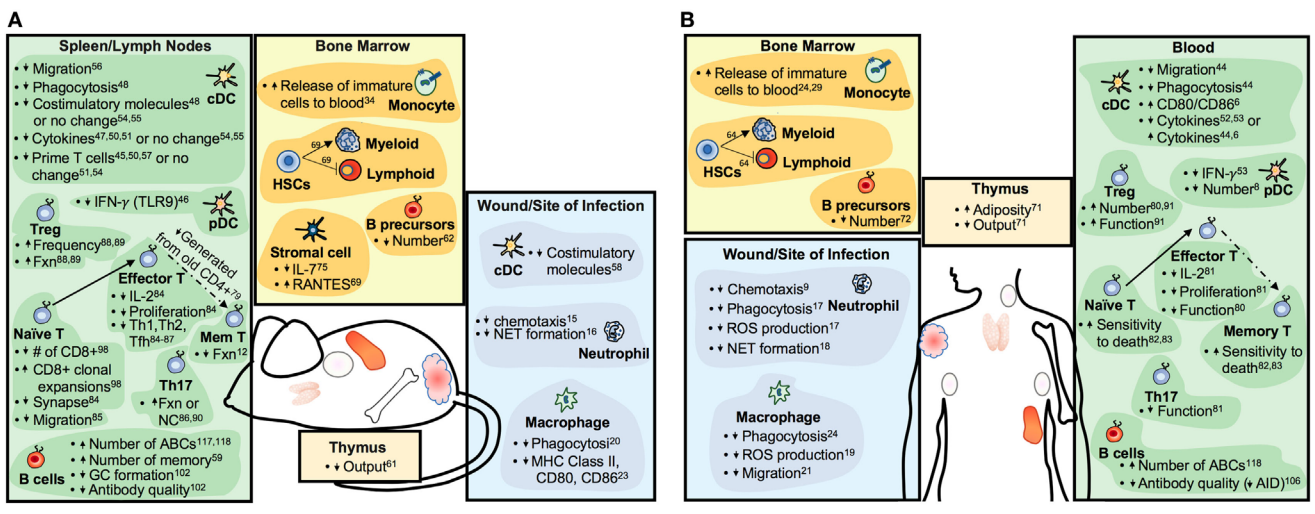

FIGURE 1 | Summary of aging-related changes to the immune systems of mice **(A)** and humans **(B)**. Increases or decreases in cell numbers or particular functions are indicated by upward- or downward-pointing arrows, respectively. Abbreviations: Fxn, function; GC, germinal center; Mem, memory; ABC, age-associated B cell; NC, no change.

secrete pro-inflammatory cytokines robustly after exposure to pathogens, LPS, or other TLR ligands (44–50). Chronic exposure to inflammatory cytokines such as IL-6 and TNF-α and dysregulated expression and/or function of TLRs have been discussed as possible causes (44, 45, 48).

Several recent reports have suggested that at least in mice, inflammaging may precede and perhaps even cause dysregulation of innate immune cells, which may further contribute to inflammation. For example, aging is also associated with increased proportions of pro-inflammatory monocytes of non-classical and intermediate phenotypes (i.e., $CD14^{+}CD16^{+}$ or $CD16^{++}$ in humans and $Ly6C^{hi}$ in mice) that are less mature, poorer phagocytes, and may be more prone to secreting pro-inflammatory cytokines at baseline and in response to stimuli (15–17, 47, 51). In mice, aged $Ly6C^{hi}$ monocytes both contribute to age-associated inflammation and are impaired by the inflammation with negative consequences for bacterial clearance (16). Circumstantial evidence indicates that in humans, premature migration of intermediate phenotype monocytes ($CD14^{++}CD16^{+}$) is driven by TNF-α-mediated upregulation of CCR2, as also occurs in mice (16), and may contribute to worsened disease outcomes in rheumatoid arthritis patients (52, 53).

Additional age-related changes to monocyte function may contribute to increased susceptibility to infection concomitant with a reduced ability to resolve inflammation. For example, the production of specialized pro-resolving mediators, including lipid signaling molecules produced by macrophages and monocytes, is reduced in aged mice and is associated with delayed resolution of acute inflammation (54). In addition, aged macrophages isolated from mice and humans phagocytose infectious agents and apoptotic cells less efficiently than young macrophages (15, 40, 55–59). The phagocytosis of infectious agents and apoptotic cells by macrophages is important for resolution of inflammation and restoration of tissue integrity, which is reduced with aging.

Age-Related Changes in Adaptive Immunity

Aging is accompanied by a decline in the production of new lymphocytes as well as increased expansion and survival of antigen-specific memory lymphocytes in mice and humans (60–72). Despite reduced lymphopoeisis (73–76), the overall number of peripheral lymphocytes is maintained in aged mice (11) and humans [reviewed in Ref. (77)], with the exception of peripheral B cell numbers being reduced in older humans (78, 79). The diminished functionality of older adaptive cells may be related to age-associated changes in lymphocyte development.

The ability of aged T cells to proliferate robustly, differentiate appropriately, and generate memory is generally diminished (10, 12, 13, 80–85). However, all T cell functions are not impaired by aging. T regulatory (T_{reg}) and, in some cases, T helper 17, cells increase in number and/or function with age (81, 85–93). It was recently proposed that naïve T cells produced in neonates form a long-lived, self-renewing population of "incumbent" naïve T cells that are resistant to replacement by T cells produced after the neonatal period (94). It is conceivable that accumulated damage in these long-lived incumbents may contribute to reduced naïve T cell function with age. In addition, accelerated homeostatic proliferation, as may be more likely to occur in aged individuals (95, 96), is associated with the selection of autoreactive T cells, at least in mice (97–99) and may also affect overall T cell functionality.

Changes in aged naïve T cell function likely contribute to defective memory generation and also partially explain the observation that antibodies elicited from older mice and humans are less protective compared with those from the young individuals (100–106), even though serum IgG levels increase with age in both mice and humans (107, 108). In addition, aged B cells demonstrate intrinsic defects in germinal center formation, class switch recombination, and somatic hypermutation (109–112). Aged B cells from mice and humans do not sufficiently upregulate expression of activation-induced cytidine deaminase (AID, the enzyme required for class switch recombination and somatic hypermutation) due to diminished levels of the necessary transcription factor (107, 110, 113, 114). With age, there are also more long-lived antigen-experienced B cells, including age-associated B cells (ABCs) (60, 115–120). ABCs are responsive to TLR7 and 9 ligands but less so to T cell-dependent signals and have been hypothesized to be generated by nucleic acid-containing antigens during inflammation (118, 121).

SEX DIFFERENCES IN AGE-RELATED CHANGES IN IMMUNE FUNCTION

Both innate and adaptive immune responses differ between males and females at young and advanced ages (summarized in **Table 1**). Most published studies of immune system differences between the sexes utilize young adults and do not address whether sex differences in immune function change with aging. Overall, the available data indicate that young adult females demonstrate a more reactive, inflammatory profile when compared with young adult males. A clear consensus has not emerged regarding whether these sex differences are maintained during advanced age, but the immune systems of aged women on hormone replacement therapy (HRT) and monocytes isolated from aged women, regardless of HRT status, appear to remain skewed toward an inflammatory phenotype (16, 122–124). The currently available data also indicate that the adaptive immune response of aged women may be preserved to a greater extent than in aged men. These studies are discussed in more detail below.

Sex Differences in Age-Related Changes to Innate Immunity

As mentioned above, at least among young adults, innate immune responses differ between the sexes. Using murine model systems, it has been shown that the activity of pattern-recognition receptors, production of inflammatory proteins (e.g., IFN-α, IFN-γ, and TNF-α), activity of macrophages, including antigen presentation and phagocytosis is higher in females than males (132–138). Studies evaluating innate immune system differences between the sexes are scarce. But, at least one small study demonstrated that aged females display elevated concentrations of inflammatory proteins compared with males, as also occurs in young men and women (139). Several cytokines show differential

TABLE 1 | Sex differences in innate and adaptive immune responses in young and aged individuals.

	 **Dendritic cells**	 **Monocytes and macrophages**	 **Granulocytes**	 **Innate lymphoid cells**	 **Natural killer cells**	 **B cells**	 **T cells**
Young adults	♀ > ♂ TLR7 activity (H) Type 1 IFN activity (H)	♀ > ♂ Activation (M) Phagocytic capacity (M) IL-10 production (M) M2 polarization (M)	♀ > ♂ Phagocytic capacity (M) Neutrophil count (M) Nitric Oxide production post stimulation (H, R, M)	♀ > ♂ Type 2 cytokine levels upon stimulation (M)		♀ > ♂ B cell numbers (H, M) Antibody production (H, M) % switched memory B cells (H)	♀ > ♂ $CD4^+$ T cell count (H, M) $CD4^+/CD8^+$ T cell ratio (H) Activated T cell count (M) T cell proliferative capacity (M) Cytotoxic T cell activity (H)
	♂ > ♀ IL-10 production (R, H)	♂ > ♀ TLR4 expression (M) Pro-inflammatory cytokine production (M) M1 polarization (M)	♂ > ♀ Neutrophil attractant chemokines (R) TLR9 expression (M)	♂ > ♀ Type 2 ILC count (H) IL-13 production upon stimulation (M)	♂ > ♀ NK cell activity (R) ♀ = ♂ NK cell count (H)		♂ > ♀ $CD8^+$ T cell count (M) T_{reg} count (M)
Aged adults	♀ > ♂ Nitric oxide synthesis (H) Mammalian family of mitogen-activated protein kinases (MAPK) signaling (H, M)	♀ > ♂ CD62L, CD115 (H) expression			♀ > ♂ NK cytotoxicity (H) Immunosurveillance (H)	♀ > ♂ Antibody production (H) Age-associated B cell count (H, M)	♀ > ♂ $CD3^+$ T cell count (H) $CD4^+$ T cell count (P) $CD4^+/CD8^+$ T cell ratio (P) T_H1 response (M)
	IL-15 production (H)	 ♂ > ♀ CD38 expression (H) Non-classical monocyte count (H)	ND	ND			T_H1 response (M) Naïve $CD8^+$ T effector memory cells (p) T cell proliferative capacity (H, P) ♂ > ♀ $CD8^+$ T cell count (P)

Data are from studies of mice (M), rats (R), non-human primates (P), and humans (H) (125–131).
ND, not determined.

levels in circulation between the sexes. For example, IL-15 is an important homeostatic cytokine in T cells, NK cell, and memory responses and is significantly upregulated in aged females when compared with age-matched males (122, 123). However, upon exclusion of individuals on HRT, such differences between sexes were no longer significant (122). After menopause, there is a significant increase in IL-1, IL-6, and TNFα, and reduction in IFNγ in women (140, 141). Testosterone has an immunosuppressive effect on inflammatory cytokine production and its decline with aging is associated with an increase in serum soluble IL-6 receptor (142). Monocyte and leukocyte subpopulations in aged males and females express different levels of receptors; males show higher CD38 expression, whereas females show higher CD62L and CD115 expression, indicating differences in their activation profiles and memory phenotypes (124). Sex differences among monocyte subsets have also been reported in aged individuals. Aged females have a higher proportion of intermediate ($CD14^{hi}CD16^{low}$) monocytes than similarly aged males, which have been shown to exhibit pro-inflammatory tendencies, as mentioned above (16, 124). Finally, NK cells in older women are superior at cancer immunosurveillance when compared with cells in older men. $CD56^{dim}$ NK cells are more cytotoxic and more responsive to leukemic cells in aged females compared with aged males, which may explain the higher incidences of cancer in aged men compared with women in populations (143).

Sex Differences in Age-Related Changes to Adaptive Immunity

Both humoral and cell-mediated immune responses to antigenic stimulation, vaccination, and infection are typically higher among females than males (135). Females also typically demonstrate higher basal levels of immunoglobulin (144) and higher antibody responses to viruses and vaccine antigens than males at any age (145–147). Among humans, absolute $CD3^+$ T cell counts, frequencies of $CD4^+$ T cells, helper T cell type 1 responses, and the ratio of $CD4^+$:$CD8^+$ T cells are all lower in men when compared with women (148–151).

As already mentioned, sex or gender has not traditionally been considered when evaluating age-related changes to the adaptive immune system [reviewed in Ref. (14)]. However, several groups have reported that in some ways, aging occurs at an accelerated rate in males when compared with females. For example, aged males experience a more dramatic decrease in total numbers of T and B cells and a larger increase in senescent $CD8^+$ T effector memory cells that re-express the naïve marker CD45 RA (T_{EMRA}) when compared with females (14, 150, 152–154). In addition, a greater proportion of aged males than females demonstrate an inverted CD4:CD8 T cell ratio, an age-related phenotype that is also associated with decreased levels of $CD19^+$ B cells and $CD8^+CD28^-$ senescent T cells (152). Also, the capacity of T cells to proliferate is preserved to a greater extent in women than men throughout the aging process (154), which may be an important consideration for infectious diseases and related interventions. On the other hand, transcriptional analyses of peripheral blood mononuclear cells from aged males and females revealed several pro-inflammatory pathways, including NF-κB signaling, NO synthesis, and p38 MAPK signaling, that are reduced to a greater extent in aged females than aged males (123). Moreover, aged females have greater numbers of ABCs than young females and males of all ages (118, 119).

THE IMPACT OF SEX HORMONES ON AGE-RELATED CHANGES IN IMMUNE RESPONSES

Immunological differences between males and females can arise from diverse mechanistic causes, including genetic, hormonal, and even microbiome differences between the sexes. Partly because of the ease of measuring and manipulating, sex steroids, particularly testosterone, estradiol, and progesterone, have been most well characterized as mediators of sex differences in immune responses and are the focus of this review. Sex steroids affect immune function by binding to specific hormone receptors expressed in diverse immune cells (155). With age, the hormonal milieu of females and even males changes, with an overall decline in concentrations of estrogens and progesterone in females and testosterone in males (156–158). We hypothesize that the changes in sex steroid concentrations and sex steroid receptor signaling with age may contribute to age-associated dysregulation of immune function (159). Although this has been considered in females through the comparison of pre- and post-menopausal women, few studies have considered hormonal changes in men as playing a role in age-associated changes in immune responses. Among women, with menopause, numbers of B and T cells are reduced and concentrations of IL-1β, IL-6, and TNF-α are significantly increased (141, 160, 161). Treatment of post-menopausal females with hormone replacement therapies that contain formulations of estrogen affects immune function by increasing circulating numbers of B cells and reducing baseline concentrations of pro-inflammatory cytokines when compared with post-menopausal females not on HRT (140, 161). Whether testosterone replacement therapy affects immune responses in aged human males has not been reported. In non-human primates, aged male rhesus macaques have lower frequencies of naïve $CD4^+$ and $CD8^+$ T cells than young males, with supplementation of androgens in aged male resulting in increased numbers of naïve T cells presumably by increasing thymic output (162). Whether treatment of aged individuals with hormone replacement therapies affects the outcome of vaccines or immunotherapies in either females or males has not been reported.

Studies in mice and humans have shown that the diversity and richness of intestinal microbiota differs between males and females after puberty, presumably due testosterone, but not estrogen (163–168). Moreover, in mice, exposure to specific microbiota at early ages also results in elevated levels of testosterone (164). Thus, testosterone appears to influence the composition of the gut microbiome and, in a positive feedback loop, specific microbes elevate testosterone levels (164). Sex-specific enrichment for particular microbes is likely to have significant influence on sex-specific immune function since particular commensals and their metabolites can dramatically modify host innate and adaptive immune function [reviewed in Ref. (169)] with serious

consequences for autoimmunity, vaccine efficacy, cancer, and cancer immunotherapy [reviewed in Ref. (170, 171)]. The composition and richness of commensal microbiota is sensitive to many environmental factors as well, including diet. Importantly, dietary effects on the relative abundance of specific microbial taxa also differ by sex in humans and, to a lesser extent, in mice (172). Sex-specific differences in microbial composition and richness have also been reported in humans over the age of 60 and aged mice (163, 165, 166, 173).

FUNCTIONAL SIGNIFICANCE OF SEX DIFFERENCES IN IMMUNE RESPONSES AMONG AGED INDIVIDUALS

Vaccine Responses

In aged individuals, sex differences in antibody responses to vaccines are less consistent and depend on the vaccine antigen. The influence of sex and age has been most well studied for inactivated influenza virus vaccines as they are administered annually. For example, among individuals 65+ years of age, hemagglutinin inhibition antibody titers to both the standard and high dose seasonal trivalent inactivated influenza (TIV) vaccine are significantly higher in aged females when compared with males (174). Because influenza virus vaccines are available on an annual basis, a greater number of exposures (i.e., the behavioral act of seeking out vaccination) combined with the slower decline in immunity that occurs in aged females (see above) may contribute to sex differences in the antibody response to the TIV vaccine. By contrast, aged males have higher antibody responses to the tetanus diphtheria and pertussis (Td/Tdap) vaccines as well as the 7-valent and 23-valent pneumococcal vaccines (175–179). There is an insufficient number of studies from which to draw conclusions to understand why sex differences in vaccine-induced antibody responses are higher in aged females than males for a viral vaccine (i.e., the TIV vaccine), but lower in females than males for bacterial vaccines (i.e., the Td/Tdap and pneumococcal vaccines). If more vaccine studies were designed with *a priori* hypotheses about sex differences in vaccine-induced immunity, then we could begin to understand discrepancies in the findings following exposure to differential vaccine antigens.

Adverse reactions to vaccines, which are typically mild to moderate, can include both local (i.e., at the site of vaccination) and systemic reactions. Adverse reactions are reported by aged women more than their male counterparts in response to the seasonal and pandemic influenza vaccines (180–188), the pneumococcal vaccines (189, 190), the herpes zoster vaccine (191), or the tetanus and pertussis vaccines (192–194). While the types of adverse reactions experienced by aged males and females are typically similar, the proportion of females reporting redness, swelling, and injection site pain locally as well as headache, fever, chills, joint or muscle pain, headache, back and abdominal pain, or hypersensitivity reactions systemically is often greater than males. The prevailing hypothesis for differences in adverse reactions among aged males and females is that this reflects a gender-based reporting bias.

The efficacy of a vaccine is measured by the percent reduction in disease incidence in a vaccinated population (195). Sex-specific differences in vaccine efficacy are rarely considered, with most data coming from studies of influenza vaccines. Vaccine efficacy, which is defined by hospitalization and mortality rates post-vaccination, is lower in aged females than males, at least for the influenza vaccine (196–200). For other vaccines that are not administered annually, including the pneumococcal and herpes zoster vaccines, there are considerably less data. Overall, the efficacy both the herpes zoster and pneumococcal vaccines tends to be higher in aged females than their male counterparts (191, 201, 202).

Autoimmunity

Most autoimmune patients are diagnosed between the ages of 20 and 60 years (203). For those whose autoimmune disease develops later, the disease tends to be milder and more easily controlled (203). Women are disproportionately affected by autoimmune disease, and this holds true for several autoimmune diseases with late-adult onset as well, including rheumatoid arthritis, polymyalgia rheumatica, and giant cell arteritis (**Table 2**). Regardless of the age of onset, the cellular and molecular basis of autoimmunity is complicated and distinct for each specific disease [reviewed

TABLE 2 | The female-to-male patient ratio for select mid-adult and late-adult onset autoimmune diseases.

Autoimmune disease	Autoimmune target	Mean age of onset (range) years	Female:male ratio	Reference
Mid-adult onset				
Multiple sclerosis	Myelin sheath	37 (25–45)	1.8:1	(205)
Myasthenia gravis	Neuromuscular junction	40	2.7:1	(205)
Systemic lupus erythematosus	Nuclear contents (systemic)	40 (30–50)	9:1	(205, 206)
Neuromyelitis optica	Optic nerve/spinal cord	32.6–45.7	2.4:1 ratio highest after age 65	(207, 208)
Graves' disease	Thyroid	48	7.3:1	(205)
Systemic sclerosis	Connective tissue (systemic)	50 (35–65)	11.5:1	(209)
Late-adult onset				
Granulomatosis with polyangiitis (GPA) (formerly Wegener's granulomatosis)	Cytoplasmic contents of neutrophils (systemic, vascular)	55 (40–70)	1:1 M > F after age 70	(205)
Rheumatoid arthritis	Joints	58 (42–74)	3:1	(210)
Polymyalgia rheumatica	Selected muscle groups	70–80	2.3:1	(211)
Giant cell arteritis	Vascular system	70–80	2.3:1	(211)

in Ref. (204)]. Here, we focus on the impact of age and sex on autoimmune conditions with late onset.

Although several theories have been proposed to explain sex differences in the cellular and molecular basis of aging [reviewed in Ref. (212)], perhaps most relevant to the sex-specific development of autoimmunity in the aged is that estrogen upregulates the activity of several antioxidant systems (213, 214). Dramatic loss of estrogen (such as during menopause) could be expected to result in increased cell death due to unchecked ROS-induced DNA damage. Indeed, fewer lymphocytes are detected in the blood of post-menopausal women compared with younger women (160, 215) and T cell apoptosis increases after natural or surgical menopause (216). This could especially explain increased female incidence of autoimmune diseases that may occur as a result of lymphopenia-induced homeostatic proliferation in the aged, although more studies are needed to test this hypothesis.

In mice, lymphopenia and the subsequent homeostatic proliferation of lymphocytes has been shown to contribute to the development of autoimmunity in many contexts [reviewed in Ref. (217)]. Certainly, there is an association between autoimmunity and lymphopenia in humans, but a strong case has not been made that lymphopenia is causative, or even occurs prior to, the onset of autoimmunity (218–224). However, evidence gathered by the laboratories of Goronzy et al. support a model whereby accelerated T cell loss in the aged, either due to telomerase deficiency, disruption to DNA repair responses, or menopause, may be sufficient to enable autoreactive T cells already present in the pool to respond to low-affinity self-antigens in rheumatoid arthritis patients [reviewed in Ref. (225)]. First, there is evidence of accelerated aging, or increased homeostatic proliferation in RA patients. The telomeres of naïve and memory T cells isolated from RA patients are shorter than age-matched controls (226) and T cell receptor diversity is reduced as well (227). Moreover, T cells from RA patients are more prone to apoptosis and are less capable of repairing dsDNA breaks (228). Finally, end-differentiated effector T cells that may be the consequence of homeostatic proliferation appear to be major participants in late onset autoimmune pathogenesis (229–232).

Cancer

Sex and age influence cancer incidence and mortality, but the specific effects vary by cancer type. It is widely accepted that the probability of developing cancer increases with age (233). Although few studies have examined cancer incidence in those with very advanced age, it seems that cancer prevalence actually declines for those over the age of 85 (234, 235). There is some evidence to indicate that tumors may also be generally less aggressive in the extremely aged (236). Indeed, breast and prostate cancer patients over the age of 55 are more likely to develop tumors with characteristics associated with favorable treatment and/or survival outcomes (237, 238). However, it is not clear that tumors associated with other types of cancer, including bladder cancer, lung cancer, and acute myeloid leukemia, are indolent in older patients (239–242).

Overall, young men generally experience higher rates of cancer incidence and mortality than women (243–245). At advanced ages, men continue to experience higher incidences of most types of cancers, especially colorectal cancer, when compared with women (245, 246), but relative cancer mortality rates between older men and women differ by the particular cancer. Mortality differences between men and women diminish with age (especially after the age of 70) for colorectal cancer, stomach cancer, and leukemia (247). However, the male-to-female mortality ratio for brain cancer and myeloma decreases after middle age, but then increases again after the age of 70 (247).

The loss of sex hormones (especially due to menopause in women), age-associated immunosuppression, and chronic inflammation may contribute to sex- and age-specific patterns of cancer incidence and mortality. Indeed, the male preponderance of cancer incidence and mortality before menopause has been at least partially attributed to the protective effect of estrogen (248), presumably due to its ability to enhance immunosurveillance, as well as tissue-specific effects (249, 250). Purim et al. suggests that it takes 20–25 years for some cancers (such as colorectal) to develop and since changes in sex-specific incidence ratios for those cancers occur approximately 25 years after menopause, the loss of estrogens at approximately age 55 contributes to increased female cancer incidence after the age of 80 (246). On the other hand, age- and sex-related diminishment of the effectiveness of the immune system may not contribute a great deal to increased cancer incidence in the aged, since the types of cancers observed in the aged are not the same of those observed in immunocompromised patients. HIV-induced immunodeficiency is associated with lymphoma and Kaposi's sarcoma, while most age-related malignancies in the aged are carcinomas (251). Finally, older persons with chronic inflammation may demonstrate increased risk of cancer, as it is clear that inflammation induced by viruses, bacteria, tobacco smoke, and obesity increases cancer risk (252–255). Overall, more studies are certainly warranted to better understand the factors that contribute to cancer incidence and mortality in older men and women.

Cancer Immunotherapy

Cancer immunotherapy trials typically involve younger patients with no co-morbidities, even though these characteristics are not representative of most cancer patients (256). This is particularly important because the effectiveness and dose of any particular immunotherapy is likely to be affected by age-associated changes in immunity and metabolism (256). In addition, few clinical trials are designed to compare the efficacy and safety of cancer immunotherapies between women and men of any age (257). The currently available data regarding the sex- and age-specific effectiveness of several immunotherapies are discussed below.

Checkpoint blockade therapies in young or middle-aged men and women appear to be beneficial, but the benefits may be stronger in men (258–261). Blockade of PD1/PDL1 with nivolumab was more effective in male melanoma and renal cell carcinoma patients than in female patients (258, 260). However, these studies were not designed to compare efficacy in male versus female patients, and the sample size for female patients was small. Preclinical studies of anti-PDL1 treatment revealed that melanoma tumor growth was more robustly reduced in female mice when compared with males (262). Estrogen upregulates PD-1 on T_{regs} and T_{effs}. The authors speculated that anti-PDL1 treatment

TABLE 3 | Variables to consider when designing clinical studies related to immunity in the aged.

	Clinical study considerations
Age	Clearly defined age categories Young: 20 to ≤45 years Old: >45 to ≤85 years Very old/elderly: >85 years
Health status	Frailty: three of the five following characteristics: weight loss, weakened handgrip, exhaustion, reduced gait speed, and reduced activity Concentrations of serum inflammatory proteins: IL-6, TNF-α, IL-1β, and C-reactive protein
Sex hormone status	Time of menopause Serum concentrations of sex hormones Hormone replacement therapy

was more effective in females because of the greater contribution of PD-1 to T_{reg} suppression of antitumor responses in females (262). In addition, as mentioned above, the microbiome varies with age and sex and has recently been shown to significantly influence cancer immunotherapy success. Indeed, recent antibiotic use and the absence of specific microbial taxa correlates with reduced efficacy of PD1/PDL1 blockade and certain immune-reliant chemotherapies in both humans and mice [reviewed in Ref. (170, 263)]. Therefore, it is critical to more formally evaluate the effect of cancer immunotherapies in men and women and to assess the suitability of various cancer models for predicting the success of particular immunotherapies in the sexes.

As already mentioned, few clinical immunotherapy trials enroll patients of advanced age and studies that did include older patients reach different conclusions about the efficacy of checkpoint blockade in the aged. Meta-analyses of heterogeneous groups of cancer patients over the age of 65 or 70 treated with immune checkpoint inhibitors (biologicals targeting PD1, PDL1, or CTLA4) compared with similarly aged patients enrolled in the control arm of the studies revealed that checkpoint inhibitors reduced the risk of death by 34–37% in patients with advanced age (264, 265). Moreover, in at least one meta-analysis, the overall survival rate of patients over the age of 65 or 70 and younger patients treated with immune checkpoint inhibitors did not differ (264). However, other studies have reported significantly worse overall survival rates in patients over the age of 75 treated with checkpoint inhibitors (266). Finally, there is concern that treatment of older cancer patients with checkpoint inhibitors could actually enhance tumor growth, as occurred in one subset of cancer patients (267) or prompt immune-related adverse events, as occurs in mouse models (268).

CONCLUSION

For most, the aging process is accompanied by alterations in the function of the immune system. Many experience chronic inflammation and a general impairment of immune cell function. The immune systems of young men and women are quite different, and it appears that aging affects the cellular composition and function of the immune system in sex-specific ways as well. This is likely because of pre-existing differences in immunity between men and women as well as differences in how menopause and andropause unfold. Age- and sex-specific changes to immunity may have consequences for late-adult onset autoimmunity and cancer, as well as for the efficacy of vaccinations and cancer immunotherapies. However, our understanding of the ways in which sex and age intersect with immune function and the consequences of this for autoimmunity, cancer, and therapeutic interventions is severely limited by the lack of inclusion of these variables in clinical and preclinical studies. Therefore, preclinical and clinical studies related to vaccination, autoimmunity, and cancer therapies must be powered to detect sex effects, in accordance with the sex and gender equity in research (SAGER) guidelines (269). Age, sex hormone concentrations, hormone replacement therapies, and health status must be considered as well, given the known impact of these variables on immune-related conditions common in the aged (**Table 3**).

AUTHOR CONTRIBUTIONS

MB conceived of the idea for this review. MB and SK outlined the content. MB, TP, AF, and SK researched and wrote sections. All authors edited and reviewed the final draft.

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REFERENCES

1. Ortman JM, Velkoff VA, Hogan H. An aging nation: the older population in the United States. *Current Population Reports*. Washington, DC: U.S. Census Bureau (2014).
2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* (2012) 380(9859):2095–128. doi:10.1016/S0140-6736(12)61728-0
3. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* (2015) 385(9963):117–71. doi:10.1016/S0140-6736(14)61682-2
4. Castelo-Branco C, Soveral I. The immune system and aging: a review. *Gynecol Endocrinol* (2014) 30(1):16–22. doi:10.3109/09513590.2013.852531
5. Cannizzo ES, Clement CC, Sahu R, Follo C, Santambrogio L. Oxidative stress, inflamm-aging and immunosenescence. *J Proteomics* (2011) 74(11):2313–23. doi:10.1016/j.jprot.2011.06.005
6. Agrawal A, Tay J, Ton S, Agrawal S, Gupta S. Increased reactivity of dendritic cells from aged subjects to self-antigen, the human DNA. *J Immunol* (2009) 182(2):1138–45. doi:10.4049/jimmunol.182.2.1138
7. Canan CH, Gokhale NS, Carruthers B, Lafuse WP, Schlesinger LS, Torrelles JB, et al. Characterization of lung inflammation and its impact on macrophage function in aging. *J Leukoc Biol* (2014) 96(3):473–80. doi:10.1189/jlb.4A0214-093RR
8. Jing Y, Shaheen E, Drake RR, Chen N, Gravenstein S, Deng Y. Aging is associated with a numerical and functional decline in plasmacytoid dendritic cells, whereas

myeloid dendritic cells are relatively unaltered in human peripheral blood. *Hum Immunol* (2009) 70(10):777–84. doi:10.1016/j.humimm.2009.07.005
9. Wenisch C, Patruta S, Daxbock F, Krause R, Horl W. Effect of age on human neutrophil function. *J Leukoc Biol* (2000) 67(1):40–5. doi:10.1002/jlb.67.1.40
10. Haynes L, Maue AC. Effects of aging on T cell function. *Curr Opin Immunol* (2009) 21(4):414–7. doi:10.1016/j.coi.2009.05.009
11. Miller JP, Cancro MP. B cells and aging: balancing the homeostatic equation. *Exp Gerontol* (2007) 42(5):396–9. doi:10.1016/j.exger.2007.01.010
12. Haynes L, Eaton SM, Burns EM, Randall TD, Swain SL. CD4 T cell memory derived from young naive cells functions well into old age, but memory generated from aged naive cells functions poorly. *Proc Natl Acad Sci U S A* (2003) 100(25):15053–8. doi:10.1073/pnas.2433717100
13. Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, et al. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. *J Immunol* (2004) 173(1):673–81. doi:10.4049/jimmunol.173.1.673
14. Gubbels Bupp MR. Sex, the aging immune system, and chronic disease. *Cell Immunol* (2015) 294(2):102–10. doi:10.1016/j.cellimm.2015.02.002
15. Hearps AC, Martin GE, Angelovich TA, Cheng WJ, Maisa A, Landay AL, et al. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging Cell* (2012) 11(5):867–75. doi:10.1111/j.1474-9726.2012.00851.x
16. Puchta A, Naidoo A, Verschoor CP, Loukov D, Thevaranjan N, Mandur TS, et al. TNF drives monocyte dysfunction with age and results in impaired anti-pneumococcal immunity. *PLoS Pathog* (2016) 12(1):e1005368. doi:10.1371/journal.ppat.1005368
17. Verschoor CP, Johnstone J, Millar J, Parsons R, Lelic A, Loeb M, et al. Alterations to the frequency and function of peripheral blood monocytes and associations with chronic disease in the advanced-age, frail elderly. *PLoS One* (2014) 9(8):e104522. doi:10.1371/journal.pone.0104522
18. Agrawal A, Agrawal S, Cao JN, Su H, Osann K, Gupta S. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. *J Immunol* (2007) 178(11):6912–22. doi:10.4049/jimmunol.178.11.6912
19. Moretto MM, Lawlor EM, Khan IA. Aging mice exhibit a functional defect in mucosal dendritic cell response against an intracellular pathogen. *J Immunol* (2008) 181(11):7977–84. doi:10.4049/jimmunol.181.11.7977
20. Stout-Delgado HW, Yang X, Walker WE, Tesar BM, Goldstein DR. Aging impairs IFN regulatory factor 7 up-regulation in plasmacytoid dendritic cells during TLR9 activation. *J Immunol* (2008) 181(10):6747–56. doi:10.4049/jimmunol.181.10.6747
21. Pereira LF, de Souza AP, Borges TJ, Bonorino C. Impaired in vivo CD4+ T cell expansion and differentiation in aged mice is not solely due to T cell defects: decreased stimulation by aged dendritic cells. *Mech Ageing Dev* (2011) 132(4):187–94. doi:10.1016/j.mad.2011.03.005
22. Li G, Smithey MJ, Rudd BD, Nikolich-Zugich J. Age-associated alterations in CD8alpha+ dendritic cells impair CD8 T-cell expansion in response to an intracellular bacterium. *Aging Cell* (2012) 11(6):968–77. doi:10.1111/j.1474-9726.2012.00867.x
23. Chougnet CA, Thacker RI, Shehata HM, Hennies CM, Lehn MA, Lages CS, et al. Loss of phagocytic and antigen cross-presenting capacity in aging dendritic cells is associated with mitochondrial dysfunction. *J Immunol* (2015) 195(6):2624–32. doi:10.4049/jimmunol.1501006
24. Grolleau-Julius A, Garg MR, Mo R, Stoolman LL, Yung RL. Effect of aging on bone marrow-derived murine CD11c+CD4-CD8alpha- dendritic cell function. *J Gerontol A Biol Sci Med Sci* (2006) 61(10):1039–47. doi:10.1093/gerona/61.10.1039
25. Wong CP, Magnusson KR, Ho E. Aging is associated with altered dendritic cells subset distribution and impaired proinflammatory cytokine production. *Exp Gerontol* (2010) 45(2):163–9. doi:10.1016/j.exger.2009.11.005
26. Qian F, Wang X, Zhang L, Lin A, Zhao H, Fikrig E, et al. Impaired interferon signaling in dendritic cells from older donors infected in vitro with West Nile virus. *J Infect Dis* (2011) 203(10):1415–24. doi:10.1093/infdis/jir048
27. Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L, et al. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. *J Immunol* (2010) 184(5):2518–27. doi:10.4049/jimmunol.0901022
28. Tesar BM, Walker WE, Unternaehrer J, Joshi NS, Chandele A, Haynes L, et al. Murine [corrected] myeloid dendritic cell-dependent toll-like receptor immunity is preserved with aging. *Aging Cell* (2006) 5(6):473–86. doi:10.1111/j.1474-9726.2006.00245.x
29. Jones SC, Brahmakshatriya V, Huston G, Dibble J, Swain SL. TLR-activated dendritic cells enhance the response of aged naive CD4 T cells via an IL-6-dependent mechanism. *J Immunol* (2010) 185(11):6783–94. doi:10.4049/jimmunol.0901296
30. Zhao J, Zhao J, Legge K, Perlman S. Age-related increases in PGD(2) expression impair respiratory DC migration, resulting in diminished T cell responses upon respiratory virus infection in mice. *J Clin Invest* (2011) 121(12):4921–30. doi:10.1172/JCI59777
31. Grolleau-Julius A, Harning EK, Abernathy LM, Yung RL. Impaired dendritic cell function in aging leads to defective antitumor immunity. *Cancer Res* (2008) 68(15):6341–9. doi:10.1158/0008-5472.CAN-07-5769
32. Tan SY, Cavanagh LL, d'Advigor W, Shackel N, Fazekas de St Groth B, Weninger W. Phenotype and functions of conventional dendritic cells are not compromised in aged mice. *Immunol Cell Biol* (2012) 90(7):722–32. doi:10.1038/icb.2011.104
33. Shaw AC, Panda A, Joshi SR, Qian F, Allore HG, Montgomery RR. Dysregulation of human toll-like receptor function in aging. *Ageing Res Rev* (2011) 10(3):346–53. doi:10.1016/j.arr.2010.10.007
34. Sapey E, Greenwood H, Walton G, Mann E, Love A, Aaronson N, et al. Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence. *Blood* (2014) 123(2):239–48. doi:10.1182/blood-2013-08-519520
35. Brubaker AL, Rendon JL, Ramirez L, Choudhry MA, Kovacs EJ. Reduced neutrophil chemotaxis and infiltration contributes to delayed resolution of cutaneous wound infection with advanced age. *J Immunol* (2013) 190(4):1746–57. doi:10.4049/jimmunol.1201213
36. Tseng CW, Kyme PA, Arruda A, Ramanujan VK, Tawackoli W, Liu GY. Innate immune dysfunctions in aged mice facilitate the systemic dissemination of methicillin-resistant *S. aureus*. *PLoS One* (2012) 7(7):e41454. doi:10.1371/journal.pone.0041454
37. Alonso-Fernandez P, Puerto M, Mate I, Ribera JM, de la Fuente M. Neutrophils of centenarians show function levels similar to those of young adults. *J Am Geriatr Soc* (2008) 56(12):2244–51. doi:10.1111/j.1532-5415.2008.02018.x
38. Hazeldine J, Harris P, Chapple IL, Grant M, Greenwood H, Livesey A, et al. Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals. *Aging Cell* (2014) 13(4):690–8. doi:10.1111/acel.12222
39. McLachlan JA, Serkin CD, Morrey-Clark KM, Bakouche O. Immunological functions of aged human monocytes. *Pathobiology* (1995) 63(3):148–59. doi:10.1159/000163946
40. De La Fuente M. Changes in the macrophage function with aging. *Comp Biochem Physiol A Comp Physiol* (1985) 81(4):935–8. doi:10.1016/0300-9629(85)90933-8
41. Fietta A, Merlini C, De Bernardi PM, Gandola L, Piccioni PD, Grassi C. Non specific immunity in aged healthy subjects and in patients with chronic bronchitis. *Aging (Milano)* (1993) 5(5):357–61.
42. Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: impact on macrophage function. *Aging Cell* (2004) 3(4):161–7. doi:10.1111/j.1474-9728.2004.00102.x
43. Herrero C, Marques L, Lloberas J, Celada A. IFN-gamma-dependent transcription of MHC class II IA is impaired in macrophages from aged mice. *J Clin Invest* (2001) 107(4):485–93. doi:10.1172/JCI11696
44. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol* (2004) 75(2):342–9. doi:10.1189/jlb.0803389
45. Boehmer ED, Meehan MJ, Cutro BT, Kovacs EJ. Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev* (2005) 126(12):1305–13. doi:10.1016/j.mad.2005.07.009
46. Montgomery RR, Shaw AC. Paradoxical changes in innate immunity in aging: recent progress and new directions. *J Leukoc Biol* (2015) 98(6):937–43. doi:10.1189/jlb.5MR0315-104R
47. Nyugen J, Agrawal S, Gollapudi S, Gupta S. Impaired functions of peripheral blood monocyte subpopulations in aged humans. *J Clin Immunol* (2010) 30(6):806–13. doi:10.1007/s10875-010-9448-8

48. van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, et al. Age-associated defect in human TLR-1/2 function. *J Immunol* (2007) 178(2):970–5. doi:10.4049/jimmunol.178.2.970
49. Agius E, Lacy KE, Vukmanovic-Stejic M, Jagger AL, Papageorgiou AP, Hall S, et al. Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. *J Exp Med* (2009) 206(9):1929–40. doi:10.1084/jem.20090896
50. Molony RD, Nguyen JT, Kong Y, Montgomery RR, Shaw AC, Iwasaki A. Aging impairs both primary and secondary RIG-I signaling for interferon induction in human monocytes. *Sci Signal* (2017) 10(509):eaan2392. doi:10.1126/scisignal.aan2392
51. Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol* (2010) 11:30. doi:10.1186/1471-2172-11-30
52. Chara L, Sanchez-Atrio A, Perez A, Cuende E, Albarran F, Turrion A, et al. Monocyte populations as markers of response to adalimumab plus MTX in rheumatoid arthritis. *Arthritis Res Ther* (2012) 14(4):R175. doi:10.1186/ar3928
53. Xia L, Lu J, Xiao W. Blockage of TNF-alpha by infliximab reduces CCL2 and CCR2 levels in patients with rheumatoid arthritis. *J Investig Med* (2011) 59(6):961–3. doi:10.2310/JIM.0b013e31821c0242
54. Arnardottir HH, Dalli J, Colas RA, Shinohara M, Serhan CN. Aging delays resolution of acute inflammation in mice: reprogramming the host response with novel nano-proresolving medicines. *J Immunol* (2014) 193(8):4235–44. doi:10.4049/jimmunol.1401313
55. Albright JW, Albright JF. Ageing alters the competence of the immune system to control parasitic infection. *Immunol Lett* (1994) 40(3):279–85. doi:10.1016/0165-2478(94)00066-2
56. Bradley SF, Kauffman CA. Aging and the response to *Salmonella* infection. *Exp Gerontol* (1990) 25(1):75–80. doi:10.1016/0531-5565(90)90012-Q
57. Aprahamian T, Takemura Y, Goukassian D, Walsh K. Ageing is associated with diminished apoptotic cell clearance in vivo. *Clin Exp Immunol* (2008) 152(3):448–55. doi:10.1111/j.1365-2249.2008.03658.x
58. Njie EG, Boelen E, Stassen FR, Steinbusch HW, Borchelt DR, Streit WJ. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiol Aging* (2012) 33(1):195.e1–12. doi:10.1016/j.neurobiolaging.2010.05.008
59. Swift ME, Burns AL, Gray KL, DiPietro LA. Age-related alterations in the inflammatory response to dermal injury. *J Invest Dermatol* (2001) 117(5):1027–35. doi:10.1046/j.0022-202x.2001.01539.x
60. Johnson SA, Rozzo SJ, Cambier JC. Aging-dependent exclusion of antigen-inexperienced cells from the peripheral B cell repertoire. *J Immunol* (2002) 168(10):5014–23. doi:10.4049/jimmunol.168.10.5014
61. Riley RL. Impaired B lymphopoiesis in old age: a role for inflammatory B cells? *Immunol Res* (2013) 57(1–3):361–9. doi:10.1007/s12026-013-8444-5
62. Berent-Maoz B, Montecino-Rodriguez E, Dorshkind K. Genetic regulation of thymocyte progenitor aging. *Semin Immunol* (2012) 24(5):303–8. doi:10.1016/j.smim.2012.04.006
63. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on early B- and T-cell development. *Immunol Rev* (2005) 205:7–17. doi:10.1111/j.0105-2896.2005.00263.x
64. Nikolich-Zugich J. Aging of the T cell compartment in mice and humans: from no naive expectations to foggy memories. *J Immunol* (2014) 193(6):2622–9. doi:10.4049/jimmunol.1401174
65. Geiger H, de Haan G, Florian MC. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol* (2013) 13(5):376–89. doi:10.1038/nri3433
66. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. *Nat Med* (1996) 2(9):1011–6. doi:10.1038/nm1296-1282
67. Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* (2000) 192(9):1273–80. doi:10.1084/jem.192.9.1273
68. Miller JP, Allman D. Linking age-related defects in B lymphopoiesis to the aging of hematopoietic stem cells. *Semin Immunol* (2005) 17(5):321–9. doi:10.1016/j.smim.2005.05.003
69. Shimazu T, Iida R, Zhang Q, Welner RS, Medina KL, Alberola-Lla J, et al. CD86 is expressed on murine hematopoietic stem cells and denotes lymphopoietic potential. *Blood* (2012) 119(21):4889–97. doi:10.1182/blood-2011-10-388736
70. Ergen AV, Boles NC, Goodell MA. Rantes/Ccl5 influences hematopoietic stem cell subtypes and causes myeloid skewing. *Blood* (2012) 119(11):2500–9. doi:10.1182/blood-2011-11-391730
71. Labrie JE III, Sah AP, Allman DM, Cancro MP, Gerstein RM. Bone marrow microenvironmental changes underlie reduced RAG-mediated recombination and B cell generation in aged mice. *J Exp Med* (2004) 200(4):411–23. doi:10.1084/jem.20040845
72. Palmer DB. The effect of age on thymic function. *Front Immunol* (2013) 4:316. doi:10.3389/fimmu.2013.00316
73. McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. *Blood* (2001) 98(8):2498–507. doi:10.1182/blood.V98.8.2498
74. Rossi MID, Yokota T, Medina KL, Garrett KP, Comp PC, Schipul AH, et al. B lymphopoiesis is active throughout human life, but there are developmental age-related changes. *Blood* (2003) 101(2):576–84. doi:10.1182/blood-2002-03-0896
75. Alter-Wolf S, Blomberg BB, Riley RL. Deviation of the B cell pathway in senescent mice is associated with reduced surrogate light chain expression and altered immature B cell generation, phenotype, and light chain expression. *J Immunol* (2009) 182(1):138–47. doi:10.4049/jimmunol.182.1.138
76. Stephan RP, Reilly CR, Witte PL. Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. *Blood* (1998) 91(1):75–88.
77. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T cell maintenance and function in human aging. *J Immunol* (2015) 194(9):4073–80. doi:10.4049/jimmunol.1500046
78. Weksler ME, Szabo P. The effect of age on the B-cell repertoire. *J Clin Immunol* (2000) 20(4):240–9. doi:10.1023/A:1006659401385
79. Sansoni P, Cossarizza A, Brianti V, Fagnoni F, Snelli G, Monti D, et al. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood* (1993) 82(9):2767–73.
80. Mattoo H, Faulkner M, Kandpal U, Das R, Lewis V, George A, et al. Naive CD4 T cells from aged mice show enhanced death upon primary activation. *Int Immunol* (2009) 21(11):1277–89. doi:10.1093/intimm/dxp094
81. van der Geest KS, Abdulahad WH, Tete SM, Lorencetti PG, Horst G, Bos NA, et al. Aging disturbs the balance between effector and regulatory CD4+ T cells. *Exp Gerontol* (2014) 60:190–6. doi:10.1016/j.exger.2014.11.005
82. Bruunsgaard H, Pedersen AN, Schroll M, Skinhoj P, Pedersen BK. Proliferative responses of blood mononuclear cells (BMNC) in a cohort of elderly humans: role of lymphocyte phenotype and cytokine production. *Clin Exp Immunol* (2000) 119(3):433–40. doi:10.1046/j.1365-2249.2000.01146.x
83. Aggarwal S, Gupta S. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. *J Immunol* (1998) 160(4):1627–37.
84. Aggarwal S, Gollapudi S, Gupta S. Increased TNF-alpha-induced apoptosis in lymphocytes from aged humans: changes in TNF-alpha receptor expression and activation of caspases. *J Immunol* (1999) 162(4):2154–61.
85. Haynes L, Linton PJ, Eaton SM, Tonkonogy SL, Swain SL. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J Exp Med* (1999) 190(7):1013–24. doi:10.1084/jem.190.7.1013
86. Lefebvre JS, Maue AC, Eaton SM, Lanthier PA, Tighe M, Haynes L. The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. *Aging Cell* (2012) 11(5):732–40. doi:10.1111/j.1474-9726.2012.00836.x
87. Maue AC, Eaton SM, Lanthier PA, Sweet KB, Blumerman SL, Haynes L. Proinflammatory adjuvants enhance the cognate helper activity of aged CD4 T cells. *J Immunol* (2009) 182(10):6129–35. doi:10.4049/jimmunol.0804226
88. Lefebvre JS, Masters AR, Hopkins JW, Haynes L. Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses. *Sci Rep* (2016) 6:25051. doi:10.1038/srep25051
89. Lages CS, Suffia I, Velilla PA, Huang B, Warshaw G, Hildeman DA, et al. Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. *J Immunol* (2008) 181(3):1835–48. doi:10.4049/jimmunol.181.3.1835

90. Raynor J, Karns R, Almanan M, Li KP, Divanovic S, Chougnet CA, et al. IL-6 and ICOS antagonize Bim and promote regulatory T cell accrual with age. *J Immunol* (2015) 195(3):944–52. doi:10.4049/jimmunol.1500443
91. Huang MC, Liao JJ, Bonasera S, Longo DL, Goetzl EJ. Nuclear factor-kappaB-dependent reversal of aging-induced alterations in T cell cytokines. *FASEB J* (2008) 22(7):2142–50. doi:10.1096/fj.07-103721
92. Rosenkranz D, Weyer S, Tolosa E, Gaenslen A, Berg D, Leyhe T, et al. Higher frequency of regulatory T cells in the elderly and increased suppressive activity in neurodegeneration. *J Neuroimmunol* (2007) 188(1–2):117–27. doi:10.1016/j.jneuroim.2007.05.011
93. Hwang KA, Kim HR, Kang I. Aging and human CD4(+) regulatory T cells. *Mech Ageing Dev* (2009) 130(8):509–17. doi:10.1016/j.mad.2009.06.003
94. Hogan T, Gossel G, Yates AJ, Seddon B. Temporal fate mapping reveals age-linked heterogeneity in naive T lymphocytes in mice. *Proc Natl Acad Sci U S A* (2015) 112(50):E6917–26. doi:10.1073/pnas.1517246112
95. Wallace DL, Zhang Y, Ghattas H, Worth A, Irvine A, Bennett AR, et al. Direct measurement of T cell subset kinetics in vivo in elderly men and women. *J Immunol* (2004) 173(3):1787–94. doi:10.4049/jimmunol.173.3.1787
96. Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, et al. The influence of age on T cell generation and TCR diversity. *J Immunol* (2005) 174(11):7446–52. doi:10.4049/jimmunol.174.11.7446
97. Kassiotis G, Zamoyska R, Stockinger B. Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells. *J Exp Med* (2003) 197(8):1007–16. doi:10.1084/jem.20021812
98. Kieper WC, Burghardt JT, Surh CD. A role for TCR affinity in regulating naive T cell homeostasis. *J Immunol* (2004) 172(1):40–4. doi:10.4049/jimmunol.172.1.40
99. Rudd BD, Venturi V, Li G, Samadder P, Ertelt JM, Way SS, et al. Nonrandom attrition of the naive CD8+ T-cell pool with aging governed by T-cell receptor:pMHC interactions. *Proc Natl Acad Sci U S A* (2011) 108(33):13694–9. doi:10.1073/pnas.1107594108
100. Weng NP. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* (2006) 24(5):495–9. doi:10.1016/j.immuni.2006.05.001
101. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* (2004) 5(2):133–9. doi:10.1038/ni1033
102. Johnson SA, Cambier JC. Ageing, autoimmunity and arthritis: senescence of the B cell compartment – implications for humoral immunity. *Arthritis Res Ther* (2004) 6(4):131–9. doi:10.1186/ar1180
103. Frasca D, Landin AM, Riley RL, Blomberg BB. Mechanisms for decreased function of B cells in aged mice and humans. *J Immunol* (2008) 180(5):2741–6. doi:10.4049/jimmunol.180.5.2741
104. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, Kondeatis E, et al. B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell* (2009) 8(1):18–25. doi:10.1111/j.1474-9726.2008.00443.x
105. Murasko DM, Bernstein ED, Gardner EM, Gross P, Munk G, Dran S, et al. Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly. *Exp Gerontol* (2002) 37(2–3):427–39. doi:10.1016/S0531-5565(01)00210-8
106. Nicoletti C, Yang X, Cerny J. Repertoire diversity of antibody response to bacterial antigens in aged mice. III. Phosphorylcholine antibody from young and aged mice differ in structure and protective activity against infection with *Streptococcus pneumoniae*. *J Immunol* (1993) 150(2):543–9.
107. Frasca D, Landin AM, Lechner SC, Ryan JG, Schwartz R, Riley RL, et al. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol* (2008) 180(8):5283–90. doi:10.4049/jimmunol.180.8.5283
108. Speziali E, Santiago AF, Fernandes RM, Vaz NM, Menezes JS, Faria AM. Specific immune responses but not basal functions of B and T cells are impaired in aged mice. *Cell Immunol* (2009) 256(1–2):1–5. doi:10.1016/j.cellimm.2009.01.010
109. Zheng B, Han S, Takahashi Y, Kelsoe G. Immunosenescence and germinal center reaction. *Immunol Rev* (1997) 160:63–77. doi:10.1111/j.1600-065X.1997.tb01028.x
110. Frasca D, Van der Put E, Riley RL, Blomberg BB. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *J Immunol* (2004) 172(4):2155–62. doi:10.4049/jimmunol.172.4.2155
111. Whisler RL, Grants IS. Age-related alterations in the activation and expression of phosphotyrosine kinases and protein kinase C (PKC) among human B cells. *Mech Ageing Dev* (1993) 71(1–2):31–46. doi:10.1016/0047-6374(93)90033-N
112. Dailey RW, Eun SY, Russell CE, Vogel LA. B cells of aged mice show decreased expansion in response to antigen, but are normal in effector function. *Cell Immunol* (2001) 214(2):99–109. doi:10.1006/cimm.2001.1894
113. Frasca D, Riley RL, Blomberg BB. Effect of age on the immunoglobulin class switch. *Crit Rev Immunol* (2004) 24(5):297–320. doi:10.1615/CritRevImmunol.v24.i5.10
114. Frasca D, Van der Put E, Landin AM, Gong D, Riley RL, Blomberg BB. RNA stability of the E2A-encoded transcription factor E47 is lower in splenic activated B cells from aged mice. *J Immunol* (2005) 175(10):6633–44. doi:10.4049/jimmunol.175.10.6633
115. Kline GH, Hayden TA, Klinman NR. B cell maintenance in aged mice reflects both increased B cell longevity and decreased B cell generation. *J Immunol* (1999) 162(6):3342–9.
116. Klinman NR, Kline GH. The B-cell biology of aging. *Immunol Rev* (1997) 160:103–14. doi:10.1111/j.1600-065X.1997.tb01031.x
117. Weksler ME. Changes in the B-cell repertoire with age. *Vaccine* (2000) 18(16):1624–8. doi:10.1016/S0264-410X(99)00497-1
118. Hao Y, O'Neill P, Naradikian MS, Scholz JL, Cancro MP. A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* (2011) 118(5):1294–304. doi:10.1182/blood-2011-01-330530
119. Rubtsov AV, Rubtsova K, Fischer A, Meehan RT, Gillis JZ, Kappler JW, et al. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c(+) B-cell population is important for the development of autoimmunity. *Blood* (2011) 118(5):1305–15. doi:10.1182/blood-2011-01-331462
120. Lee-Chang C, Bodogai M, Moritoh K, Olkhanud PB, Chan AC, Croft M, et al. Accumulation of 4-1BBL+ B cells in the elderly induces the generation of granzyme-B+ CD8+ T cells with potential antitumor activity. *Blood* (2014) 124(9):1450–9. doi:10.1182/blood-2014-03-563940
121. Naradikian MS, Hao Y, Cancro MP. Age-associated B cells: key mediators of both protective and autoreactive humoral responses. *Immunol Rev* (2016) 269(1):118–29. doi:10.1111/imr.12380
122. Al-Attar A, Presnell SR, Peterson CA, Thomas DT, Lutz CT. Data correlations between gender, cytomegalovirus infection and T cells, NK cells, and soluble immune mediators in elderly humans. *Data Brief* (2016) 8:536–44. doi:10.1016/j.dib.2016.06.006
123. Marttila S, Jylhävä J, Nevalainen T, Nykter M, Jylhä M, Hervonen A, et al. Transcriptional analysis reveals gender-specific changes in the aging of the human immune system. *PLoS One* (2013) 8(6):e66229. doi:10.1371/journal.pone.0066229
124. Al-Attar A, Presnell SR, Peterson CA, Thomas DT, Lutz CT. The effect of sex on immune cells in healthy aging: elderly women have more robust natural killer lymphocytes than do elderly men. *Mech Ageing Dev* (2016) 156:25–33. doi:10.1016/j.mad.2016.04.001
125. Yovel G, Shakhar K, Ben-Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. *Gynecol Oncol* (2001) 81(2):254–62. doi:10.1006/gyno.2001.6153
126. Bain BJ. Ethnic and sex differences in the total and differential white cell count and platelet count. *J Clin Pathol* (1996) 49(8):664–6. doi:10.1136/jcp.49.8.664
127. Sánchez-Ramón S, Radigan L, Joyce EY, Bard S, Cunningham-Rundles C. Memory B cells in common variable immunodeficiency: clinical associations and sex differences. *Clin Immunol* (2008) 128(3):314–21. doi:10.1016/j.clim.2008.02.013
128. Warren KJ, Sweeter JM, Pavlik JA, Nelson AJ, Devasure JM, Dickinson JD, et al. Sex differences in activation of lung-related type 2 innate lymphoid cells in experimental asthma. *Ann Allergy Asthma Immunol* (2017) 118(2):233–4. doi:10.1016/j.anai.2016.11.011
129. Les Laboratoires Servier. *Lymphatic system Illustrations* (2018) Available from: https://smart.servier.com/ (Accessed: March 15, 2018).
130. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* (2016) 16(10):626–38. doi:10.1038/nri.2016.90
131. Traub S, Demaria O, Chasson L, Serra F, Desnues B, Alexopoulou L. Sex bias in susceptibility to MCMV infection: implication of TLR9. *PLoS One* (2012) 7(9):e45171. doi:10.1371/journal.pone.0045171

132. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* (2006) 312(5780):1669–72. doi:10.1126/science.1124978
133. Berghofer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-alpha production in females. *J Immunol* (2006) 177(4):2088–96. doi:10.4049/jimmunol.177.4.2088
134. Da Silva JA. Sex hormones, glucocorticoids and autoimmunity: facts and hypotheses. *Ann Rheum Dis* (1995) 54(1):6–16. doi:10.1136/ard.54.1.6
135. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol* (2008) 8(9):737–44. doi:10.1038/nri2394
136. Spitzer JA. Gender differences in some host defense mechanisms. *Lupus* (1999) 8(5):380–3. doi:10.1177/096120339900800510
137. Mondal S, Rai U. Sexual dimorphism in phagocytic activity of wall lizard's splenic macrophages and its control by sex steroids. *Gen Comp Endocrinol* (1999) 116(2):291–8. doi:10.1006/gcen.1999.7370
138. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *J Immunol* (1984) 132(2):656–61.
139. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci U S A* (2014) 111(2):869–74. doi:10.1073/pnas.1321060111
140. Deguchi K, Kamada M, Irahara M, Maegawa M, Yamamoto S, Ohmoto Y, et al. Postmenopausal changes in production of type 1 and type 2 cytokines and the effects of hormone replacement therapy. *Menopause* (2001) 8(4):266–73. doi:10.1097/00042192-200107000-00008
141. Kumru S, Godekmerdan A, Yılmaz B. Immune effects of surgical menopause and estrogen replacement therapy in peri-menopausal women. *J Reprod Immunol* (2004) 63(1):31–8. doi:10.1016/j.jri.2004.02.001
142. Maggio M, Basaria S, Ble A, Lauretani F, Bandinelli S, Ceda GP, et al. Correlation between testosterone and the inflammatory marker soluble interleukin-6 receptor in older men. *J Clin Endocrinol Metab* (2006) 91(1):345–7. doi:10.1210/jc.2005-1097
143. Siegel R, Naishadham D, Jemal A. Cancer statistics for Hispanics/Latinos 2012. *CA Cancer J Clin* (2012) 62:10–29. doi:10.3322/caac.21153
144. Butterworth M, McClellan B, Allansmith M. Influence of sex in immunoglobulin levels. *Nature* (1967) 214(5094):1224–5. doi:10.1038/2141224a0
145. Klein SL, Huber S. Sex differences in susceptibility to viral infection. In: Klein SL, Roberts CW, editors. *Sex Hormones and Immunity to Infection*. Berlin: Springer-Verlag (2009) 93–122.
146. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. *Vaccine* (2008) 26(29–30):3551–5. doi:10.1016/j.vaccine.2008.04.054
147. Klein SL, Jedlicka A, Pekosz A. The Xs and Y of immune responses to viral vaccines. *Lancet Infect Dis* (2010) 10(5):338–49. doi:10.1016/S1473-3099(10)70049-9
148. Wikby A, Mansson IA, Johansson B, Strindhall J, Nilsson SE. The immune risk profile is associated with age and gender: findings from three Swedish population studies of individuals 20–100 years of age. *Biogerontology* (2008) 9(5):299–308. doi:10.1007/s10522-008-9138-6
149. Villacres MC, Longmate J, Auge C, Diamond DJ. Predominant type 1 CMV-specific memory T-helper response in humans: evidence for gender differences in cytokine secretion. *Hum Immunol* (2004) 65(5):476–85. doi:10.1016/j.humimm.2004.02.021
150. Amadori A, Zamarchi R, De Silvestro G, Forza G, Cavatton G, Danieli GA, et al. Genetic control of the CD4/CD8 T-cell ratio in humans. *Nat Med* (1995) 1(12):1279–83. doi:10.1038/nm1295-1279
151. Das BR, Bhanushali AA, Khadapkar R, Jeswani KD, Bhavsar M, Dasgupta A. Reference ranges for lymphocyte subsets in adults from western India: influence of sex, age and method of enumeration. *Indian J Med Sci* (2008) 62(10):397–406. doi:10.4103/0019-5359.42725
152. Strindhall J, Skog M, Ernerudh J, Bengner M, Lofgren S, Matussek A, et al. The inverted CD4/CD8 ratio and associated parameters in 66-year-old individuals: the Swedish HEXA immune study. *Age (Dordr)* (2013) 35(3):985–91. doi:10.1007/s11357-012-9400-3
153. Lee OJ, Cho YN, Kee SJ, Kim MJ, Jin HM, Lee SJ, et al. Circulating mucosal-associated invariant T cell levels and their cytokine levels in healthy adults. *Exp Gerontol* (2014) 49:47–54. doi:10.1016/j.exger.2013.11.003
154. Hirokawa K, Utsuyama M, Hayashi Y, Kitagawa M, Makinodan T, Fulop T. Slower immune system aging in women versus men in the Japanese population. *Immun Ageing* (2013) 10(1):19. doi:10.1186/1742-4933-10-19
155. Kovats S, Carreras E, Agrawal H. Sex steroid receptors in immune cells. In: Klein SL, Roberts CW, editors. *Sex Hormones and Immunity to Infection*. Berlin: Springer-Verlag (2010) 53–92.
156. Perheentupa A, Huhtaniemi I. Aging of the human ovary and testis. *Mol Cell Endocrinol* (2009) 299(1):2–13. doi:10.1016/j.mce.2008.11.004
157. Bain J. Testosterone and the aging male: to treat or not to treat? *Maturitas* (2010) 66(1):16–22. doi:10.1016/j.maturitas.2010.01.009
158. Neal-Perry G, Nejat E, Dicken C. The neuroendocrine physiology of female reproductive aging: an update. *Maturitas* (2010) 67(1):34–8. doi:10.1016/j.maturitas.2010.04.016
159. Giefing-Kröll C, Berger P, Lepperdinger G, Grubeck-Loebenstein B. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell* (2015) 14(3):309–21. doi:10.1111/acel.12326
160. Giglio T, Imro MA, Filaci G, Scudeletti M, Puppo F, De Cecco L, et al. Immune cell circulating subsets are affected by gonadal function. *Life Sci* (1994) 54(18):1305–12. doi:10.1016/0024-3205(94)00508-7
161. Kamada M, Irahara M, Maegawa M, Yasui T, Yamano S, Yamada M, et al. B cell subsets in postmenopausal women and the effect of hormone replacement therapy. *Maturitas* (2001) 37(3):173–9. doi:10.1016/S0378-5122(00)00180-8
162. Rais M, Wilson RM, Urbanski HF, Messaoudi I. Androgen supplementation improves some but not all aspects of immune senescence in aged male macaques. *GeroScience* (2017) 39:373–84. doi:10.1007/s11357-017-9979-5
163. Elderman M, Sovran B, Hugenholtz F, Graversen K, Huijskes M, Houtsma E, et al. The effect of age on the intestinal mucus thickness, microbiota composition and immunity in relation to sex in mice. *PLoS One* (2017) 12(9):e0184274. doi:10.1371/journal.pone.0184274
164. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* (2013) 339(6123):1084–8. doi:10.1126/science.1233521
165. Haro C, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, Delgado-Lista J, et al. Intestinal microbiota is influenced by gender and body mass index. *PLoS One* (2016) 11(5):e0154090. doi:10.1371/journal.pone.0154090
166. Singh P, Manning SD. Impact of age and sex on the composition and abundance of the intestinal microbiota in individuals with and without enteric infections. *Ann Epidemiol* (2016) 26(5):380–5. doi:10.1016/j.annepidem.2016.03.007
167. Kozik AJ, Nakatsu CH, Chun H, Jones-Hall YL. Age, sex, and TNF associated differences in the gut microbiota of mice and their impact on acute TNBS colitis. *Exp Mol Pathol* (2017) 103(3):311–9. doi:10.1016/j.yexmp.2017.11.014
168. Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, et al. Gender bias in autoimmunity is influenced by microbiota. *Immunity* (2013) 39(2):400–12. doi:10.1016/j.immuni.2013.08.013
169. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* (2014) 157(1):121–41. doi:10.1016/j.cell.2014.03.011
170. Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy. *Cancer Cell* (2018) 33(4):570–80. doi:10.1016/j.ccell.2018.03.015
171. Gomez A, Luckey D, Taneja V. The gut microbiome in autoimmunity: sex matters. *Clin Immunol* (2015) 159(2):154–62. doi:10.1016/j.clim.2015.04.016
172. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, et al. Individual diet has sex-dependent effects on vertebrate gut microbiota. *Nat Commun* (2014) 5:4500. doi:10.1038/ncomms5500
173. Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, et al. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol* (2006) 72(2):1027–33. doi:10.1128/AEM.72.2.1027-1033.2006
174. Falsey AR, Treanor JJ, Tornieporth N, Capellan J, Gorse GJ. Randomized, double-blind controlled phase 3 trial comparing the immunogenicity of high-dose and standard-dose influenza vaccine in adults 65 years of age and older. *J Infect Dis* (2009) 200(2):172–80. doi:10.1086/599790
175. Brandão AP, De Oliveira TC, de Cunto Brandileone MC, Gonçalves JE, Yara TI, Simonsen V. Persistence of antibody response to pneumococcal capsular polysaccharides in vaccinated long term-care residents in Brazil. *Vaccine* (2004) 23(6):762–8. doi:10.1016/j.vaccine.2004.07.024

176. Goldblatt D, Southern J, Andrews N, Ashton L, Burbidge P, Woodgate S, et al. The immunogenicity of 7-valent pneumococcal conjugate vaccine versus 23-valent polysaccharide vaccine in adults aged 50–80 years. *Clin Infecti Dis* (2009) 49(9):1318–25. doi:10.1086/606046
177. Hainz U, Jenewein B, Asch E, Pfeiffer K-P, Berger P, Grubeck-Loebenstein B. Insufficient protection for healthy elderly adults by tetanus and TBE vaccines. *Vaccine* (2005) 23(25):3232–5. doi:10.1016/j.vaccine.2005.01.085
178. Stark K, Schönfeld C, Barg J, Molz B, Vornwald A, Bienzle U. Seroprevalence and determinants of diphtheria, tetanus and poliomyelitis antibodies among adults in Berlin, Germany. *Vaccine* (1999) 17(7):844–50. doi:10.1016/S0264-410X(98)00269-2
179. Marlovits S, Stocker R, Efstratiou A, Broughton K, Kaider A, Vecsei V, et al. Effect on diphtheria immunity of combined tetanus and diphtheria booster vaccination in adults. *Eur J Clin Microbiol Infect Dis* (2000) 19(7):506–13. doi:10.1007/s100960000305
180. Cook IF. Sex differences in injection site reactions with human vaccines. *Hum Vaccin* (2009) 5(7):441–9. doi:10.4161/hv.8476
181. Nichol KL, Margolis KL, Lind A, Murdoch M, McFadden R, Hauge M, et al. Side effects associated with influenza vaccination in healthy working adults. A randomized, placebo-controlled trial. *Arch Intern Med* (1996) 156(14):1546–50. doi:10.1001/archinte.1996.00440130090009
182. Govaert T, Dinant G, Aretz K, Masurel N, Sprenger M, Knottnerus J. Adverse reactions to influenza vaccine in elderly people: randomised double blind placebo controlled trial. *BMJ* (1993) 307(6910):988–90. doi:10.1136/bmj.307.6910.988
183. Honkanen PO, Keistinen T, Kivelä S-L. Reactions following administration of influenza vaccine alone or with pneumococcal vaccine to the elderly. *Arch Intern Med* (1996) 156(2):205–8. doi:10.1001/archinte.1996.00440020115015
184. Donalisio MR, Ramalheira RM, Cordeiro R. Eventos adversos após vacinação contra influenza em idosos, Distrito de Campinas, SP, 2000. *Rev Soc Bras Med Trop* (2003) 36(4):467–71. doi:10.1590/S0037-86822003000400006
185. Couch RB, Winokur P, Brady R, Belshe R, Chen WH, Cate TR, et al. Safety and immunogenicity of a high dosage trivalent influenza vaccine among elderly subjects. *Vaccine* (2007) 25(44):7656–63. doi:10.1016/j.vaccine.2007.08.042
186. Beyer WP, Palache A, Kerstens R, Masurel N. Gender differences in local and systemic reactions to inactivated influenza vaccine, established by a meta-analysis of fourteen independent studies. *Eur J Clin Microbiol Infect Dis* (1996) 15(1):65–70. doi:10.1007/BF01586187
187. Engler RJ, Nelson MR, Klote MM, VanRaden MJ, Huang C-Y, Cox NJ, et al. Half-vs full-dose trivalent inactivated influenza vaccine (2004–2005): age, dose, and sex effects on immune responses. *Arch Intern Med* (2008) 168(22):2405–14. doi:10.1001/archinternmed.2008.513
188. Jackson LA, Austin G, Chen RT, Stout R, DeStefano F, Gorse GJ, et al. Safety and immunogenicity of varying dosages of trivalent inactivated influenza vaccine administered by needle-free jet injectors. *Vaccine* (2001) 19(32):4703–9. doi:10.1016/S0264-410X(01)00225-0
189. Cook IF, Pond D, Hartel G. Comparative reactogenicity and immunogenicity of 23 valent pneumococcal vaccine administered by intramuscular or subcutaneous injection in elderly adults. *Vaccine* (2007) 25(25):4767–74. doi:10.1016/j.vaccine.2007.04.017
190. Sočan M, Frelih T, Janet E, Petraš T, Peternelj B. Reactions after pneumococcal vaccine alone or in combination with influenza vaccine. *Vaccine* (2004) 22(23):3087–91. doi:10.1016/j.vaccine.2004.02.003
191. Hillebrand K, Bricout H, Schulze-Rath R, Schink T, Garbe E. Incidence of herpes zoster and its complications in Germany, 2005–2009. *J Infect* (2015) 70(2):178–86. doi:10.1016/j.jinf.2014.08.018
192. Gergen PJ, McQuillan GM, Kiely M, Ezzati-Rice TM, Sutter RW, Virella G. A population-based serologic survey of immunity to tetanus in the United States. *N Engl J Med* (1995) 332(12):761–7. doi:10.1056/NEJM199503233321201
193. White W, Barnes G, Barker E, Gall D, Knight P, Griffith A, et al. Reactions to tetanus toxoid. *J Hyg (Lond)* (1973) 71(02):283–97. doi:10.1017/S0022172400022750
194. Bayas J, Vilella A, Bertran M, Vidal J, Batalla J, Asenjo M, et al. Immunogenicity and reactogenicity of the adult tetanus–diphtheria vaccine. How many doses are necessary? *Epidemiol Infect* (2001) 127(03):451–60. doi:10.1017/S095026880100629X
195. Weinberg GA, Szilagyi PG. Vaccine epidemiology: efficacy, effectiveness, and the translational research roadmap. *J Infect Dis* (2010) 201(11):1607–10. doi:10.1086/652404
196. Wang CS, Wang ST, Chou P. Efficacy and cost-effectiveness of influenza vaccination of the elderly in a densely populated and unvaccinated community. *Vaccine* (2002) 20(19–20):2494–9. doi:10.1016/S0264-410X(02)00181-0
197. Vila-Córcoles A, Rodriguez T, de Diego C, Ochoa O, Valdivieso A, Salsench E, et al. Effect of influenza vaccine status on winter mortality in Spanish community-dwelling elderly people during 2002–2005 influenza periods. *Vaccine* (2007) 25(37):6699–707. doi:10.1016/j.vaccine.2007.07.015
198. Fleming D, Watson J, Nicholas S, Smith G, Swan A. Study of the effectiveness of influenza vaccination in the elderly in the epidemic of 1989–90 using a general practice database. *Epidemiol Infect* (1995) 115(3):581–9. doi:10.1017/S095026880005874X
199. Nichol KL, Nordin JD, Nelson DB, Mullooly JP, Hak E. Effectiveness of influenza vaccine in the community-dwelling elderly. *N Engl J Med* (2007) 357(14):1373–81. doi:10.1056/NEJMoa070844
200. Nichol K, Margolis K, Wuorenma J, Von Sternberg T. The efficacy and cost effectiveness of vaccination against influenza among elderly persons living in the community. *N Engl J Med* (1994) 331(12):778–84. doi:10.1056/NEJM199409223311206
201. Soneji S, Metlay J. Mortality reductions for older adults differ by race/ethnicity and gender since the introduction of adult and pediatric pneumococcal vaccines. *Public Health Rep* (2011) 126(2):259. doi:10.1177/003335491112600217
202. Wiemken TL, Carrico RM, Klein SL, Jonsson CB, Peyrani P, Kelley RR, et al. The effectiveness of the polysaccharide pneumococcal vaccine for the prevention of hospitalizations due to *Streptococcus pneumoniae* community-acquired pneumonia in the elderly differs between the sexes: results from the Community-Acquired Pneumonia Organization (CAPO) international cohort study. *Vaccine* (2014) 32(19):2198–203. doi:10.1016/j.vaccine.2014.02.048
203. Vadasz Z, Haj T, Kessel A, Toubi E. Age-related autoimmunity. *BMC Med* (2013) 11:94. doi:10.1186/1741-7015-11-94
204. Rosenblum MD, Remedios KA, Abbas AK. Mechanisms of human autoimmunity. *J Clin Invest* (2015) 125(6):2228–33. doi:10.1172/JCI78088
205. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. *Autoimmun Rev* (2003) 2(3):119–25. doi:10.1016/S1568-9972(03)00006-5
206. Kotzin BL. Systemic lupus erythematosus. *Cell* (1996) 85(3):303–6. doi:10.1016/S0092-8674(00)81108-3
207. Pandit L, Asgari N, Apiwattanakul M, Palace J, Paul F, Leite MI, et al. Demographic and clinical features of neuromyelitis optica: a review. *Mult Scler* (2015) 21(7):845–53. doi:10.1177/1352458515572406
208. Quek AM, McKeon A, Lennon VA, Mandrekar JN, Iorio R, Jiao Y, et al. Effects of age and sex on aquaporin-4 autoimmunity. *Arch Neurol* (2012) 69(8):1039–43. doi:10.1001/archneurol.2012.249
209. Steen VD, Oddis CV, Conte CG, Janoski J, Casterline GZ, Medsger TA Jr. Incidence of systemic sclerosis in Allegheny county, Pennsylvania. A twenty-year study of hospital-diagnosed cases, 1963–1982. *Arthritis Rheum* (1997) 40(3):441–5. doi:10.1002/art.1780400309
210. Linos A, Worthington JW, O'Fallon WM, Kurland LT. The epidemiology of rheumatoid arthritis in Rochester, Minnesota: a study of incidence, prevalence, and mortality. *Am J Epidemiol* (1980) 111(1):87–98. doi:10.1093/oxfordjournals.aje.a112878
211. Weyand CM, Goronzy JJ. Giant-cell arteritis and polymyalgia rheumatica. *N Engl J Med* (2014) 371(17):1653. doi:10.1056/NEJMcp1214825
212. Ostan R, Monti D, Gueresi P, Bussolotto M, Franceschi C, Baggio G. Gender, aging and longevity in humans: an update of an intriguing/neglected scenario paving the way to a gender-specific medicine. *Clin Sci (Lond)* (2016) 130(19):1711–25. doi:10.1042/CS20160004
213. Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* (2003) 34(5):546–52. doi:10.1016/S0891-5849(02)01356-4
214. Vina J, Borras C, Gambini J, Sastre J, Pallardo FV. Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. *FEBS Lett* (2005) 579(12):2541–5. doi:10.1016/j.febslet.2005.03.090
215. Engelmann F, Rivera A, Park B, Messerle-Forbes M, Jensen JT, Messaoudi I. Impact of estrogen therapy on lymphocyte homeostasis and the response to seasonal influenza vaccine in post-menopausal women. *PLoS One* (2016) 11(2):e0149045. doi:10.1371/journal.pone.0149045

216. Zhang J, Chen X, Zhang S, Zhou G, Xia X, Lu L. Effects of transdermal estrogen therapy on expressions of estrogen receptors and T-lymphocyte apoptosis in surgically menopausal women. *Cell Mol Immunol* (2009) 6(4):277–83. doi:10.1038/cmi.2009.37
217. Schulze-Koops H. Lymphopenia and autoimmune diseases. *Arthritis Res Ther* (2004) 6(4):178–80. doi:10.1186/ar1208
218. Viguier M, Fouere S, de la Salmoniere P, Rabian C, Lebbe C, Dubertret L, et al. Peripheral blood lymphocyte subset counts in patients with dermatomyositis: clinical correlations and changes following therapy. *Medicine (Baltimore)* (2003) 82(2):82–6. doi:10.1097/00005792-200303000-00002
219. Iannone F, Cauli A, Yanni G, Kingsley GH, Isenberg DA, Corrigall V, et al. T-lymphocyte immunophenotyping in polymyositis and dermatomyositis. *Br J Rheumatol* (1996) 35(9):839–45. doi:10.1093/rheumatology/35.9.839
220. Kirtava Z, Blomberg J, Bredberg A, Henriksson G, Jacobsson L, Manthorpe R. CD4+ T-lymphocytopenia without HIV infection: increased prevalence among patients with primary Sjogren's syndrome. *Clin Exp Rheumatol* (1995) 13(5):609–16.
221. Mandl T, Bredberg A, Jacobsson LT, Manthorpe R, Henriksson G. CD4+ T-lymphocytopenia – a frequent finding in anti-SSA antibody seropositive patients with primary Sjogren's syndrome. *J Rheumatol* (2004) 31(4):726–8.
222. Kaaba SA, Al-Harbi SA. Abnormal lymphocyte subsets in Kuwaiti patients with type-1 insulin-dependent diabetes mellitus and their first-degree relatives. *Immunol Lett* (1995) 47(3):209–13. doi:10.1016/0165-2478(95)00088-5
223. Brandt L, Stenstam M. Letter: subnormal lymphocyte-counts in adult coeliac disease. *Lancet* (1975) 1(7913):978–9. doi:10.1016/S0140-6736(75)92041-3
224. Heimann TM, Bolnick K, Aufses AH Jr. Prognostic significance of severe preoperative lymphopenia in patients with Crohn's disease. *Ann Surg* (1986) 203(2):132–5. doi:10.1097/00000658-198602000-00004
225. Goronzy JJ, Li G, Yang Z, Weyand CM. The Janus head of T cell aging – autoimmunity and immunodeficiency. *Front Immunol* (2013) 4:131. doi:10.3389/fimmu.2013.00131
226. Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM. T cell homeostasis in patients with rheumatoid arthritis. *Proc Natl Acad Sci U S A* (2000) 97(16):9203–8. doi:10.1073/pnas.97.16.9203
227. Wagner UG, Koetz K, Weyand CM, Goronzy JJ. Perturbation of the T cell repertoire in rheumatoid arthritis. *Proc Natl Acad Sci U S A* (1998) 95(24):14447–52. doi:10.1073/pnas.95.24.14447
228. Shao L, Fujii H, Colmegna I, Oishi H, Goronzy JJ, Weyand CM. Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis. *J Exp Med* (2009) 206(6):1435–49. doi:10.1084/jem.20082251
229. Martens PB, Goronzy JJ, Schaid D, Weyand CM. Expansion of unusual CD4+ T cells in severe rheumatoid arthritis. *Arthritis Rheum* (1997) 40(6):1106–14. doi:10.1002/art.1780400615
230. Goronzy JJ, Matteson EL, Fulbright JW, Warrington KJ, Chang-Miller A, Hunder GG, et al. Prognostic markers of radiographic progression in early rheumatoid arthritis. *Arthritis Rheum* (2004) 50(1):43–54. doi:10.1002/art.11445
231. Komocsi A, Lamprecht P, Csernok E, Mueller A, Holl-Ulrich K, Seitzer U, et al. Peripheral blood and granuloma CD4(+)CD28(-) T cells are a major source of interferon-gamma and tumor necrosis factor-alpha in Wegener's granulomatosis. *Am J Pathol* (2002) 160(5):1717–24. doi:10.1016/S0002-9440(10)61118-2
232. McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DR, Willcocks LC, et al. A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med* (2010) 16(5):586–91. doi:10.1038/nm.2130
233. Jemal A, Siegel R, Ward E, Hao YP, Xu JQ, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* (2009) 59(4):225–49. doi:10.3322/caac.20006
234. Kanapuru B, Posani K, Muller D, Ershler WB. Decreased cancer prevalence in the nursing home. *J Am Geriatr Soc* (2008) 56(11). doi:10.1111/j.1532-5415.2008.01864.x
235. Stanta G, Campagner L, Cavalieri F, Giarelli L. Cancer of the oldest old – what we have learned from autopsy studies. *Clin Geriatr Med* (1997) 13(1):55.
236. Ershler WB, Longo DL. Aging and cancer: issues of basic and clinical science. *J Natl Cancer Inst* (1997) 89(20):1489–97. doi:10.1093/jnci/89.20.1489
237. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J Natl Cancer Inst* (2000) 92(7):550–6. doi:10.1093/jnci/92.7.550
238. Jedroszka D, Orzechowska M, Hamouz R, Gorniak K, Bednarek AK. Markers of epithelial-to-mesenchymal transition reflect tumor biology according to patient age and Gleason score in prostate cancer. *PLoS One* (2017) 12(12):e0188842. doi:10.1371/journal.pone.0188842
239. Shariat SF, Sfakianos JP, Droller MJ, Karakiewicz PI, Meryn S, Bochner BH. The effect of age and gender on bladder cancer: a critical review of the literature. *BJU Int* (2010) 105(3):300–8. doi:10.1111/j.1464-410X.2009.09076.x
240. Ferrara F, Schiffer CA. Acute myeloid leukaemia in adults. *Lancet* (2013) 381(9865):484–95. doi:10.1016/S0140-6736(12)61727-9
241. Chian CF, Hwang YT, Terng HJ, Lee SC, Chao TY, Chang H, et al. Panels of tumor-derived RNA markers in peripheral blood of patients with non-small cell lung cancer: their dependence on age, gender and clinical stages. *Oncotarget* (2016) 7(31):50582–95. doi:10.18632/oncotarget.10558
242. Snaebjornsson P, Jonasson L, Jonsson T, Moller PH, Theodors A, Jonasson JG. Colon cancer in Iceland-a nationwide comparative study on various pathology parameters with respect to right and left tumor location and patients age. *Int J Cancer* (2010) 127(11):2645–53. doi:10.1002/ijc.25258
243. Cook MB, Dawsey SM, Freedman ND, Inskip PD, Wichner SM, Quraishi SM, et al. Sex disparities in cancer incidence by period and age. *Cancer Epidemiol Biomarkers Prev* (2009) 18(4):1174–82. doi:10.1158/1055-9965.EPI-08-1118
244. Cook MB, McGlynn KA, Devesa SS, Freedman ND, Anderson WF. Sex disparities in cancer mortality and survival. *Cancer Epidemiol Biomarkers Prev* (2011) 20(8):1629–37. doi:10.1158/1055-9965.EPI-11-0246
245. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* (2018) 68(1):7–30. doi:10.3322/caac.21442
246. Purim O, Gordon N, Brenner B. Cancer of the colon and rectum: potential effects of sex-age interactions on incidence and outcome. *Med Sci Monit* (2013) 19:203–9. doi:10.12659/MSM.883842
247. Yang Y, Li T, Nielsen ME. Aging and cancer mortality: dynamics of change and sex differences. *Exp Gerontol* (2012) 47(9):695–705. doi:10.1016/j.exger.2012.06.009
248. Yang Y, Kozloski M. Sex differences in age trajectories of physiological dysregulation: inflammation, metabolic syndrome, and allostatic load. *J Gerontol A Biol Sci Med Sci* (2011) 66(5):493–500. doi:10.1093/gerona/glr003
249. Barzi A, Lenz AM, Labonte MJ, Lenz HJ. Molecular pathways: estrogen pathway in colorectal cancer. *Clin Cancer Res* (2013) 19(21):5842–8. doi:10.1158/1078-0432.CCR-13-0325
250. Caiazza F, Ryan EJ, Doherty G, Winter DC, Sheahan K. Estrogen receptors and their implications in colorectal carcinogenesis. *Front Oncol* (2015) 5:19. doi:10.3389/fonc.2015.00019
251. DePinho RA. The age of cancer. *Nature* (2000) 408(6809):248–54. doi:10.1038/35041694
252. de Martel C, Franceschi S. Infections and cancer: established associations and new hypotheses. *Crit Rev Oncol Hematol* (2009) 70(3):183–94. doi:10.1016/j.critrevonc.2008.07.021
253. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cell* (2010) 17(1):89–97. doi:10.1016/j.ccr.2009.12.008
254. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* (2010) 140(2):197–208. doi:10.1016/j.cell.2009.12.052
255. Khasawneh J, Schulz MD, Walch A, Rozman J, Hrabe de Angelis M, Klingenspor M, et al. Inflammation and mitochondrial fatty acid beta-oxidation link obesity to early tumor promotion. *Proc Natl Acad Sci U S A* (2009) 106(9):3354–9. doi:10.1073/pnas.0802864106
256. Pal SK, Hurria A. Impact of age, sex, and comorbidity on cancer therapy and disease progression. *J Clin Oncol* (2010) 28(26):4086–93. doi:10.1200/JCO.2009.27.0579
257. Mirandola L, Wade R, Verma R, Pena C, Hosiriluck N, Figueroa JA, et al. Sex-driven differences in immunological responses: challenges and opportunities for the immunotherapies of the third millennium. *Int Rev Immunol* (2015) 34(2):134–42. doi:10.3109/08830185.2015.1018417
258. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med* (2015) 373(19):1803–13. doi:10.1056/NEJMoa1510665
259. Hodi FS. Improved survival with ipilimumab in patients with metastatic melanoma (vol 363, pg 711, 2010). *N Engl J Med* (2010) 363(13):1290. doi:10.1056/NEJMx100063

260. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* (2015) 372(4):320–30. doi:10.1056/NEJMoa1412082
261. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet* (2016) 387(10031):1909–20. doi:10.1016/S0140-6736(16)00561-4
262. Lin PY, Sun LS, Thibodeaux SR, Ludwig SM, Vadlamudi RK, Hurez VJ, et al. B7-H1-dependent sex-related differences in tumor immunity and immunotherapy responses. *J Immunol* (2010) 185(5):2747–53. doi:10.4049/jimmunol.1000496
263. Patel J, Crawford JM. Microbiota-regulated outcomes of human cancer immunotherapy via the PD-1/PD-L1 axis. *Biochemistry* (2018) 57(6):901–3. doi:10.1021/acs.biochem.7b01249
264. Nishijima TF, Muss HB, Shachar SS, Moschos SJ. Comparison of efficacy of immune checkpoint inhibitors (ICIs) between younger and older patients: a systematic review and meta-analysis. *Cancer Treat Rev* (2016) 45:30–7. doi:10.1016/j.ctrv.2016.02.006
265. Ferrara R, Mezquita L, Auclin E, Chaput N, Besse B. Immunosenescence and immunecheckpoint inhibitors in non-small cell lung cancer patients: does age really matter? *Cancer Treat Rev* (2017) 60:60–8. doi:10.1016/j.ctrv.2017.08.003
266. Landre T, Taleb C, Nicolas P, Guetz GD. Is there a clinical benefit of anti-PD-1 in patients older than 75 years with previously treated solid tumour? *J Clin Oncol* (2016) 34(15):3070. doi:10.1200/JCO.2016.34.15_suppl.3070
267. Champiat S, Dercle L, Ammari S, Massard C, Hollebecque A, Postel-Vinay S, et al. Hyperprogressive disease is a new pattern of progression in cancer patients treated by anti-PD-1/PD-L1. *Clin Cancer Res* (2017) 23(8):1920–8. doi:10.1158/1078-0432.CCR-16-1741
268. Bouchlaka MN, Murphy WJ. Impact of aging in cancer immunotherapy the importance of using accurate preclinical models. *Oncoimmunology* (2013) 2(12):e27186. doi:10.4161/onci.27186
269. Clayton JA. Applying the new SABV (sex as a biological variable) policy to research and clinical care. *Physiol Behav* (2018) 187:2–5. doi:10.1016/j.physbeh.2017.08.012

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OPINION
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Sex Hormones Determine Immune Response

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Keywords: sex hormones, autoimmune diseases, X-linked genetic disease, immune system, TLRs (toll-like receptors)

Females and males differ in the energy consumption and nutritional requirements which are based on the interactions between environmental factors and sex hormones (1). The studies in early 1940s ascertained that females have enhanced capability of producing antibodies (2, 3). This enhanced immune reactivity in females helps mount an effective resistance to infection and therefore females are less susceptible to viral infections, but can develop immune-pathogenic effects and predisposition to autoimmunity due to hyper immune responses (4, 5). Sex hormones can also control the immune response via circadian rhythm. Many hormones like cortisol, known to regulate T cell mediated inflammation, have a circadian rhythm with a maximum peak at 8:00 a.m. and progressively lower levels as the day progresses (6). Interaction between sex hormones and environmental factors like cigarette smoke and infections lead to variable responses in both genders (5, 7, 8). There is emerging evidence that sex hormones impact microbial composition and the resulting immune response via secondary metabolites binding with receptors like estrogen receptors (ERs), peroxisome proliferator-activated receptors (PPARs) etc. (9). These differences in immune response can lead to variability in disease phenotypes with autoimmunity occurring more often in females and cancers occurring more in males (**Figure 1**).

SEX HORMONES AND IMMUNITY

Conserved pathogen-associated molecular patterns (PAMPs) of microbes can bind various pathogen recognition receptors like toll like receptors (TLRs). Since TLR expression differs between sexes, TLR3, 7, and 9 are expressed higher in females and TLR2 and TLR4 in males, it can influence strength of TLR-dependent responses. Macrophages from male mice generate a higher TLR4- and TLR2-dependent Th1 response to clear infections, while estrogen regulates immune response via modulation of endosomal TLRs and TLR8 expression thus hormonal balance determines the overall response in females (10–13). TLR3, 7, and TLR9 recognize viral RNA or DNA while TLR2 and TLR4 are known to bind bacterial cell wall proteins. In humans, mononuclear cells from men produce lower levels of type I IFNs in response to TLR7 ligands and higher IL-10 in response to TLR9 ligands as compared to females (14, 15). The differential immune response may also be associated with the differences in immune cell populations between sexes. CD4 and CD8 cells decline during aging in both sexes, though aged women showed lower NK cells and memory Tregs as compared to aged men (16, 17), which may partially explain the sex-biased immune response and cytokine production. Immune responses to environmental factors like infections and vaccinations are also sex-biased (18). Women maintain a high immune reactivity post-viral infections (2). Since females generate higher antibody responses, vaccinations also result in higher antibody levels in women than men and provide efficient protection (19); however this can lead to

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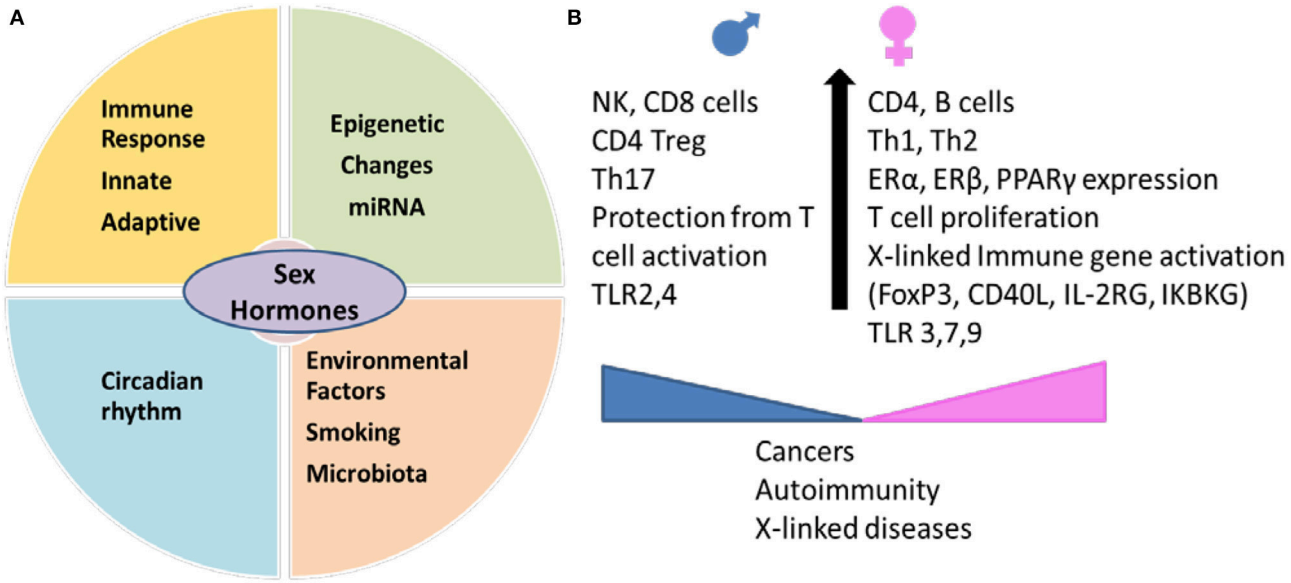

FIGURE 1 | Sex hormones interact with genetic and environmental factors and determine immunity in an individual. **(A)** Environmental factors like smoking and gut microbiome generate sex-hormones dependent immunity leading to differences in circadian rhythm, innate and adaptive immune response and epigenetic changes between males and females. Sexual dimorphism between miRNA expression contributes to sex-specific regulation of function in various tissues. **(B)** Sex determines expression of cell markers involved in innate and adaptive immunity. Females have higher expression of genes on X chromosome which include immune markers like regulatory marker FoxP3, CD40L. Females produce higher Th2 response and antibodies and better protection from infections but the hyperimmune response makes them susceptible to autoimmune diseases. Males generate more of Th17 response and are less likely to develop autoimmunity but have higher percent of non-reproductive cancers. miRNA, microRNA; TLR, Toll like receptor; ER, estrogen receptor; PPAR, peroxisome proliferator-activated receptor; IKBKG, inhibitor of nuclear factor kappa B kinase; IL2RG, interleukin receptor subunit gamma.

worse side effects than men due to enhanced immune reactivity. This augmented immune response can perpetuate and precipitate inflammation in many ways including bystander effect, production of pro-inflammatory cytokines and if antigen shares mimicry with a self-protein, an autoimmune response.

Sexual dimorphism in immunity has been described in both arms of immunity, innate and adaptive (20). Generally testosterone has an immunosuppressive effect while estrogen has an immunoenhancing effect on the immune system. Estrogen has been shown to regulate immune response by impairing negative selection of high affinity auto-reactive B cells, modulating B cell function and leading to Th2 response (21, 22). Estrogen influences physiological functions via ERs which are expressed in brain, gut epithelial cells, lymphoid tissue cells as well as immune cells (23, 24). Estrogen also induces T cell homing by enhancing the expression of CCR5, a homing marker (25). Based on the relative numbers of various immune cells in males and females, overall immune response is sexually dimorphic and determines pathogenicity (**Figure 1**). On the other hand, immune regulation by androgens such as testosterone impacts the immune system by augmenting Th1 response and activation of CD8 cells while down-regulating natural killer (NK) cells response, tumor necrosis factor-alpha (TNFα) and increasing the production of anti-inflammatory IL-10 (26, 27). This is supported by studies showing that *in vitro* presence of testosterone leads to a higher production of Th1 by peripheral blood cells with a higher Th1:Th2 ratio in men (28, 29). The dichotomy of sex-specific response was shown in a humanized mouse model of inflammation where exogenous supply of estradiol and castration in male mice led to an increase in autoimmunity (30) by augmenting Major Histocompatibility Complex II (MHCII) expression and modulating B cells function (31). B cells are targets for treatment in many diseases including rheumatoid arthritis (RA), lupus and multiple Sclerosis (MS). Depletion of B cells in ongoing arthritis in female mice showed higher efficacy as compared to males (32). Similar observations were reported in patients treated with Rituximab where women achieved remission more frequently than men (33). A predominant role of sex hormones has been suggested as the main cause of sex-biased autoimmune diseases like RA and MS (5). Remission of RA and lupus during pregnancy further support a role of female sex hormones in immune response. Although consideration of patient's sex for treatment is not a practice, sex differences in immune response suggest that sex-based treatments would be optimal.

Recently, evidence has emerged on the critical role played by environmental factors like smoking and the gut microbiota in controlling immune responses locally as well as systemically. Gut microbial composition is influenced by many factors including genetic, diet and sex hormones (34–36). Sex-dependent effects of diet were shown on the gut microbial composition in two fish populations (37). In humans, diet-based effects on

the microbiome were much more prominent in men than women (38, 39); suggesting diet can further influence sex-bias immune responses by impacting colonic ecosystem. In a study in 1998, women treated with hormonal contraceptives for 3 weeks showed an increase in *Prevotella* species suggesting a direct role of hormones on the gut microbiota (40). The lower abundance of *Prevotella* and *Bacteroides* in females compared to males further supports sex-dependent differences in microbial composition (41), which impact intestinal and systemic immune responses. Metabolites generated by the gut commensals can bind epithelial cells and other immune cells via ERs and PPARs that are expressed differentially in both sexes (42). There is compelling evidence that sex hormones regulate the hippocampal serotonergic system of the gut-brain axis in a sexually dimorphic manner (43). The gut microbiota can impact systemic levels of testosterone via 17β reduction of androgen (44–46) consequently changing the intestinal metabolic landscape. Evidence for this was demonstrated in an experimental model of diabetes where females were protected from diabetes when microbiota from male mice was transferred, which was dependent on an increase in the testosterone levels (47). There is limited information on the mechanism by which microbiome-derived sex steroids impact host immunity. One can speculate that the interaction of sex hormones with environmental factors as well as epigenetic changes caused by the microbiota determine the immune response by cells of innate and adaptive immune cells and the overall sex-biased difference in immune-mediated cytokine responses.

GENETIC FACTORS IN SEXUAL DIMORPHIC IMMUNITY

Gene diversity or dosage may be one of the factors that can explain the sex-bias in immune responses and female predominance of autoimmune diseases. Women carry two copies of X chromosome, one of which is randomly transcriptionally inactivated while men have only one X. Many genes on X chromosome are associated with regulation of immune functions; IL-2R γ chain, IL-3R α chain, IL-13 α chain, IL-1R associated kinase 1 (IRAK1) TLR7, GATA1, FOXP3, and CD40L. It is surmised that skewed inactivation, mutations or under certain physiological conditions, approximately 10–15% of these genes may be activated (48, 49). In females, maternal or paternal X chromosome inactivation in different cell types combined with the fact that X chromosomes have genes associated with immune functions, it is reasonable to assume that some of these genes may be involved in sex-biased abnormalities in immune responses. X chromosome involvement in sex-bias immunity is supported by the inherited disorders such as Klinefelter with XXY in males and Turner syndrome with XO in females, both with hormonal and immune abnormalities (50). The X chromosome also contains 10% of the microRNA (miRNA) in the human genome as compared to 2 miRNA on the Y chromosome (51, 52). The X-linked miRNAs have also been shown to contribute to sex differences in immune responses, leading to much higher responses in females.

Sex steroid levels change rapidly for women when they are menopausal while in males the change is gradual. While aging is associated with changes in immune cells in both sexes (53), in women heightened immune response and accumulation of antibodies over a period can cause a low grade inflammation which can predispose to sex-bias in inflammatory diseases. MHC molecules present antigens from pathogens and generate immune response. While testosterone has been suggested to decrease the MHC II expression on DCs, estrogen increases the expression (54). As DCs are important for generation of immune responses and T cell differentiation, it may determine the quantitative as well-specific TH cytokines in a sex-specific manner. Thus, even in the presence of similar MHC II, women pay the price of higher incidence of sex-biased diseases but generate a superior response to infections. Interestingly, sex-specific immune response by MHCII molecules in humanized mice showed that males generated higher response to antigens presented by HLA-DQ alleles while females showed higher immune response to HLA-DR-presented antigens (30, 32). HLA-DR and DQ molecules select T cells with different cytokine producing abilities which may dictate the sexually-dimorphic immune response (4). Differential upregulation of MHC expression and antigen presentation leading to differential cytokines milieu in both sexes will determine the outcome of infections and diseases.

Besides the known inherited genes, there is some evidence that non-inherited maternal antigens (NIMA) that are not encoded by the offspring but passed along from the mother may have a role in sex-biased immune response. However, the role of NIMA in various diseases has not been consistent (55). The strongest association for NIMA was observed in RA patients negative for RA-susceptible HLA alleles (56). Besides NIMA, the presence of allogeneic male fetal cells (Fetal microchimerism) in women may also be involved in generating immune response. Although the data is not consistent in most diseases, studies in MS and systemic sclerosis provide some evidence that it is a possibility (57, 58).

The reason why sex-bias immunity exists may lie in the evolution and preservation of mankind. Evolutionarily, during reproductive years, an enhanced response to infections should help maintain health for reproduction. In aged women, reproductive function is not required, enhanced immune reactivity along with changes in immune cells during aging causes sex-specific differences in immunity. The sex-specific expression of genes may explain why women with a similar genetic background show higher immune reactivity or develop autoimmunity at a higher rate than men. Also, the circadian rhythm of sex-hormone-dependent immune system and microbiome could control metabolic profile of an individual. Microbial-metabolites are involved in various signaling pathways as well as immune pathways like differentiation of T cells via binding to receptors of gut immune cells and epithelium. Similar functions also occur in other tissues. Thus, combined with variable X inactivation in cells and pleiotropic nature of many

genes, it is likely that sex-hormones impact immune system and its ability to break tolerance to pathogens, environmental or endogenous. Although there is a plethora of evidence suggesting a sex-bias in innate and adaptive immunity that can impact response to infections, vaccinations and onset of various diseases, there is no consensus on treating diseases based on the sex of a patient. The challenge is to be able to define the role of a single receptor or hormone in humans. Animal models have provided some information though more research is required to define the pathways that determine sex-specific immune response during inflammation.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REFERENCES

1. Wu BN, O'Sullivan AJ. Sex differences in energy metabolism need to be considered with lifestyle modifications in humans. *J Nutr Metab*. (2011) 2011:391809. doi: 10.1155/2011/391809
2. Butterworth M, McClellan B, Allansmith M. Influence of sex in immunoglobulin levels. *Nature* (1967) 214:1224–5.
3. Von Haam E, Rosenfeld I. The effect of estrone on antibody-production. *J Immunol.* (1942) 43:109–117.
4. Mangalam AK, Taneja V, David CS. HLA class II molecules influence susceptibility versus protection in inflammatory diseases by determining the cytokine profile. *J Immunol*. (2013) 190:513–8. doi: 10.4049/jimmunol.1201891
5. Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. *Front Neuroendocrinol*. (2014) 35:347–69. doi: 10.1016/j.yfrne.2014.04.004
6. Sherman B, Wysham C, Pfohl B. Age-related changes in the circadian rhythm of plasma cortisol in man. *J Clin Endocrinol Metab*. (1985) 61:439–43.
7. Sugiyama D, Nishimura K, Tamaki K, Tsuji G, Nakazawa T, Morinobu A, et al. Impact of smoking as a risk factor for developing rheumatoid arthritis: a meta-analysis of observational studies. *Ann Rheum Dis.* (2010) 69:70–81. doi: 10.1136/ard.2008.096487
8. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. *J Immunol.* (2017) 198:1782–90. doi: 10.4049/jimmunol.1601166
9. Park HJ, Choi JM. Sex-specific regulation of immune responses by PPARs. *Exp Mol Med*. (2017) 49:e364. doi: 10.1038/emm.2017.102
10. Marriott I, Bost KL, Huet-Hudson YM. Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: a possible mechanism for gender-based differences in endotoxic shock susceptibility. *J Reprod Immunol.* (2006) 71:12–27. doi: 10.1016/j.jri.2006.01.004
11. Garcia-Gomez E, Gonzalez-Pedrajo B, Camacho-Arroyo I. Role of sex steroid hormones in bacterial-host interactions. *Biomed Res Int*. (2013) 2013:928290. doi: 10.1155/2013/928290
12. Young NA, Wu L-C, Burd CJ, Friedman AK, Kaffenberger BH, Rajaram MV, et al. Estrogen modulation of endosome-associated toll-like receptor 8: an IFNalpha-independent mechanism of sex-bias in systemic lupus erythematosus. *Clin Immunol.* (2014) 151:66–77. doi: 10.1016/j.clim.2014.01.006
13. Roberts BJ, Dragon JA, Moussawi M, Huber SA. Sex-specific signaling through Toll-like receptors 2 and 4 contributes to survival outcome of coxsackievirus B3 infection in C57Bl/6 mice. *Biol Sex Differ*. (2012) 3:25. doi: 10.1186/2042-6410-3-25
14. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences in the Toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. *Nat Med.* (2009) 15:955–9. doi: 10.1038/nm.2004
15. Torcia MG, Nencioni L, Clemente AM, Civitelli L, Celestino I, Limongi D, et al. Sex differences in the response to viral infections: TLR8 and TLR9 ligand stimulation induce higher IL10 production in males. *PLoS ONE* (2012) 7:e39853. doi: 10.1371/journal.pone.0039853
16. Hirokawa K, Utsuyama M, Hayashi Y, Kitagawa M, Makinodan T, Fulop T. Slower immune system aging in women versus men in the Japanese population. *Immun Ageing* (2013) 10:19. doi: 10.1186/1742-4933-10-19
17. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GS, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. *Cytometry* (1996) 26:8–15. doi: 10.1002/(SICI)1097-0320(19960315)26:1<8::AID-CYTO2>3.0.CO;2-E
18. Ruggieri A, Anticoli S, D'Ambrosio A, Giordani L, Viora M. The influence of sex and gender on immunity, infection and vaccination. *Ann Ist Super Sanita* (2016) 52:198–204. doi: 10.4415/ANN_16_02_11
19. Klein SL, Marriott I, Fish EN. Sex-based differences in immune function and responses to vaccination. *Trans R Soc Trop Med Hyg.* (2015) 109:9–15. doi: 10.1093/trstmh/tru167
20. Markle JG, Fish EN. SeXX matters in immunity. *Trends Immunol.* (2014) 35:97–104. doi: 10.1016/j.it.2013.10.006
21. Cutolo M, Capellino S, Sulli A, Serioli B, Secchi ME, Villaggio B, et al. Estrogens and autoimmune diseases. *Ann N Y Acad Sci.* (2006) 1089:538–47. doi: 10.1196/annals.1386.043
22. Grimaldi CM, Jeganathan V, Diamond B. Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *J Immunol.* (2006) 176:2703–10. doi: 10.4049/jimmunol.176.5.2703
23. Klein SL. Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunol.* (2004) 26:247–64. doi: 10.1111/j.0141-9838.2004.00710.x
24. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev.* (2000) 24:627–38. doi: 10.1016/S0149-7634(00)00027-0
25. Mo R, Chen J, Grolleau-Julius A, Murphy HS, Richardson BC, Yung RL. Estrogen regulates CCR gene expression and function in T lymphocytes. *J Immunol.* (2005) 174:6023–9. doi: 10.4049/jimmunol.174.10.6023
26. Gleicher N, Barad DH. Gender as risk factor for autoimmune diseases. *J Autoimmun.* (2007) 28:1–6. doi: 10.1016/j.jaut.2006.12.004
27. Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunol Med Microbiol.* (2003) 38:13–22. doi: 10.1016/S0928-8244(03)00202-5
28. Bouman A, Schipper M, Heineman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. *Am J Reprod Immunol.* (2004) 52:19–26. doi: 10.1111/j.1600-0897.2004.00177.x
29. Giron-Gonzalez JA, Moral FJ, Elvira J, Garcia-Gil D, Guerrero F, Gavilan I, et al. Consistent production of a higher TH1:TH2 cytokine ratio by stimulated T cells in men compared with women. *Eur J Endocrinol.* (2000) 143:31–6. doi: 10.1530/eje.0.1430031
30. Behrens M, Trejo T, Luthra H, Griffiths M, David CS, Taneja V. Mechanism by which HLA-DR4 regulates sex-bias of arthritis in humanized mice. *J Autoimmun.* (2010) 35:1–9. doi: 10.1016/j.jaut.2009.12.007
31. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest.* (2002) 109:1625–33. doi: 10.1172/JCI0214873
32. Behrens M, Luckey D, Luthra H, David C, Taneja V. B cells influence sex specificity of arthritis via myeloid suppressors and chemokines in humanized mice. *Clin Immunol.* (2017) 178:10–9. doi: 10.1016/j.clim.2015.05.015
33. Couderc M, Gottenberg JE, Mariette X, Pereira B, Bardin T, Cantagrel A, et al. Influence of gender on response to rituximab in patients

with rheumatoid arthritis: results from the Autoimmunity and Rituximab registry. *Rheumatology (Oxford)* (2014) 53:1788–93. doi: 10.1093/rheumatology/keu176
34. Gomez A, Luckey D, Taneja V. The gut microbiome in autoimmunity: sex matters. *Clin Immunol.* (2015) 159:154–62. doi: 10.1016/j.clim.2015.04.016
35. Marietta E, Rishi A, Taneja V. Immunogenetic control of the intestinal microbiota. *Immunology* (2015) 145:313–22. doi: 10.1111/imm.12474
36. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* (2011) 334:105–8. doi: 10.1126/science.1208344
37. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, et al. Individual diet has sex-dependent effects on vertebrate gut microbiota. *Nat Commun.* (2014) 5:4500. doi: 10.1038/ncomms5500
38. Leone VA, Cham CM, Chang EB. Diet, gut microbes, and genetics in immune function: can we leverage our current knowledge to achieve better outcomes in inflammatory bowel diseases? *Curr Opin Immunol.* (2014) 31:16–23. doi: 10.1016/j.coi.2014.08.004
39. Nielsen DS, Krych L, Buschard K, Hansen CH, Hansen AK. Beyond genetics. Influence of dietary factors and gut microbiota on type 1 diabetes. *FEBS Lett.* (2014) 588:4234–43. doi: 10.1016/j.febslet.2014.04.010
40. Klinger G, Eick S, Pfister W, Graser T, Moore C, Oettel M. Influence of hormonal contraceptives on microbial flora of gingival sulcus. *Contraception* (1998) 57:381–4.
41. Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, et al. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol.* (2006) 72:1027–33. doi: 10.1128/AEM.72.2.1027-1033.2006
42. Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, et al. Peroxisome proliferator-activated receptor (PPAR)alpha and-gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way. *Proc Natl Acad Sci USA.* (2012) 109:9505–10. doi: 10.1073/pnas.1118458109
43. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* (2013) 18:666–73. doi: 10.1038/mp.2012.77
44. Adlercreutz H, Pulkkinen MO, Hamalainen EK, Korpela JT. Studies on the role of intestinal bacteria in metabolism of synthetic and natural steroid hormones. *J Steroid Biochem.* (1984) 20:217–29.
45. Hughes DT, Sperandio V. Inter-kingdom signalling: communication between bacteria and their hosts. *Nat Rev Microbiol.* (2008) 6:111–20. doi: 10.1038/nrmicro1836
46. Ridlon JM, Ikegawa S, Alves JM, Zhou B, Kobayashi A, Iida T, et al. Clostridium scindens: a human gut microbe with a high potential to convert glucocorticoids into androgens. *J Lipid Res.* (2013) 54:2437–49. doi: 10.1194/jlr.M038869
47. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* (2013) 339:1084–8. doi: 10.1126/science.1233521
48. Rubtsov AV, Rubtsova K, Kappler JW, Marrack P. Genetic and hormonal factors in female-biased autoimmunity. *Autoimmun Rev.* (2010) 9:494–8. doi: 10.1016/j.autrev.2010.02.008
49. Quintero OL, Amador-Patarroyo MJ, Montoya-Ortiz G, Rojas-Villarraga A, Anaya JM. Autoimmune disease and gender: plausible mechanisms for the female predominance of autoimmunity. *J Autoimmun.* (2012) 38:J109–19. doi: 10.1016/j.jaut.2011.10.003
50. Abramowitz LK, Olivier-Van Stichelen S, Hanover JA. Chromosome imbalance as a driver of sex disparity in disease. *J Genomics* (2014) 2:77–88. doi: 10.7150/jgen.8123
51. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *Bioessays* (2011) 33:791–802. doi: 10.1002/bies.201100047
52. Dai R, McReynolds S, Leroith T, Heid B, Liang Z, Ahmed SA. Sex differences in the expression of lupus-associated miRNAs in splenocytes from lupus-prone NZB/WF1 mice. *Biol Sex Differ.* (2013) 4:19. doi: 10.1186/2042-6410-4-19
53. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and age-related diseases: role of inflammation triggers and cytokines. *Front Immunol.* (2018) 9:586. doi: 10.3389/fimmu.2018.00586
54. Kovats S, Carreras E. Regulation of dendritic cell differentiation and function by estrogen receptor ligands. *Cell Immunol.* (2008) 252:81–90. doi: 10.1016/j.cellimm.2007.10.008
55. Bronson PG, Komorowski LK, Ramsay PP, May SL, Noble J, Lane JA, et al. Analysis of maternal-offspring HLA compatibility, parent-of-origin effects, and noninherited maternal antigen effects for HLA-DRB1 in systemic lupus erythematosus. *Arthritis Rheum.* (2010) 62:1712–7. doi: 10.1002/art.27426
56. Harney S, Newton J, Milicic A, Brown MA, Wordsworth BP. Non-inherited maternal HLA alleles are associated with rheumatoid arthritis. *Rheumatology (Oxford)* (2003) 42:171–4. doi: 10.1093/rheumatology/keg059
57. Nelson JL, Furst DE, Maloney S, Gooley T, Evans PC, Smith A, et al. Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *Lancet* (1998) 351:559–62. doi: 10.1016/S0140-6736(97)08357-8
58. Willer CJ, Herrera BM, Morrison KME, Sadovnick AD, Ebers GC, Canadian Collaborative Study on Genetic Susceptibility to Multiple S. Association between microchimerism and multiple sclerosis in Canadian twins. *J Neuroimmunol.* (2006) 179:145–51. doi: 10.1016/j.jneuroim.2006.06.011

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Glucocorticoids, Sex Hormones, and Immunity

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Glucocorticoid hormones regulate essential body functions in mammals, control cell metabolism, growth, differentiation, and apoptosis. Importantly, they are potent suppressors of inflammation, and multiple immune-modulatory mechanisms involving leukocyte apoptosis, differentiation, and cytokine production have been described. Due to their potent anti-inflammatory and immune-suppressive activity, synthetic glucocorticoids (GCs) are the most prescribed drugs used for treatment of autoimmune and inflammatory diseases. It is long been noted that males and females exhibit differences in the prevalence in several autoimmune diseases (AD). This can be due to the role of sexual hormones in regulation of the immune responses, acting through their endogenous nuclear receptors to mediate gene expression and generate unique gender-specific cellular environments. Given the fact that GCs are the primary physiological anti-inflammatory hormones, and that sex hormones may also exert immune-modulatory functions, the link between GCs and sex hormones may exist. Understanding the nature of this possible crosstalk is important to unravel the reason of sexual disparity in AD and to carefully prescribe these drugs for the treatment of inflammatory diseases. In this review, we discuss similarities and differences between the effects of sex hormones and GCs on the immune system, to highlight possible axes of functional interaction.

Keywords: glucocorticoids, sex hormones, innate immunity, adaptive immunity, glucocorticoid receptor, estrogen receptor alpha

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INTRODUCTION

The interaction between endocrine and immune systems ensures the correct function of immune system. Women mount stronger immune responses against foreign but also against self-antigens, and the prevalence of most autoimmune diseases (AD) is greatly increased in women compared to men (1–4). An important role underlying the difference in activity of immune cells in men and women is attributed to sex hormones (4, 5).

Steroid hormones, such as estrogens, prolactin, progesterone, and glucocorticoids (GCs) modulate the development and activity of both innate and adaptive immunity differently in men and women (2, 5–8). Therefore, characterization of the mechanisms of hormonal regulation of different immune cell types is important for understanding the regulatory circuits critical for keeping a competent and a healthy immune system and to improve therapy of AD.

The degree and the duration of the immune response is influenced by the number and the type of circulating immune cells; therefore, the effect of steroid hormones on survival and differentiation of T and B lymphocytes and cells of innate immune system will define the numeric leukocyte output in the periphery. Hormonal regulation of cytokine production impacts on differentiation of naïve T cells into the particular effector subtypes, thus defining the type of the mounted immune responses.

Interestingly, since sex hormones and GCs are acting on the same cellular pathways that regulate leukocytes growth, differentiation, and survival, their simultaneous action would likely enhance or abrogate the effects elicited by individual factors. Therefore, biologic differences in endogenous GCs levels, as well as exposure of an individual to specific environmental stimuli, including presence of chronic inflammatory disease, prolonged stress, metabolic challenges or injuries, as well as pharmacologic administration of exogenous GCs, will alter the expected gender-related effects on immunity and ADs. The converging and diverging effects of GCs and sex hormones on different cells types of the immune system are discussed in this minireview.

STEROID HORMONES: MECHANISMS OF ACTION

Corticosteroids and sex hormones are derived from cholesterol through the same sterodoigenic pathway, with common metabolic intermediate, progesterone, and are under the control of the hypothalamus–pituitary–adrenal gland (HPA) axis (9). The main natural GCs (i.e., cortisol) are produced in the cortical part of the adrenal gland (9). Biosynthesis of androgens, including testosterone, occurs mainly in Leydig cells in male gonads, and small amounts are produced by the ovary and adrenal cortex in females (9). The androgens dehydroepiandrosterone (DHEA), androstenedione, and testosterone are the precursors of estrogens, produced in females primarily in ovaries (9).

Glucocorticoids

Glucocorticoids are essential endocrine regulators of body functions in homeostasis and adaptation to environmental changes. One important feature of GCs regulation is the circadian control of GCs secretion by the HPA axis. The rhythmically released GCs may have an impact on immunity regulation (10). Endogenous GCs act on a variety of cell types to regulate the expression of genes controlling cellular metabolism, growth, differentiation, and apoptosis (11, 12). Thus, proper production and activity of the endogenous GCs is critical for the regulation of inflammatory events during tissue repair and pathogens elimination. Due to their potent immune-suppressive and anti-inflammatory function, synthetic GCs are extensively used in clinic to treat acute and chronic inflammation (13, 14).

The GCs act *via* genomic (transcriptional) and non-genomic (transcription-independent) mechanisms. Most cellular actions of GCs are mediated by binding to its cognate intracellular receptor (GR), transcription factor of the nuclear receptors (NR) superfamily (15). GR shares functional domains with other NR that include an N-terminal transactivation domain, a highly conserved central DNA binding domain, and a C-terminal ligand-binding domain (11). After ligand-induced conformational changes, GR translocates into the nucleus where it regulates transcription of hundreds of genes. It may bind directly to DNA *via* glucocorticoid recognition elements, or regulate gene expression *via* indirect mechanisms (16, 17). GR may directly interact with NF-κB (17, 18), a key transcription factor that activates many pro-inflammatory genes (19), as well as with other transcription factors (TFs), such as STAT-3 and -5 (20, 21), AP-1 (22), and CREB (23). GR does not interfere with the DNA binding activity of NF-κB, but inhibits its transcriptional activation function *via* preventing its nuclear translocation (17, 18), or interfering with transcriptional machinery by competition with co-factors such as p300/CBP (24), thus repressing the expression of pro-inflammatory genes, such as TNF-α, IL-1, and ICAM-1 (13, 25). Mechanisms of GCs actions also include induction of proteins with anti-inflammatory activities, such as glucocorticoid-induced leucine zipper (GILZ) (26, 27), which mediates many of the GCs' activities (28–31), including inhibition of RAS/RAF/MAPK pathways (32, 33), and of NF-κB and AP-1 activities (34–37). "Non-genomic" effects of GCs include direct interaction of liganded GR with diverse intracellular mediators and modulating several signaling pathways, including protein kinase C, phosphatidylinositol-specific phospholipase C, and src kinase pathways (38–42).

Sex Hormones

Sex hormones regulate reproductive and metabolic body functions throughout the life of the subjects. Sex hormones influence immune cell function and inflammation: androgens are mainly anti-inflammatory (7), whereas estrogens have both pro- and anti-inflammatory roles, depending on several factors, such as type of immune response or variability of expression of different estrogen receptor (ER) isoforms (8).

Estrogens exert their effects through binding to ERα or ERβ, TFs of NR superfamily that regulate expression of genes involved in cell survival, proliferation, differentiation, and reproductive functions (6, 43). Similar to GR, nuclear ERs bind DNA either directly through estrogen response elements, or indirectly, *via* ERE-independent TFs, such as NF-κB, SP1, AP-1, C/EBPβ (43–45), to induce or repress gene expression. Estrogens also elicit rapid ("non-genomic") signal transduction effects, *via* modulation of intracellular calcium, cAMP, potassium currents, phospholipase C activation, and stimulation of PI3K/AKT and ERK pathways (44).

Estrogen receptors are expressed in various types of immune cells, including lymphocytes, macrophages, and dendritic cells (DC) (5, 8). Estrogens were shown to exert both, anti- and pro-inflammatory effects, depending on the context and combination of factors that include: the type of the immune cell target, the concentration of the hormone, the type of immune stimulus (foreign or auto- antigens), the target organ microenvironment, and the relative expression of ERα and ERβ. Estrogens may promote inflammation *via* regulation of the expression of inflammatory mediators *via* Akt/mTOR pathway (46, 47). However, pregnancy or higher doses of ectopic estrogens typically suppress immune responses (4), by repressing the expression of multiple NF-κB- and c-Jun-driven cytokine genes (45, 48–50), similar to GCs. ERα may displace p65 and CREB and their associated co-regulators from NF-κB binding site (51). Progesterone (P4) is produced at high levels during the menstrual cycle and during pregnancy. P4 signals through the progesterone receptor (PR) and to a lesser extent, through GR and mineralocorticoid receptors. P4 is expressed in different immune cells types, including NK, macrophages, DCs, and T cells (52), and have broad anti-inflammatory effects of the immune system by decreasing leukocytes activation and

production of pro-inflammatory mediators (5). NF-κB inhibition is also suggested to play a role in these effects of P4 (53).

Similar to GCs, male steroid hormones demonstrate mostly the suppressive role in immune function (2, 54–57), *via* binding to androgen receptor (AR), also a member of NR superfamily, and regulation of target gene expression (58). AR recognizes directly the androgen response elements in the regulatory regions of AR target genes (59). Androgens, including dihydrotestosterone and testosterone, generally suppress immune cell activity, by reducing the inflammatory and promoting anti-inflammatory mediators' expression by macrophages and T cells (5, 60–62). The levels of androgen DHEA are reduced in patients with RA (63) and inflammatory bowel disease (64) suggesting that DHEA may cover many aspects of immune regulatory effects of sex hormones.

Moreover, an indirect immunomodulatory effect of androgens may be related to the anti-inflammatory activity of endogenous GCs due to their effect on the HPA axis (65).

MODULATION OF IMMUNE RESPONSES BY STEROID HORMONES

Effect of Steroid Hormones on Innate Immunity

Both GCs and sex steroid modulate the development and function of various cells of innate immunity, including neutrophils, macrophages, natural killer cells, and DC (**Table 1**). GCs actions include regulation of apoptosis in many cell types: they exert a protective effect in macrophages, by inhibiting activation of caspases and contributing to inflammation resolution (66); however, prolonged usage of GCs promotes apoptosis in macrophages (67), natural killer cells (68), DC (69–71), neutrophils (72, 73), and eosinophils (72). To the contrary, estrogens and androgens increase the number of neutrophils (74, 75).

Glucocorticoids have mainly suppressive effects on the cells of innate immunity (**Table 1**). High doses of GCs inhibit most of the functions of tissue macrophages, such as chemotaxis, phagocytosis, proliferation, and antigen presentation (67, 76). GCs suppress the expression of various pro-inflammatory cytokines released by macrophages, such as IFN-γ, IL-1α, and IL-1β (76). In addition, the synthesis of anti-inflammatory molecules annexin A1 and GILZ in macrophages contributes to the anti-inflammatory and immunosuppressive action of GCs (28, 29, 77). GCs also inhibit monocytes' chemotaxis by reducing expression of chemokines, such as CXCL-1, IL-8 and CXCL-2, and CCL2 (78, 79), control granulocyte trafficking by reducing expression of adhesion molecules, such as Mac-1 and L-selectin on neutrophils (80–82), thus limiting the inflammatory response. In addition, GCs prevent neutrophils migration into inflamed tissues *via* the upregulation of GILZ and annexin A1 (82). Similar to GCs, treatment with estrogens inhibits neutrophils' activity by restricting their recruitment (83–88) and inhibiting the NFκB-dependent production of major neutrophil chemoattractants CXCL-1, CXCL2, CXCL3, and CXCL8 in experimental models of tissue injury (86–91). Estradiol inhibits neutrophil activation through a reduction in oxidative metabolism (92), adhesion to endothelial cells *via* upregulation of the anti-inflammatory protein annexin A1 (93), and attenuates the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in human peripheral blood mononuclear cells (94, 95) and in neutrophils and macrophages (96–99). Interestingly, the inhibition of NFκB activity is a central mechanism underlying these actions (83).

Glucocorticoids and estrogens have both convergent and divergent actions in DCs. GCs inhibit DC function in several ways: by promoting apoptosis (76), disturbing maturation of immature DCs (100), and inducing a tolerogenic phenotype, *via* downregulating the expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules and cytokines, such as IL-1, IL-6, and IL-12 (101, 102). Such changes are associated with reduced proliferative and T helper 1 (Th1) responses by T cells (103), and increase in immunosuppressive regulatory T (Treg) cells (101). Instead, estrogens promote DC cell differentiation and MHCII expression, and induce the expression

TABLE 1 | Effect of steroid hormones on innate and adaptive immunity.

Immune target	Glucocorticoids	Estrogens	Androgens
Macrophages	High dose, activity ↓ (67, 76) Apoptosis ↓↑ (66, 67) Inflamm cytokines ↓ (76)	Inflamm cytokines↓ (94, 95)	Inflamm cytokines ↓ (5)
DC	Apoptosis ↑ (76) Maturation ↓ (100) Inflamm cytokines↓ (101–103) IL-10, TGF-β ↑ (101–103)	Maturation ↑ (107) Inflamm cytokines↑↓ (104–107) IL-10, TGF-β ↑ (109–112)	ND
Neutrophils	Number ↓ (72, 73) Trafficking ↓ (80–82) Apoptosis ↑ (72, 73)	Number ↑ (74, 75) Trafficking ↓ (83–88) Activation ↓ (92, 93) Inflamm cytokines↑↓ (96–99)	Number ↑ (62) Trafficking ↑ (62) Inflamm cytokines↑
Thymocytes	Apoptosis ↑ (38, 39, 113–116) Proliferation ↓ (13)	Proliferation ↓ (123–126)	Proliferation ↓ (127, 128)
Th1 cells	Apoptosis ↑ (130) Th1 cytokines ↓ (131–133)	Activation ↓ Th1 cytokines ↑ (149–153) High levels ↓ (155–157)	Activation ↓ Th1 cytokines ↓ (60)
Th2 cells	Apoptosis ↑↓ Th2 cytokines ↑	Th2 cytokines↑ (155–158)	Activation ↓ Th2 cytokines ↓↑ (5, 60)
Th17 cells	Apoptosis ↑↓ (135) Th17 cytokines ↓ (134)	Number ↓ (161) Th17 cytokines ↑↓ (159–161)	Number ↓ Th17 cytokines ↑ (5)
Treg cells	Number ↑ (136–140) Function ↑ (101, 141–143)	Number ↑ (162–166)	Number ↑ (5)
B cells	Apoptosis ↑ (167, 168) Number ↓ Ig ↓↑ (171–173)	Apoptosis↓ (175–177) Ig ↑ (5)	Number ↓ (5, 62) Ig ↓ (5)
ILCs	ILC2 ↓ (180)	Uterine ILC2 ↑ (179)	Lung ILC2 ↓ (181)

DC, dendritic cells; Th, T helper cells; Treg, regulatory T cells; ILC, innate lymphoid cells; ND, not determined; Ig, immunoglobulins.

of IL-6, IL-23, IL-12, and IL-1β (104–107), thus increasing the type-1 responses (108). On the other hand, similar to GCs, estrogens induce a tolerogenic phenotype in DC, by decreasing the expression of pro-inflammatory cytokines and chemokines, such as IL-6, IFN-γ, IL-12, CXCL8, and CCL2 (109–111), and upregulating inhibitory molecules PD-L1 and PD-L2, and regulatory cytokines IL-10 and TGF-β, thus also leading to a decrease in the Th1 cells activation and a shift toward production of Th2-type cytokines (109, 112).

Effect of Steroid Hormones on Adaptive Immunity

Controlled elimination of T cells during thymocyte development and T cell-mediated immune responses is essential to prevent immunopathologies, such as autoimmunity and cancer. GCs induce apoptosis in developing thymocytes (113–116) and regulate both "death by neglect" and positive selection (117). The GC-induced apoptosis in thymocytes is also attributed to non-genomic effects of GR (38, 39, 118, 119). Their role in positive selection is inferred from the antagonism between GCs and T cell repertoire (TCR)-activated signals, which allows cells with intermediate TCR affinity to be positively selected (120–122).

The growth suppressive effect of GCs on thymocytes is common to the action of female and male sex hormones. Similar to GCs, estrogens inhibit thymocyte proliferation (123) and induce thymic atrophy (124). Pregnancy is associated with accelerated thymic involution (125, 126). Androgens also restrain active cell cycling and the number of immature thymocytes (127). The number of CD4+ and CD8+ T cells is lower in males around 50–75 of age compared to females, and the diversity of the TCR in females is larger than in males of the same age (128).

Upon TCR activation and stimulation with particular cytokines, naïve mature CD4+ T cells differentiate in the periphery into one of several lineages of T helper (Th) cells that include Th1, Th2, Th17, and Treg cells (129). GCs promote the shift from Th1 to Th2 type immune responses by differentially regulating apoptosis of Th1 and Th2 cells (130), and by interfering with the activity of their master regulators T-bet and GATA-3, respectively (131–133). GCs can also suppress the production of TNF-α, IL-12, and IFN-γ and induce the production of IL-4, IL-10, and IL-13 (13, 130). GCs inhibit the production of Th17-type cytokines in AD (134), although the sensitivity of Th17 cells to GC-induced apoptosis varies dependent on disease-specific microenvironment (135). Treg cells play a critical role in regulating immune responses and peripheral tolerance. GCs upregulate expression of FoxP3, the master regulator of Treg cells, expand Treg cell population (136–140), and increase Treg function in AD (141–143). Expression of GCs' target gene GILZ also contributes to the GC-mediated regulation of Th1/Th2 balance (144, 145), and the induction of Treg cells by promoting TGF-β-dependent FoxP3 expression (136).

Sex steroids also modulate the differentiation and function of all subsets of T cells (75, 146–148). Contrary to GCs, estrogens promote INF-γ production by Th1 cells in both human and mice (149–151), via potentially direct interaction of ER with the *Ifng* promoter (150, 152), upregulation of Th-1 transcription factor T-bet (151, 153), or via microRNA-dependent suppression of IFN-γ expression (154). However, high levels of estrogen skew the immune response from Th1 to Th2-type (155–157), similar to GCs. Estradiol also regulates anti-inflammatory Th2 shift by activating SGK1 kinase (158). The effects of estrogens on Th17 subset are different depending on experimental model, leading to enhancement (159, 160) or decrease (161) of Th17-dependent inflammation. Like GCs, estrogens promote the expansion of Treg cells (162, 163) by upregulating the expression of FoxP3, PD-1, and CTLA-4 (162–166), therefore, GCs and estrogens may co-operate in promoting the Treg development and activity.

Glucocorticoids and estrogens elicit opposite effects on B cells. GCs have a pro-apoptotic effect on developing B lymphocytes in the bone marrow (167, 168). On the other hand, B-lymphoblastic leukemia cells are resistant to GC-induced apoptosis, due to enhanced expression of B-cell lymphoma-2 protein (167, 169). GILZ mediates GC-induced apoptosis in B cells as shown by the accumulation of B cells in the bone marrow and in the periphery in GILZ-deficient mice, due to reduced B cell apoptosis (170). GCs affect directly humoral response by reducing circulating immunoglobulins (Igs) although some studies have shown an increase of IgE production in conjunction with IL-4 (171–173). Instead, enhanced antibody production is observed in women, suggesting that female sex hormones stimulate B cell-mediated responses. Estrogen treatment also interferes with normal tolerance of naive DNA-reactive B cells, thus contributing to the development of AD. Elevated estrogen alters the negative selection of DNA-reactive B cells in the periphery (174, 175), interfering with proper B cell receptor signaling and regulation of B cells activation and apoptosis (176, 177). Thus, pharmacologic treatment with synthetic GCs may be useful in suppression of the enhanced B cell activities in AD.

Effect of Steroid Hormones on Innate Lymphoid Cells (ILC)

Innate lymphoid cell is a most recently identified immune cell type, which contributes to inflammation, immunity, and the maintenance of tissue integrity and homeostasis (178). Recent evidence demonstrated that group 2 ILC2s are present in the uterus under control of estrogens and are increased upon estrogen administration (179). It is, therefore, possible that estrogens modulate tolerance *via* ILC2-mediated modulation of the protective Th2 shift in pregnancy. Instead, ILC2 promote lung inflammation during asthma (180). Interestingly, male mice have reduced numbers of ILC2s in peripheral tissues compared to females, and the number of ILC2s in the lungs is negatively regulated by androgens (181), consistent with the overall suppressive role of male steroids in immunity. GCs were shown to modulate the cytokine production by the ILC2s, thus these finding suggests a modulatory role of steroid hormones in ILCs and homeostasis of specific tissues.

INTERACTION BETWEEN GR AND SEX HORMONE RECEPTORS

GR, ER, and AR are ligand-activated TFs belonging to the NR superfamily (182). Experimental evidence shows that GR and sex hormone receptors share some common mechanisms of gene

regulation, but they also exploit different mechanisms to repress pro-inflammatory genes depending on the target gene, cell type, and interactions with other TFs.

Estrogen receptor, AR, and GR induce expression of genes that control proliferation, differentiation, and cell death by directly binding to specific hormone response elements or by indirectly tethering through TFs, such as AP1 (22, 183–185), Sp1 (186, 187), Stat1 (188), and NFκB (189, 190). The potential crosstalk in the regulation of gene expression by GR and ER was studied mostly in non-immune cell types, which may, however, provide mechanistic evidence of the mechanisms potentially operating in cells of the immune system.

The functional interaction between GR and ER signaling has been observed in several cancer cell lines, and requires additional factors, such as MED14, SRC-2, and SRC-3 in the same complex, resulting in either cooperative or mutually inhibiting effects (191). GR may inhibit the action of ER *via* distinct mechanisms. GCs may inhibit the estrogen signaling indirectly, by inducing the estrogen-metabolizing enzyme in breast cancer cells (192). Synthetic GC dexamethasone (Dex) antagonizes ERα-regulated target gene expression in breast cancer cells treated with estrogen and Dex simultaneously *via* the direct protein–protein interaction and the recruitment of GR to ERα binding sites (193). This recruitment is facilitated by AP-1 and leads to a displacement of ERα from DNA and repression of its target genes transcription (193, 194).

Reciprocally, the ER-mediated inhibition of the GR function was also described. Treatment of the breast cancer cell line with estrogen agonists downregulates GR protein levels *via* proteosome-mediated degradation (195). On the other hand, in an experimental lung inflammation rat model, the ER antagonist (ICI 182780) blocked the anti-inflammatory effects of GCs, suggesting that GR and ER co-operate in this setting in their anti-inflammatory activity (196). A subset of pro-inflammatory genes was repressed by both ER and GR (CD69, MCP-1, IL-6, IL-8), and the ER antagonist blocked Dex-mediated repression of these genes (197), by preventing the recruitment of nuclear coactivator 2 by the GR necessary for trans-repression. These data suggest that GR and ER functionally cooperate on selected promoters.

The functional effect of the interaction of GR with PR and ER is less characterized. PR and GR were shown to interact *in vitro*, and *in vivo.* Progesterone acts *via* GR to repress IL-1β-driven COX-2 activation (198, 199). The AR and the GR form heterodimers at a common DNA site both *in vitro* and *in vivo*, and this interaction leads to mutual inhibition of transcriptional activity (200).

CONCLUSIONS

Most of the mechanistic insights into synergistic and antagonizing effects of GR and sex steroid receptors in gene expression were obtained in non-immune cells, and, to our knowledge, the interactions between GCs and sex hormones in immune cells have not been studied *in vitro*. However, receptors for both classes of hormones are present in variety of immune cells, where, as reviewed above, they have been separately shown to influence various aspect of immune cell activity, ranging from cell survival to differentiation and expression of pro- and anti-inflammatory molecules (**Table 1**). Thus, the investigation of possible mutual influence of GCs and sex hormones in immune cells and its mechanisms is warranted.

The effects of female sex hormones on cells of adaptive immune system such as Th cell differentiation and B cells may underlie the higher predisposition of women to AD (4). The GC-mediated suppression of Th1 and promotion of Treg cell activity, as well as apoptotic effects on B cells may explain in part the cellular basis of GCs' efficacy in dampening the symptoms of many AD (13). Development of novel therapies for immune cell type- and gender-specific modulation of immune system may represent future direction for treatment of AD.

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REFERENCES

1. Grossman CJ. Interactions between the gonadal steroids and the immune system. *Science* (1985) 227(4684):257–61. doi:10.1126/science.3871252
2. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. *Endocr Rev* (1996) 17(4):369–84. doi:10.1210/edrv-17-4-369
3. Talal N. Sex steroid hormones and systemic lupus erythematosus. *Arthritis Rheum* (1981) 24(8):1054–6. doi:10.1002/art.1780240811
4. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita* (2016) 52(2):205–12. doi:10.4415/ANN_16_02_12
5. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* (2016) 16(10):626–38. doi:10.1038/nri.2016.90
6. Hewitt SC, Winuthayanon W, Korach KS. What's new in estrogen receptor action in the female reproductive tract. *J Mol Endocrinol* (2016) 56(2):R55–71. doi:10.1530/JME-15-0254
7. Gilliver SC. Sex steroids as inflammatory regulators. *J Steroid Biochem Mol Biol* (2010) 120(2–3):105–15. doi:10.1016/j.jsbmb.2009.12.015
8. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* (2007) 28(5):521–74. doi:10.1210/er.2007-0001
9. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* (2011) 32(1):81–151. doi:10.1210/er.2010-0013
10. Dumbell R, Matveeva O, Oster H. Circadian clocks, stress, and immunity. *Front Endocrinol* (2016) 7:37. doi:10.3389/fendo.2016.00037
11. Godowski PJ, Rusconi S, Miesfeld R, Yamamoto KR. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* (1987) 325(6102):365–8. doi:10.1038/325365a0
12. Grad I, Picard D. The glucocorticoid responses are shaped by molecular chaperones. *Mol Cell Endocrinol* (2007) 275(1–2):2–12. doi:10.1016/j.mce.2007.05.018
13. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol* (2017) 17(4):233–47. doi:10.1038/nri.2017.1
14. Riccardi C, Bruscoli S, Migliorati G. Molecular mechanisms of immunomodulatory activity of glucocorticoids. *Pharmacol Res* (2002) 45(5):361–8. doi:10.1006/phrs.2002.0969

15. Vandewalle J, Luypaert A, De Bosscher K, Libert C. Therapeutic mechanisms of glucocorticoids. *Trends Endocrinol Metab* (2018) 29(1):42–54. doi:10.1016/j.tem.2017.10.010
16. Guido EC, Delorme EO, Clemm DL, Stein RB, Rosen J, Miner JN. Determinants of promoter-specific activity by glucocorticoid receptor. *Mol Endocrinol* (1996) 10(10):1178–90. doi:10.1210/me.10.10.1178
17. McEwan IJ, Wright AP, Gustafsson JA. Mechanism of gene expression by the glucocorticoid receptor: role of protein-protein interactions. *Bioessays* (1997) 19(2):153–60. doi:10.1002/bies.950190210
18. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* (1998) 16:225–60. doi:10.1146/annurev.immunol.16.1.225
19. Smale ST. Selective transcription in response to an inflammatory stimulus. *Cell* (2010) 140(6):833–44. doi:10.1016/j.cell.2010.01.037
20. Zhang Z, Jones S, Hagood JS, Fuentes NL, Fuller GM. STAT3 acts as a co-activator of glucocorticoid receptor signaling. *J Biol Chem* (1997) 272(49):30607–10. doi:10.1074/jbc.272.49.30607
21. Stocklin E, Wissler M, Gouilleux F, Groner B. Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* (1996) 383(6602):726–8. doi:10.1038/383726a0
22. Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, et al. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* (1990) 62(6):1189–204. doi:10.1016/0092-8674(90)90395-U
23. Imai E, Miner JN, Mitchell JA, Yamamoto KR, Granner DK. Glucocorticoid receptor-cAMP response element-binding protein interaction and the response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids. *J Biol Chem* (1993) 268(8):5353–6.
24. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, et al. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* (1996) 85(3):403–14. doi:10.1016/S0092-8674(00)81118-6
25. Van Bogaert T, De Bosscher K, Libert C. Crosstalk between TNF and glucocorticoid receptor signaling pathways. *Cytokine Growth Factor Rev* (2010) 21(4):275–86. doi:10.1016/j.cytogfr.2010.04.003
26. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, et al. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* (1997) 7(6):803–12. doi:10.1016/S1074-7613(00)80398-2
27. Cannarile L, Zollo O, D'Adamio F, Ayroldi E, Marchetti C, Tabilio A, et al. Cloning, chromosomal assignment and tissue distribution of human GILZ, a glucocorticoid hormone-induced gene. *Cell Death Differ* (2001) 8(2):201–3. doi:10.1038/sj.cdd.4400798
28. Berrebi D, Bruscoli S, Cohen N, Foussat A, Migliorati G, Bouchet-Delbos L, et al. Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. *Blood* (2003) 101(2):729–38. doi:10.1182/blood-2002-02-0538
29. Hoppstadter J, Kessler SM, Bruscoli S, Huwer H, Riccardi C, Kiemer AK. Glucocorticoid-induced leucine zipper: a critical factor in macrophage endotoxin tolerance. *J Immunol* (2015) 194(12):6057–67. doi:10.4049/jimmunol.1403207
30. Ronchetti S, Migliorati G, Riccardi C. GILZ as a mediator of the anti-inflammatory effects of glucocorticoids. *Front Endocrinol* (2015) 6:170. doi:10.3389/fendo.2015.00170
31. Vago JP, Tavares LP, Garcia CC, Lima KM, Perucci LO, Vieira EL, et al. The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *J Immunol* (2015) 194(10):4940–50. doi:10.4049/jimmunol.1401722
32. Ayroldi E, Zollo O, Macchiarulo A, Di Marco B, Marchetti C, Riccardi C. Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Mol Cell Biol* (2002) 22(22):7929–41. doi:10.1128/MCB.22.22.7929-7941.2002
33. Bruscoli S, Velardi E, Di Sante M, Bereshchenko O, Venanzi A, Coppo M, et al. Long glucocorticoid-induced leucine zipper (L-GILZ) protein interacts with ras protein pathway and contributes to spermatogenesis control. *J Biol Chem* (2012) 287(2):1242–51. doi:10.1074/jbc.M111.316372
34. Asselin-Labat ML, David M, Biola-Vidamment A, Lecoeuche D, Zennaro MC, Bertoglio J, et al. GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. *Blood* (2004) 104(1):215–23. doi:10.1182/blood-2003-12-4295
35. Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L, et al. Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* (2001) 98(3):743–53. doi:10.1182/blood.V98.3.743
36. Riccardi C, Bruscoli S, Ayroldi E, Agostini M, Migliorati G. GILZ, a glucocorticoid hormone induced gene, modulates T lymphocytes activation and death through interaction with NF-kB. *Adv Exp Med Biol* (2001) 495:31–9. doi:10.1007/978-1-4615-0685-0_5
37. Di Marco B, Massetti M, Bruscoli S, Macchiarulo A, Di Virgilio R, Velardi E, et al. Glucocorticoid-induced leucine zipper (GILZ)/NF-kappaB interaction: role of GILZ homo-dimerization and C-terminal domain. *Nucleic Acids Res* (2007) 35(2):517–28. doi:10.1093/nar/gkl1080
38. Cifone MG, Migliorati G, Parroni R, Marchetti C, Millimaggi D, Santoni A, et al. Dexamethasone-induced thymocyte apoptosis: apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. *Blood* (1999) 93(7):2282–96.
39. Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C. Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. *Blood* (2003) 101(2):585–93. doi:10.1182/blood-2002-06-1779
40. Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. *Pharmacol Rev* (2000) 52(4):513–56.
41. Bruscoli S, Di Virgilio R, Donato V, Velardi E, Baldoni M, Marchetti C, et al. Genomic and non-genomic effects of different glucocorticoids on mouse thymocyte apoptosis. *Eur J Pharmacol* (2006) 529(1–3):63–70. doi:10.1016/j.ejphar.2005.10.053
42. Croxtall JD, Choudhury Q, Flower RJ. Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *Br J Pharmacol* (2000) 130(2):289–98. doi:10.1038/sj.bjp.0703272
43. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* (2007) 87(3):905–31. doi:10.1152/physrev.00026.2006
44. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol* (2015) 294(2):63–9. doi:10.1016/j.cellimm.2015.01.018
45. Cvoro A, Tzagarakis-Foster C, Tatomer D, Paruthiyil S, Fox MS, Leitman DC. Distinct roles of unliganded and liganded estrogen receptors in transcriptional repression. *Mol Cell* (2006) 21(4):555–64. doi:10.1016/j.molcel.2006.01.014
46. Calippe B, Douin-Echinard V, Delpy L, Laffargue M, Lelu K, Krust A, et al. 17Beta-estradiol promotes TLR4-triggered proinflammatory mediator production through direct estrogen receptor alpha signaling in macrophages in vivo. *J Immunol* (2010) 185(2):1169–76. doi:10.4049/jimmunol.0902383
47. Pratap UP, Sharma HR, Mohanty A, Kale P, Gopinath S, Hima L, et al. Estrogen upregulates inflammatory signals through NF-kappaB, IFN-gamma, and nitric oxide via Akt/mTOR pathway in the lymph node lymphocytes of middle-aged female rats. *Int Immunopharmacol* (2015) 29(2):591–8. doi:10.1016/j.intimp.2015.09.024
48. Cvoro A, Tatomer D, Tee MK, Zogovic T, Harris HA, Leitman DC. Selective estrogen receptor-beta agonists repress transcription of proinflammatory genes. *J Immunol* (2008) 180(1):630–6. doi:10.4049/jimmunol.180.1.630
49. Stein B, Yang MX. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol* (1995) 15(9):4971–9. doi:10.1128/MCB.15.9.4971
50. An J, Ribeiro RC, Webb P, Gustafsson JA, Kushner PJ, Baxter JD, et al. Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators. *Proc Natl Acad Sci U S A* (1999) 96(26):15161–6. doi:10.1073/pnas.96.26.15161
51. Nettles KW, Gil G, Nowak J, Metivier R, Sharma VB, Greene GL. CBP is a dosage-dependent regulator of nuclear factor-kappaB suppression by the estrogen receptor. *Mol Endocrinol* (2008) 22(2):263–72. doi:10.1210/me.2007-0324
52. Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST. Expression and localization of the progesterone receptor in mouse and

human reproductive organs. *J Endocrinol* (2006) 191(3):525–35. doi:10.1677/joe.1.06565
53. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. *Mol Endocrinol* (2006) 20(11):2724–33. doi:10.1210/me.2006-0112
54. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J Exp Med* (1978) 147(6):1568–83. doi:10.1084/jem.147.6.1568
55. Steinberg AD, Roths JB, Murphy ED, Steinberg RT, Raveche ES. Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. *J Immunol* (1980) 125(2):871–3.
56. Ariga H, Edwards J, Sullivan DA. Androgen control of autoimmune expression in lacrimal glands of MRL/Mp-lpr/lpr mice. *Clin Immunol Immunopathol* (1989) 53(3):499–508. doi:10.1016/0090-1229(89)90011-1
57. Fox HS. Androgen treatment prevents diabetes in nonobese diabetic mice. *J Exp Med* (1992) 175(5):1409–12. doi:10.1084/jem.175.5.1409
58. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* (1988) 240(4850):324–6. doi:10.1126/science.3353726
59. MacLean HE, Warne GL, Zajac JD. Localization of functional domains in the androgen receptor. *J Steroid Biochem Mol Biol* (1997) 62(4):233–42. doi:10.1016/S0960-0760(97)00049-6
60. Liva SM, Voskuhl RR. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. *J Immunol* (2001) 167(4):2060–7. doi:10.4049/jimmunol.167.4.2060
61. D'Agostino P, Milano S, Barbera C, Di Bella G, La Rosa M, Ferlazzo V, et al. Sex hormones modulate inflammatory mediators produced by macrophages. *Ann N Y Acad Sci* (1999) 876:426–9. doi:10.1111/j.1749-6632.1999.tb07667.x
62. Lai JJ, Lai KP, Zeng W, Chuang KH, Altuwaijri S, Chang C. Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol* (2012) 181(5):1504–12. doi:10.1016/j.ajpath.2012.07.008
63. Straub RH, Harle P, Atzeni F, Weidler C, Cutolo M, Sarzi-Puttini P. Sex hormone concentrations in patients with rheumatoid arthritis are not normalized during 12 weeks of anti-tumor necrosis factor therapy. *J Rheumatol* (2005) 32(7):1253–8.
64. Straub RH, Vogl D, Gross V, Lang B, Scholmerich J, Andus T. Association of humoral markers of inflammation and dehydroepiandrosterone sulfate or cortisol serum levels in patients with chronic inflammatory bowel disease. *Am J Gastroenterol* (1998) 93(11):2197–202. doi:10.1111/j.1572-0241.1998.00535.x
65. Da Silva JA. Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* (1999) 876:102–17; discussion 17–8. doi:10.1111/j.1749-6632.1999.tb07628.x
66. Barczyk K, Ehrchen J, Tenbrock K, Ahlmann M, Kneidl J, Viemann D, et al. Glucocorticoids promote survival of anti-inflammatory macrophages via stimulation of adenosine receptor A3. *Blood* (2010) 116(3):446–55. doi:10.1182/blood-2009-10-247106
67. Zhou JY, Zhong HJ, Yang C, Yan J, Wang HY, Jiang JX. Corticosterone exerts immunostimulatory effects on macrophages via endoplasmic reticulum stress. *Br J Surg* (2010) 97(2):281–93. doi:10.1002/bjs.6820
68. Migliorati G, Nicoletti I, D'Adamio F, Spreca A, Pagliacci C, Riccardi C. Dexamethasone induces apoptosis in mouse natural killer cells and cytotoxic T lymphocytes. *Immunology* (1994) 81(1):21–6.
69. Brokaw JJ, White GW, Baluk P, Anderson GP, Umemoto EY, McDonald DM. Glucocorticoid-induced apoptosis of dendritic cells in the rat tracheal mucosa. *Am J Respir Cell Mol Biol* (1998) 19(4):598–605. doi:10.1165/ajrcmb.19.4.2870
70. Kim KD, Choe YK, Choe IS, Lim JS. Inhibition of glucocorticoid-mediated, caspase-independent dendritic cell death by CD40 activation. *J Leukoc Biol* (2001) 69(3):426–34.
71. Cao Y, Bender IK, Konstantinidis AK, Shin SC, Jewell CM, Cidlowski JA, et al. Glucocorticoid receptor translational isoforms underlie maturational stage-specific glucocorticoid sensitivities of dendritic cells in mice and humans. *Blood* (2013) 121(9):1553–62. doi:10.1182/blood-2012-05-432336
72. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* (1996) 156(11):4422–8.
73. Saffar AS, Ashdown H, Gounni AS. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. *Curr Drug Targets* (2011) 12(4):556–62. doi:10.2174/138945011794751555
74. Jilma B, Eichler HG, Breiteneder H, Wolzt M, Aringer M, Graninger W, et al. Effects of 17 beta-estradiol on circulating adhesion molecules. *J Clin Endocrinol Metab* (1994) 79(6):1619–24. doi:10.1210/jcem.79.6.7527406
75. Robinson DP, Hall OJ, Nilles TL, Bream JH, Klein SL. 17beta-estradiol protects females against influenza by recruiting neutrophils and increasing virus-specific CD8 T cell responses in the lungs. *J Virol* (2014) 88(9):4711–20. doi:10.1128/JVI.02081-13
76. Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. *J Steroid Biochem Mol Biol* (2010) 120(2–3):69–75. doi:10.1016/j.jsbmb.2010.03.058
77. Perretti M, Flower RJ. Measurement of lipocortin 1 levels in murine peripheral blood leukocytes by flow cytometry: modulation by glucocorticoids and inflammation. *Br J Pharmacol* (1996) 118(3):605–10. doi:10.1111/j.1476-5381.1996.tb15444.x
78. Shah S, King EM, Chandrasekhar A, Newton R. Roles for the mitogen-activated protein kinase (MAPK) phosphatase, DUSP1, in feedback control of inflammatory gene expression and repression by dexamethasone. *J Biol Chem* (2014) 289(19):13667–79. doi:10.1074/jbc.M113.540799
79. Cho H, Park OH, Park J, Ryu I, Kim J, Ko J, et al. Glucocorticoid receptor interacts with PNRC2 in a ligand-dependent manner to recruit UPF1 for rapid mRNA degradation. *Proc Natl Acad Sci U S A* (2015) 112(13):E1540–9. doi:10.1073/pnas.1409612112
80. Nakagawa M, Bondy GP, Waisman D, Minshall D, Hogg JC, van Eeden SF. The effect of glucocorticoids on the expression of L-selectin on polymorphonuclear leukocyte. *Blood* (1999) 93(8):2730–7.
81. Caramori G, Adcock I. Anti-inflammatory mechanisms of glucocorticoids targeting granulocytes. *Curr Drug Targets Inflamm Allergy* (2005) 4(4):455–63. doi:10.2174/1568010054526331
82. Ricci E, Ronchetti S, Pericolini E, Gabrielli E, Cari L, Gentili M, et al. Role of the glucocorticoid-induced leucine zipper gene in dexamethasone-induced inhibition of mouse neutrophil migration via control of annexin A1 expression. *FASEB J* (2017) 31(7):3054–65. doi:10.1096/fj.201601315R
83. Nadkarni S, McArthur S. Oestrogen and immunomodulation: new mechanisms that impact on peripheral and central immunity. *Curr Opin Pharmacol* (2013) 13(4):576–81. doi:10.1016/j.coph.2013.05.007
84. MacNeil LG, Baker SK, Stevic I, Tarnopolsky MA. 17beta-estradiol attenuates exercise-induced neutrophil infiltration in men. *Am J Physiol Regul Integr Comp Physiol* (2011) 300(6):R1443–51. doi:10.1152/ajpregu.00689.2009
85. Chandrasekaran VR, Periasamy S, Liu LL, Liu MY. 17beta-Estradiol protects against acetaminophen-overdose-induced acute oxidative hepatic damage and increases the survival rate in mice. *Steroids* (2011) 76(1–2):118–24. doi:10.1016/j.steroids.2010.09.008
86. Sheh A, Ge Z, Parry NM, Muthupalani S, Rager JE, Raczynski AR, et al. 17beta-Estradiol and tamoxifen prevent gastric cancer by modulating leukocyte recruitment and oncogenic pathways in *Helicobacter pylori*-infected INS-GAS male mice. *Cancer Prev Res (Phila)* (2011) 4(9):1426–35. doi:10.1158/1940-6207.CAPR-11-0219
87. Shih HC, Huang MS, Lee CH. Estrogen augments the protection of hypertonic saline treatment from mesenteric ischemia-reperfusion injury. *Shock* (2011) 35(3):302–7. doi:10.1097/SHK.0b013e3181f8b420
88. Yang SJ, Chen HM, Hsieh CH, Hsu JT, Yeh CN, Yeh TS, et al. Akt pathway is required for oestrogen-mediated attenuation of lung injury in a rodent model of cerulein-induced acute pancreatitis. *Injury* (2011) 42(7):638–42. doi:10.1016/j.injury.2010.07.242
89. Pioli PA, Jensen AL, Weaver LK, Amiel E, Shen Z, Shen L, et al. Estradiol attenuates lipopolysaccharide-induced CXC chemokine ligand 8 production by human peripheral blood monocytes. *J Immunol* (2007) 179(9):6284–90. doi:10.4049/jimmunol.179.9.6284
90. Doucet D, Badami C, Palange D, Bonitz RP, Lu Q, Xu DZ, et al. Estrogen receptor hormone agonists limit trauma hemorrhage shock-induced gut and lung injury in rats. *PLoS One* (2010) 5(2):e9421. doi:10.1371/journal.pone.0009421

91. Xing D, Gong K, Feng W, Nozell SE, Chen YF, Chatham JC, et al. O-GlcNAc modification of NFkappaB p65 inhibits TNF-alpha-induced inflammatory mediator expression in rat aortic smooth muscle cells. *PLoS One* (2011) 6(8):e24021. doi:10.1371/journal.pone.0024021
92. Golecka-Bakowska M, Mierzwinska-Nastalska E, Bychawska M. Influence of hormone supplementation therapy on the incidence of denture stomatitis and on chemiluminescent activity of polymorphonuclear granulocytes in blood of menopausal-aged women. *Eur J Med Res* (2010) 15(Suppl 2): 46–9.
93. Nadkarni S, Cooper D, Brancaleone V, Bena S, Perretti M. Activation of the annexin A1 pathway underlies the protective effects exerted by estrogen in polymorphonuclear leukocytes. *Arterioscler Thromb Vasc Biol* (2011) 31(11):2749–59. doi:10.1161/ATVBAHA.111.235176
94. Rogers A, Eastell R. The effect of 17beta-estradiol on production of cytokines in cultures of peripheral blood. *Bone* (2001) 29(1):30–4. doi:10.1016/S8756-3282(01)00468-9
95. Asai K, Hiki N, Mimura Y, Ogawa T, Unou K, Kaminishi M. Gender differences in cytokine secretion by human peripheral blood mononuclear cells: role of estrogen in modulating LPS-induced cytokine secretion in an ex vivo septic model. *Shock* (2001) 16(5):340–3. doi:10.1097/00024382-200116050-00003
96. Toyoda Y, Miyashita T, Endo S, Tsuneyama K, Fukami T, Nakajima M, et al. Estradiol and progesterone modulate halothane-induced liver injury in mice. *Toxicol Lett* (2011) 204(1):17–24. doi:10.1016/j.toxlet.2011.03.031
97. Murphy AJ, Guyre PM, Pioli PA. Estradiol suppresses NF-kappa B activation through coordinated regulation of let-7a and miR-125b in primary human macrophages. *J Immunol* (2010) 184(9):5029–37. doi:10.4049/jimmunol.0903463
98. Hsu JT, Kan WH, Hsieh CH, Choudhry MA, Schwacha MG, Bland KI, et al. Mechanism of estrogen-mediated attenuation of hepatic injury following trauma-hemorrhage: Akt-dependent HO-1 up-regulation. *J Leukoc Biol* (2007) 82(4):1019–26. doi:10.1189/jlb.0607355
99. Cuzzocrea S, Genovese T, Mazzon E, Esposito E, Di Paola R, Muia C, et al. Effect of 17beta-estradiol on signal transduction pathways and secondary damage in experimental spinal cord trauma. *Shock* (2008) 29(3):362–71. doi:10.1097/shk.0b013e31814545dc
100. Woltman AM, de Fijter JW, Kamerling SW, Paul LC, Daha MR, van Kooten C. The effect of calcineurin inhibitors and corticosteroids on the differentiation of human dendritic cells. *Eur J Immunol* (2000) 30(7):1807–12. doi:10.1002/1521-4141(200007)30:7<1807::AID-IMMU1807>3.0.CO;2-N
101. Chen L, Hasni MS, Jondal M, Yakimchuk K. Modification of anti-tumor immunity by tolerogenic dendritic cells. *Autoimmunity* (2017) 50(6):370–6. doi:10.1080/08916934.2017.1344837
102. Bros M, Jahrling F, Renzing A, Wiechmann N, Dang NA, Sutter A, et al. A newly established murine immature dendritic cell line can be differentiated into a mature state, but exerts tolerogenic function upon maturation in the presence of glucocorticoid. *Blood* (2007) 109(9):3820–9. doi:10.1182/blood-2006-07-035576
103. Vanderheyde N, Verhasselt V, Goldman M, Willems F. Inhibition of human dendritic cell functions by methylprednisolone. *Transplantation* (1999) 67(10):1342–7. doi:10.1097/00007890-199905270-00009
104. Seillet C, Rouquie N, Foulon E, Douin-Echinard V, Krust A, Chambon P, et al. Estradiol promotes functional responses in inflammatory and steady-state dendritic cells through differential requirement for activation function-1 of estrogen receptor alpha. *J Immunol* (2013) 190(11):5459–70. doi:10.4049/jimmunol.1203312
105. Siracusa MC, Overstreet MG, Housseau F, Scott AL, Klein SL. 17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells. *J Immunol* (2008) 180(3):1423–31. doi:10.4049/jimmunol.180.3.1423
106. Cunningham MA, Naga OS, Eudaly JG, Scott JL, Gilkeson GS. Estrogen receptor alpha modulates toll-like receptor signaling in murine lupus. *Clin Immunol* (2012) 144(1):1–12. doi:10.1016/j.clim.2012.04.001
107. Douin-Echinard V, Laffont S, Seillet C, Delpy L, Krust A, Chambon P, et al. Estrogen receptor alpha, but not beta, is required for optimal dendritic cell differentiation and [corrected] CD40-induced cytokine production. *J Immunol* (2008) 180(6):3661–9. doi:10.4049/jimmunol.180.6.3661
108. Delpy L, Douin-Echinard V, Garidou L, Bruand C, Saoudi A, Guery JC. Estrogen enhances susceptibility to experimental autoimmune myasthenia gravis by promoting type 1-polarized immune responses. *J Immunol* (2005) 175(8):5050–7. doi:10.4049/jimmunol.175.8.5050
109. Liu HY, Buenafe AC, Matejuk A, Ito A, Zamora A, Dwyer J, et al. Estrogen inhibition of EAE involves effects on dendritic cell function. *J Neurosci Res* (2002) 70(2):238–48. doi:10.1002/jnr.10409
110. Bachy V, Williams DJ, Ibrahim MA. Altered dendritic cell function in normal pregnancy. *J Reprod Immunol* (2008) 78(1):11–21. doi:10.1016/j.jri.2007.09.004
111. Papenfuss TL, Powell ND, McClain MA, Bedarf A, Singh A, Gienapp IE, et al. Estriol generates tolerogenic dendritic cells in vivo that protect against autoimmunity. *J Immunol* (2011) 186(6):3346–55. doi:10.4049/jimmunol.1001322
112. Subramanian S, Yates M, Vandenbark AA, Offner H. Oestrogen-mediated protection of experimental autoimmune encephalomyelitis in the absence of Foxp3+ regulatory T cells implicates compensatory pathways including regulatory B cells. *Immunology* (2011) 132(3):340–7. doi:10.1111/j.1365-2567.2010.03380.x
113. Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol* (2002) 169(4):1837–43. doi:10.4049/jimmunol.169.4.1837
114. Vacchio MS, Ashwell JD. Glucocorticoids and thymocyte development. *Semin Immunol* (2000) 12(5):475–85. doi:10.1006/smim.2000.0265
115. Caron-Leslie LM, Schwartzman RA, Gaido ML, Compton MM, Cidlowski JA. Identification and characterization of glucocorticoid-regulated nuclease(s) in lymphoid cells undergoing apoptosis. *J Steroid Biochem Mol Biol* (1991) 40(4–6):661–71. doi:10.1016/0960-0760(91)90288-G
116. Brunetti M, Martelli N, Colasante A, Piantelli M, Musiani P, Aiello FB. Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes. *Blood* (1995) 86(11):4199–205.
117. Stephens GL, Ignatowicz L. Decreasing the threshold for thymocyte activation biases CD4+ T cells toward a regulatory (CD4+CD25+) lineage. *Eur J Immunol* (2003) 33(5):1282–91. doi:10.1002/eji.200323927
118. Cohen JJ, Duke RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* (1984) 132(1):38–42.
119. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* (1980) 284(5756):555–6. doi:10.1038/284555a0
120. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function*. *Annu Rev Immunol* (2000) 18:309–45. doi:10.1146/annurev.immunol.18.1.309
121. Van Laethem F, Baus E, Smyth LA, Andris F, Bex F, Urbain J, et al. Glucocorticoids attenuate T cell receptor signaling. *J Exp Med* (2001) 193(7):803–14. doi:10.1084/jem.193.7.803
122. Chen Y, Qiao S, Tuckermann J, Okret S, Jondal M. Thymus-derived glucocorticoids mediate androgen effects on thymocyte homeostasis. *FASEB J* (2010) 24(12):5043–51. doi:10.1096/fj.10-168724
123. Aboussaouira T, Marie C, Brugal G, Idelman S. Inhibitory effect of 17 beta-estradiol on thymocyte proliferation and metabolic activity in young rats. *Thymus* (1991) 17(3):167–80.
124. Okuyama R, Abo T, Seki S, Ohteki T, Sugiura K, Kusumi A, et al. Estrogen administration activates extrathymic T cell differentiation in the liver. *J Exp Med* (1992) 175(3):661–9. doi:10.1084/jem.175.3.661
125. Phuc LH, Papiernik M, Berrih S, Duval D. Thymic involution in pregnant mice. I. Characterization of the remaining thymocyte subpopulations. *Clin Exp Immunol* (1981) 44(2):247–52.
126. Phuc LH, Papiernik M, Dardenne M. Thymic involution in pregnant mice. II. Functional aspects of the remaining thymocytes. *Clin Exp Immunol* (1981) 44(2):253–61.
127. Olsen NJ, Viselli SM, Shults K, Stelzer G, Kovacs WJ. Induction of immature thymocyte proliferation after castration of normal male mice. *Endocrinology* (1994) 134(1):107–13. doi:10.1210/endo.134.1.8275924
128. Britanova OV, Shugay M, Merzlyak EM, Staroverov DB, Putintseva EV, Turchaninova MA, et al. Dynamics of individual T cell repertoires: from cord blood to centenarians. *J Immunol* (2016) 196(12):5005–13. doi:10.4049/jimmunol.1600005

129. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* (2010) 28:445–89. doi:10.1146/annurev-immunol-030409-101212
130. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci* (2004) 1024:138–46. doi:10.1196/annals.1321.010
131. Refojo D, Liberman AC, Giacomini D, Carbia Nagashima A, Graciarena M, Echenique C, et al. Integrating systemic information at the molecular level: cross-talk between steroid receptors and cytokine signaling on different target cells. *Ann N Y Acad Sci* (2003) 992:196–204. doi:10.1111/j.1749-6632.2003.tb03150.x
132. Almawi WY, Melemedjian OK, Rieder MJ. An alternate mechanism of glucocorticoid anti-proliferative effect: promotion of a Th2 cytokine-secreting profile. *Clin Transplant* (1999) 13(5):365–74. doi:10.1034/j.1399-0012.1999.130501.x
133. Liberman AC, Refojo D, Druker J, Toscano M, Rein T, Holsboer F, et al. The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction. *FASEB J* (2007) 21(4):1177–88. doi:10.1096/fj.06-7452com
134. Liu M, Hu X, Wang Y, Peng F, Yang Y, Chen X, et al. Effect of high-dose methylprednisolone treatment on Th17 cells in patients with multiple sclerosis in relapse. *Acta Neurol Scand* (2009) 120(4):235–41. doi:10.1111/j.1600-0404.2009.01158.x
135. Banuelos J, Cao Y, Shin SC, Lu NZ. Immunopathology alters Th17 cell glucocorticoid sensitivity. *Allergy* (2017) 72(3):331–41. doi:10.1111/all.13051
136. Bereshchenko O, Coppo M, Bruscoli S, Biagioli M, Cimino M, Frammartino T, et al. GILZ promotes production of peripherally induced Treg cells and mediates the crosstalk between glucocorticoids and TGF-beta signaling. *Cell Rep* (2014) 7(2):464–75. doi:10.1016/j.celrep.2014.03.004
137. Ugor E, Prenek L, Pap R, Berta G, Ernszt D, Najbauer J, et al. Glucocorticoid hormone treatment enhances the cytokine production of regulatory T cells by upregulation of Foxp3 expression. *Immunobiology* (2018) 223(4–5):422–31. doi:10.1016/j.imbio.2017.10.010
138. Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM. Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+) CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE. *Eur J Immunol* (2006) 36(8):2139–49. doi:10.1002/eji.200635873
139. Chung IY, Dong HF, Zhang X, Hassanein NM, Howard OM, Oppenheim JJ, et al. Effects of IL-7 and dexamethasone: induction of CD25, the high affinity IL-2 receptor, on human CD4+ cells. *Cell Immunol* (2004) 232(1–2):57–63. doi:10.1016/j.cellimm.2005.01.011
140. Stary G, Klein I, Bauer W, Koszik F, Reininger B, Kohlhofer S, et al. Glucocorticosteroids modify Langerhans cells to produce TGF-beta and expand regulatory T cells. *J Immunol* (2011) 186(1):103–12. doi:10.4049/jimmunol.1002485
141. Suarez A, Lopez P, Gomez J, Gutierrez C. Enrichment of CD4+ CD25high T cell population in patients with systemic lupus erythematosus treated with glucocorticoids. *Ann Rheum Dis* (2006) 65(11):1512–7. doi:10.1136/ard.2005.049924
142. de Paz B, Alperi-Lopez M, Ballina-Garcia FJ, Prado C, Gutierrez C, Suarez A. Cytokines and regulatory T cells in rheumatoid arthritis and their relationship with response to corticosteroids. *J Rheumatol* (2010) 37(12):2502–10. doi:10.3899/jrheum.100324
143. Hu Y, Tian W, Zhang LL, Liu H, Yin GP, He BS, et al. Function of regulatory T-cells improved by dexamethasone in Graves' disease. *Eur J Endocrinol* (2012) 166(4):641–6. doi:10.1530/EJE-11-0879
144. Cannarile L, Cuzzocrea S, Santucci L, Agostini M, Mazzon E, Esposito E, et al. Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. *Gastroenterology* (2009) 136(2):530–41. doi:10.1053/j.gastro.2008.09.024
145. Cannarile L, Fallarino F, Agostini M, Cuzzocrea S, Mazzon E, Vacca C, et al. Increased GILZ expression in transgenic mice up-regulates Th-2 lymphokines. *Blood* (2006) 107(3):1039–47. doi:10.1182/blood-2005-05-2183
146. Lelu K, Laffont S, Delpy L, Paulet PE, Perinat T, Tschanz SA, et al. Estrogen receptor alpha signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J Immunol* (2011) 187(5):2386–93. doi:10.4049/jimmunol.1101578
147. Priyanka HP, Krishnan HC, Singh RV, Hima L, Thyagarajan S. Estrogen modulates in vitro T cell responses in a concentration- and receptor-dependent manner: effects on intracellular molecular targets and antioxidant enzymes. *Mol Immunol* (2013) 56(4):328–39. doi:10.1016/j.molimm.2013.05.226
148. Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar Ahmed S. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine* (2001) 14(4):208–17. doi:10.1006/cyto.2001.0876
149. Grasso G, Muscettola M. The influence of beta-estradiol and progesterone on interferon gamma production in vitro. *Int J Neurosci* (1990) 51(3–4):315–7. doi:10.3109/00207459008999730
150. Fox HS, Bond BL, Parslow TG. Estrogen regulates the IFN-gamma promoter. *J Immunol* (1991) 146(12):4362–7.
151. Karpuzoglu-Sahin E, Hissong BD, Ansar Ahmed S. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol* (2001) 52(1–2):113–27. doi:10.1016/S0165-0378(01)00117-6
152. Maret A, Coudert JD, Garidou L, Foucras G, Gourdy P, Krust A, et al. Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development in vivo. Essential role of estrogen receptor alpha expression in hematopoietic cells. *Eur J Immunol* (2003) 33(2):512–21. doi:10.1002/immu.200310027
153. Karpuzoglu E, Phillips RA, Gogal RM Jr, Ansar Ahmed S. IFN-gamma-inducing transcription factor, T-bet is upregulated by estrogen in murine splenocytes: role of IL-27 but not IL-12. *Mol Immunol* (2007) 44(7):1808–14. doi:10.1016/j.molimm.2006.08.005
154. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA. Suppression of LPS-induced interferon-gamma and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. *Blood* (2008) 112(12):4591–7. doi:10.1182/blood-2008-04-152488
155. Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, et al. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin Exp Immunol* (1996) 106(1):127–33. doi:10.1046/j.1365-2249.1996.d01-809.x
156. Matalka KZ. The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. *Neuro Endocrinol Lett* (2003) 24(3–4):185–91.
157. Sabahi F, Rola-Plesczcynski M, O'Connell S, Frenkel LD. Qualitative and quantitative analysis of T lymphocytes during normal human pregnancy. *Am J Reprod Immunol* (1995) 33(5):381–93. doi:10.1111/j.1600-0897.1995.tb00907.x
158. Lou Y, Hu M, Wang Q, Yuan M, Wang N, Le F, et al. Estradiol suppresses TLR4-triggered apoptosis of decidual stromal cells and drives an anti-inflammatory TH2 shift by activating SGK1. *Int J Biol Sci* (2017) 13(4):434–48. doi:10.7150/ijbs.18278
159. Konermann A, Winter J, Novak N, Allam JP, Jager A. Verification of IL-17A and IL-17F in oral tissues and modulation of their expression pattern by steroid hormones. *Cell Immunol* (2013) 285(1–2):133–40. doi:10.1016/j.cellimm.2013.10.004
160. Wang Y, Cela E, Gagnon S, Sweezey NB. Estrogen aggravates inflammation in *Pseudomonas aeruginosa* pneumonia in cystic fibrosis mice. *Respir Res* (2010) 11:166. doi:10.1186/1465-9921-11-166
161. Molnar I, Bohaty I, Somogyine-Vari E. High prevalence of increased interleukin-17A serum levels in postmenopausal estrogen deficiency. *Menopause* (2014) 21(7):749–52. doi:10.1097/GME.0000000000000125
162. Polanczyk MJ, Hopke C, Huan J, Vandenbark AA, Offner H. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *J Neuroimmunol* (2005) 170(1–2):85–92. doi:10.1016/j.jneuroim.2005.08.023
163. Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, et al. Induction of regulatory T cells by physiological level estrogen. *J Cell Physiol* (2008) 214(2):456–64. doi:10.1002/jcp.21221
164. Adurthi S, Kumar MM, Vinodkumar HS, Mukherjee G, Krishnamurthy H, Acharya KK, et al. Oestrogen receptor-alpha binds the FOXP3 promoter and modulates regulatory T-cell function in human cervical cancer. *Sci Rep* (2017) 7(1):17289. doi:10.1038/s41598-017-17102-w
165. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF, et al. Cutting edge: estrogen drives expansion of the CD4+CD25+

regulatory T cell compartment. *J Immunol* (2004) 173(4):2227–30. doi:10.4049/jimmunol.173.4.2227
166. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *Int Immunol* (2007) 19(3):337–43. doi:10.1093/intimm/dxl151
167. Gruver-Yates AL, Quinn MA, Cidlowski JA. Analysis of glucocorticoid receptors and their apoptotic response to dexamethasone in male murine B cells during development. *Endocrinology* (2014) 155(2):463–74. doi:10.1210/en.2013-1473
168. Lill-Elghanian D, Schwartz K, King L, Fraker P. Glucocorticoid-induced apoptosis in early B cells from human bone marrow. *Exp Biol Med (Maywood)* (2002) 227(9):763–70. doi:10.1177/153537020222700907
169. Smith LK, Cidlowski JA. Glucocorticoid-induced apoptosis of healthy and malignant lymphocytes. *Prog Brain Res* (2010) 182:1–30. doi:10.1016/S0079-6123(10)82001-1
170. Bruscoli S, Biagioli M, Sorcini D, Frammartino T, Cimino M, Sportoletti P, et al. Lack of glucocorticoid-induced leucine zipper (GILZ) deregulates B-cell survival and results in B-cell lymphocytosis in mice. *Blood* (2015) 126(15):1790–801. doi:10.1182/blood-2015-03-631580
171. Barnes PJ. Corticosteroids, IgE, and atopy. *J Clin Invest* (2001) 107(3):265–6. doi:10.1172/JCI12157
172. Jabara HH, Brodeur SR, Geha RS. Glucocorticoids upregulate CD40 ligand expression and induce CD40L-dependent immunoglobulin isotype switching. *J Clin Invest* (2001) 107(3):371–8. doi:10.1172/JCI10168
173. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* (2005) 67:259–84. doi:10.1146/annurev.physiol.67.040403.120816
174. Bynoe MS, Grimaldi CM, Diamond B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci U S A* (2000) 97(6):2703–8. doi:10.1073/pnas.040577497
175. Grimaldi CM, Michael DJ, Diamond B. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J Immunol* (2001) 167(4):1886–90. doi:10.4049/jimmunol.167.4.1886
176. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest* (2002) 109(12):1625–33. doi:10.1172/JCI0214873
177. Grimaldi CM, Jeganathan V, Diamond B. Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *J Immunol* (2006) 176(5):2703–10. doi:10.4049/jimmunol.176.5.2703
178. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* (2016) 17(7):765–74. doi:10.1038/ni.3489
179. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus. *J Immunol* (2018) 200(1):229–36. doi:10.4049/jimmunol.1602085
180. Li BW, Hendriks RW. Group 2 innate lymphoid cells in lung inflammation. *Immunology* (2013) 140(3):281–7. doi:10.1111/imm.12153
181. Laffont S, Blanquart E, Savignac M, Cenac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. *J Exp Med* (2017) 214(6):1581–92. doi:10.1084/jem.20161807
182. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* (1988) 240(4854):889–95. doi:10.1126/science.3283939
183. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. *Physiol Rev* (2001) 81(4):1535–65. doi:10.1152/physrev.2001.81.4.1535
184. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* (2000) 74(5):311–7. doi:10.1016/S0960-0760(00)00108-4
185. Kerppola TK, Luk D, Curran T. Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. *Mol Cell Biol* (1993) 13(6):3782–91. doi:10.1128/MCB.13.6.3782
186. Safe S. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* (2001) 62:231–52. doi:10.1016/S0083-6729(01)62006-5
187. Ou XM, Chen K, Shih JC. Glucocorticoid and androgen activation of monoamine oxidase A is regulated differently by R1 and Sp1. *J Biol Chem* (2006) 281(30):21512–25. doi:10.1074/jbc.M600250200
188. Wyszomierski SL, Yeh J, Rosen JM. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. *Mol Endocrinol* (1999) 13(2):330–43. doi:10.1210/mend.13.2.0232
189. Biswas DK, Singh S, Shi Q, Pardee AB, Iglehart JD. Crossroads of estrogen receptor and NF-kappaB signaling. *Sci STKE* (2005) 2005(288):pe27. doi:10.1126/stke.2882005pe27
190. McKay LI, Cidlowski JA. Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol* (1998) 12(1):45–56. doi:10.1210/me.12.1.45
191. Bolt MJ, Stossi F, Newberg JY, Orjalo A, Johansson HE, Mancini MA. Coactivators enable glucocorticoid receptor recruitment to fine-tune estrogen receptor transcriptional responses. *Nucleic Acids Res* (2013) 41(7):4036–48. doi:10.1093/nar/gkt100
192. Gong H, Jarzynka MJ, Cole TJ, Lee JH, Wada T, Zhang B, et al. Glucocorticoids antagonize estrogens by glucocorticoid receptor-mediated activation of estrogen sulfotransferase. *Cancer Res* (2008) 68(18):7386–93. doi:10.1158/0008-5472.CAN-08-1545
193. Karmakar S, Jin Y, Nagaich AK. Interaction of glucocorticoid receptor (GR) with estrogen receptor (ER) alpha and activator protein 1 (AP1) in dexamethasone-mediated interference of ERalpha activity. *J Biol Chem* (2013) 288(33):24020–34. doi:10.1074/jbc.M113.473819
194. Uht RM, Anderson CM, Webb P, Kushner PJ. Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* (1997) 138(7):2900–8. doi:10.1210/endo.138.7.5244
195. Kinyamu HK, Archer TK. Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression. *Mol Cell Biol* (2003) 23(16):5867–81. doi:10.1128/MCB.23.16.5867-5881.2003
196. Cuzzocrea S, Bruscoli S, Crisafulli C, Mazzon E, Agostini M, Muia C, et al. Estrogen receptor antagonist fulvestrant (ICI 182,780) inhibits the anti-inflammatory effect of glucocorticoids. *Mol Pharmacol* (2007) 71(1):132–44. doi:10.1124/mol.106.029629
197. Cvoro A, Yuan C, Paruthiyil S, Miller OH, Yamamoto KR, Leitman DC. Cross talk between glucocorticoid and estrogen receptors occurs at a subset of proinflammatory genes. *J Immunol* (2011) 186(7):4354–60. doi:10.4049/jimmunol.1002205
198. Lei K, Chen L, Georgiou EX, Sooranna SR, Khanjani S, Brosens JJ, et al. Progesterone acts via the nuclear glucocorticoid receptor to suppress IL-1beta-induced COX-2 expression in human term myometrial cells. *PLoS One* (2012) 7(11):e50167. doi:10.1371/journal.pone.0050167
199. Demirpence E, Semlali A, Oliva J, Balaguer P, Badia E, Duchesne MJ, et al. An estrogen-responsive element-targeted histone deacetylase enzyme has an antiestrogen activity that differs from that of hydroxytamoxifen. *Cancer Res* (2002) 62(22):6519–28.
200. Chen S, Wang J, Yu G, Liu W, Pearce D. Androgen and glucocorticoid receptor heterodimer formation. A possible mechanism for mutual inhibition of transcriptional activity. *J Biol Chem* (1997) 272(22):14087–92. doi:10.1074/jbc.272.22.14087

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REVIEW
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Sex Hormones Regulate Innate Immune Cells and Promote Sex Differences in Respiratory Virus Infection

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Sex differences in the incidence and severity of respiratory virus infection are widely documented in humans and murine models and correlate with sex biases in numbers and/or functional responses of innate immune cells in homeostasis and lung infection. Similarly, changes in sex hormone levels upon puberty, pregnancy, and menopause/aging are associated with qualitative and quantitative differences in innate immunity. Immune cells express receptors for estrogens (ERα and ERβ), androgens (AR), and progesterone (PR), and experimental manipulation of sex hormone levels or receptors has revealed that sex hormone receptor activity often underlies sex differences in immune cell numbers and/or functional responses in the respiratory tract. While elegant studies have defined mechanistic roles for sex hormones and receptors in innate immune cells, much remains to be learned about the cellular and molecular mechanisms of action of ER, PR, and AR in myeloid cells and innate lymphocytes to promote the initiation and resolution of antiviral immunity in the lung. Here, we review the literature on sex differences and sex hormone regulation in innate immune cells in the lung in homeostasis and upon respiratory virus infection.

Keywords: sex hormones, respiratory virus, lung, estrogen, androgen, innate immunity

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INTRODUCTION

Respiratory virus infections lead to significant health problems worldwide (1). Humans show marked sex differences in the severity, prevalence, and outcome of inflammatory lung diseases including viral infection (2, 3). Innate immune responses have crucial roles in early defense against viruses but also shape antigen-specific adaptive immune responses and promote tissue repair. A number of recent reviews highlight sex differences in innate immune pathways during infectious disease (4–6). Here, we review literature reports on the sex differences in numbers and functional responses of innate immune cells in the lung and their regulation by sex hormones in homeostasis and during viral lung infection. Specifically, we highlight ways in which sex differences in innate cells may influence both the proinflammatory/effector phase and the resolution/tissue repair phase important in the host response to respiratory virus infection (**Figure 1**).

SEX HORMONES AND RECEPTOR SIGNALING

Sex Hormone Levels

Endogenous estrogens include estrone (E1), 17-β-estradiol (E2), and estriol (E3), with E2 being the major form present in adult females and males while E3 is present at high levels in pregnancy (7).

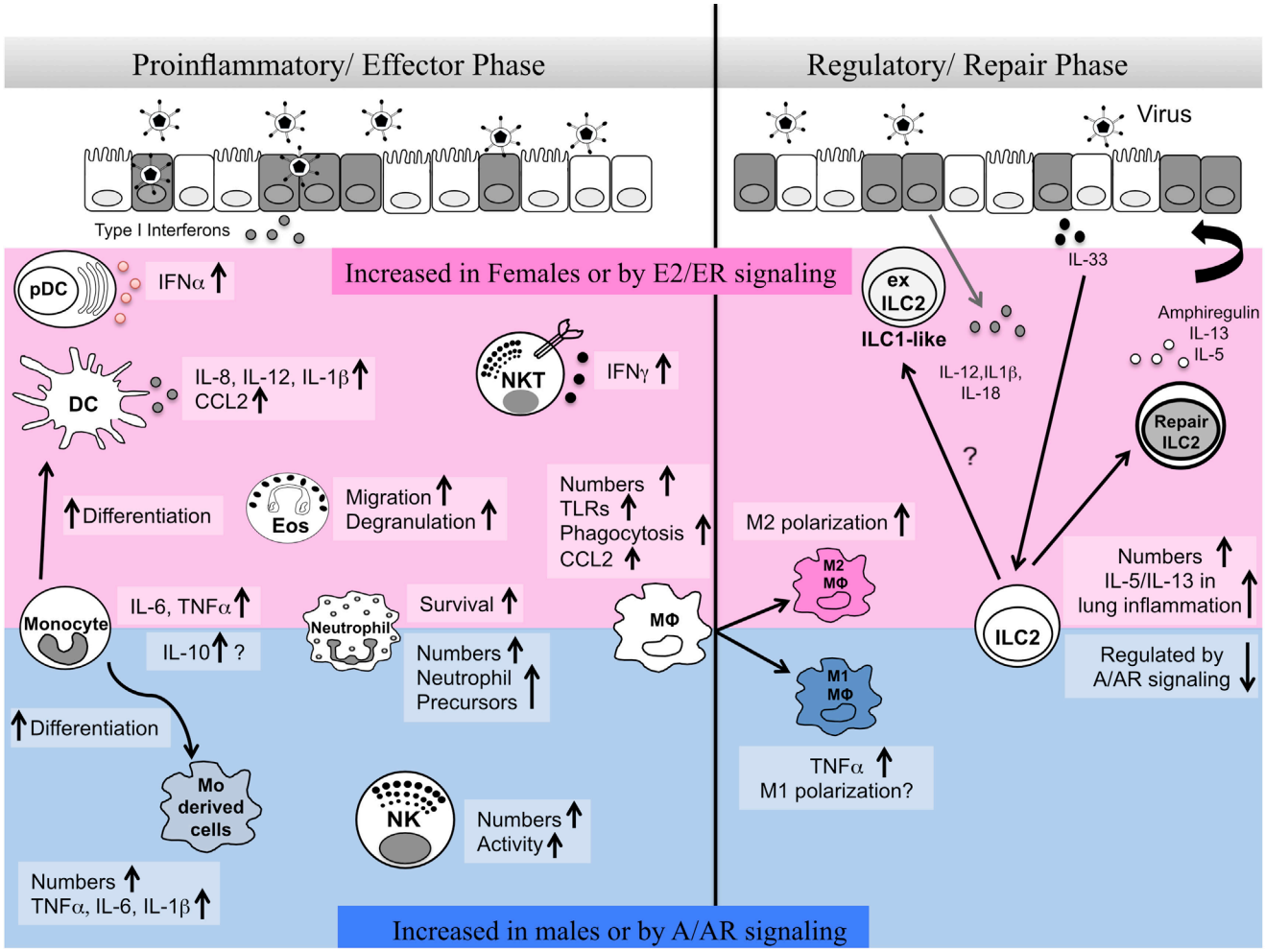

FIGURE 1 | Sex differences in innate immune responses during the effector and repair phases of respiratory virus infection. Here, we summarize reports of sex differences or sex hormone receptor regulation of innate immune cells. The pink shaded area indicates cells and pathways reported to be elevated in females and/or upon estrogen/ER activity. The blue shaded area indicates cells and pathways reported to be increased in males and/or upon androgen/AR activity.
A balanced type 1 immune response involving different innate immune cells is required early post-infection in the lung for viral clearance. At later stages of infection, regulatory immune responses mediated by alveolar macrophages and innate lymphoid cells are important for the repair of damaged tissues and renewal of barrier integrity. Sex differences in numbers, functional responses, plasticity, and survival of innate immune cells regulate the proinflammatory/effector and regulatory/repair phases of infection.

Testosterone is synthesized in both females and males and is converted by 5α-reductase to the physiologically active metabolite dihydrotestosterone (DHT) or by aromatase to estradiol (8). Progesterone (P4) is present in both sexes and, in females, varies during the menstrual cycle and is produced by the placenta at high levels during pregnancy (9). The levels of circulating sex hormones vary in both sexes throughout the lifespan, with the highest levels of estrogens and progesterone in females and testosterone in males during the reproductive years (10, 11). In pregnancy, estrogens and progesterone reach significantly higher levels (8, 12). Sex hormone levels in humans in phases of the menstrual cycle and pregnancy are nicely summarized in a recent review (13). The dramatic changes in sex hormone levels at puberty are correlated with changes in immune function and susceptibility to immune-mediated disease. Sex hormones also are present *in utero* and immediately post-birth, and this may influence immune cell differentiation and neonatal immunity. The developing testes in male fetuses produce testosterone, and both sexes are exposed to high levels of maternal estrogens *in utero* (14, 15). In the first weeks after birth, both human and rodent males have a "mini-puberty," in which testosterone levels approach those of adults (15–17).

Sex steroids are synthesized in the gonads and adrenal cortex, and in peripheral tissues such as liver, fat, and kidney (8, 18). Little information is available about local synthesis in the lung (8). Activated macrophages may increase local estrogen levels since cytokine receptor signaling induces their synthesis of aromatase, the enzyme that converts testosterone to estradiol (19). Few studies of immune cells in tissues have correlated tissue levels of sex hormones with immune function.

Sex Hormone Receptors

Sex hormones mediate their effects through estrogen receptors (ERα and ERβ), androgen receptor (AR), and progesterone receptors (PR-A and PR-B) (20–22). Splice variants of ER leading to truncated but functional proteins such as ERα46 have been identified in myeloid cells (23). Sex steroid receptors are ligand-dependent transcription factors that recruit transcriptional coregulators such as SRC1 and histone-modifying enzymes such as p300/CBP into multi-protein complexes that bind DNA [reviewed in Ref. (20, 24)]. ERs, PRs, and AR bind to their respective response elements at specific DNA sites leading to epigenetic modifications of chromatin and changes in transcription of target genes. Nuclear sex hormone receptors also may be tethered indirectly to DNA *via* their ability to bind transcription factors such as SP1. Ligand-free receptors also can recruit corepressors such as NCOR and histone deacetylases to repress transcription. Rapid "nongenomic" sex steroid signaling occurs via inner plasma membrane-localized ER or AR, and possibly *via* the G protein-coupled receptor GPR30 (also termed GPER) (20, 25).

Innate immune cells express ERs (*Esr1*, *Esr2*), AR (*Ar*), and PRs (*Pgr*) to varying degrees. *Esr1* and *Ar* RNAs also are expressed at high levels in hematopoietic progenitors in bone marrow (BM), consistent with documented effects of sex hormones on immune cell differentiation and numbers in homeostasis (26). Based on our literature review and data from the Immunological Genome Project (www.immgen.org), **Table 1** summarizes the relative expression of sex steroid receptor RNA or protein in hematopoietic progenitors and innate cells of the lymphoid and myeloid lineages. Since limited information is available about sex steroid receptor expression in lung-resident immune cells, **Table 1** includes information for the cell type regardless of tissue location or activation state. Patterns of receptor expression may underlie the effects of the sex hormones on numbers and functional responses of innate immune cells. Some mature innate cells do not apparently express significant levels of the sex hormone receptors, but they may still function differently in the sexes due to epigenetic imprinting of developmental precursors or because their responses are influenced indirectly *via* other cell types responding to sex hormones.

Sex differences in lung development, structure, and function have been identified (57). The lungs of human females are smaller than males of the same height; however, airway size and capacity do not always correlate with lung size, and the extent and type of sex differences in lung function vary throughout the lifespan (58). Smooth muscle, fibroblasts, and epithelial cells express sex steroid receptors (8), and their functional responses in different sex hormone environments may alter the immune response or modulate infection severity.

Methods to Study Effects of Sex Hormones and Receptors on Immunity *In Vivo*

Investigators have taken multiple approaches to understand the impact of sex differences and sex hormone receptor signaling on immunity. Diverse approaches in different experimental models have often led to conflicting results. Age should be carefully

TABLE 1 | Expression of sex steroid receptors in human and murine innate immune cells.

Cell type	ERα	ERβ	Other ERs	PRs	AR	ERα	ERβ	PRs	AR
	Human					Murine			
Type II innate lymphoid cells						Yes (27)/No (28)	Yes (27)/No (28)	–/+	Yes (28–30)
Natural killer cells (NK)	Yes (31, 32)	Yes (31, 32)	ERα46 (31)	Yes (33)	–	Yes (34)	Yes (34)	–	–/+
Gamma delta T cells (γδ T)				Yes (35)		–/+ or +	–/+	–	+ or –
Natural killer T cells	–	–/+		–	–	Yes (36)	–/+	–	– or –/+
Neutrophils	Yes (37)	Yes (37)	GPER (38)	No (39)	Yes (40)	–/+	–	–	Yes (41)
Eosinophils	–/+	–/+	GPER (42)	No (39)	No (40)	–/+	–	–	–
Plasmacytoid dendritic cells (pDC)	Yes (32)	Yes (32)		–	–	Yes (43)	–	–/+	–
Monocytes	Yes (23, 32, 44, 45)	Yes (23, 32, 44, 45)	ERα46 (23)	–	–	+	–/+	–	–
Dendritic cell (tissue-resident, monocyte-derived, BM-derived)	Yes (32, 45)	Yes (32, 45)		–	–	Yes (46, 47)	Yes (46, 47)/No (48)	–/+	–
Macrophage (alveolar, BM-derived, peritoneal)	Yes (23, 49, 50)	Yes (23, 49, 51)	ERα46 (23)	Yes (49)	Yes (40, 52)	Yes (48, 53)	Yes (50)/No (48, 53)	Yes (54)	Yes (55)
Hematopoietic stem cell	Yes (26)	Yes (26)		–	Yes (26)	Yes (56)	No (56)	No (56)	No (56)

The presence of sex steroid receptors in each cell type (located in any tissue and regardless of activation state) is indicated by "Yes" or "No" and the literature reference. Some cell types differ in receptor expression in different tissues, and this is indicated by "Yes/No." If a literature report was not found, we consulted the Immunological Genome Project, and the presence or absence of receptor RNA is indicated in blue if available.

–, <50 counts; –/+, 50–100 counts; +, 100–300 counts; + or – represents positive or negative value depending upon the tissue location.

considered when studying sex differences in immune cell numbers or functional responses in humans and rodents since sex hormone levels vary over the lifespan (2). Littermate or colony matching will reduce variables such as diet and microbiome and help to identify sex differences. Studies of sex differences in human immunity would be improved by direct measurements of hormone levels in each individual (particularly in women) because age alone does not predict hormone levels modulated by oral contraceptives or hormone replacement therapy. A common approach is to gonadectomize young mice and replace estrogens or androgens by implantation of time release pellets. While this strategy has led to many answers, the absence of sex hormones in young gonadectomized mice may alter immune cell development and numbers prior to infection. In addition, replacement of hormones to a constant level does not mimic the cyclic variation that occurs particularly in females. Similarly, it is difficult to recapitulate accurate *in vivo* exposures of sex hormones in cell culture models. Another approach is to impose male levels of DHT in a female mouse (or female levels of estradiol in a male mouse) to help elucidate sex hormone interactions and their effects independent of chromosomal sex and developmental programming.

Mice lacking sex hormone receptors also have informed our understanding of sex differences in immunity. However, global deletion of sex hormone receptors can lead to abnormal levels of estrogens and androgens; for example, global *Esr1* deficiency leads to high levels of circulating testosterone (59–61). Furthermore, global loss of receptor signaling may alter the function of non-immune cell types in ways that impact immune responses. To circumvent this issue, investigators are beginning to study mice bearing conditional deletion of *Esr1* or *Ar* along with lineage-restricted Cre drivers to understand the effects of sex hormone receptor deficiency on numbers and function of specific cell types. This approach will help to identify direct effects of sex hormone receptor signaling in immune cells. Use of emerging technologies such as single cell RNA-sequencing, assay of transposase-accessible chromatin-sequencing, multiplex mass cytometry, or chip cytometry (62–64) to monitor the transcriptome, epigenome, or proteome at the single cell level will help us to understand sex differences in immune function and how sex hormone receptors regulate immune cells in homeostasis and during viral infection. These approaches will be especially valuable to dissect the diversity of responses of rare immune cell types in peripheral tissues such as the lung. More precise methods and attention to age and hormonal cycles and levels will help to clarify the roles of sex hormones and receptors in immune responses.

SEX DIFFERENCES IN INNATE IMMUNE RESPONSES IN RESPIRATORY VIRUS INFECTION

Despite the lung's structural and chemical barriers to pathogen entry, many viruses subvert these barriers and efficiently infect and replicate within lung epithelial cells [reviewed in Ref. (65)]. Damage to host lungs may be directly induced by the virus or be secondary to a strong immune response. Upon respiratory virus infection, immune cells typically participate in three phases: (i) innate immune cells sense presence of the virus and initiate early antiviral responses and prime the adaptive response; (ii) effector or adaptive immune cells clear virus by killing infected cells and producing antiviral antibodies, followed by conversion of a subset to memory lymphocytes; and (iii) innate immune cells act in concert with epithelial regeneration pathways to repair injured tissue and produce mediators that return the immune system to homeostasis (65). Herein, we focus on responses of innate immune cells in the initiation and repair phases of respiratory virus infection.

Epidemiological studies of humans and experimental models with rodents show that it is difficult to arrive at a universal paradigm regarding effects of sex or sex hormones on immune responses to respiratory viruses. vom Steeg and Klein hypothesize that sex differences in infection outcome are a function of the strength of the immune response and resulting host tissue damage (66). In this model, a male bias in risk occurs when weak immune responses underlie significant host damage, while a female bias in risk occurs when strong immune responses promote host damage. Experimental manipulation of sex hormones and their receptors in rodents has shown that sex differences in systemic estrogen and androgen levels often underlie differential immune function and infection outcome. Depending on the role of the sex hormone to promote or inhibit inflammation or immunity, sex differences may arise due to either the predominance of, or the absence of, estrogen or androgen in one sex.

In the initiation phase, lung-resident dendritic cells (DCs) and macrophages (alveolar and interstitial) respond to viral molecules (nucleic acids and glycoproteins) *via* cell surface or intracellular receptors that are linked to signaling pathways resulting in production of interferons (IFN), cytokines, and chemokines (65, 67). Coupled with these viral "pathogen-associated molecular patterns," damage to host cells results in the release of host molecules such as ATP, heat shock proteins, or HMGB1, termed "danger-associated molecular patterns," which also trigger innate immune receptors and inflammasomes. Innate lymphocytes respond to cytokines produced by activated myeloid cells or alarmins released by damaged tissue and in turn produce type 1 (IFNs, IL-12, IL-1β, TNFα) or type 2 or regulatory (IL-5, IL-10) cytokines that direct subsequent innate or adaptive responses. Type I and III IFNs elicit expression of molecules that are directly antiviral. DCs acquire and present viral antigens, migrate to draining lymph nodes and prime adaptive responses through interactions with naïve T. Activated T cells then return to the lung where they interact again with resident or recruited myeloid cells, produce pro- or anti-inflammatory cytokines, and lyse infected cells.

Respiratory viruses typically elicit strong type 1 immune responses involving myeloid cell production of type I and III IFN and proinflammatory mediators such as IL-12, TNFα, and CCL2 and lymphocyte production of IFNγ (67). As described in detail in later sections, there is some evidence for sex differences in (or sex hormone regulation of) the function of myeloid cells and innate lymphocytes during respiratory virus infection. However, more often, reports of sex differences or sex hormone regulation involve studies of immune cells at other tissue sites, in autoimmune or other pathogen models or performed *in vitro*. In brief,

type I IFN synthesis is promoted by estrogen and ERα signaling, and multiple reports show that female plasmacytoid DCs (pDCs) produce more type I IFN than male pDCs (32, 43, 68, 69). Sex hormone regulation of proinflammatory cytokines (IL-12, IL-6, IL-1β) seems more complex, but a number of studies show that lower physiological levels of estrogens enhance their production while higher physiological levels dampen their production and instead promote regulatory cytokines such as IL-10 [reviewed in Ref. (24)]. In contrast, reports show that testosterone decreases cytokines such as IFNγ and TNFα and increases IL-10 (21).

Regulatory and type 2 immune responses are important in later stages of respiratory viral infection. It is now recognized that while type 1 responses are important for viral clearance, type 2 responses also are elicited and promote repair of injured tissue and resolution of the immune response upon influenza virus and respiratory syncytial virus (RSV) infection (70–72). In murine models of allergic asthma, estrogen and ERα signaling promote type 2 responses of alveolar macrophages (AM) (73), while androgens and AR signaling attenuate type 2 responses promoted by innate lymphoid cells (ILC2s) and myeloid cells (74). These ER and AR regulated pathways also may be important in respiratory virus infection. Indeed, the chronic elevation of type 2 responses in asthmatic individuals can lead to a milder course of influenza virus infection and reduced lung injury (75), while ILC2 activity in influenza virus infection can exacerbate asthma (76).

Influenza Virus A (IAV) Infection

Sex differences in the incidence and severity of IAV infection in the human population have been well documented (13, 77). However, given the strong impact of age on morbidity and mortality, it is often difficult to separate effects of sex and age since sex hormone levels change dramatically with age (78). Furthermore, social and cultural differences in gender norms also may influence ascertainment or self-reporting of infection symptoms or access to medical care. While the incidence of infection is often higher in males, females often show greater morbidity. Increased infection severity in females may result from stronger innate and adaptive responses in females that lead to more extensive immunopathology. Epidemiological studies from the 1957 H2N2, 2005 H1N1, and 2009 H1N1 pandemic IAV infections showed that the mortality and hospitalization of patients following viral infection was higher for females than males during their reproductive years (77, 79–81). This suggests that adult levels of sex hormones modulate immunity to IAV infection; however, these studies did not measure immune responses at the molecular or cellular level. Females in their reproductive years also have increased asthma incidence (58), which may alter immune responses and increase IAV-induced pathology. In contrast, infection of young males (<age 20) and elderly adults (>age 80) led to greater hospitalization or mortality (80). While this might suggest that lower levels of androgens in young boys and elderly men correlate with increased infection severity, information about comorbidities and measurement of androgen levels coupled with more precise information regarding susceptibility of males pre- and post-puberty would be needed to make this correlation.

We also lack information regarding differential susceptibility to IAV infection in distinct phases of the menstrual cycle and in women taking oral contraceptives. These hormonal variables may modulate susceptibility or severity of IAV infection, as epidemiological data from asthmatic women and girls show premenstrual aggravation of asthma symptoms and alleviation of this cyclical effect while taking the oral combined contraceptive pill (58). Pregnancy was highly associated with increased mortality and morbidity following IAV infection, and one factor may be immune suppression by elevated estrogens and progesterone (82–84).

Studies of mice infected with mouse adapted and pandemic H1N1, and avian H3N1 and H7N9, viruses have provided valuable insights into sex differences in susceptibility and immunity to IAV. Morbidity, mortality, and the associated inflammatory response is greater in female than male mice at moderate IAV loads, but mortality of both sexes is similar at higher loads (85–87). At sublethal doses, females showed higher levels of TNFα, IFNγ, and CCL2 (85, 88) and neutralizing anti-influenza antibodies, which correlated with greater protection upon heterosubtypic virus challenge (86). At viral doses lethal in females, but not males, estrogen protected from mortality, as shown by comparing ovariectomized mice supplemented with estradiol or placebo (85). Estrogen replacement correlated with reduced TNFα and CCL2 production, yet increased numbers of neutrophils and $CD8^+$ viral antigen-specific T cells producing IFNγ (89). Overall, gonadally intact and gonadectomized females produced greater inflammatory responses and showed increased morbidity following infection, suggesting that low levels of estrogens promote excessive inflammatory responses. In contrast, replacement of higher levels of estradiol to gonadectomized mice ameliorated inflammation and promoted adaptive immunity. This is consistent with anti-inflammatory effects of replaced estrogen in autoimmune disease models (90) and the ability of ERα to negatively regulate NF-κB signaling [reviewed in Ref. (20)].

Ovariectomy of females followed by progesterone replacement to luteal phase levels also reduced morbidity upon IAV infection (91). Progesterone led to increased tissue repair due to upregulation of the epidermal growth factor amphiregulin (Areg) in the lung (91). These studies suggest that progesterone-based contraceptives may promote recovery from respiratory virus infection (9).

Gonadectomy of young males increased morbidity and pathology upon IAV infection, and replacement of testosterone or DHT, which cannot be metabolized to estradiol, reduced morbidity, mortality, and inflammation (85, 92). In contrast, testosterone treatment of old male mice, which have decreased testosterone levels as in humans, increased survival but did not alter pathology (92). These data are consistent with the ability of testosterone to suppress inflammation (21, 93).

In murine models of sublethal IAV infection, morbidity typically is most related to immune-mediated pathology rather than failure to clear virus (67). Thus, data from the above studies suggest that the increased morbidity and mortality of females is secondary to a strong proinflammatory response that leads to extensive tissue damage, while the lesser morbidity in males is the result of a more balanced immune response that clears virus with less tissue damage. Sex hormones that suppress inflammation (testosterone, progesterone, or high levels of estrogens) may

attenuate antiviral immune responses to an optimal level, while lower levels of estrogens and androgens may permit excessive inflammation in some cases. The evolutionary benefit of this disparate effect of female and male sex hormones on immunity remains unclear.

Other Respiratory Viruses

Infection by two other respiratory viruses leads to increased morbidity in males. RSV is a common respiratory tract infection that most often progresses to the lower respiratory tract with severe consequences in infants and the elderly. The overall incidence of RSV is higher in young boys than girls (94, 95); however, the possible immunological basis of this sex difference and the role of sex hormones remains unknown. In outbreaks of pathogenic coronaviruses including the severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome (MERS-CoV), males showed increased infection incidence and severity (96, 97). Male mice showed enhanced susceptibility to SARS-CoV including elevated viral titers and increased accumulation of inflammatory monocytes and neutrophils in the lungs (98). ER signaling in females may be protective in this infection since ovariectomy or treatment with an ER antagonist increased mortality, while male gonadectomy did not alter disease outcome.

SEX DIFFERENCES IN INNATE IMMUNE CELLS DURING RESPIRATORY VIRUS INFECTION

During respiratory viral infection, responses of lymphoid and myeloid innate cells play a crucial role in early antiviral protection and promote the generation of adaptive immune responses including effector and memory T and B cells. Here, we highlight studies demonstrating sex differences and effects of sex hormones in the number, function, and development of innate cells in the respiratory tract (**Figure 1**). We also review reports of sex differences and sex hormone regulation in innate cells in other tissues, which may inform our understanding of sex-dependent regulatory mechanisms in the respiratory tract. This topic is the subject of excellent recent reviews (74, 93, 99, 100).

INNATE LYMPHOCYTES

Type II ILC2s

Innate lymphoid cells are tissue-resident cells that develop from lymphoid progenitors but lack antigen specific receptors. Like T cells, ILCs are divided into the ILC1, ILC2, ILC3, and natural killer cell (NK) subsets based on expression of fate-determining transcription factors and cytokine production (101). In homeostasis, ILC2s are the predominant ILC subset in the murine lung (102), and both ILC2s and ILC3s are predominant in human lung (103). NKs (see below), ILC1s, and ILC2s generate innate responses during IAV infection while the role of ILC3s has not been investigated. Notably, ILC2s in murine lung, BM, and small intestine express high levels of *Ar* but little *Esr1* or *Esr2* (27–30).

Respiratory viral infections cause death of lung epithelium mediated by viral toxicity and immune cell activation, and appropriate remodeling of lung tissue to maintain barrier integrity is crucial (70). ILC2s are important for tissue repair following IAV infection as they expand and secrete Areg, IL-13, and IL-5 (76, 104, 105). Areg promotes regeneration of the bronchial epithelium, and IL-13 promotes barrier integrity by inducing epithelial cell proliferation and survival (91, 106). IL-5 recruits eosinophils that promote antiviral immunity and lung tissue regeneration in the resolution phase (105, 107, 108). *Via* these pathways, ILC2s facilitate tissue repair in IAV and RSV infection (104, 109).

We and others reported sex differences in murine lung ILC2 numbers, with female mice harboring more lung ILC2s compared to males in homeostasis (28, 30, 110). A functional subset of lung ILC2s that lack the inhibitory E-cadherin-binding receptor KLRG1 is uniquely present in females (28, 110). Experiments involving hormone replacement in gonadectomized mice and mice bearing global or conditional deficiency in *Esr1* or *Ar* showed that the sex difference in ILC2s is regulated by androgens and AR but not estrogens or progesterone (28, 30, 110). Males have increased numbers of ILC precursors in BM, suggesting that androgens attenuate the progression from ILC precursor to mature ILC2 (110). In humans, sex differences in lung ILC2s have not yet been investigated; however, increased numbers of ILC2s are present in the blood of asthmatic females compared to males (30). Interestingly, sex hormones may regulate ILC2s differently in each tissue. Estrogen and ERα signaling sustain uterine ILC2s, which express high levels of *Esr1* compared to lung ILC2s (27). Fewer ILC2s accumulate in the central nervous system of female mice in the EAE model of multiple sclerosis (111). A lower proportion of ILC2s are present in cord blood of human female neonates compared to males (112).

Innate lymphoid cells in gonadectomized males produce more IL-5 and IL-13 after stimulation (28). Similarly, DHT treatment *in vivo* decreases IL-5 and IL-13 production by ILC2s (30), although a direct role for AR was not tested in these studies. Together with the finding that progesterone increases Areg expression (91), these data suggest that IAV-infected females may show superior tissue repair due to increased numbers of ILC2s capable of producing IL-13, IL-5, and Areg.

Alternately, the higher number of ILC2s in females may induce more pathology due to their functional plasticity. ILC2s convert to ILC1-like cells producing IFNγ in response to IL-12 and IL-18 produced during IAV infection and lung inflammation triggered by smoking or chronic obstructive pulmonary disease (113–115). Although sex differences in ILC2 plasticity during IAV infection have not been reported, higher numbers of ILC2s that are capable of converting to ILC1s at the peak of infection may contribute to more severe immunopathology in females.

Natural Killer (NK) Cells

Natural killers are cytotoxic innate lymphocytes that control viral burden *via* their early production of IFNγ (116). NKs enhance DC migration and T cell recruitment upon infection with a sublethal IAV dose, but depletion of NKs was protective after infection with a lethal dose (116). Human and murine NKs express ERs and PR but not AR (**Table 1**). Human studies revealed higher numbers and cytotoxic activity of blood

NK cells in males compared to females (117–119). This sex difference was reversed in old age (120). Studies show that NK numbers in blood correlate with stages of the menstrual cycle, suggesting regulation by sex hormones (121–123). In pregnancy, the recruitment of NKs from the blood to the uterine mucosa coincides with the dramatic rise in estriol and progesterone (124, 125). However, the effect of estrogen or progesterone on NK cell activity is unclear since some studies showed that *in vitro* (human) or *in vivo* exposure to estrogen or progesterone decreases NK cell activity while others found no effect (33, 126–129). Sex differences in NK numbers or function during IAV infection have not been reported.

Gamma Delta (γδ) T Cells

Innate γδ T cells bear TCRs with limited junctional diversity that recognize intact protein antigens and small phosphate or amine containing molecules (130). γδ T cells are divided into different tissue-specific subsets based on predominant pairings of particular Vγ or Vδ genes (131). γδ T cells show important functional responses during infection by RSV and IAV. In murine RSV infection, γδ T cells are recruited to the lungs and produce IFNγ, IL-17A, IL-10, and IL-4 resulting in the activation of other innate cells (132). Vγ4$^+$ T cells also secrete IL-17A during IAV H1N1 infection to aggravate acute lung immunopathology (133). Levels of circulating Vγ9/Vδ2 T cells in adult women were significantly higher than in men (134); however, another study showed the opposite trend (135). Sex differences in γδ T cells in mice have not been reported.

Natural Killer T Cells (NKT)

Natural killer T cells facilitate cross-talk between the innate and adaptive immune system during viral infection. NKT cells are a subset of T lymphocytes expressing a restricted αβ TCR that recognizes CD1d-bound lipids. NKT cells play a protective role in IAV infection through their secretion of IL-22 and IFNγ to activate NK cells and CD8$^+$ T cells (67). The absence of NKTs in a murine model of RSV infection led to a delay in viral clearance, suggesting a protective role in infection (136). In mice, estrogen acting *via* ERα regulates a sexual dimorphism in NKT function. Administration of estradiol to ovariectomized mice increased NKT IFNγ production upon *in vivo* stimulation by IL-12 + IL-18 and a CD1d ligand, and NKTs in ERα−/− mice produced less IFNγ (36). Reports of sex differences in human NKTs are inconsistent, and data from the Immunological Genome Project show only low levels of sex hormone receptor RNA in human NKTs (**Table 1**). Increased blood NKT cell numbers in women relative to men was reported in some studies (137–139). Sex differences in NKT cells in respiratory virus infection have not been reported, but in view of the above studies, analyses of possible sex disparate responses in NKT function in murine models of IAV or RSV may yield important insights.

Innate Lymphocyte Summary

Innate lymphocytes express sex hormone receptor RNAs at varying levels depending on the subset and tissue location (**Table 1**). While NK and NKT cells primarily express ERs, lung ILC2s predominantly express AR, suggesting regulation of the classes of innate lymphocytes by distinct sex hormone-mediated mechanisms. However, we lack information about sex differences in numbers and function of these diverse subsets in the murine lung during respiratory virus infection. Recent studies have shown a profound sex difference in numbers and functional responses of murine lung-resident ILC2s, and AR signaling decreases numbers of ILC2s in males. Future work will determine if this numerical disparity in ILC2s leads to sex differences in the resolution of respiratory virus infection. Reports of sex differences in numbers or function of innate lymphocytes in human blood are often conflicting, and more studies that carefully correlate gender, age, and sex hormone status with lymphocyte numbers and function in blood or tissues are needed to clarify the field.

MYELOID CELLS

Neutrophils

Neutrophils are the predominant infiltrating innate cell type during respiratory viral infection in both humans and mice. Neutrophils mediate antiviral defense *via* their production of proinflammatory cytokines and reactive oxygen species (140). Their role in respiratory viral infection remains unclear, as they cause pathology and susceptibility to secondary infections in mice. Neutrophil numbers and neutrophil extracellular trap (NET) formation directly correlate with the severity of RSV infection (141, 142).

Neutrophils express ER and AR (**Table 1**), and sex differences in the number and function of neutrophils in humans have been reported. Neutrophil numbers in blood are increased during pregnancy and the luteal phase of the menstrual cycle, suggesting that higher levels of progesterone or estrogens promote neutrophil numbers (143–145). Neutrophils from healthy women of reproductive age show improved survival *in vitro* compared to those of healthy men. Estradiol and progesterone contribute to the delay in neutrophil apoptosis by decreasing expression of the pro-apoptotic protein caspase 3 (146). Other studies showed that sex hormones modulate neutrophil function *in vitro*, including chemotaxis and nitric oxide and superoxide production (147–149).

Sex hormones also regulate neutrophil numbers in homeostasis and infection in murine models. AR-deficient mice show reduced numbers of neutrophils and neutrophil precursors in BM (41). Consistent with regulation of neutrophil numbers by AR signaling, the enhanced susceptibility of male mice to SARS-CoV infection was associated with increased accumulation of neutrophils in the lung (98). In contrast, estradiol treatment of ovariectomized females elevated neutrophil chemoattractants and recruitment of neutrophils into the lungs, thereby increasing protection in IAV infection (89).

Eosinophils

Eosinophils enhance antiviral immunity during RSV infection by sensing viral RNA *via* TLR7 and producing nitric oxide (150). In IAV infection, eosinophil degranulation and activation of viral antigen specific CD8$^+$ T cells increases protection against

infection (151). Estrogen increases eosinophil migration adhesion, survival, and degranulation both *in vitro* (42, 152) and *in vivo* (153). Furthermore, the number of eosinophils in female rats peaks with higher levels of estrogen during estrus, and ovariectomy significantly reduces uterine eosinophils (154, 155). These studies suggest that female sex hormones regulate eosinophil numbers, but sex differences in the numbers or function of eosinophil during respiratory viral infection have not been reported. Since eosinophils were reported to express very little *Esr1* and no *Ar* or *Pgr* RNA (**Table 1**), sex differences in eosinophil numbers may be secondary to the sex differences in numbers of IL-5-producing ILC2s.

Alveolar Macrophages

Alveolar macrophages are lung-resident phagocytic cells that induce protective antiviral immune responses *via* production of soluble mediators (156). In viral infection, AMs produce high levels of type I IFN important for viral clearance and chemokines that recruit inflammatory monocyte into the lung (156). Sex differences or the effect of sex hormones in AM function during respiratory virus infection have not been described, although murine AMs express both ERα and AR (73). Studies of peritoneal macrophages, which also express ERβ, offer some insight into how AMs may be regulated by sex hormones during virus-induced inflammation. Increased numbers of macrophages were present in the pleural and peritoneal cavities of female mice, and they showed higher levels of TLRs and phagocytic capacity, which was associated with stronger acute inflammatory responses (157). Consistent with this, inflammatory TLR-mediated responses of human monocyte-derived macrophages and murine peritoneal macrophages were enhanced by estrogen and reduced by testosterone exposure (158–161).

The roles of sex hormones in AM function during allergic asthma may provide insight into sex differences in AM functional responses in viral infection. AMs are polarized to an M1 phenotype in a type 1 environment involving IFN or to an M2 phenotype in a type 2 environment involving IL-4/IL-13. In allergy models, female mice show an increased type 2 polarized AM response, and estrogen signaling *via* ERα in AMs was an important driver of the allergic response *in vivo* (73, 99, 162). This is consistent with other reports that female sex and/or ERα signaling promotes M2 macrophage function in cutaneous wound healing (163), Coxsackievirus-induced myocarditis (164), and atherosclerosis (53). In contrast, AR activity in macrophages suppresses wound healing by enhancing local TNFα expression (165). These data suggest that estrogens and ERα may promote, while AR may attenuate, the type 2 responses that promote tissue repair in the resolution phase of a viral infection.

Monocytes and Monocyte-Derived Cells

Monocytes respond to viral infection by secreting cytokines and chemokines. They also are precursors to "inflammatory" macrophages or DCs in tissues. Following virus infection, $CCR2^+$ monocytes are recruited *via* the chemokine CCL2 from blood to the lung, where they differentiate into DC- or macrophage-like cells often producing proinflammatory cytokines such as TNFα and IL-12 (166). Physiological levels of estradiol decrease expression of CCR2 and CXCR3 on murine monocytes *in vivo*, suggesting that ER signaling might reduce monocyte recruitment to tissues (167). Indeed, systemic estradiol treatment of ovariectomized mice reduced CCL2 induction and numbers of infiltrating monocytes during IAV infection, although no differences in numbers of inflammatory monocyte-derived DCs (Mo-DCs) were noted (89). Consistent with this, SARS-CoV infection of more susceptible male mice led to increased accumulation of monocyte-derived cells ($Ly6C^+$ $CD11b^+$) producing proinflammatory cytokines relative to female mice, and depletion of the monocyte-derived cells partially protected mice from a lethal infection (98). In this model, ovarian hormones and ER signaling in female mice were protective while orchidectomy of male mice had no effect, suggesting estrogens rather than androgens regulate pathogenic monocyte responses.

Reports of sex differences in human monocyte numbers and cytokine production are inconsistent and may reflect the diversity of the human population. Postmenopausal women showed increased numbers of monocytes compared to premenopausal women (168). Other work showed that monocyte counts were higher in the luteal phase associated with higher progesterone levels than in the follicular phase (143). Pregnancy also was associated with higher monocyte numbers, yet reduced capacity for IL-12 and TNFα production (169). Peripheral monocytes from healthy females produced more IL-6 upon LPS stimulation as compared to males (170). However, studies to determine if estrogens regulate pro-inflammatory cytokine production by female monocytes and monocyte-derived macrophages showed either negative (167, 171) or positive regulation (172). Macrophages and monocytes exposed to testosterone decreased their production of proinflammatory cytokines and increased synthesis of IL-10 (173–175).

Dendritic Cells

Dendritic cells are professional antigen-presenting cells classified by phenotype and functional capacity into distinct subsets including (pDCs), conventional DCs (cDCs), and Mo-DCs. While the lung harbors at least three subsets of tissue-resident cDCs (176), pDCs and Mo-DCs enter the lung in significant numbers upon infection. Murine lung-resident DCs express *Esr1* but little *Ar* (**Table 1**). The direct effect of sex hormone receptor signaling in these DC subsets in the lung during respiratory virus infection has not been reported. However, studies of sex differences and sex hormone effects on DCs in other tissues may provide some clues about lung DC subsets (100, 177).

Upon infection, lung-resident cDCs migrate to the draining mediastinal lymph nodes and prime naïve T cells. While sex differences in the numbers or function of these DCs during virus infection have not been reported, no differences in lung cDC numbers were found in ovariectomized mice treated with placebo or estradiol and infected with IAV (89). Functional studies with murine BM-derived DCs showed that estradiol and ERα signaling promote the TLR dependent production of proinflammatory

cytokines of cDCs in the Flt3L-driven model and inflammatory DCs in the GM-CSF model (178–181). Estradiol also increased the production of IL-8 and CCL2 from human Mo-DCs (182). Other studies have shown that estradiol promotes GM-CSF-driven DC differentiation *in vitro* [reviewed in Ref. (177)]. Estradiol acts *via* ERα in murine myeloid progenitors to promote DC differentiation by upregulating the transcription factor IRF4 (183). In contrast, progesterone decreased TNFα and IL-1β but not IL-10 production by rat BM-derived DCs (184) and reversed estradiol-mediated changes in differentiation and function of BM-derived murine DCs (185). Progesterone modulated TLR-induced activation and cytokine production by murine BM-derived DCs (186).

Plasmacytoid DCs rapidly respond to viral particles *via* endosomal and cytosolic sensors of viral nucleic acids and produce type I IFN and IFN-induced proteins that are directly antiviral. Female pDCs produce significantly more IFNα in response to viral nucleic acids or synthetic TLR7 ligands than male pDCs (68, 69), and this correlates with higher levels of ERα-regulated IRF5 in female cells (187). Estrogen signaling and XX chromosome dosage promoted sex differences in TLR7-mediated IFNα production by human pDC (32), and estradiol treatment of postmenopausal women enhanced their production of IFNα (43). Models of conditional *Esr1* deficiency in DCs showed that ERα signaling drives sex differences in pDC functions (43, 188). Consistent with greater production of type I IFN by pDCs or other innate cells, female rats infected with respiratory Hantavirus showed greater expression of genes encoding viral nucleic acid sensors and type I IFN compared to males (189).

Testosterone and progesterone may suppress pDC responses, although pDCs do not apparently express significant levels of *Ar* or *Pgr* RNA in homeostasis (**Table 1**). Progesterone inhibits IFNα production by pDCs (190). Upon stimulation with a TLR7/8 agonist, human infant male infant pDC responses were significantly lower than those of females (191), which may be due to increased testosterone (or lower estrogen) levels in infants post-birth. Male PBMCs produced similar amounts of IFNα, yet greater amounts of IL-10 than female PBMCs upon IAV stimulation, and the IL-10 may dampen type 1 inflammation in males (192, 193). Taken together, these studies show that female pDCs produce higher levels of type I IFNs, consistent with stronger antiviral immune responses, yet more immunopathology in females.

Myeloid Cell Summary

Sex differences in the numbers or functional responses of myeloid cells in murine models of IAV and coronavirus infection have been reported. Manipulation of sex hormone signaling through gonadectomy −/+ sex hormone replacement, or ER or AR deficiency, has provided evidence for sex hormone-mediated regulation of neutrophils, pDCs, monocytes, and monocyte-derived cells in the lung during infection. Sex differences in lung-resident cDCs during infection have not been reported, but these DCs do express *Esr1* suggesting estrogens may regulate their important role in initiation of innate and adaptive responses to viruses. In asthma models in which females exhibit more disease, sex hormones regulate AM type 2 responses, suggesting that sex differences in AM function during the resolution phase of respiratory virus infection also may occur. Overall, more research is needed to fully understand mechanisms of sex hormone regulation of myeloid cells during respiratory virus infection and how these may contribute to sex differences in antiviral defense.

CONCLUDING REMARKS

Sex differences in immunity to respiratory viruses are evident in humans and experimental rodent models. Sex hormones may act directly in innate immune cells or their precursors to promote or attenuate their function, but it is also probable that innate cells are indirectly modulated by actions of other immune or non-immune cells responding to sex hormones. Differential regulation of innate cells by sex hormones during the proinflammatory/effector phase and resolution/repair phase is likely to shape the mechanisms of viral clearance and the host capacity to resolve inflammation and repair damaged tissue. For example, estrogens and ER signaling may promote IFN production by pDCs and NKT cells early post-infection, but also type 2 or regulatory responses of AMs important for optimal resolution of the infection. Sex or sex hormones may not have universal effects during respiratory virus infection. Indeed, although endogenous estrogens in gonad-intact murine females promoted inflammation during IAV, they were protective in coronavirus infection.

While elegant studies of sex differences and the role of sex hormones have informed the field of innate antiviral immunity, we still lack information on how sex hormone receptors act in individual cell types to regulate functional responses. Many reports of sex differences or sex hormone effects in immunity are conflicting, most likely because of experimental approaches that do not fully take into account sex hormone levels varying due to age or cycle, difficulty in reproducing natural sex hormone levels *via* manipulation *in vitro* or *in vivo*, or hormone imbalances in globally *Ar* or *Esr1* deficient mice. Our understanding of sex biases in the antiviral responses of innate lymphoid and myeloid cells of the respiratory tract will be greatly facilitated by more precise approaches and measurements enabled by emerging technologies. When possible, careful studies of innate immune cells in the respiratory tract of infected humans would also contribute greatly to our understanding of sex-specific molecular and cellular pathways that underlie population data on incidence and severity of viral infections.

Whether sex differences in immunity confer an advantage at the population level remains unclear. Ideally, the capacity for strong immune responses to infection or tumors would be balanced by a lesser propensity for autoimmunity. Studies suggest this continuum differs between the sexes, with females often capable of superior immunity to pathogens but more susceptible to autoimmunity (2), although not all reported data fit into this simple model. Sex differences in immune function may arise as a byproduct of the distinct levels of androgens and estrogens that specify biological sex and gonad development. Consistent with their ability to bind DNA and regulate chromatin conformation, sex hormone receptors may

act early in the pre- or postnatal period or during puberty to imprint sex-specific epigenetic patterns in the genome (5, 20). Epigenetically imprinted regions of open or closed chromatin in hematopoietic progenitors may differ between the sexes, and a sex divergent epigenome may be reinforced in mature immune cells in response to the sex hormone environment. The challenge of the field is to understand how sex hormones and their receptors regulate the epigenome and transcriptome in innate immune cells to mediate sex-divergent pathways that govern antiviral immune responses.

AUTHOR CONTRIBUTIONS

S Kadel and S Kovats reviewed the literature and wrote the manuscript.

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REFERENCES

1. Mizgerd JP. Lung infection – a public health priority. *PLoS Med* (2006) 3(2):e76. doi:10.1371/journal.pmed.0030076
2. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* (2016) 16(10):626–38. doi:10.1038/nri.2016.90
3. Chamekh M, Deny M, Romano M, Lefevre N, Corazza F, Duchateau J, et al. Differential susceptibility to infectious respiratory diseases between males and females linked to sex-specific innate immune inflammatory response. *Front Immunol* (2017) 8:1806. doi:10.3389/fimmu.2017.01806
4. Fischer J, Jung N, Robinson N, Lehmann C. Sex differences in immune responses to infectious diseases. *Infection* (2015) 43(4):399–403. doi:10.1007/s15010-015-0791-9
5. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. *J Immunol* (2017) 198(5):1782–90. doi:10.4049/jimmunol.1601166
6. Jaillon S, Berthenet K, Garlanda C. Sexual dimorphism in innate immunity. *Clin Rev Allergy Immunol* (2017). doi:10.1007/s12016-017-8648-x
7. Frump AL, Lahm T. Sex hormone signaling in the lung in health and disease: airways, parenchyma, and pulmonary vasculature. In: Hemnes AR, editor. *Gender, Sex Hormones and Respiratory Disease: A Comprehensive Guide*. Cham: Springer International Publishing (2016). p. 27–62.
8. Sathish V, Martin YN, Prakash YS. Sex steroid signaling: implications for lung diseases. *Pharmacol Ther* (2015) 150:94–108. doi:10.1016/j.pharmthera.2015.01.007
9. Hall OJ, Klein SL. Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunol* (2017) 10(5):1097–107. doi:10.1038/mi.2017.35
10. Decaroli MC, Rochira V. Aging and sex hormones in males. *Virulence* (2017) 8(5):545–70. doi:10.1080/21505594.2016.1259053
11. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Hum Reprod Update* (2005) 11(4):411–23. doi:10.1093/humupd/dmi008
12. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *Am J Obstet Gynecol* (1972) 112(8):1095–100. doi:10.1016/0002-9378(72)90185-8
13. Gabriel G, Arck PC. Sex, immunity and influenza. *J Infect Dis* (2014) 209(Suppl_3):S93–9. doi:10.1093/infdis/jiu020
14. vom Saal FS. Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. *J Anim Sci* (1989) 67(7):1824–40. doi:10.2527/jas1989.6771824x
15. Pang SF, Tang F. Sex differences in the serum concentrations of testosterone in mice and hamsters during their critical periods of neural sexual differentiation. *J Endocrinol* (1984) 100(1):7–11. doi:10.1677/joe.0.1000007
16. Quigley CA. Editorial: the postnatal gonadotropin and sex steroid surge-insights from the androgen insensitivity syndrome. *J Clin Endocrinol Metab* (2002) 87(1):24–8. doi:10.1210/jc.87.1.24
17. Motelica-Heino I, Castanier M, Corbier P, Edwards DA, Roffi J. Testosterone levels in plasma and testes of neonatal mice. *J Steroid Biochem* (1988) 31(3):283–6. doi:10.1016/0022-4731(88)90351-2
18. Labrie F, Luu-The V, Belanger A, Lin SX, Simard J, Pelletier G, et al. Is dehydroepiandrosterone a hormone? *J Endocrinol* (2005) 187(2):169–96. doi:10.1677/joe.1.06264
19. Capellino S, Straub RH, Cutolo M. Aromatase and regulation of the estrogen-to-androgen ratio in synovial tissue inflammation: common pathway in both sexes. *Ann N Y Acad Sci* (2014) 1317:24–31. doi:10.1111/nyas.12398
20. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol* (2015) 294(2):63–9. doi:10.1016/j.cellimm.2015.01.018
21. Trigunaite A, Dimo J, Jorgensen TN. Suppressive effects of androgens on the immune system. *Cell Immunol* (2015) 294(2):87–94. doi:10.1016/j.cellimm.2015.02.004
22. Buskiewicz IA, Huber SA, Fairweather D. Chapter 4 – sex hormone receptor expression in the immune system A2. In: Neigh GN, Mitzelfelt MM, editors. *Sex Differences in Physiology*. Boston: Academic Press (2016). p. 45–60.
23. Murphy AJ, Guyre PM, Wira CR, Pioli PA. Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages. *PLoS One* (2009) 4(5):e5539. doi:10.1371/journal.pone.0005539
24. Kovats S, Carreras E, Agrawal H. Sex steroid receptors in immune cells. In: Klein SL, Roberts C, editors. *Sex Hormones and Immunity to Infection*. Berlin, Heidelberg: Springer Berlin Heidelberg (2010). p. 53–91.
25. Langer G, Bader B, Meoli L, Isensee J, Delbeck M, Noppinger PR, et al. A critical review of fundamental controversies in the field of GPR30 research. *Steroids* (2010) 75(8–9):603–10. doi:10.1016/j.steroids.2009.12.006
26. Igarashi H, Kouro T, Yokota T, Comp PC, Kincade PW. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. *Proc Natl Acad Sci U S A* (2001) 98(26):15131–6. doi:10.1073/pnas.011513098
27. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus. *J Immunol* (2018) 200(1):229–36. doi:10.4049/jimmunol.1602085
28. Laffont S, Blanquart E, Savignac M, Cenac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. *J Exp Med* (2017) 214(6):1581–92. doi:10.1084/jem.20161807
29. Robinette ML, Fuchs A, Cortez VS, Lee JS, Wang Y, Durum SK, et al. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat Immunol* (2015) 16(3):306–17. doi:10.1038/ni.3094
30. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone attenuates group 2 innate lymphoid cell-mediated airway inflammation. *Cell Rep* (2017) 21(9):2487–99. doi:10.1016/j.celrep.2017.10.110
31. Pierdominici M, Maselli A, Colasanti T, Giammarioli AM, Delunardo F, Vacirca D, et al. Estrogen receptor profiles in human peripheral blood lymphocytes. *Immunol Lett* (2010) 132(1):79–85. doi:10.1016/j.imlet.2010.06.003
32. Laffont S, Rouquie N, Azar P, Seillet C, Plumas J, Aspord C, et al. X-Chromosome complement and estrogen receptor signaling independently contribute to the enhanced TLR7-mediated IFN-alpha production of plasmacytoid dendritic cells from women. *J Immunol* (2014) 193(11):5444–52. doi:10.4049/jimmunol.1303400
33. Arruvito L, Giulianelli S, Flores AC, Paladino N, Barboza M, Lanari C, et al. NK cells expressing a progesterone receptor are susceptible to progesterone-induced apoptosis. *J Immunol* (2008) 180(8):5746–53. doi:10.4049/jimmunol.180.8.5746
34. Curran EM, Berghaus LJ, Vernetti NJ, Saporita AJ, Lubahn DB, Estes DM. Natural killer cells express estrogen receptor-α and estrogen receptor-β and can respond to estrogen via a non-estrogen receptor-α-mediated pathway. *Cell Immunol* (2001) 214(1):12–20. doi:10.1006/cimm.2002.1886

35. Polgar B, Barakonyi A, Xynos I, Szekeres-Bartho J. The role of gamma/delta T cell receptor positive cells in pregnancy. *Am J Reprod Immunol* (1999) 41(4):239–44. doi:10.1111/j.1600-0897.1999.tb00433.x
36. Gourdy P, Araujo LM, Zhu R, Garmy-Susini B, Diem S, Laurell H, et al. Relevance of sexual dimorphism to regulatory T cells: estradiol promotes IFN-gamma production by invariant natural killer T cells. *Blood* (2005) 105(6):2415–20. doi:10.1182/blood-2004-07-2819
37. Molero L, Garcia-Duran M, Diaz-Recasens J, Rico L, Casado S, Lopez-Farre A. Expression of estrogen receptor subtypes and neuronal nitric oxide synthase in neutrophils from women and men: regulation by estrogen. *Cardiovasc Res* (2002) 56(1):43–51. doi:10.1016/S0008-6363(02)00505-9
38. Rodenas MC, Tamassia N, Cabas I, Calzetti F, Meseguer J, Cassatella MA, et al. G protein-coupled estrogen receptor 1 regulates human neutrophil functions. *Biomed Hub* (2017) 2(1):2. doi:10.1159/000454981
39. Aerts JL, Christiaens MR, Vandekerckhove P. Evaluation of progesterone receptor expression in eosinophils using real-time quantitative PCR. *Biochim Biophys Acta* (2002) 1571(3):167–72. doi:10.1016/S0304-4165(02)00192-7
40. Mantalaris A, Panoskaltsis N, Sakai Y, Bourne P, Chang C, Messing EM, et al. Localization of androgen receptor expression in human bone marrow. *J Pathol* (2001) 193(3):361–6. doi:10.1002/1096-9896(0000)9999:9999<::AID-PATH803>3.0.CO;2-W
41. Chuang K-H, Altuwaijri S, Li G, Lai J-J, Chu C-Y, Lai K-P, et al. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *J Exp Med* (2009) 206(5):1181–99. doi:10.1084/jem.20082521
42. Tamaki M, Konno Y, Kobayashi Y, Takeda M, Itoga M, Moritoki Y, et al. Expression and functional roles of G-protein-coupled estrogen receptor (GPER) in human eosinophils. *Immunol Lett* (2014) 160(1):72–8. doi:10.1016/j.imlet.2014.03.012
43. Seillet C, Laffont S, Tremollieres F, Rouquie N, Ribot C, Arnal JF, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. *Blood* (2012) 119(2):454–64. doi:10.1182/blood-2011-08-371831
44. Pioli PA, Jensen AL, Weaver LK, Amiel E, Shen Z, Shen L, et al. Estradiol attenuates lipopolysaccharide-induced CXC chemokine ligand 8 production by human peripheral blood monocytes. *J Immunol* (2007) 179(9):6284–90. doi:10.4049/jimmunol.179.9.6284
45. Komi J, Lassila O. Nonsteroidal anti-estrogens inhibit the functional differentiation of human monocyte-derived dendritic cells. *Blood* (2000) 95(9):2875–82.
46. Mao A, Paharkova-Vatchkova V, Hardy J, Miller MM, Kovats S. Estrogen selectively promotes the differentiation of dendritic cells with characteristics of Langerhans cells. *J Immunol* (2005) 175(8):5146–51. doi:10.4049/jimmunol.175.8.5146
47. Paharkova-Vatchkova V, Maldonado R, Kovats S. Estrogen preferentially promotes the differentiation of CD11c+ CD11b(intermediate) dendritic cells from bone marrow precursors. *J Immunol* (2004) 172(3):1426–36. doi:10.4049/jimmunol.172.3.1426
48. Lambert KC, Curran EM, Judy BM, Lubahn DB, Estes DM. Estrogen receptor-alpha deficiency promotes increased TNF-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. *J Leukoc Biol* (2004) 75(6):1166–72. doi:10.1189/jlb.1103589
49. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Matsuyama T, et al. Estrogen and progesterone receptor expression in macrophages and regulation of hepatocyte growth factor by ovarian steroids in women with endometriosis. *Hum Reprod* (2005) 20(7):2004–13. doi:10.1093/humrep/deh897
50. Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, et al. Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J Neurosci* (2001) 21(6):1809–18. doi:10.1523/JNEUROSCI.21-06-01809.2001
51. Kramer PR, Wray S. 17-Beta-estradiol regulates expression of genes that function in macrophage activation and cholesterol homeostasis. *J Steroid Biochem Mol Biol* (2002) 81(3):203–16. doi:10.1016/S0960-0760(02)00065-1
52. McCrohon JA, Death AK, Nakhla S, Jessup W, Handelsman DJ, Stanley KK, et al. Androgen receptor expression is greater in macrophages from male than from female donors. A sex difference with implications for atherogenesis. *Circulation* (2000) 101(3):224–6. doi:10.1161/01.CIR.101.3.224
53. Ribas V, Drew BG, Le JA, Soleymani T, Daraei P, Sitz D, et al. Myeloid-specific estrogen receptor α deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *Proc Natl Acad Sci U S A* (2011) 108(39):16457–62. doi:10.1073/pnas.1104533108
54. Lu J, Reese J, Zhou Y, Hirsch E. Progesterone-induced activation of membrane-bound progesterone receptors in murine macrophage cells. *J Endocrinol* (2015) 224(2):183–94. doi:10.1530/JOE-14-0470
55. Ashcroft GS, Mills SJ. Androgen receptor-mediated inhibition of cutaneous wound healing. *J Clin Invest* (2002) 110(5):615–24. doi:10.1172/JCI0215704
56. Nakada D, Oguro H, Levi BP, Ryan N, Kitano A, Saitoh Y, et al. Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. *Nature* (2014) 505(7484):555–8. doi:10.1038/nature12932
57. Thurlbeck WM. Postnatal human lung growth. *Thorax* (1982) 37(8):564–71. doi:10.1136/thx.37.8.564
58. Becklake MR, Kauffmann F. Gender differences in airway behaviour over the human life span. *Thorax* (1999) 54(12):1119–38. doi:10.1136/thx.54.12.1119
59. Elliot SJ, Berho M, Korach K, Doublier S, Lupia E, Striker GE, et al. Gender-specific effects of endogenous testosterone: female alpha-estrogen receptor-deficient C57Bl/6J mice develop glomerulosclerosis. *Kidney Int* (2007) 72(4):464–72. doi:10.1038/sj.ki.5002328
60. Akingbemi BT, Ge R, Rosenfeld CS, Newton LG, Hardy DO, Catterall JF, et al. Estrogen receptor-alpha gene deficiency enhances androgen biosynthesis in the mouse Leydig cell. *Endocrinology* (2003) 144(1):84–93. doi:10.1210/en.2002-220292
61. Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone* (2002) 30(1):18–25. doi:10.1016/S8756-3282(01)00643-3
62. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* (2013) 10(12):1213–8. doi:10.1038/nmeth.2688
63. Cheung P, Vallania F, Warsinske HC, Donato M, Schaffert S, Chang SE, et al. Single-cell chromatin modification profiling reveals increased epigenetic variations with aging. *Cell* (2018) 173(6):1385–97.e14. doi:10.1016/j.cell.2018.03.079
64. Happle C, Lachmann N, Skuljec J, Wetzke M, Ackermann M, Brennig S, et al. Pulmonary transplantation of macrophage progenitors as effective and long-lasting therapy for hereditary pulmonary alveolar proteinosis. *Sci Transl Med* (2014) 6(250):250ra113. doi:10.1126/scitranslmed.3009750
65. Yoo JK, Kim TS, Hufford MM, Braciale TJ. Viral infection of the lung: host response and sequelae. *J Allergy Clin Immunol* (2013) 132(6):1263–76; quiz 77. doi:10.1016/j.jaci.2013.06.006
66. vom Steeg LG, Klein SL. SeXX matters in infectious disease pathogenesis. *PLoS Pathog* (2016) 12(2):e1005374. doi:10.1371/journal.ppat.1005374
67. Newton AH, Cardani A, Braciale TJ. The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. *Semin Immunopathol* (2016) 38(4):471–82. doi:10.1007/s00281-016-0558-0
68. Berghofer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-alpha production in females. *J Immunol* (2006) 177(4):2088–96. doi:10.4049/jimmunol.177.4.2088
69. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences in the toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. *Nat Med* (2009) 15(8):955–9. doi:10.1038/nm.2004
70. Gorski SA, Hufford MM, Braciale TJ. Recent insights into pulmonary repair following virus-induced inflammation of the respiratory tract. *Curr Opin Virol* (2012) 2(3):233–41. doi:10.1016/j.coviro.2012.04.006
71. Guo XJ, Thomas PG. New fronts emerge in the influenza cytokine storm. *Semin Immunopathol* (2017) 39(5):541–50. doi:10.1007/s00281-017-0636-y
72. Sang Y, Miller LC, Blecha F. Macrophage polarization in virus-host interactions. *J Clin Cell Immunol* (2015) 6(2):311–21. doi:10.4172/2155-9899.1000311
73. Keselman A, Fang X, White PB, Heller NM. Estrogen signaling contributes to sex differences in macrophage polarization during asthma. *J Immunol* (2017) 199(5):1573–83. doi:10.4049/jimmunol.1601975
74. Laffont S, Blanquart E, Guery JC. Sex differences in asthma: a key role of androgen-signaling in group 2 innate lymphoid cells. *Front Immunol* (2017) 8:1069. doi:10.3389/fimmu.2017.01069
75. Samarasinghe AE, Woolard SN, Boyd KL, Hoselton SA, Schuh JM, McCullers JA. The immune profile associated with acute allergic asthma accelerates clearance

of influenza virus. *Immunol Cell Biol* (2014) 92(5):449–59. doi:10.1038/icb.2013.113
76. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* (2011) 12(7):631–8. doi:10.1038/ni.2045
77. Klein SL, Hodgson A, Robinson DP. Mechanisms of sex disparities in influenza pathogenesis. *J Leukoc Biol* (2012) 92(1):67–73. doi:10.1189/jlb.0811427
78. Gubbels Bupp MR, Potluri T, Fink AL, Klein SL. The confluence of sex hormones and aging on immunity. *Front Immunol* (2018) 9:1269. doi:10.3389/fimmu.2018.01269
79. Serfung RE, Sherman IL, Houseworth WJ. Excess pneumonia-influenza mortality by age and sex in three major influenza A2 epidemics, United States, 1957–58, 1960 and 1963. *Am J Epidemiol* (1967) 86(2):433–41. doi:10.1093/oxfordjournals.aje.a120753
80. Eshima N, Tokumaru O, Hara S, Bacal K, Korematsu S, Tabata M, et al. Sex- and age-related differences in morbidity rates of 2009 pandemic influenza A H1N1 virus of swine origin in Japan. *PLoS One* (2011) 6(4):e19409. doi:10.1371/journal.pone.0019409
81. Kumar A, Zarychanski R, Pinto R, Cook DJ, Marshall J, Lacroix J, et al. Critically ill patients with 2009 influenza A(H1N1) infection in Canada. *JAMA* (2009) 302(17):1872–9. doi:10.1001/jama.2009.1496
82. Satpathy HK, Lindsay M, Kawwass JF. Novel H1N1 virus infection and pregnancy. *Postgrad Med* (2009) 121(6):106–12. doi:10.3810/pgm.2009.11.2080
83. Louie JK, Acosta M, Jamieson DJ, Honein MA. Severe 2009 H1N1 influenza in pregnant and postpartum women in California. *N Engl J Med* (2010) 362(1):27–35. doi:10.1056/NEJMoa0910444
84. Jamieson DJ, Honein MA, Rasmussen SA, Williams JL, Swerdlow DL, Biggerstaff MS, et al. H1N1 2009 influenza virus infection during pregnancy in the USA. *Lancet* (2009) 374(9688):451–8. doi:10.1016/S0140-6736(09)61304-0
85. Robinson DP, Lorenzo ME, Jian W, Klein SL. Elevated 17beta-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses. *PLoS Pathog* (2011) 7(7):e1002149. doi:10.1371/journal.ppat.1002149
86. Lorenzo ME, Hodgson A, Robinson DP, Kaplan JB, Pekosz A, Klein SL. Antibody responses and cross protection against lethal influenza A viruses differ between the sexes in C57BL/6 mice. *Vaccine* (2011) 29(49):9246–55. doi:10.1016/j.vaccine.2011.09.110
87. Hoffmann J, Otte A, Thiele S, Lotter H, Shu Y, Gabriel G. Sex differences in H7N9 influenza A virus pathogenesis. *Vaccine* (2015) 33(49):6949–54. doi:10.1016/j.vaccine.2015.08.044
88. Larcombe AN, Foong RE, Bozanich EM, Berry LJ, Garratt LW, Gualano RC, et al. Sexual dimorphism in lung function responses to acute influenza A infection. *Influenza Other Respi Viruses* (2011) 5(5):334–42. doi:10.1111/j.1750-2659.2011.00236.x
89. Robinson DP, Hall OJ, Nilles TL, Bream JH, Klein SL. 17beta-estradiol protects females against influenza by recruiting neutrophils and increasing virus-specific CD8 T cell responses in the lungs. *J Virol* (2014) 88(9):4711–20. doi:10.1128/JVI.02081-13
90. Offner H, Polanczyk M. A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis. *Ann N Y Acad Sci* (2006) 1089:343–72. doi:10.1196/annals.1386.021
91. Hall OJ, Limjunyawong N, Vermillion MS, Robinson DP, Wohlgemuth N, Pekosz A, et al. Progesterone-based therapy protects against influenza by promoting lung repair and recovery in females. *PLoS Pathog* (2016) 12(9):e1005840. doi:10.1371/journal.ppat.1005840
92. vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, et al. Age and testosterone mediate influenza pathogenesis in male mice. *Am J Physiol Lung Cell Mol Physiol* (2016) 311(6):L1234–44. doi:10.1152/ajplung.00352.2016
93. Gubbels Bupp MR, Jorgensen TN. Androgen-induced immunosuppression. *Front Immunol* (2018) 9:794. doi:10.3389/fimmu.2018.00794
94. Glezen WP, Loda FA, Clyde WA Jr, Senior RJ, Sheaffer CI, Conley WG, et al. Epidemiologic patterns of acute lower respiratory disease of children in a pediatric group practice. *J Pediatr* (1971) 78(3):397–406. doi:10.1016/S0022-3476(71)80218-4
95. Simoes EA. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. *J Pediatr* (2003) 143(5 Suppl):S118–26. doi:10.1067/S0022-3476(03)00511-0
96. Karlberg J, Chong DS, Lai WY. Do men have a higher case fatality rate of severe acute respiratory syndrome than women do? *Am J Epidemiol* (2004) 159(3):229–31. doi:10.1093/aje/kwh056
97. Alghamdi IG, Hussain II, Almalki SS, Alghamdi MS, Alghamdi MM, El-Sheemy MA. The pattern of Middle East respiratory syndrome coronavirus in Saudi Arabia: a descriptive epidemiological analysis of data from the Saudi Ministry of Health. *Int J Gen Med* (2014) 7:417–23. doi:10.2147/IJGM.S67061
98. Channappanavar R, Fett C, Mack M, Ten Eyck PP, Meyerholz DK, Perlman S. Sex-based differences in susceptibility to severe acute respiratory syndrome coronavirus infection. *J Immunol* (2017) 198(10):4046–53. doi:10.4049/jimmunol.1601896
99. Keselman A, Heller N. Estrogen signaling modulates allergic inflammation and contributes to sex differences in asthma. *Front Immunol* (2015) 6:568. doi:10.3389/fimmu.2015.00568
100. Laffont S, Seillet C, Guéry J-C. Estrogen receptor-dependent regulation of dendritic cell development and function. *Front Immunol* (2017) 8:108. doi:10.3389/fimmu.2017.00108
101. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) 517(7534):293–301. doi:10.1038/nature14189
102. Lai D-M, Shu Q, Fan J. The origin and role of innate lymphoid cells in the lung. *Military Med Res* (2016) 3:25. doi:10.1186/s40779-016-0093-2
103. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and quantification of innate lymphoid cell subsets in human lung. *PLoS One* (2016) 11(1):e0145961. doi:10.1371/journal.pone.0145961
104. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* (2011) 12(11):1045–54. doi:10.1031/ni.2131
105. Califano D, Furuya Y, Roberts S, Avram D, McKenzie ANJ, Metzger DW. IFN-gamma increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal Immunol* (2018) 11(1):209–19. doi:10.1038/mi.2017.41
106. Zaiss DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity* (2015) 42(2):216–26. doi:10.1016/j.immuni.2015.01.020
107. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog* (2013) 9(9):e1003615. doi:10.1371/journal.ppat.1003615
108. Stevens RL. Viral infections: beneficial role of eosinophils. *Blood* (2007) 110(5):1406. doi:10.1182/blood-2007-05-091389
109. Liu J, Wu J, Qi F, Zeng S, Xu L, Hu H, et al. Natural helper cells contribute to pulmonary eosinophilia by producing IL-13 via IL-33/ST2 pathway in a murine model of respiratory syncytial virus infection. *Int Immunopharmacol* (2015) 28(1):337–43. doi:10.1016/j.intimp.2015.05.035
110. Kadel S, Ainsua-Enrich E, Hatipoglu I, Turner S, Singh S, Khan S, et al. A major population of functional KLRG1(-) ILC2s in female lungs contributes to a sex bias in ILC2 numbers. *Immunohorizons* (2018) 2(2):74–86. doi:10.4049/immunohorizons.1800008
111. Russi AE, Walker-Caulfield ME, Ebel ME, Brown MA. Cutting edge: c-Kit signaling differentially regulates type 2 innate lymphoid cell accumulation and susceptibility to central nervous system demyelination in male and female SJL mice. *J Immunol* (2015) 194(12):5609–13. doi:10.4049/jimmunol.1500068
112. Forsberg A, Bengtsson M, Eringfalt A, Ernerudh J, Mjosberg J, Jenmalm MC. GATA binding protein 3(+) group 2 innate lymphoid cells are present in cord blood and in higher proportions in male than in female neonates. *J Allergy Clin Immunol* (2014) 134(1):228–30. doi:10.1016/j.jaci.2014.01.027
113. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol* (2016) 17(6):626–35. doi:10.1038/ni.3443
114. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in

human airway inflammation in the lungs. *Nat Immunol* (2016) 17(6):636–45. doi:10.1038/ni.3444
115. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol* (2016) 17(6):646–55. doi:10.1038/ni.3447
116. Lam VC, Lanier LL. NK cells in host responses to viral infections. *Curr Opin Immunol* (2017) 44:43–51. doi:10.1016/j.coi.2016.11.003
117. Abdullah M, Chai PS, Chong MY, Tohit ER, Ramasamy R, Pei CP, et al. Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cell Immunol* (2012) 272(2):214–9. doi:10.1016/j.cellimm.2011.10.009
118. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GS, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. *Cytometry* (1996) 26(1):8–15. doi:10.1002/(SICI)1097-0320(19960315)26:1<8::AID-CYTO2>3.0.CO;2-E
119. Chng WJ, Tan GB, Kuperan P. Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single-platform flow cytometry: influence of age, sex, and race and comparison with other published studies. *Clin Diagn Lab Immunol* (2004) 11(1):168–73. doi:10.1128/CDLI.11.1.168-173.2004
120. Al-Attar A, Presnell S, Peterson CA, Travis Thomas D, Lutz CT. The effect of sex on immune cells in healthy aging: elderly women have more robust natural killer lymphocytes than do elderly men. *Mech Ageing Dev* (2016) 156:25–33. doi:10.1016/j.mad.2016.04.001
121. McDonald FG, Ferguson MM. Variation in natural killer activity in peripheral blood during the menstrual cycle. *Br Med J (Clin Res Ed)* (1985) 290(6480):1514–5. doi:10.1136/bmj.290.6480.1514-b
122. Souza SS, Castro FA, Mendonca HC, Palma PV, Morais FR, Ferriani RA, et al. Influence of menstrual cycle on NK activity. *J Reprod Immunol* (2001) 50(2):151–9. doi:10.1016/S0165-0378(00)00091-7
123. Yovel G, Shakhar K, Ben-Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. *Gynecol Oncol* (2001) 81(2):254–62. doi:10.1006/gyno.2001.6153
124. King A, Jokhi PP, Burrows TD, Gardner L, Sharkey AM, Loke YW. Functions of human decidual NK cells. *Am J Reprod Immunol* (1996) 35(3):258–60. doi:10.1111/j.1600-0897.1996.tb00041.x
125. Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, et al. Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* (2008) 111(6):3108–15. doi:10.1182/blood-2007-08-105965
126. Sulke AN, Jones DB, Wood PJ. Hormonal modulation of human natural killer cell activity in vitro. *J Reprod Immunol* (1985) 7(2):105–10. doi:10.1016/0165-0378(85)90064-6
127. Hou J, Zheng WF. Effect of sex hormones on NK and ADCC activity of mice. *Int J Immunopharmacol* (1988) 10(1):15–22. doi:10.1016/0192-0561(88)90145-2
128. Ferguson MM, McDonald FG. Oestrogen as an inhibitor of human NK cell cytolysis. *FEBS Lett* (1985) 191(1):145–8. doi:10.1016/0014-5793(85)81011-5
129. Hao S, Zhao J, Zhou J, Zhao S, Hu Y, Hou Y. Modulation of 17beta-estradiol on the number and cytotoxicity of NK cells in vivo related to MCM and activating receptors. *Int Immunopharmacol* (2007) 7(13):1765–75. doi:10.1016/j.intimp.2007.09.017
130. Allison TJ, Garboczi DN. Structure of γδ T cell receptors and their recognition of non-peptide antigens. *Mol Immunol* (2002) 38(14):1051–61. doi:10.1016/S0161-5890(02)00034-2
131. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* (2000) 18:975–1026. doi:10.1146/annurev.immunol.18.1.975
132. Dodd J, Riffault S, Kodituwakku JS, Hayday AC, Openshaw PJ. Pulmonary V gamma 4+ gamma delta T cells have proinflammatory and antiviral effects in viral lung disease. *J Immunol* (2009) 182(2):1174–81. doi:10.4049/jimmunol.182.2.1174
133. Xue C, Wen M, Bao L, Li H, Li F, Liu M, et al. Vgamma4(+)gammadelta T cells aggravate severe H1N1 influenza virus infection-induced acute pulmonary immunopathological injury via secreting interleukin-17A. *Front Immunol* (2017) 8:1054. doi:10.3389/fimmu.2017.01054
134. Caccamo N, Dieli F, Wesch D, Jomaa H, Eberl M. Sex-specific phenotypical and functional differences in peripheral human Vgamma9/Vdelta2 T cells. *J Leukoc Biol* (2006) 79(4):663–6. doi:10.1189/jlb.1105640
135. Michishita Y, Hirokawa M, Guo YM, Abe Y, Liu J, Ubukawa K, et al. Age-associated alteration of gammadelta T-cell repertoire and different profiles of activation-induced death of Vdelta1 and Vdelta2 T cells. *Int J Hematol* (2011) 94(3):230–40. doi:10.1007/s12185-011-0907-7
136. Johnson TR, Hong S, Van Kaer L, Koezuka Y, Graham BS. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* (2002) 76(9):4294–303. doi:10.1128/JVI.76.9.4294-4303.2002
137. Bernin H, Fehling H, Marggraff C, Tannich E, Lotter H. The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. *Med Microbiol Immunol* (2016) 205(4):321–32. doi:10.1007/s00430-016-0449-y
138. Kee SJ, Park YW, Cho YN, Jin HM, Kim MJ, Lee SJ, et al. Age- and gender-related differences in circulating natural killer T cells and their subset levels in healthy Korean adults. *Hum Immunol* (2012) 73(10):1011–6. doi:10.1016/j.humimm.2012.07.335
139. Sandberg JK, Bhardwaj N, Nixon DF. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. *Eur J Immunol* (2003) 33(3):588–96. doi:10.1002/eji.200323707
140. Camp JV, Jonsson CB. A role for neutrophils in viral respiratory disease. *Front Immunol* (2017) 8:550. doi:10.3389/fimmu.2017.00550
141. Funchal GA, Jaeger N, Czepielewski RS, Machado MS, Muraro SP, Stein RT, et al. Respiratory syncytial virus fusion protein promotes TLR-4-dependent neutrophil extracellular trap formation by human neutrophils. *PLoS One* (2015) 10(4):e0124082. doi:10.1371/journal.pone.0124082
142. Emboriadou M, Hatzistilianou M, Magnisali C, Sakelaropoulou A, Exintari M, Conti P, et al. Human neutrophil elastase in RSV bronchiolitis. *Ann Clin Lab Sci* (2007) 37(1):79–84.
143. Mathur S, Mathur RS, Goust JM, Williamson HO, Fudenberg HH. Cyclic variations in white cell subpopulations in the human menstrual cycle: correlations with progesterone and estradiol. *Clin Immunol Immunopathol* (1979) 13(3):246–53. doi:10.1016/0090-1229(79)90069-2
144. Bain BJ, England JM. Variations in leucocyte count during menstrual cycle. *Br Med J* (1975) 2(5969):473–5. doi:10.1136/bmj.2.5969.473
145. Chandra S, Tripathi AK, Mishra S, Amzarul M, Vaish AK. Physiological changes in hematological parameters during pregnancy. *Indian J Hematol Blood Transfus* (2012) 28(3):144–6. doi:10.1007/s12288-012-0175-6
146. Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, et al. Sex-specific alterations in neutrophil apoptosis: the role of estradiol and progesterone. *Blood* (2003) 102(7):2653–9. doi:10.1182/blood-2003-02-0649
147. Bekesi G, Kakucs R, Varbiro S, Racz K, Sprintz D, Feher J, et al. In vitro effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. *Steroids* (2000) 65(12):889–94. doi:10.1016/S0039-128X(00)00183-5
148. Marczell I, Hrabak A, Nyiro G, Patocs A, Stark J, Dinya E, et al. 17-beta-estradiol decreases neutrophil superoxide production through Rac1. *Exp Clin Endocrinol Diabetes* (2016) 124(10):588–92. doi:10.1055/s-0042-105556
149. Garcia-Duran M, de Frutos T, Diaz-Recasens J, Garcia-Galvez G, Jimenez A, Monton M, et al. Estrogen stimulates neuronal nitric oxide synthase protein expression in human neutrophils. *Circ Res* (1999) 85(11):1020–6. doi:10.1161/01.RES.85.11.1020
150. Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. *Blood* (2007) 110(5):1578–86. doi:10.1182/blood-2007-01-071340
151. Samarasinghe AE, Melo RC, Duan S, LeMessurier KS, Liedmann S, Surman SL, et al. Eosinophils promote antiviral immunity in mice infected with influenza A virus. *J Immunol* (2017) 198(8):3214–26. doi:10.4049/jimmunol.1600787
152. Hamano N, Terada N, Maesako K, Numata T, Konno A. Effect of sex hormones on eosinophilic inflammation in nasal mucosa. *Allergy Asthma Proc* (1998) 19(5):263–9. doi:10.2500/108854198778557773
153. Katayama ML, Federico MH, Brentani RR, Brentani MM. Eosinophil accumulation in rat uterus following estradiol administration is modulated by laminin and its integrin receptors. *Cell Adhes Commun* (1998) 5(5):409–24. doi:10.3109/15419069809010785

154. Perez MC, Furth EE, Matzumura PD, Lyttle CR. Role of eosinophils in uterine responses to estrogen. *Biol Reprod* (1996) 54(1):249–54. doi:10.1095/biolreprod54.1.249
155. Luque EH, Munoz de Toro MM, Ramos JG, Rodriguez HA, Sherwood OD. Role of relaxin and estrogen in the control of eosinophilic invasion and collagen remodeling in rat cervical tissue at term. *Biol Reprod* (1998) 59(4): 795–800. doi:10.1095/biolreprod59.4.795
156. Goritzka M, Makris S, Kausar F, Durant LR, Pereira C, Kumagai Y, et al. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *J Exp Med* (2015) 212(5):699–714. doi:10.1084/jem.20140825
157. Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood* (2011) 118(22):5918–27. doi:10.1182/blood-2011-03-340281
158. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod* (2008) 78(3):432–7. doi:10.1095/biolreprod.107.063545
159. Chao TC, Chao HH, Chen MF, Greager JA, Walter RJ. Female sex hormones modulate the function of LPS-treated macrophages. *Am J Reprod Immunol* (2000) 44(5):310–8. doi:10.1111/j.8755-8920.2000.440511.x
160. Calippe B, Douin-Echinard V, Laffargue M, Laurell H, Rana-Poussine V, Pipy B, et al. Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: involvement of the phosphatidylinositol 3-kinase pathway. *J Immunol* (2008) 180(12):7980–8. doi:10.4049/jimmunol.180.12.7980
161. Corcoran MP, Meydani M, Lichtenstein AH, Schaefer EJ, Dillard A, Lamon-Fava S. Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women. *J Endocrinol* (2010) 206(2):217–24. doi:10.1677/JOE-10-0057
162. Melgert BN, Oriss TB, Qi Z, Dixon-McCarthy B, Geerlings M, Hylkema MN, et al. Macrophages: regulators of sex differences in asthma? *Am J Respir Cell Mol Biol* (2010) 42(5):595–603. doi:10.1165/rcmb.2009-0016OC
163. Campbell L, Emmerson E, Williams H, Saville CR, Krust A, Chambon P, et al. Estrogen receptor-alpha promotes alternative macrophage activation during cutaneous repair. *J Invest Dermatol* (2014) 134(9):2447–57. doi:10.1038/jid.2014.175
164. Li K, Xu W, Guo Q, Jiang Z, Wang P, Yue Y, et al. Differential macrophage polarization in male and female BALB/c mice infected with coxsackievirus B3 defines susceptibility to viral myocarditis. *Circ Res* (2009) 105(4): 353–64. doi:10.1161/CIRCRESAHA.109.195230
165. Lai JJ, Lai KP, Chuang KH, Chang P, Yu IC, Lin WJ, et al. Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression. *J Clin Invest* (2009) 119(12):3739–51. doi:10.1172/JCI39335
166. Lauvau G, Chorro L, Spaulding E, Soudja SMH. Inflammatory monocyte effector mechanisms. *Cell Immunol* (2014) 291(1-2):32–40. doi:10.1016/j.cellimm.2014.07.007
167. Janis K, Hoeltke J, Nazareth M, Fanti P, Poppenberg K, Aronica SM. Estrogen decreases expression of chemokine receptors, and suppresses chemokine bioactivity in murine monocytes. *Am J Reprod Immunol* (2004) 51(1): 22–31. doi:10.1046/j.8755-8920.2003.00117.x
168. Ben-Hur H, Mor G, Insler V, Blickstein I, Amir-Zaltsman Y, Sharp A, et al. Menopause is associated with a significant increase in blood monocyte number and a relative decrease in the expression of estrogen receptors in human peripheral monocytes. *Am J Reprod Immunol* (1995) 34(6):363–9. doi:10.1111/j.1600-0897.1995.tb00965.x
169. Elenkov IJ, Wilder RL, Bakalov VK, Link AA, Dimitrov MA, Fisher S, et al. IL-12, TNF-α, and hormonal changes during late pregnancy and early postpartum: implications for autoimmune disease activity during these times. *J Clin Endocrinol Metab* (2001) 86(10):4933–8. doi:10.1210/jc.86.10.4933
170. O'Connor MF, Motivala SJ, Valladares EM, Olmstead R, Irwin MR. Sex differences in monocyte expression of IL-6: role of autonomic mechanisms. *Am J Physiol Regul Integr Comp Physiol* (2007) 293(1):R145–51. doi:10.1152/ajpregu.00752.2006
171. Kramer PR, Kramer SF, Guan G. 17 beta-estradiol regulates cytokine release through modulation of CD16 expression in monocytes and monocyte-derived macrophages. *Arthritis Rheum* (2004) 50(6):1967–75. doi:10.1002/art.20309
172. Miyagi M, Aoyama H, Morishita M, Iwamoto Y. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *J Periodontol* (1992) 63(1):28–32. doi:10.1902/jop.1992.63.1.28
173. Angele MK, Knoferl MW, Schwacha MG, Ayala A, Cioffi WG, Bland KI, et al. Sex steroids regulate pro- and anti-inflammatory cytokine release by macrophages after trauma-hemorrhage. *Am J Physiol* (1999) 277(1 Pt 1): C35–42. doi:10.1152/ajpcell.1999.277.1.C35
174. D'Agostino P, Milano S, Barbera C, Di Bella G, La Rosa M, Ferlazzo V, et al. Sex hormones modulate inflammatory mediators produced by macrophages. *Ann N Y Acad Sci* (1999) 876:426–9. doi:10.1111/j.1749-6632.1999.tb07667.x
175. Li ZG, Danis VA, Brooks PM. Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clin Exp Rheumatol* (1993) 11(2):157–62.
176. Bajana S, Turner S, Paul J, Ainsua-Enrich E, Kovats S. IRF4 and IRF8 act in CD11c+ cells to regulate terminal differentiation of lung tissue dendritic cells. *J Immunol* (2016) 196(4):1666–77. doi:10.4049/jimmunol.1501870
177. Kovats S. Estrogen receptors regulate an inflammatory pathway of dendritic cell differentiation: mechanisms and implications for immunity. *Horm Behav* (2012) 62(3):254–62. doi:10.1016/j.yhbeh.2012.04.011
178. Seillet C, Rouquie N, Foulon E, Douin-Echinard V, Krust A, Chambon P, et al. Estradiol promotes functional responses in inflammatory and steady-state dendritic cells through differential requirement for activation function-1 of estrogen receptor alpha. *J Immunol* (2013) 190(11):5459–70. doi:10.4049/jimmunol.1203312
179. Siracusa MC, Overstreet MG, Housseau F, Scott AL, Klein SL. 17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells. *J Immunol* (2008) 180(3):1423–31. doi:10.4049/jimmunol.180.3.1423
180. Carreras E, Turner S, Paharkova-Vatchkova V, Mao A, Dascher C, Kovats S. Estradiol acts directly on bone marrow myeloid progenitors to differentially regulate GM-CSF or Flt3 ligand-mediated dendritic cell differentiation. *J Immunol* (2008) 180(2):727–38. doi:10.4049/jimmunol.180.2.727
181. Cunningham MA, Naga OS, Eudaly JG, Scott JL, Gilkeson GS. Estrogen receptor alpha modulates toll-like receptor signaling in murine lupus. *Clin Immunol* (2012) 144(1):1–12. doi:10.1016/j.clim.2012.04.001
182. Bengtsson AK, Ryan EJ, Giordano D, Magaletti DM, Clark EA. 17beta-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. *Blood* (2004) 104(5):1404–10. doi:10.1182/blood-2003-10-3380
183. Carreras E, Turner S, Frank MB, Knowlton N, Osban J, Centola M, et al. Estrogen receptor signaling promotes dendritic cell differentiation by increasing expression of the transcription factor IRF4. *Blood* (2010) 115(2):238–46. doi:10.1182/blood-2009-08-236935
184. Butts CL, Shukair SA, Duncan KM, Bowers E, Horn C, Belyavskaya E, et al. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. *Int Immunol* (2007) 19(3):287–96. doi:10.1093/intimm/dxl145
185. Xiu F, Anipindi VC, Nguyen PV, Boudreau J, Liang H, Wan Y, et al. High physiological concentrations of progesterone reverse estradiol-mediated changes in differentiation and functions of bone marrow derived dendritic cells. *PLoS One* (2016) 11(4):e0153304. doi:10.1371/journal.pone.0153304
186. Jones LA, Kreem S, Shweash M, Paul A, Alexander J, Roberts CW. Differential modulation of TLR3- and TLR4-mediated dendritic cell maturation and function by progesterone. *J Immunol* (2010) 185(8):4525–34. doi:10.4049/jimmunol.0901155
187. Griesbeck M, Ziegler S, Laffont S, Smith N, Chauveau L, Tomezsko P, et al. Sex differences in plasmacytoid dendritic cell levels of IRF5 drive higher IFN-alpha production in women. *J Immunol* (2015) 195(11):5327–36. doi:10.4049/jimmunol.1501684
188. Scott JL, Cunningham MA, Naga OS, Wirth JR, Eudaly JG, Gilkeson GS. Estrogen receptor alpha deficiency modulates TLR ligand-mediated

PDC-TREM expression in plasmacytoid dendritic cells in lupus-prone mice. *J Immunol* (2015) 195(12):5561–71. doi:10.4049/jimmunol.1500315
189. Hannah MF, Bajic VB, Klein SL. Sex differences in the recognition of and innate antiviral responses to Seoul virus in Norway rats. *Brain Behav Immun* (2008) 22(4):503–16. doi:10.1016/j.bbi.2007.10.005
190. Hughes GC, Thomas S, Li C, Kaja MK, Clark EA. Cutting edge: progesterone regulates IFN-alpha production by plasmacytoid dendritic cells. *J Immunol* (2008) 180(4):2029–33. doi:10.4049/jimmunol.180.4.2029
191. Wang JP, Zhang L, Madera RF, Woda M, Libraty DH. Plasmacytoid dendritic cell interferon-α production to R-848 stimulation is decreased in male infants. *BMC Immunol* (2012) 13:35. doi:10.1186/1471-2172-13-35
192. Torcia MG, Nencioni L, Clemente AM, Civitelli L, Celestino I, Limongi D, et al. Sex differences in the response to viral infections: TLR8 and TLR9 ligand stimulation induce higher IL10 production in males. *PLoS One* (2012) 7(6):e39853. doi:10.1371/journal.pone.0039853
193. Sun K, Torres L, Metzger DW. A detrimental effect of interleukin-10 on protective pulmonary humoral immunity during primary influenza A virus infection. *J Virol* (2010) 84(10):5007–14. doi:10.1128/JVI.02408-09

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MINI REVIEW
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Sex-Dependent Outcome of Hepatitis B and C Viruses Infections: Synergy of Sex Hormones and Immune Responses?

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Hepatitis B virus (HBV) and hepatitis C virus (HCV) are hepatotropic viruses that differ in their genomic content, life cycle and molecular prognosis. HBV and HCV establish chronic lifespan infections that can evolve to fibrosis, cirrhosis and hepatocellular carcinoma (HCC). This malignant liver cancer affects more commonly male patients than females, with a male-to-female incidence ratio of <Capword>2</Capword>:1 up to 7:1. Sex significantly contributes to shape the immune responses, contributing to differences in the pathogenesis of infectious diseases, in males and females patients. Females usually develop more intense innate, humoral and cellular immune responses to viral infections and to vaccination compared to male subjects. Sex hormones, in turn, differentially affect the immune responses to viruses, by specific binding to the hormone receptors expressed on the immune cells. In general, estrogens have immune-stimulating effect, while androgens are immune-suppressing. However, sex hormones, such as androgen, can also directly interact with HBV genome integrated into the cell nucleus and activate transcription of HBV oncoproteins. On the other side, estradiol and estrogen receptors protect liver cells from inflammatory damage, apoptosis and oxidative stress, which contribute to fibrosis and malignant transformation preceding HCC. In HCV-associated cirrhosis and HCC the decreased expression of estrogen receptor alfa (ERα) in male patients may explain the worse outcome of HCV infection in men than in women. The synergistic action of male and female sex hormones and of immune responses, together with viral factors contribute to the mechanism of sex/gender disparity in the outcome and progression of hepatitis viruses infection.

Keywords: sex, HBV, HCV, estrogens, androgens, progesterone, innate immune response, adaptive immune response

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INTRODUCTION

Men and women are extremely different in health and disease. Women usually live longer than men but not healthier; in fact, women are often sicker than men. In addition, sex-related differences in the frequency of side effects have been reported for several drugs, with women experiencing more adverse events than men (1). The higher rate of side effects in women than in men may be due, at least in part, to the fact that results from clinical trials derive mainly from male subjects, being women inadequately represented

in clinical trials (2). In the last 18 years gender-specific medicine has been recognized as the study of how diseases differ between sexes in terms of susceptibility, prevention, clinical manifestations, therapy, prognosis and mechanisms of pathogenesis (3). In this context, viral infections have been recognized to differ between males and females for prevalence, intensity, outcome and pathogenetic mechanisms (4). In this minireview, we introduce some mechanisms that determine sexual dimorphism in immune function in males and females. We then discuss the impact of the synergy between sex hormones and sexually dimorphic immune responses on pathogenesis during Hepatitis B (HBV) and Hepatitis C (HCV) viral infections.

SEX DISPARITY IN IMMUNE RESPONSES TO VIRAL INFECTIONS

It is well-known that sexual dimorphism occurs in humans and animals with regard to immune responses and viral infections (4). Female individuals usually are less susceptible to viral infections than males, since they mount a more efficient, intense and prolonged immune response, either innate, as well as humoral and cell-mediated (5, 6). The innate immune response is the first line of defense against viruses and it is mediated by Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RIG-I) and nucleotide oligomerization domain-like receptors (NOD-like receptors). These, named pattern recognition receptors (PRRs), recognize viral components (such as DNA, dsRNA, ssRNA, and viral proteins) and activate production of type 1 interferon (IFN) and inflammatory cytokines (IL-1, TNFs). In rodents and in humans expression of TLRs (such as TLR7) as well as number of monocytes, macrophages and dendritic cells, that are innate immune response players, have been reported to be significantly higher in females than in males (7, 8), thus accomplishing the more intense inflammatory responses in female subjects than in males (9).

In general, once a viral infection is established, the activation, by the Antigen Presenting Cells (APCs), of adaptive immune responses and of B cells, with subsequent rise of the antibodies specific for viral antigens, in most cases is greater in female animals and humans compared to males (10). In addition, females have higher number of $CD4^+$T cells than males, that induces a greater number of T cells activated by viral antigens engagement of the T cell receptor (9–13); moreover, stronger cytotoxic T cell activity along with overexpression of antiviral and pro-inflammatory genes, many of which have estrogen response elements in their promoters, have been reported in women (14).

In most viral infections, following viral clearance, when the immune system returns to the homeostasis, basal immune responses are higher in females than in males. This can result in a higher risk of developing immunopathologies associated to viral infections in female individuals. In contrast, the lower antiviral immune responses at homeostasis in males can be responsible for the increased risk to undergo to persistent viral infections (9).

Based on this, it is deductible that female are less prone than males to be virus infected, due to their more effective antiviral immune defenses, but more frequently they develop more severe symptoms, due to the more intense inflammatory responses (9).

The immunological dimorphism between sexes, besides being shaped by the individual genetic background (15, 16), is mostly regulated by sex steroids hormones, particularly by estrogens, progesterone and androgens, that affect function of the immune cells. Due to the expression of the sex hormone receptors on immune cells, including lymphocytes, monocytes and dendritic cells, the interaction of sex hormones and immune cells affects release of cytokines and chemokines, which determine differentiation, maturation and proliferation of the immune cells (17).

Presence of different sex hormones, which circulate at different levels in males and females, makes sense of the fact that the immune responses are differentially modulated in individuals of different sexes (18). There are three classes of sex hormones: androgens (testosterone), estrogens (17-beta-estradiol) and progesterone. The level of estrogens in females fluctuates during menstrual cycle and declines with menopause; in males the testosterone level is stable up to almost 60 years of age, before age declining. Consequently, sex disparity in immune responses to viral infections may vary with aging.

Several studies have been published so far to clarify the various roles of estrogens on immune system, whereas much less is known about the roles of androgens (19–21). In general, testosterone has been demonstrated to have suppressive effect on the immune function, either in animal models and in human trials (21); conversely the effect of estrogen varies depending on their levels and on the immune measure used (20).

Androgens have been shown to suppress pro-inflammatory responses in rodents, by increasing production of anti-inflammatory cytokines (IL-10, TGF-β) (22–24). In humans, androgens deficiency in men has been reported to induce increased levels of inflammatory cytokines (IL-1β, IL-2, and TNF), an increased $CD4^+/CD8^+$ T cell ratio and higher antibodies titers, compared to male subjects with normal level of testosterone (25, 26). Actually, several studies (21, 27) have indicated that androgens exert an overall inhibitory effect on differentiation of Th1 arm of the immune system, with consequent reduced production of IFN-γ that may explain the enhanced susceptibility to viral infections in males than in females (28).

Estrogens (17β-estradiol) act by binding to the estrogen receptors (ER) -alfa or -beta, which are expressed differentially among the subsets of immune cells: ERα is highly expressed on T cells and ERβ highly expressed on B cells (19). Estrogens affect different activity of the innate and adaptive immune responses, and they have opposite effects on the immune system based on their concentration. In humans, low doses of estrogens have been reported to induce monocytes differentiation into inflammatory DCs with consequent high production of IL-4 and IFN-α. Conversely, high doses of estrogen have inhibitory activity on innate and pro-inflammatory immune responses (19, 29).

In addition, the low level of estrogens activates Th1-type and cell-mediated immune responses, whereas high levels enhance

Th2-type responses and humoral immune responses in diverse species and *in vitro*. Treg cell populations are also positively affected by estrogens either in mouse model and in women (30). At physiological concentration estrogens also stimulate humoral response to viral infections, by inducing higher levels of antibodies and activating antibodies-producing cells. From the above it is deductable that the fluctuation of the estrogen levels during the menstrual cycle in female subjects can make women differently immune-reactive before ovulation, when the antibody levels are highest (31).

Progesterone's effect on immune system is similar to androgen's immune suppression of both innate and cell mediated immune responses. It is known that progesterone suppresses Th1 response and favors the Th2 cytokines production, inhibits cytotoxic T cells and modulate function of NK cells (5).

SEX DISPARITY IN HEPATITIS B VIRUS (HBV) AND HEPATITIS C VIRUS (HCV) INFECTIONS

HBV and HCV are two hepatotropic viruses belonging, respectively, to Hepadnaviridae (HBV) and Flaviviridae (HCV) families, that differ in their genomic content, life cycle and molecular prognosis. HBV is the DNA virus that has the ability to integrate into host cell DNA, establishing persistent infection and whose replication cycle involves an RNA intermediate. HCV is the RNA virus that replicates in host cell cytoplasmic membranous webs (vescicle-like cytoplasmic membranes). Escaping from innate and adaptive immune responses is the main mechanism involved in establishment of persistent HCV infection (32).

Both viruses are responsible for chronic infections and represent a major risk factor for the development of hepatocellular carcinoma (HCC). Male sex is a risk factor for HBV and HCV prevalence and for HCC development subsequent to HBV and/or HCV infection. HBV-associated HCC develops more frequently in men than in women, with a female/male ratio ranging from 1:4 to 1:7 (33). In addition, female HBV carriers have lower viral loads than male carriers (34, 35) and the prevalence of serum HBV surface antigen (HBsAg) has been reported higher in men than in women (36). Some studies suggested that high levels of serum testosterone could associate with an increased risk of HCC development in male carriers of HBV (37). This and similar observations suggested that HBV infection and pathogenesis could be regulated by sex hormones. As reported above, male and female sex hormones affect the release of inflammatory cytokines in opposite way, as estrogens induce pro-inflammatory cytokine whereas androgens suppress pro-inflammatory responses, and if this occurs in the HCC microenvironment it can contribute to the epigenetic changes responsible for malignant transformation in different or opposite ways between sexes.

As described in the previous paragraph, the innate immunity response is the first line of defense against viral infections. The impact of sex differences and, in particular, of sex hormones on innate immune response to HBV are largely unknown, due, at least in part, to the lack of adequate models. A little more is known regarding gender-related differences in adaptive immune response to HBV infection. After prophylactic vaccination against HBV, women have higher anti-HBs antibody titer than men (38). In addition, a more frequent seroconversion to HBeAg (HBV e antigen) and HBsAg antibodies has been reported in female HBV chronic carriers than in males (39). According to a study conducted in mouse models of acute and persistent HBV infection, sex-related discrepancies in the adaptive response to HBV infection may be explained by different $CD8^+$-T cells activity. In both murine models higher $CD8^+$-T cells activity has been reported in females than in males and correlated to lower number of intrahepatic Treg cells in female mice than in male ones (40). However, the role of androgens and estrogens in regulating T-cells response to HBV infection is still unclear, and only some clues are available. For example, susceptibility to chronic HBV infection has been associated to a particular ERα polymorphism; one possible explanation is that this polymorphism affects ESR1 (Estrogen Receptor 1) gene transcription with the consequence of a defective response of immune cells to estrogens (41). Given the reported effect of sex hormones on immune system, it is reasonable to speculate that this can also account for the sex different susceptibility to HBV infection. Antiviral immune response modulation by sex hormones may also contribute to explain HCC prevalence in male gender, as in the case of chemically induced HCC that is worse in male than in female mice, due to the increased production of IL-6 by Kupffer cells in the males liver (42). Same Authors showed that estrogens transcriptionally inhibited IL-6, through reduction of Myd 88-dependent induction of NF-κB (42, 43).

Besides the effect on immune responses sex hormones can also directly influence virus activity, in some instances, being some viruses directly responsive to male or female sex hormones. As a consequence the viral load and the outcome of several viral infections are different in male and female individuals (44–50). In general HBV surface antigen (HBsAg) circulates at higher level in serum of male adult mice than in female (34) and its level decreases upon castration of the animals, thus indicating that viral antigen expression and viral replication are regulated by androgens (35). The androgens do their biological functions by binding to their cognate receptor (androgen receptor, AR), that dimerizes and translocates into the cell nucleus, where it binds to the cellular DNA, to specific Androgen Responsive Elements (ARE). This binding activates the transcriptional expression of various target genes that are associated with male phenotype. The HBV genome integrated into the host cell DNA contains two ARE elements within the enhancer I region. When the AR-Androgen complex is internalized into the hepatocytes it binds either to the nuclear and viral ARE sequences, thus activating HBV genome transcription and production of the HBV X protein (**Figure 1**). This latter, in turn, facilitates dimerization of the AR and enhances AR transactivation activity, through activation of Src kinase activity, thus establishing a positive feedback loop that can promote hepatocarcinogenesis. The AR further acts in conjunction with other molecules, such as cell cycle-related kinases (CCRK) that in turn activates

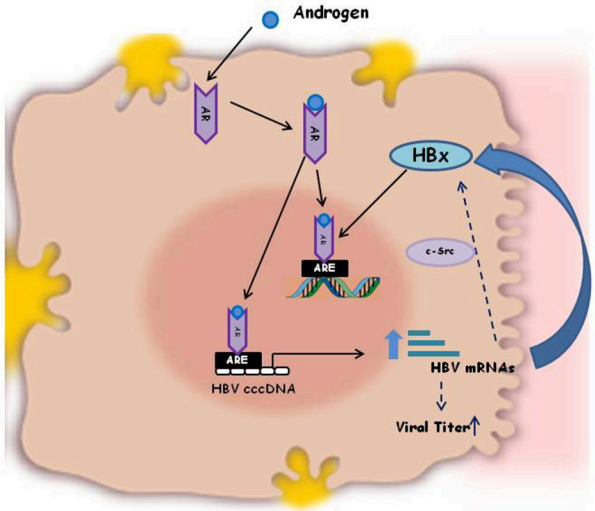

FIGURE 1 | Schematic illustration of the mechanism of Androgen activation of HBV replication. HBV genome integrated in the host cell DNA contains two Androgen Responsive Elements (ARE) within the enhancer I region. The AR-Androgen complex binds either to the nuclear and viral ARE sequences, thus activating HBV genome transcription and production of the viral HBx oncoprotein. HBx, in turn, enhances AR dimerization and androgen-stimulated AR activity, through the c-Src kinase. This positive feedback loop is one of the molecular explanations for the increased HBV titers in male patients compared to females and for the different outcome of HBV infection in men and women.

oncogenic β-catenin in hepatocytes. This mechanism indicates that Androgen/AR signaling may promote HBV-related HCC development and explains the higher frequency of HCC as well as the higher HBV titers in serum of male sex than in female (51). Conversely, the estrogens signaling has been reported to probably suppress hepatocarcinogenesis and to be protective against HBV associated progression to HCC. The molecular mechanism for estrogen is mediated through binding of estradiol to the nuclear estrogen receptor-alfa, that inhibits the HBV enhancer I and transcription of the integrated viral genomes (51, 52).

HCV infection causes, in 80% of cases, chronic infection that usually is asymptomatic and lasts lifetime, but in a small percentage of patients can evolve to fibrosis, cirrhosis and the end stage HCC. The pathogenic mechanisms involved in the establishment of HCV chronicity and in the disease outcome are only partially described. Several factors, like age, gender, alcohol consumption, body mass index and HIV or HBV co-infection may be involved (32).

Epidemiological and clinical reports have indicated that chronic HCV infections are more prevalent in men than in women and HCV-associated disease progression to fibrosis, cirrhosis to the end- stage HCC is more rapid and more common in male patients than in female (53). Women, on the other side, are more likely to clear the virus spontaneously, after initial infection. Host factors, such as Il-28 genotype and virus genotype 1a, together with female sex have been reported to be predictor of spontaneous viral clearance of acute HCV infection (54).

A strong and efficient innate inflammatory response is considered necessary for spontaneous clearance of HCV. A critical role in host innate immune response to HCV is played by TRL7, whose activation leads to IFN-α induction and interferon-stimulated genes (ISGs) expression, subsequent to janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway induction by IFN-α (55–57). Activation of TRL7 by a synthetic ligand (SM360320) has been reported to induce HCV-specific immune response and to decrease HCV RNA levels in a replicon system *in vitro* (58); furthermore, treatment with isatoribine, a selective TLR7 agonist, caused a significant drop in plasma HCV levels in chronic HCV patients (59).

Little is known regarding the influence of sex on TRL7 expression and activation during HCV infection. A study conducted on a Maroccan chronic hepatitis C patients has reported a higher rate of spontaneous HCV clearance in women than in men, due to a particular polymorphism in TLR7 gene (60). Expression of MxA gene, one of the ISGs induced byTLR7, has been found higher in premenopausal women compared to both postmenopausal women and men (57). In addition, TLR7 activation by synthetic agonists induces a significantly higher IFN-α production in healthy women than in men (61).

However, women in postmenopausal period, when estrogens levels significantly decrease, have been reported to experience more rapid progression of hepatic fibrosis and HCC development (62) and lower response to antiviral therapy (63). Thus, pointing out for an important role of the estrogen level in determining the fate of HCV infection in female subjects and also pointing to the age effect on HCV pathogenesis.

Normal liver express estrogen receptor of both type, alfa and beta (ERα and ERβ), thus it is responsive to the estrogenic stimulus, however normal male livers have higher expression of ERα with respect to normal female livers. In contrast, in HCV associated cirrhosis and HCC ERα level has been reported to decrease only in male patients compared to normal male livers. The Authors correlated the ERα changes associated to HCV disease with the increase of inflammation markers and proliferation that are involved in the pathogenesis of liver cirrhosis and HCC, thus explaining the worse outcome of chronic HCV infection in male patients than in female (64).

The worse outcome of HCV infection in men may also be explained by the direct influence of sex hormones on HCV itself. 17β-estradiol was found to inhibit production of mature HCV virions, through ERα binding (65, 66) and to inhibit HCV entry, through down-regulation of occludin (one of the receptors used by HCV to access the hepatocytes), in infected cell cultures (67).

Studies analyzing the effect of testosterone on HCV replication are lacking so far. However, it was reported an increased expression of scavenger receptors, which are necessary for viral entry, in both HepG2 cell lines and human macrophages treated with testosterone (68, 69). Interestingly, estrogen decreased the expression of hepatic scavenger receptors in rat livers (70). The different effect of estrogens and testosterone on HCV replication may explain, at least in part, the lower incidence of HCV infection and the less common progression of liver disease to cirrhosis and HCC, in premenopausal women than in postmenopausal women and in men.

CONCLUSIONS AND FUTURE DIRECTIONS

From the above, the direct and diverse effects of male and female sex hormones on HBV virus genome replication and on HCV disease progression, act jointly to the effect of sex hormones on the anti-viral immune response, thus favoring the hypothesis of an interplayed action among sex-hormones, virus and immune system that determines the sex-dependent final outcome of chronic infections of hepatitis viruses. The data available to date, on the potential mechanisms determining the different susceptibility and outcome of HBV and HCV infections, either immunologic and hormonal, are fragmented and not exhaustive, but it is encouraging the disclosure in order to identify sex-specific molecular pathways involved. Molecular mechanisms of sex bias in infectious diseases is in its infancy, identification of the key players in sex-related outcome of hepatitis and of the molecular factors involved, will provide disclosure of new targets to personalized medicine and vaccinology.

AUTHOR CONTRIBUTIONS

AR conceived and wrote the manuscript, with the support of SA. MCG contributed to write the paragraph on immune responses to hepatitis viruses.

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REFERENCES

1. Soldin OP, Mattison DR. Sex differences in pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet*. (2009) 48:143–57. doi: 10.2165/00003088-200948030-00001
2. Mazure CM, Jones DP. Twenty years and still counting: including women as participants and studying sex and gender in biomedical research. *BMC Womens Health* (2015) 15:94. doi: 10.1186/s12905-015-0251-9
3. Baggio G, Corsini A, Floreani A, Giannini S, Zagonel V. Gender medicine: a task for the third millennium. *Clin Chem Lab Med*. (2013) 51:713–27. doi: 10.1515/cclm-2012-0849
4. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. *J Immunol*. (2017) 198:1782–90. doi: 10.4049/jimmunol.1601166
5. Giefing-Kröll C, Berger P, Lepperdinger G, Grubeck-Loebenstein B. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell* (2015) 14:309–21. doi: 10.1111/acel.12326
6. Ruggieri A, Anticoli S, D'Ambrosio A, Giordani L, Viora M. The influence of sex and gender on immunity, infection and vaccination. *Ann Ist Super Sanita* (2016) 52:198–204. doi: 10.4415/ANN_16_02_11
7. Melgert BN, Oriss TB, Qi Z, Dixon-McCarthy B, Geerlings M, Hylkema MN, et al. Macrophages: regulators of sex differences in asthma? *Am J Respir Cell Mol Biol*. (2010) 42:595–603. doi: 10.1165/rcmb.2009-0016OC
8. Klein SL. Immune cells have sex and so should journal articles. *Endocrinology* (2012) 153:2544–50. doi: 10.1210/en.2011-2120
9. Klein SL. Sex influences immune responses to viruses, and efficacy of prophylaxis and treatments for viral diseases. *Bioessays* (2012) 34:1050–9. doi: 10.1002/bies.201200099
10. Klein SL, Marriott I, Fish EN. Sex-based differences in immune function and responses to vaccination. *Trans R Soc Trop Med Hyg*. (2015) 109:9–15. doi: 10.1093/trstmh/tru167
11. Donnelly CA, Bartley LM, Ghani AC, Le Fevre AM, Kwong GP, Cowling BJ, et al. Gender difference in HIV-1 RNA viral loads. *HIV Med*. (2005) 6:170–8. doi: 10.1111/j.1468-1293.2005.00285.x
12. Hsieh AR, Fann CS, Yeh CT, Lin HC, Wan SY, Chen YC, et al. Effects of sex and generation on hepatitis B viral load in families with hepatocellular carcinoma. *World J Gastroenterol*. (2017) 23:876–84. doi: 10.3748/wjg.v23.i5.876
13. Park HJ, Choi JM. Sex-specific regulation of immune responses by PPARs. *Exp Mol Med*. (2017) 49:e364. doi: 10.1038/emm.2017.102
14. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes Immun*. (2009) 10:509–16. doi: 10.1038/gene.2009.12
15. Patin E, Hasan M, Bergstedt J, Rouilly V, Libri V, Urrutia A, et al. Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors. *Nat Immunol*. (2018) 19:302–14. doi: 10.1038/s41590-018-0049-7
16. Aguirre-Gamboa R, Joosten I, Urbano PCM, van der Molen RG, van Rijssen E, van Cranenbroek B, et al. Differential effects of environmental and genetic factors on T and B Cell immune traits. *Cell Rep*. (2016) 17:2474–87. doi: 10.1016/j.celrep.2016.10.053
17. Bhatia A, Sekhon HK, Kaur G. Sex hormones and immune dimorphism. *Sci World J*. (2014) 2014:159150. doi: 10.1155/2014/159150
18. Kovats S, Carreras E, Agrawal H. Sex steroid receptors in immune cells. In: Klein SL, Roberts CW, editors. *Sex Hormones And Immunity to Infection*. Berlin: Springer-Verlag (2010). p. 53–92.
19. Khan D, Ansar Ahmed S. The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol*. (2016) 6:635. doi: 10.3389/fimmu.2015.00635
20. Foo YZ, Nakagawa S, Rhodes G, Simmons LW. The effects of sex hormones on immune function: a meta-analysis. *Biol Rev Camb Philos Soc*. (2017) 92:551–71. doi: 10.1111/brv.12243
21. Trigunaite A, Dimo J, Jørgensen TN. Suppressive effects of androgens on the immune system. *Cell Immunol*. (2015) 294:87–94. doi: 10.1016/j.cellimm.2015.02.004
22. D'Agostino P, Milano S, Barbera C, Di Bella G, La Rosa M, Ferlazzo V, et al. Sex hormones modulate inflammatory mediators produced by macrophages. *Ann NY Acad Sci*. (1999) 876:426–9. doi: 10.1111/j.1749-6632.1999.tb07667.x
23. Liva SM, Voskuhl RR. Testosterone acts directly on $CD4^+$ T lymphocytes to increase IL-10 production. *J Immunol*. (2001) 167:2060–7. doi: 10.4049/jimmunol.167.4.2060
24. Pergola C, Dodt G, Rossi A, Neunhoeffer E, Lawrenz B, Northoff H, et al. ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. *Proc Natl Acad Sci USA*. (2008) 105:19881–6. doi: 10.1073/pnas.0809120105
25. Malkin CJ, Pugh PJ, Jones RD, Kapoor D, Channer KS, Jones TH. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. *J Clin Endocrinol Metab*. (2004) 89:3313–8. doi: 10.1210/jc.2003-031069
26. Bobjer J, Katrinaki M, Tsatsanis C, Lundberg Giwercman Y, Giwercman A. Negative association between testosterone concentration and inflammatory markers in young men: a nested cross-sectional study. *PLoS ONE* (2013) 8:e61466. doi: 10.1371/journal.pone.0061466
27. Kissick HT, Sanda MG, Dunn LK, Pellegrini KL, On ST, Noel JK, et al. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. *Proc Natl Acad Sci USA*. (2014) 111:9887–92. doi: 10.1073/pnas.1402468111
28. Kumar N, Shan LX, Hardy MP, Bardin CW, Sundaram K. Mechanism of androgen-induced thymolysis in rats. *Endocrinology* (1995) 136:4887–93. doi: 10.1210/endo.136.11.7588221

29. Seillet C, Laffont S, Trémollières F, Rouquié N, Ribot C, Arnal JF, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol *in vivo* through cell-intrinsic estrogen receptor α signaling. *Blood* (2012) 119:454–64. doi: 10.1182/blood-2011-08-371831
30. Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, et al. Induction of regulatory T cells by physiological level estrogen. *J Cell Physiol*. (2008) 214:456–64. doi: 10.1002/jcp.21221
31. Lü FX, Abel K, Ma Z, Rourke T, Lu D, Torten J, et al. The strength of B cell immunity in female rhesus macaques is controlled by $CD8^+$ T cells under the influence of ovarian steroid hormones. *Clin Exp Immunol*. (2002) 128:10–20. doi: 10.1046/j.1365-2249.2002.01780.x
32. Shlomai A, de Jong YP, Rice CM. Virus associated malignancies: the role of viral hepatitis in hepatocellular carcinoma. *Semin Cancer Biol*. (2014) 26:78–88. doi: 10.1016/j.semcancer.2014.01.004
33. Ruggieri A, Barbati C, Malorni W. Cellular and molecular mechanisms involved in hepatocellular carcinoma gender disparity. *Int J Cancer* (2010) 127:499–504. doi: 10.1002/ijc.25298
34. Farza H, Salmon AM, Hadchouel M, Moreau JL, Babinet C, Tiollais P, et al. Hepatitis B surface antigen gene expression is regulated by sex steroids and glucocorticoids in transgenic mice. *Proc Natl Acad Sci USA*. (1987) 84:1187–91. doi: 10.1073/pnas.84.5.1187
35. Breidbart S, Burk RD, Saenger P. Hormonal regulation of hepatitis B virus gene expression: influence of androgen receptor. *Pediatr Res*. (1993) 34:300–2. doi: 10.1203/00006450-199309000-00012
36. Tsay PK, Tai DI, Chen YM, Yu CP, Wan SY, Shen YJ, et al. Impact of gender, viral transmission and aging in the prevalence of hepatitis B surface antigen. *Chang Gung Med J*. (2009) 32:155–64.
37. Yu MW, Chen CJ. Elevated serum testosterone levels and risk of hepatocellular carcinoma. *Cancer Res*. (1993) 53:790–4.
38. Su FH, Chen JD, Cheng SH, Lin CH, Liu YH, Chu FY. Seroprevalence of Hepatitis-B infection amongst Taiwanese university students 18 years following the commencement of a national Hepatitis-B vaccination program. *J Med Virol*. (2007) 79:138–43. doi: 10.1002/jmv.20771
39. Alward WL, McMahon BJ, Hall DB, Heyward WL, Francis DP, Bender TR. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *J Infect Dis*. (1985) 151:604–9. doi: 10.1093/infdis/151.4.604
40. Kosinska AD, Pishraft-Sabet L, Wu W, Fang Z, Lenart M, Chen J, et al. Low hepatitis B virus-specific T-cell response in males correlates with high regulatory T-cell numbers in murine models. *Hepatology* (2017) 66:69–83. doi: 10.1002/hep.29155
41. Yan Z, Tan W, Dan Y, Zhao W, Deng C, Wang Y, et al. Estrogen receptor alpha gene polymorphisms and risk of HBV-related acute liver failure in the Chinese population. *BMC Med Genet*. (2012) 13:49. doi: 10.1186/1471-2350-13-49
42. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* (2007) 317:121–4. doi: 10.1126/science.1140485
43. Prieto J. Inflammation, HCC and sex: IL-6 in the centre of the triangle. *J Hepatol*. (2008). 48:380–1. doi: 10.1016/j.jhep.2007.11.007
44. Scully EP. Sex differences in HIV infection. *Curr HIV/AIDS Rep*. (2018) 15:136–46. doi: 10.1007/s11904-018-0383-2
45. Ziegler SM, Altfeld M. Human immunodeficiency virus 1 and Type I interferons-where sex makes a difference. *Front Immunol*. (2017) 8:1224. doi: 10.3389/fimmu.2017.01224
46. Hassan MM, Botrus G, Abdel-Wahab R, Wolff RA, Li D, Tweardy D, et al. Estrogen replacement reduces risk and increases survival times of women with hepatocellular carcinoma. *Clin Gastroenterol Hepatol*. (2017) 15:1791–9. doi: 10.1016/j.cgh.2017.05.036
47. Weyn C, Vanderwinden JM, Rasschaert J, Englert Y, Fontaine V. Regulation of human papillomavirus type 16 early gene expression in trophoblastic and cervical cells. *Virology* (2011) 412:146–55. doi: 10.1016/j.virol.2010.12.056
48. Itaborahy RM, Mancini DA, de Medeiros SF. Response to the influenza vaccine based on estradiol use in menopausal women. *Vaccine* (2016) 34:1358–62. doi: 10.1016/j.vaccine.2016.01.052
49. Peretz J, Pekosz A, Lane AP, Klein SL. Estrogenic compounds reduce influenza A virus replication in primary human nasal epithelial cells derived from female, but not male, donors. *Am J Physiol Lung Cell Mol Physiol*. (2016) 310:L415–25. doi: 10.1152/ajplung.00398.2015
50. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiébaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci USA*. (2014) 111:869–74. doi: 10.1073/pnas.1321060111
51. Wang SH, Yeh SH, Lin WH, Wang HY, Chen DS, Chen PJ. Identification of androgen response elements in the enhancer I of hepatitis B virus: a mechanism for sex disparity in chronic hepatitis B. *Hepatology* (2009) 50:1392–402. doi: 10.1002/hep.23163
52. Wang SH, Chen PJ, Yeh SH. Gender disparity in chronic hepatitis B: mechanisms of sex hormones. *J Gastroenterol Hepatol*. (2015) 30:1237–45. doi: 10.1111/jgh.12934
53. Di Martino V, Lebray P, Myers RP, Pannier E, Paradis V, Charlotte F, et al. Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. *Hepatology* (2004) 40:1426–33. doi: 10.1002/hep.20463
54. Grebely J, Page K, Sacks-Davis R, van der Loeff MS, Rice TM, Bruneau J, et al. The effects of female sex, viral genotype, and IL28B genotype on spontaneous clearance of acute hepatitis C virus infection. *Hepatology* (2014) 59:109–20. doi: 10.1002/hep.26639
55. Chang S, Kodys K, Szabo G. Impaired expression and function of toll-like receptor 7 in hepatitis C virus infection in human hepatoma cells. *Hepatology* (2010) 51:35–42. doi: 10.1002/hep.23256
56. Zhang YL, Guo YJ, Bin Li, Sun SH. Hepatitis C virus single- stranded RNA induces innate immunity via toll-like receptor 7. *J Hepatol*. (2009) 51:29–38. doi: 10.1016/j.jhep.2009.03.012
57. Mekky RY, Hamdi N, El-Akel W, Esmat G, Abdelaziz AI. Estrogen-related MxA transcriptional variation in hepatitis C virus-infected patients. *Transl Res*. (2012) 159:190–6. doi: 10.1016/j.trsl.2011.08.002
58. Lee J, Wu CC, Lee KJ, Chuang TH, Katakura K, Liu YT, et al. Activation of anti-hepatitis C virus responses via toll-like receptor 7. *Proc Natl Acad Sci USA*. (2006) 103:1828–33. doi: 10.1073/pnas.0510801103
59. Horsmans Y, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, et al. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology* (2005) 42:724–31. doi: 10.1002/hep.20839
60. Fakhir FZ, Lkhider M, Badre W, Alaoui R, Meurs EF, Pineau P, et al. Genetic variations in toll-like receptors 7 and 8 modulate natural hepatitis C outcomes and liver disease progression. *Liver Int*. (2018) 38:432–42. doi: 10.1111/liv.13533
61. Berghöfer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-alpha production in females. *J Immunol*. (2006) 177:2088–96.
62. Villa E, Karampatou A, Cammà C, Di Leo A, Luongo M, Ferrari A, et al. Early menopause is associated with lack of response to antiviral therapy in women with chronic hepatitis C. *Gastroenterology* (2011) 140:818–29. doi: 10.1053/j.gastro.2010.12.027
63. Yu JW, Sun LJ, Zhao YH, Kang P, Yan BZ. Impact of sex on virologic response rates in genotype 1 chronic hepatitis C patients with peginterferon alpha-2a and ribavirin treatment. *Int J Infect Dis*. (2011) 15:e740–6. doi: 10.1016/j.ijid.2011.05.018
64. Iyer JK, Kalra M, Kaul A, Payton ME, Kaul R. Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis. *World J Gastroenterol*. (2017) 23:6802–16. doi: 10.3748/wjg.v23.i37.6802
65. Hayashida K, Shoji I, Deng L, Jiang DP, Ide YH, Hotta H. 17β-estradiol inhibits the production of infectious particles of hepatitis C virus. *Microbiol Immunol*. (2010) 54:684–90. doi: 10.1111/j.1348-0421.2010.00268.x
66. Magri A, Barbaglia MN, Foglia CZ, Boccato E, Burlone ME, Cole S, et al. 17, β-estradiol inhibits hepatitis C virus mainly by interference with the release phase of its life cycle. *Liver Int*. (2017) 37:669–77. doi: 10.1111/liv.13303
67. Ulitzky L, Lafer MM, KuKuruga MA, Silberstein E, Cehan N, Taylor DR. A new signaling pathway for HCV inhibition by estrogen: GPR30 activation leads to cleavage of occludin by MMP-9. *PLoS ONE* (2016) 11:e0145212. doi: 10.1371/journal.pone.0145212

68. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J*. (2002):5017–25. doi: 10.1093/emboj/cdf529
69. Langer C, Gansz B, Goepfert C, Engel T, Uehara Y, von Dehn G, et al. Testosterone up-regulates scavenger receptor BI and stimulates cholesterol efflux from macrophages. *Biochem Biophys Res Commun*. (2002) 296:1051–7. doi: 10.1016/S0006-291X(02)02038-7
70. Stangl H, Graf GA, Yu L, Cao G, Wyne K. Effect of estrogen on scavenger receptor BI expression in the rat. *J Endocrinol*. (2002) 175:663–72. doi: 10.1677/joe.0.1750663

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REVIEW
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Sex Bias in Asthma Prevalence and Pathogenesis

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Sex-related differences in asthma prevalence are well established and change through the reproductive phases of life. As children, boys have increased prevalence of asthma compared to girls. However, as adults, women have increased prevalence of asthma compared to men. Many factors, including genetics, environment, immunological responses, and sex hormones, affect the sex disparity associated with the development and control of asthma and other allergic diseases. Fluctuations of hormones during puberty, menstruation, pregnancy, and menopause, alter asthma symptoms and severity. In this article, we review clinical and epidemiological studies that examined the sex disparity in asthma and other allergic diseases as well as the role of sex hormones on asthma pathogenesis.

Keywords: asthma, allergic disease, sex hormones, puberty, pregnancy, menopause

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INTRODUCTION

There is a sexual dimorphism in asthma and allergic disease that changes through life. Among children, boys have an increased prevalence of asthma and allergic disease compared to girls. Interestingly, around puberty the frequency of asthma and allergic disease starts to change from being higher in males to higher in females. By adulthood, the prevalence of asthma and allergic disease is increased in women compared to men (1). This change in prevalence around puberty suggests sex hormones and other factors alter pathways important in asthma pathogenesis and allergic disease.

Asthma is a heterogeneous disease characterized by episodes of airway narrowing or hyper responsiveness, obstruction, inflammation and mucous production. Asthma clinically presents as wheezing, coughing, chest tightness and shortness of breath (2, 3), and different inflammatory pathways drive the airway inflammation and hyperresponsiveness associated with asthma. Patients with allergic asthma have increased eosinophils in the airway or bronchoalveolar lavage fluid (BAL) caused by increased type 2 inflammation. Type 2 inflammation is characterized by increased production of interleukin (IL)-4, IL-5, and IL-13, increased IgE antibody production, and mast cell or basophil degranulation (4–6). However, some patients with more severe phenotypes of asthma have increased neutrophils in the airway and BAL fluid that is driven by IFN-γ or IL-17A-mediated pathways (4, 5). Patients with increased airway neutrophils may or may not have increased airway eosinophils as well. Multi-variate cluster analyses on adults with asthma and healthy controls determined a female predominance in two clusters: (1) less atopic, less corticosteroid responsive patients and (2) late-onset, more severe phenotypes of asthma in obese patients (4, 7). As summarized in **Figure 1**, studies also highlighted the many different factors, including genetics and epigenetics, environment, respiratory mechanics, immunological responses, sex hormones and obesity, regulated asthma pathophysiology and the various endotypes seen in asthma throughout

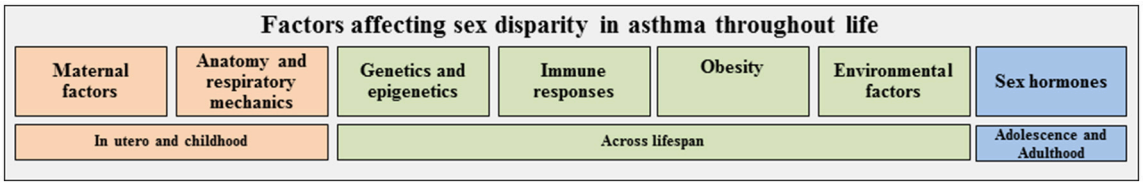

FIGURE 1 | Multiple factors are associated with asthma and may contribute to the sex disparity seen in asthma throughout the life span. These factors may independently or jointly be associated with asthma or regulate each other (e.g., genetics may impact immune response and/or obesity). Factors are color-coded based on importance in asthma at various phases of life: orange, *in utero* and childhood; green, throughout life; blue, adolescence and adulthood.

the lifespan. Therefore, understanding how sex hormones and other factors regulate asthma pathogenesis is important since asthma affects approximately 235 million people with a global annual health care burden in 2007 of approximately $56 billion for medical costs, lost school and work days and early deaths (8). In this review, we primarily focused on the sex differences and sex hormone regulation in asthma pathogenesis in the clinical and epidemiological literature organized by maternal factors, childhood asthma, and asthma after puberty (**Table 1**). At the end of the review, there will be a brief overview of the sex disparity in other allergic diseases.

MATERNAL FACTORS, EPIGENETICS, THE MICROBIOME, AND ASTHMA

Maternal factors, such as smoking, antibiotic or corticosteroid use, and prenatal stress, are associated with increased development of asthma or wheeze in children (33–36). While boys have increased development of asthma and wheeze as children, no sex specific association was determined with maternal smoking or antibiotic use and the development of wheeze in children (34, 35). Further, a prospective study showed no sex differences in maternal asthma control during pregnancy and asthma risk in offspring (37). However, previous studies showed that female infants from mothers not taking inhaled corticosteroids had reduced birth weights compared to female infants from mothers administered inhaled corticosteroids, with no change in birth weights in male infants from these groups (38). A female fetus was also associated with increased circulating monocytes compared to the male fetus in this study. A sex bias, with more boys compared to girls, was also determined with prenatal maternal stress and the development of asthma or wheeze (39). However, not all maternal exposures are adverse, as maternal exposure to farming and farm milk is protective against the development of asthma (40). Women who farmed during pregnancy and had exposure to multiple animal species and consumed farm milk led to enhanced innate immune responses through increased expression of pattern recognition receptors, upregulated IFN-γ production, upregulated T regulatory cell function and reduced Th2 cell dependent allergic inflammation in early childhood (41–43). Further, breastfeeding is known to decrease the risk of asthma in infants (44). However, sex was not addressed as a variable in some breastfeeding studies (37, 38), or was listed as a co-variate that did not impact the findings in other studies (45–49). Combined, these studies suggest that prenatal stress and potentially maternal asthma control may differentially affect male and female offspring in childhood development of asthma/wheeze.

Microbiome formation in early life is vital for educating the immune system and many environmental factors, including mode of delivery, formula feeding, antibiotic use, and exposure to animals, are important in microbiome formation and are associated with asthma. The Canadian Healthy Infant Longitudinal Development (CHILD) study determined that the microbiome composition, specifically reductions in bacterial genres *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia,* at 3 months of age (but not 1 year) increased the risk of developing asthma or allergic diseases (50). No sex differences were observed in the abundance of these bacterial genres and development of asthma or allergic disease. However, additional analysis of this cohort showed a decreased abundance of *Lactobacillus* in Caucasian male infants at 3–4 months of age born to mothers' with asthma compared to female infants (51). *Lactobacillus* are associated with increased production of anti-inflammatory cytokines in cord blood and *Lactobacillus johnsonii* reduced ovalbumin or cockroach allergen-mediated airway inflammation in mouse models of asthma (52, 53).

Gene transcription can be altered by epigenetic modifications that are established *in utero* or developed due to environmental exposures, disease states, antibiotic or medication use, and lifestyle choices. Alterations in the epigenome *in utero* or during childhood may also drive asthma development (54, 55). Maternal smoking alters DNA methylation and maternal smoking is a known risk factor for childhood asthma. Further, asthmatic children have differences in DNA methylation levels at 19 different loci depending on *in utero* exposure to maternal smoking (54, 56, 57). However, none of these studies showed

Abbreviations: ACQ, asthma control questionnaire; ADR, adverse drug reactions; BAL, bronchoalveolar lavage fluid; BMI, body mass index; CAMP, childhood asthma management program; CHILD, canadian healthy infant longitudinal development; DHEA-S, dehydroepiandrosterone-sulfate; ECHRS, european community respiratory healthy survey; FEV1, forced expiratory volume in 1 s; GINA, global initiative for asthma; HRT, hormone replacement therapy; IFN, interferon; IL, interleukin; ILC2, group 2 innate lymphoid cells; PBMCs, Peripheral blood mononuclear cells; PIAMA, prevention and incidence of asthma and mite allergy; PMA, pre or perimenstrual asthma symptoms; SAPALDIA, swiss study on air pollution and lung diseases in adults; TRAILS, tracking adolescents' individual lives survey.

TABLE 1 | Summary of clinical findings for gender disparities in asthma during various reproductive phases of life.

Authors	Methods	Demographics and number of subjects	Conclusions
ASTHMA AND PUBERTY			
Fu et al. (9)	- Longitudinal study for asthma symptom diary scores (CAMP data) - Tanner stage scores	- 5 to 17 yr olds - 418 subjects - 564,518 records	- 5–6 yrs: asthma severity: M > F - 7–9 yrs: no sex difference in severity - 10–17 yrs: asthma severity: F > M
Wijga et al. (10)	- Longitudinal study, questionnaire based data collected during pregnancy, 3 months, 1 yr and yearly thereafter (PIAMA data)	- 3,308 children followed birth-8 yrs	- 0–3 yrs: incidence of asthmatic wheeze: M >F - 4–7yrs: no significant difference - 8 yrs: atopy prevalence: M > F
Vink et al. (11)	- Longitudinal study, questionnaire based data	- 2,230 dutch adolescents	- At mean age 11.1: asthma prevalence: boys = girls - At mean age 16.3: asthma prevalence: F > M
Chen et al. (1)	- Retrospective study on canadian hospital records over 3 yrs	- 288,977 asthma-related records - 204,304 asthma patients	- 3 yrs cumulative incidence of asthma hospitalization: boys> girls; reversed in adults - 25–34 yrs of age, incidence ratio for asthma hospitalization 2.8 F:M
Schatz el al. (12)	- Retrospective study on computerized data used to identify and analyze asthmatic patients with regard to asthma related HCU	- 60,694 subjects (2–64 yrs)	- Ages 2–13 yrs: asthma HCU and severity: M > F - Ages 14–22: asthma HCU and severity: F > M - Ages 23–64: asthma HCU and severity: F > M
Nicolai et al. (13)	- Longitudinal study, asthmatic and control patients recruited at age 10, re-evaluated at age 14 and 20	- 274 asthmatics and 1,000 healthy controls (ages 10–20)	- At 20 yrs, 24.5% still had symptoms (M > F) and 4.8% had developed asthma (F > M)
ASTHMA AND THE MENSTRUAL CYCLE			
Shames et al. (14)	- Daily asthma symptoms, medication use, PEFRs, spirometry and methacholine challenges longitudinally over 6 menstrual cycles	- 32 asthmatic women	- 28.2% of subjects reported PMA. - Women with PMA had increased perimenstrual use inhaled SABA and decreased morning PEFRs.
Pauli et al. (15)	- Daily asthma symptom diaries, PEFRs, spirometry and methacholine challenges longitudinally during 3–4 menstrual cycles	- 11 asthmatic women and 29 healthy controls	- AM PEFRs and asthma symptoms from follicular to luteal phase: asthmatics > controls - No significant changes in spirometry and airway reactivity
Rao et al. (16)	- Questionnaire based data (SARP), - Inflammatory markers, spirometry	- 756 women; 483 self-reported PMA	- Use of oral corticosteroid bursts and HCU: women with self-reported PMA > women without PMA
Brenner et al. (17)	- ED interview - Medical record review - Visits classified by menstrual phase	- 792 asthmatic women (18–54 yrs)	- Acute asthma exacerbations do not markedly increase during perimenstrual phase -Preovulatory + perimenstrual phases may have adverse impact
Zimmerman et al. (18)	- ED interview - Medical record review - Visits classified by menstrual phase	- 288 asthmatic women	- Menstrual phase at time of ED visit: 33% preovulatory, 26% periovulatory, 20% postovulatory, and 21% perimenstrual
Eliasson et al. (19)	- Survey based data	- 57 asthmatic women	- 33% had increased pre or perimenstrual pulmonary symptoms
Gibbs et al. (20)	- Questionnaires and twice daily PEFRs	- 126 asthmatic women (14–46 yrs)	- 40% reported premenstrual increase in symptoms, data confirmed by PEFRs.
Agarwal et al. (21)	- Questionnaires based data PEFRs	- 100 asthmatic women	- 23% patients had increase in symptoms with menstruation - Decreased mean AM and PM PEFR values during pre or perimenstrual phase
Juniper et al. (22)	- Methacholine challenge performed longitudinally during 2 consecutive menstrual cycles	- 17 asthmatics (10 natural cycles, 7 OCP)	- No difference in FEV, medication use or methacholine challenge -Symptoms worse during menstruation
ASTHMA and OCP USE			
Macsali et al. (23)	- Postal questionnaire based data	- 5,791 nordic-baltic women (25–54yrs) - 961/5791 used OCP	- OCPs associated with increase risk of asthma symptoms - Associations present only among normal and overweight women, not lean women.
Juniper et al. (22)	- Methacholine challenge performed longitudinally during 2 consecutive menstrual cycles	- 17 controlled asthmatics (7 on OCP)	- No difference in FEV, medication use or methacholine challenge - Symptoms worse during menstruation
Nwaru et al. (24)	- Longitudinal, serial survey based data (serial Scottish Health Surveys)	- 3,257 scottish women, 16–45 yrs	- Use of hormonal contraceptive associated with increased risk of current physician diagnosed asthma (OR 0.68) and increased risk of asthma HCU (OR 0.45)

(Continued)

TABLE 1 | Continued

Authors	Methods	Demographics and number of subjects	Conclusions
ASTHMA AND PREGNANCY			
Schatz et al. (25)	- Women monitored for HCU, lung function and med use before, during and after pregnancy.	- 1,739 pregnant asthmatic women	- Risk of asthma exacerbation during pregnancy: severe asthmatic women > mild and moderate asthmatic women
Schatz et al. (26)	- Asthma symptom and medication diaries - Spirometry during pregnancy and 3 months postpartum	- 366 pregnancies in 330 women	- During pregnancy, asthma symptoms increased in 33% women - 73% of these women reverted back to pre - pregnancy control by 3 months postpartum.
Belanger et al. (27)	- Interview based symptom and medication data	−872 asthmatic women	- Patients who continued to use their prescribed medication had no change in asthma severity during pregnancy
Juniper et al. (28)	- Airway responsiveness, FEV1, FVC, medication use	−20 asthmatic women	- A majority of women had decreased asthma symptoms and severity during pregnancy
ASTHMA AND MENOPAUSE			
Gomez et al. (29)	- Postal questionnaire based data	- 2,206 nordic-baltic women 46–54 yrs - 884 OCP users and 540 HRT users	- Women taking HRT: increased risk of asthma - Women not taking HRTs: no difference in self-reported asthma between pre-menopausal and post-menopausal women
Real et al. (30)	- Questionnaire data (ECRHS II) - Lung function and hormonal serum markers measured	- 4,529 women (45–56 yrs)	- Decline in lung function and asthma symptoms: women in the menopause transition (amenorrheic for 6 months) > pre-menopausal women had lower lung function
Troisi et al. (31)	- Questionnaire data (NHS data)	- 41,202 premenopausal and 23,035 postmenopausal women	- Asthma incidence: pre-menopausal women> postmenopausal - Higher incidence among postmenopausal women who had never used HRT compared to women who reported current or previous use.
Triebner et al. (32)	- Questionnaire data (RHINE)	- 2,322 women aged 45–65 yrs	- A new phenotype of asthma described with onset after menopause.

AM, morning; CAMP, The childhood asthma management program; ECRHS, european community respiratory health survey; ED, emergency department; FEV, forced expiratory volume; FVC, forced vital capacity; PIAMA, prevention and incidence of asthma and mite allergy; HCU, healthcare utilization; NHS, nurses' health study; HRT, hormone replacement therapy; RHINE, respiratory health in northern europe; PEFR, peak expiratory flow rate; PM, evening; PMA, perimenstrual asthma; pub, publication; OCP, oral contraceptive pill; SABA, short acting beta agonist; SARP, severe asthma research program; yr, year.

a sex disparity in maternal smoking and the development of asthma. Studies looking at maternal stress during pregnancy and maternal obesity have also shown similar findings, with differences in DNA methylation depending on the mother's status (54). A study investigating maternal immune status during pregnancy found that while maternal production of IL-13, IL-4, IL-5, IFN-γ, IL-10, and IL-17 during pregnancy was unrelated to childhood asthma, the ratios of IFN-γ/IL-13 and IFN-γ/IL-4 during pregnancy were associated with a decreased risk of asthma (58). *In utero* and early exposures to farm and animal barns is protective against asthma even in genetically similar Amish and Hutterite populations (59). However, no sex difference in the development of asthma was seen the Amish or Hutterite children ($n = 30$ from each population), although the sample size may not have provided the power needed to detect sex differences within and between the groups. Taken together, these studies show that maternal factors are important in the development of asthma, but it is not yet clear if there is a sex bias in the offspring developing asthma for some of these risk factors and additional studies are warranted.

SEX DISPARITY IN CHILDHOOD ASTHMA

Maternal environment and genetics are important in development of asthma during childhood. However, it is still unclear why boys have an increased prevalence of asthma compared to girls. A potential explanation is that boys have dysynaptic growth of their large airways, meaning the growth of their airway lags behind the growth of the lung parenchyma, leading to narrower airways in boys compared to girls (60). Peripheral blood mononuclear cells (PBMCs) from boys have also been shown to have increased IFN-γ in response to phytohaemagglutin stimulation compared to PMBCs from girls (61). Atopy, defined as the genetic tendency to develop allergic disease, as evidenced by specific IgE and skin prick testing to common allergens is increased in also boys compared to girls (62–64). Some studies have also shown that boys have an increased immunological response compared to girls. One study analyzing 81 infants hospitalized with a diagnosis of RSV bronchiolitis found that boys were more severely affected than girls using a severity index based on heart rate, respiratory rate, wheezing, and oxygen saturation (65). Multiple other studies

found that in children, the male sex was a risk factor for acute and chronic otitis media (66–68). These different factors contribute to the sex disparity in childhood asthma and may explain the sex differences in childhood asthma.

Longitudinal studies tracking children for asthma symptoms and diagnoses of asthma through childhood, adolescence, and adulthood determined that increasing sex hormones were important for change in asthma prevalence observed in males and females. The (Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort tracked sex differences in asthma through the first 8 years of life from questionnaires. In the PIAMA cohort, the prevalence of asthmatic wheeze, defined as wheeze that resulted in diagnosis of asthma by age 8, was increased in boys compared to girls from the first year of life. This study also showed that atopy, defined as a specific IgE > 0.70 IU/mL for at least one standard inhalant allergen (house dust mite, cat, dog, birch, grass, or mold), was more prevalent in boys than girls at age 8 years (36.4% vs. 24.0%: OR, 1.8; 95% CI, 1.3–2.5) (10). While a longitudinal study tracking early life development of wheeze and asthma, this study stopped at age 8, prior to puberty. Additional longitudinal studies have tracked asthma symptoms and prevalence from childhood through adolescence. The Childhood Asthma Management Program (CAMP) study longitudinally tracked asthma symptoms in children ages 4–18 alongside the progression of puberty, recorded by Tanner stage measurements. Prior to the start of puberty, when the Tanner stage was at 1 (ages<7), boys had increased reporting of asthma symptoms compared to girls. However, when Tanner stages began increasing in girls, starting around age 9–10, there were also increased asthma symptoms in girls. Asthma symptoms in the boys did not increase as puberty progressed and actually declined at the end of puberty (Tanner stages 4–5) (9). Similar results were determined from the Tracking Adolescents' Individual Lives Survey (TRIALS) study. In TRIALS, the prevalence of asthma was similar in boys and girls at a mean age of 11.1. However, by age 16.3, the prevalence of asthma was significantly higher in females compared to males. This shift in the prevalence of asthma was thought to be due to an increased incidence and decreased remission of asthma in females compared to males (11). Further, in a longitudinal study, a German cohort of 274 children with current asthma at age 10 were asked about asthma and symptoms via a questionnaire at ages 14 and 20. In this cohort of children with asthma (age 10), males continued to have the higher percentage of asthma at ages 14 and 20. However, in the control group, with no asthma at age 10, females had a significant development of asthma by age 20 (6.4% of females compared to 3.3% of males) (13). Combined, these longitudinal studies suggest that during puberty, the sex associated switch in asthma prevalence may be driven by increased incidence in females.

Using large retrospective cohort data and hospitalization records, investigators were able to look at asthma care utilization during childhood and adolescence. Retrospective Kaiser-Permanent care computerized data from a cohort of 60,694 patients determined asthma care utilization and severity was higher in males compared to females from ages 2 to 13, was similar between males and females from ages 14 to 22, and was higher in females compared to males from ages 23 to 64 (12). Further, a review of 288,977 asthma related hospitalization records among the Canadian population determined that the 3 year cumulative incidence of asthma hospitalization was substantially higher for males compared to females less than 15 years of age and this pattern was reversed for adults (1). The longitudinal and retrospective studies show there is a shift in asthma prevalence and hospital utilization during puberty, but the age at which female prevalence becomes higher than male prevalence varies in the studies. This variation is likely from different patient population demographics, variations in outcome measures, and the time of sampling for each study. Nevertheless, these studies strongly suggest that the prevalence of asthma in females increases during puberty and adolescence.

The association of obesity and asthma is well-established in both pediatric and adult populations and there is increasing interest in obesity and the sex discrepancy in asthma. A study evaluating 5,984 children in Israel determined obesity was associated with increased incidence of asthma in both boys and girls. However, chest symptoms, including wheezing and shortness of breath, and asthma were reported more frequently in obese boys compared to obese girls (69). As children age, the sex and obesity associated pattern changes. A study tracking body mass index (BMI) from birth to adolescence among children with and without asthma found that BMI development differed between girls with and without asthma, with the highest BMI seen among females with persistent asthma (70). Among males in this cohort, there were no clear associations between asthma and BMI (70). Castro-Rodriguez et al. also looked at the relationship between obesity and asthma. Their study showed that females who became overweight or obese between ages 6 and 11 were seven times more likely to develop new asthma symptoms at age 11 or 13 while this pattern was not seen in females who remained lean. Conversely, males who became overweight or obese in this time frame had a similar prevalence of asthma symptoms as those who did not become overweight or obese (71). These studies showed that in the pediatric populations obesity and asthma are associated with boys, but that as adolescents the association of obesity and asthma shifts to females.

Sex-related differences in the lung development, obesity, nutrition, and responses to viral infection and environmental exposures, including farm and second-hand smoke exposure, should be considered when determining sex differences in asthma pathophysiology. While sex hormones are important in regulating the inflammatory response in asthma, other factors are also important, particularly in children prior to puberty when sex hormone levels are minimal.

ASTHMA AFTER PUBERTY

As discussed above, asthma incidence begins to increase in females during late adolescence and as adults women have an increased prevalence of asthma compared to men. This increased prevalence of asthma is true in the Hutterite farming population that has a similar lifestyle, except for farming practices, and asthma risk factors as the Amish, but asthma prevalence rates

similar to westernized populations (59). Over a 10–13 year study period, 1,325 Hutterites between the ages of 6 and 91 (with a mean age of 26) were assessed using asthma questionnaires, pulmonary function tests, methacholine challenges and measures of atopy (72). This study found that the overall prevalence of asthma increased over the study period while prevalence of atopy stayed the same. Even further, the rise in asthma was only found among females while the prevalence among males did not change, suggesting a sex-specific response (72). Sex hormone fluctuations are frequent for women during the reproductive phase of life, and asthma symptoms are known to vary during the menstrual cycle, pregnancy, and menopause.

Asthma and the Menstrual Cycle

Approximately 30–40% of women with asthmatic report worsening of asthma symptoms during the pre or perimenstrual phase of their menstrual cycle (14). Women with pre or perimenstrual asthma symptoms (PMA) self-reported increased use of inhaled short acting beta agonist and decreased morning peak expiratory flow rates during the perimenstrual interval of their menstrual cycle compared to women with asthma without PMA. However, there were no differences in FEV1 or methacholine-induced bronchoprovocation between the two groups (14). A study by Pauli et al. collected daily records of asthma symptoms and peak expiratory flow rates from both women with asthma and healthy controls through the follicular, mid-luteal and late luteal phases of the menstrual cycle. Within the healthy control group as well as the group of asthmatic women, there were no significant changes in spirometry and airway reactivity at any point of the menstrual cycle. However, in asthmatic women, morning peak flows and asthma symptoms (shortness of breath, cough, wheeze, and chest tightness) deteriorated significantly from the follicular to luteal phase (15). A survey conducted among women with asthma described 33% of the women had worsening pulmonary symptoms during the premenstrual period, menstrual period or both with the most significant symptom worsening noted in dyspnea, wheezing and chest tightness in the premenstrual period. Of these women whose asthma was affected by menses, 68% had noted a history of previously being hospitalized for their asthma (19). Multiple other studies have also shown premenstrual deterioration of symptoms and decrease in premenstrual peak expiratory flow rate values (20). Clinical asthma symptoms may appear after ovarian hormones have impacted inflammatory pathways, explaining why increased asthma symptoms are noted during the pre-menstrual and menstrual phases of menses when ovarian hormones levels are not at peak levels.

Data from the Severe Asthma Research Program also revealed that women with self-reported pre-menstrual worsening of asthma had increased use of oral corticosteroid bursts and increased health care utilization compared to women without PMA (16). However, studies determining emergency department utilization for asthma exacerbations during various times of the menstrual cycle are discordant. No difference in emergency department visits for asthma during the perimenstrual phase compared to other points in the menstrual cycle was determined in 792 women with acute asthma exacerbations (17). Increased emergency department visits during the preovulatory phase were determined by interviews and medical record review in 288 women with asthma (18). While the menstrual cycle clearly impacts women with asthma, the mechanisms underlying the cyclical changes and worsening of symptoms is poorly understood (73).

With variations in asthma symptoms during the menstrual cycle, investigators also evaluated changes in asthma symptoms and pulmonary function in women taking hormonal oral contraceptives. A cross sectional study using a postal survey found that contraceptive pill use in premenopausal women was associated with an increased reporting of asthma symptoms and wheezing in normal and overweight women, but not lean women (BMI < 25) (23). However, another study determined no difference in FEV1, response to methacholine, or use of asthma medications in women taking oral contraceptives compared to those with a natural menstrual cycle (22). Both the Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) and data from the national Scottish Health Surveys showed similar data. The SAPALDIA study reported that women taking oral contraceptives had a decrease in airway responsiveness compared to women not on oral contraceptives and the Scottish data reported that women taking oral contraceptives had a reduced risk of physician diagnosed asthma and urgent care use (24, 74). These discordant findings suggest that additional studies are needed to determine the effects of birth control medications on asthma.

Asthma Control Varies During Pregnancy

During pregnancy, studies have reported discordant findings in pregnancy and changing of asthma symptoms. Increased asthma symptoms, tracked by daily diaries and monthly spirometries, were reported in approximately one third of women with asthma (26). The increase in asthma symptoms was maintained until 3-month post-partum when 73% of the women had asthma symptoms revert to the pre-pregnancy course. Importantly in this study, the course of asthma was studied in successive pregnancies in 34 of these women and a statistically significant concordance in the course of asthma symptoms during the two pregnancies was found in 58.8% of the women (26). A later study showed that more severe asthma was linked to worsening of asthma symptoms during pregnancy as 12.6% of patients initially classified with mild asthma had exacerbations while pregnant, while 25.7% of patients classified as moderate and 51.9% of patients classified as severe suffered from exacberations (25). No change in asthma symptoms and asthma medication use during pregnancy was determined in symptom and medication data collected by in-person and telephone interviews from 800 women with physician diagnosed asthma during any trimester of pregnancy when patients continued to use their prescribed medications (27). Further, viral infections and non-adherence to medications were determined to be the primary triggers for asthma exacerbations during pregnancy (75). However, in a prospective study on 16 women during pregnancy showed improved airway responsiveness and asthma severity during pregnancy and a return to preconception levels 1 month after delivery (28). The National Heart, Lung, and Blood Institute

and the Global Initiative for Asthma (GINA)'s current guidelines designate that during pregnancy, women should maintain their current regimen of asthma medications (76). A better understanding of how asthma symptoms will change will enable a more personalized approach for managing asthma and educating women on the importance of medication adherence during pregnancy.

Asthma and Menopause

There are variable findings related to menopause and asthma. The European Community Respiratory Healthy Survey I (ECHRS I) analyzed questionnaire results from 2,206 women aged 46–54 years of whom 884 were menopausal and 540 had used hormone replacement therapy (HRT). This study found an increased risk of asthma in lean women taking HRT, but no differences in self-reported asthma between pre-menopausal and postmenopausal women not taking HRT (29).The ECHRS II trial then reported that women who were in the menopause transition, those who had been amenorrheic for 6 months) had lower lung function and increased asthma symptoms compared to pre-menopausal women (30). The US Nurses' Health Study data revealed that postmenopausal had a decreased incidence of asthma than pre-menopausal women (31). The magnitude of this difference was noted to be higher among the postmenopausal women who had never used HRT compared to women who reported current or previous use (31). BMI was not addressed in this study data. The etiology of the discrepancy of these studies is unclear but may be related to other associated factors including symptom reporting rates and health care seeking behavior, concordant smoking and time course of HRT initiation in relation to diagnosis of asthma. The RHINE study in addition to others have also described a new phenotype of asthma in a subset of women who have onset of the disease after menopause (32, 77). These studies illustrate that fluctuations of hormones during menopause, including the use of HRT, may contribute to asthma symptoms. However, more studies are needed to determine the extent of this effect.

Testosterone May Be Protective Against Asthma

Given the sex disparity in asthma, male sex hormones have also been a topic of interest when studying the disease process. Using the SARP database of patients, one study looked at hormone data which was collected by blood draws assessing estradiol, progesterone, testosterone, and dehydroepiandrosterone-sulfate (DHEA-S) levels in 187 children while simultaneously analyzing Tanner stages, lung function and asthma symptom control using an asthma control questionnaire (ACQ6) (78). In males, higher DHEA-S levels were associated positively with pre and post bronchodilator FEV1% and pre-bronchodilator FVC% as well as improved ACQ6 scores while in females, higher estradiol levels were associated negatively with pre-bronchodilator FEV1% and FVC% (78). In another study evaluating 2,143 adult men, it was found that higher early morning serum testosterone and dihydrotestosterone levels were associated with a higher FEV1 and FVC (79). Our lab has shown that in humans, women with asthma had higher numbers of lung ILC2 (type 2 innate lymphoid cells) compared to men with asthma (80). Further, in adult mice, testosterone negatively affected, or lowered, the number of ILC2s showing that sex hormones do play a role in mediating inflammation in asthma (80). More studies are needed to evaluate a potential protective effect of testosterone as this would provide a better understanding of the underlying mechanism for the sex disparity that is seen in asthma.

Obesity and Adult Asthma

As adults, obesity and asthma are associated in women but not men. The National Population Health Survey in Canada determined data from 9,149 men and women tracked longitudinally from ages 20 to 64, that a baseline BMI greater than 30 had a 1.9 odds ratio of asthma incidence compared to a baseline between BMI of 20-24.9 (normal BMI) (81). No significant association between asthma and baseline BMI was determined in men (81). Further, cross-sectional and longitudinal data from 5,114 adults found the prevalence ratio of asthma was 1.93 in obese women compared to normal weight women with no difference in men that were obese or had a normal BMI (82). A cluster analysis of adults in the Severe Asthma Research Program showed that obese women with late onset of asthma had a more severe asthma phenotype with an average forced expiratory volume in 1 s (FEV1) of 75% (a moderate reduction) and this cluster of women also required frequent oral corticosteroid use to manage exacerbations (4, 7). Similar findings, with a female predominance in clusters that had obese patients with either controlled or uncontrolled asthma, were found in a multi-variable cluster analysis with patients from the Asthma Clinical Research Network (83). Further, increased neutrophils in the sputum were also found in obese women compared to non-obese women with asthma, and no differences were determined in obese and non-obese men with asthma (84). Therefore, a sex disparity in associations with obesity and asthma was seen in cross-sectional and longitudinal studies as well as with multi-variable cluster analyses. Overall, it is unclear why obesity in women, but not men, is associated with asthma and increased asthma severity. With obesity, there are increases in adipose tissue, which is known to secrete estrogen, as well as increased leptin, an energy-regulating hormone that promotes inflammation. No differences in estrogen concentrations were determined in the serum of obese and non-obese women, (84) but increased leptin levels were detected in women compared to men, at any given measure of BMI (85). Select studies in patients with asthma undergoing bariatric surgery have also determined that reducing BMI improved asthma symptoms and FEV1 (86). However, these small studies were not powered to determine if women had a more dramatic improvement compared to men. Therefore, additional research is needed to determine the mechanisms driving the sex differences in obese asthma.

A SEX DISPARITY IN OTHER ATOPIC DISEASES

A sex disparity is also described in other atopic conditions such as rhinitis, eczema, food allergy, vernal conjunctivitis, and drug

hypersensitivity (87, 88). A systematic meta-analysis looking at 67 cross-sectional population based studies internationally found that as children (age less than 11), boys had a higher reported rate of rhinitis compared to girls (89). In this same review, in adolescents (ages between 11 and 18), significantly more females than males were affected (89). Questionnaire data amongst the Isle of Wight cohort in the UK showed that atopic rhinitis prevalence was more common in boys at age 18 and was associated with a greater positive transition in boys from age 10 to 18 (90). This study also showed that non-atopic rhinitis was greater in girls at age 18 and was associated with a greater positive transition in girls from age 10 to 18 (90). The meta-analysis did not differentiate between allergic and non-allergic rhinitis. A study in the United States and multiple studies in Europe have indicated a higher prevalence of eczema in boys vs. girls (91–93). In some studies of preschool aged children, boys were more often atopic and girls suffered significantly more from non-atopic or "intrinsic" eczema (94, 95). However, other studies showed no sex difference in prevalence of eczema (96, 97). In school aged children, there have been discordant findings with one study in Taiwan showing no sex differences in the prevalence of eczema and another from Germany showing more girls than boys suffering from eczema (98, 99). In adulthood, few studies have shown a higher female prevalence of the disease (100, 101). Similar to eczema, with food allergy, the data regarding sex is variable. A systematic literature review revealed that among children with food allergies, 64.4% were males and 35.6% were females but among adults, 34.8% were males and 64.2% were females (102). Another disease of interest in terms of sex differences is vernal conjunctivitis, a condition caused by allergies. This condition typically occurs in the younger population, between 4 and 12 years of age and more frequently amongst boys with the male to female ration ranging from 3 to 5:1. Interestingly, after puberty, the disease spontaneously disappears in majority of patients (87, 103). Sex differences have also been described in drug hypersensitivity. Overall, females have increased allergic and non-allergic drug reactions (104).A common cause of adverse drug reactions (ADRs) is anaphylaxis during anesthesia, and interestingly, there were some differences with specific medications; males were more likely to have an ADR to atracurium during surgery while females were more likely to react to suxamethonium during surgery (104). Combined, these study show a sex disparity in other atopic diseases, but a better understanding of how sex hormones regulates these diseases is warranted.

CONCLUSION

Clinical studies showed a sexual dimorphism in asthma along different hormonal points of life. Prior to puberty, asthma symptoms are increased in boys compared to girls. However, after puberty, sex differences are variable during menstruation, pregnancy, and menopause. Deciphering how sex hormones regulate airway inflammation may also personalize treatment strategies for asthma-based therapeutics (including neutralizing biologics), repurpose androgens as asthma therapeutics, and determine the percentage of women and men with different asthma phenotypes are needed to test new asthma therapeutics. Further, a greater understanding of how sex hormones regulate different asthma phenotypes would enable the use (or avoidance) of hormonal therapy, help predict asthma symptoms during pregnancy, and help determine ways to control peri-menstrual and menstrual asthma (73, 105). Finally, understanding how sex hormones regulate asthma pathogenesis is crucial for treating patients during various phases of life (e.g., puberty or pregnancy), asthmatic women on hormonal birth control medications or hormone replacement therapy, or patients with comorbidities, like obesity.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Chen Y, Stewart P, Johansen H, McRae L, Taylor G. Sex difference in hospitalization due to asthma in relation to age. *J Clin Epidemiol*. (2003) 56:180–7. doi: 10.1016/S0895-4356(02)00593-0
2. NIH: National Institute of Allergy and Infectious Diseases (2018). NIH Statement on World Asthma Day. Available online at https://www.niaid.nih.gov/news-events/nih-statement-world-asthma-day-2018 (Accessed July 26, 2018).
3. Baldaçara RP de C, Silva I, Baldaçara RP de C, Silva I. Association between asthma and female sex hormones. *Sao Paulo Med J*. (2017) 135:4–14. doi: 10.1590/1516-3180.2016.011827016
4. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the severe asthma research program. *Am J Respir Crit Care Med*. (2010) 181:315–23. doi: 10.1164/rccm.200906-0896OC
5. Trejo Bittar HE, Yousem SA, Wenzel SE. Pathobiology of severe asthma. *Annu Rev Pathol*. (2014) 10:511–45. doi: 10.1146/annurev-pathol-012414-040343
6. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. *J Allergy Clin Immunol*. (2015) 135:299–310. doi: 10.1016/j.jaci.2014.12.1871
7. Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of severe asthma research program participants using expanded lung data. *J Allergy Clin Immunol*. (2014) 133:1280–8. doi: 10.1016/j.jaci.2013.11.042
8. Centers for Disease Control and Prevention. *Asthma in the US, VitalSigns CDC* (2011). Available online at: https://www.cdc.gov/vitalsigns/asthma/index.html
9. Fu L, Freishtat RJ, Gordish-Dressman H, Teach SJ, Resca L, Hoffman EP, et al. Natural progression of childhood asthma symptoms and strong influence of sex and puberty. *Ann Am Thorac Soc*. (2014) 11:939–44. doi: 10.1513/AnnalsATS.201402-084OC
10. Wijga A, Tabak C, Postma DS, Kerkhof M, Wieringa MH, Hoekstra MO, et al. Sex differences in asthma during the first 8 years of life: the prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study. *J Allergy Clin Immunol*. (2011) 127:275–7. doi: 10.1016/j.jaci.2010.09.022

11. Vink NM, Postma DS, Schouten JP, Rosmalen JGM, Boezen HM. Gender differences in asthma development and remission during transition through puberty: the tracking adolescents' individual lives survey (TRAILS) study. *J Allergy Clin Immunol*. (2010) 126:498–504.e6. doi: 10.1016/j.jaci.2010.06.018
12. Schatz M, Camargo Jr. CA. The relationship of sex to asthma prevalence, health care utilization, and medications in a large managed care organization. *Ann Allergy Asthma Immunol*. (2003) 91:553–8. doi: 10.1016/S1081-1206(10)61533-5
13. Nicolai T, Pereszlenyiova-Bliznakova L, Illi S, Reinhardt D, von Mutius E. Longitudinal follow-up of the changing gender ratio in asthma from childhood to adulthood: role of delayed manifestation in girls. *Pediatr Allergy Immunol*. (2003) 14:280–3. doi: 10.1034/j.1399-3038.2003.00047.x
14. Shames RS, Heilbron DC, Janson SL, Kishiyama JL, Au DS, Adelman DC. Clinical differences among women with and without self-reported perimenstrual asthma. *Ann Allergy Asthma Immunol*. (1998) 81:65–72. doi: 10.1016/S1081-1206(10)63111-0
15. Pauli BD, Reid RL, Munt PW, Wigle RD, Forkert L. Influence of the menstrual cycle on airway function in asthmatic and normal subjects. *Am Rev Respir Dis*. (1989) 140:358–62. doi: 10.1164/ajrccm/140.2.358
16. Rao CK, Moore CG, Bleecker E, Busse WW, Calhoun W, Castro M, et al. Characteristics of perimenstrual asthma and its relation to asthma severity and control: data from the severe asthma research program. *Chest* (2013) 143:984–92. doi: 10.1378/chest.12-0973
17. Brenner BE, Holmes TM, Mazal B, Camargo Jr. CA. Relation between phase of the menstrual cycle and asthma presentations in the emergency department. *Thorax* (2005) 60:806–9. doi: 10.1136/thx.2004.033928
18. Zimmerman JL, Woodruff PG, Clark S, Camargo CA.Relation between phase of menstrual cycle and emergency department visits for acute asthma. *Am J Respir Crit Care Med*. (2000) 162:512–5. doi: 10.1164/ajrccm.162.2.9910105
19. Eliasson O, Scherzer HH, DeGraff Jr. AC. Morbidity in asthma in relation to the menstrual cycle. *J Allergy Clin Immunol*. (1986) 77(1 Pt 1):87–94. doi: 10.1016/0091-6749(86)90328-3
20. Gibbs CJ, Coutts II, Lock R, Finnegan OC, White RJ. Premenstrual exacerbation of asthma. *Thorax* (1984) 39:833–6. doi: 10.1136/thx.39.11.833
21. Agarwal AK, Shah A. Menstrual-linked asthma. *J Asthma*. (1997) 34:539–45. doi: 10.3109/02770909709055398
22. Juniper EF, Kline PA, Roberts RS, Hargreave FE, Daniel EE. Airway responsiveness to methacholine during the natural menstrual cycle and the effect of oral contraceptives. *Am Rev Respir Dis*. (1987) 135:1039–42.
23. Macsali F, Real FG, Omenaas ER, Bjorge L, Janson C, Franklin K, et al. Oral contraception, body mass index, and asthma: a cross-sectional Nordic-Baltic population survey. *J Allergy Clin Immunol*. (2009) 123:391–7. doi: 10.1016/j.jaci.2008.10.041
24. Nwaru BI, Sheikh A. Hormonal contraceptives and asthma in women of reproductive age: analysis of data from serial national Scottish health surveys. *J R Soc Med*. (2015) 108:358–71. doi: 10.1177/0141076815588320
25. Schatz M, Dombrowski MP, Wise R, Thom EA, Landon M, Mabie W, et al. Asthma morbidity during pregnancy can be predicted by severity classification. *J Allergy Clin Immunol*. (2003) 112:283–8. doi: 10.1067/mai.2003.1516
26. Schatz M, Harden K, Forsythe A, Chilingar L, Hoffman C, Sperling W, et al. The course of asthma during pregnancy, post partum, and with successive pregnancies: a prospective analysis. *J Allergy Clin Immunol*. (1988) 81:509–17. doi: 10.1016/0091-6749(88)90187-X
27. Belanger K, Hellenbrand ME, Holford TR, Bracken M. Effect of pregnancy on maternal asthma symptoms and medication use. *Obs Gynecol*. (2010) 115:559–67. doi: 10.1097/AOG.0b013e3181d06945
28. Juniper EF, Daniel EE, Roberts RS, Kline PA, Hargreave FE, Newhouse MT. Improvement in airway responsiveness and asthma severity during pregnancy. A prospective study. *Am Rev Respir Dis*. (1989) 140:924–31. doi: 10.1164/ajrccm/140.4.924
29. Gomez Real F, Svanes C, Bjornsson EH, Franklin KA, Gislason D, Gislason T, et al. Hormone replacement therapy, body mass index and asthma in perimenopausal women: a cross sectional survey. *Thorax* (2006) 61:34–40. doi: 10.1136/thx.2005.040881
30. Real FG, Svanes C, Omenaas ER, Antò JM, Plana E, Jarvis D, et al. Lung function, respiratory symptoms, and the menopausal transition. *J Allergy Clin Immunol*. (2008) 121:72–80.e3. doi: 10.1016/j.jaci.2007.08.057
31. Troisi RJ, Speizer FE, Willett WC, Trichopoulos D, Rosner B. Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study. *Am J Respir Crit Care Med*. (1995) 152:1183–8.
32. Triebner K, Johannessen A, Puggini L, Benediktsdóttir B, Bertelsen RJ, Bifulco E, et al. Menopause as a predictor of new-onset asthma: a longitudinal Northern European population study. *J Allergy Clin Immunol*. (2016) 137:50–57e6. doi: 10.1016/j.jaci.2015.08.019
33. Duijts L. Fetal and infant origins of asthma. *Eur J Epidemiol*. (2012) 27:5–14. doi: 10.1007/s10654-012-9657-y
34. Duijts L, Jaddoe VWV, van der Valk RJP, Henderson JA, Hofman A, Raat H, et al. Fetal exposure to maternal and paternal smoking and the risks of wheezing in preschool children. *Chest* (2012) 141:876–85. doi: 10.1378/chest.11-0112
35. Lux AL, Henderson AJ, Pocock SJ. Wheeze associated with prenatal tobacco smoke exposure: a prospective, longitudinal study. ALSPAC study team. *Arch Dis Child*. (2000) 83:307–12. doi: 10.1136/adc.83.4.307
36. McKeever TM, Lewis SA, Smith C, Hubbard R. The importance of prenatal exposures on the development of allergic disease. *Am J Respir Crit Care Med*. (2002) 166:827–32. doi: 10.1164/rccm.200202-158OC
37. Liu X, Agerbo E, Schlünssen V, Wright RJ, Li J, Munk-Olsen T. Maternal asthma severity and control during pregnancy and risk of offspring asthma. *J Allergy Clin Immunol*. (2018) 141:886–92.e3. doi: 10.1016/j.jaci.2017.05.016
38. Murphy VE, Gibson PG, Giles WB, Zakar T, Smith R, Bisits AM, et al. Maternal asthma is associated with reduced female fetal growth. *Am J Respir Crit Care Med*. (2003) 168:1317–23. doi: 10.1164/rccm.200303-374OC
39. Sutherland S, Brunwasser SM. Sex differences in vulnerability to prenatal stress: a review of the recent literature. *Curr Psychiatry Rep*. (2018) 20:102. doi: 10.1007/s11920-018-0961-4
40. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358:1129–33. doi: 10.1016/S0140-6736(01)06252-3
41. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol*. (2010) 10:861–8. doi: 10.1038/nri2871
42. Pfefferle PI, Büchele G, Blümer N, Roponen M, Ege MJ, Krauss-Etschmann S, et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE study. *J Allergy Clin Immunol*. (2010) 125:108–15.e3. doi: 10.1016/j.jaci.2009.09.019
43. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol*. (2006) 117:817–23. doi: 10.1016/j.jaci.2005.12.1307
44. Abreo A, Gebretsadik T, Stone CA, Hartert T V. The impact of modifiable risk factor reduction on childhood asthma development. *Clin Transl Med*. (2018) 7:15. doi: 10.1186/s40169-018-0195-4
45. Scholtens S, Wijga AH, Brunekreef B, Kerkhof M, Hoekstra MO, Gerritsen J, et al. Breast feeding, parental allergy and asthma in children followed for 8 years. The PIAMA birth cohort study. *Thorax* (2009) 64:604–9. doi: 10.1136/thx.2007.094938
46. Wright AL, Holberg CJ, Taussig LM, Martinez FD. Factors influencing the relation of infant feeding to asthma and recurrent wheeze in childhood. *Thorax* (2001) 56:192–7. doi: 10.1136/thorax.56.3.192
47. Oddy WH, Holt PG, Sly PD, Read AW, Landau LI, Stanley FJ, et al. Association between breast feeding and asthma in 6 year old children: findings of a prospective birth cohort study. *BMJ* (1999) 319:815–9. doi: 10.1136/bmj.319.7213.815
48. Kull I, Wickman M, Lilja G, Nordvall SL, Pershagen G. Breast feeding and allergic diseases in infants-a prospective birth cohort study. *Arch Dis Child*. (2002) 87:478–81. doi: 10.1136/adc.87.6.478
49. Klopp A, Vehling L, Becker AB, Subbarao P, Mandhane PJ, Turvey SE, et al. Modes of infant feeding and the risk of childhood asthma: a prospective birth cohort study. *J Pediatr*. (2017) 190:192–9.e2. doi: 10.1016/j.jpeds.2017.07.012
50. Arrieta M-C, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations

affect risk of childhood asthma. *Sci Transl Med*. (2015) 7:307ra152. doi: 10.1126/scitranslmed.aab2271
51. Koleva PT, Tun HM, Konya T, Guttman DS, Becker AB, Mandhane PJ, et al. Sex-specific impact of asthma during pregnancy on infant gut microbiota. *Eur Respir J*. (2017) 50:1700280. doi: 10.1183/13993003.00280-2017
52. Prescott SL, Wickens K, Westcott L, Jung W, Currie H, Black PN, et al. Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-γ and breast milk transforming growth factor-β and immunoglobin A detection. *Clin Exp Allergy* (2008) 38:1606–14. doi: 10.1111/j.1365-2222.2008.03061.x
53. Fujimura KE, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, et al. House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. *Proc Natl Acad Sci USA*. (2014) 111:805–10. doi: 10.1073/pnas.1310750111
54. DeVries A, Vercelli D. The neonatal methylome as a gatekeeper in the trajectory to childhood asthma. *Epigenomics* (2017) 9:585–93. doi: 10.2217/epi-2016-0149
55. Vercelli D. Are we what our mothers made us? Lessons from epigenetics. *J Allergy Clin Immunol*. (2018) 141:525–6. doi: 10.1016/j.jaci.2017.12.973
56. Breton C V, Siegmund KD, Joubert BR, Wang X, Qui W, Carey V, et al. Prenatal tobacco smoke exposure is associated with childhood DNA CpG methylation. *PLoS ONE* (2014) 9:e99716. doi: 10.1371/journal.pone.0099716
57. Gunawardhana LP, Baines KJ, Mattes J, Murphy VE, Simpson JL, Gibson PG. Differential DNA methylation profiles of infants exposed to maternal asthma during pregnancy. *Pediatr Pulmonol*. (2014) 49:852–62. doi: 10.1002/ppul.22930
58. Rothers J, Stern DA, Lohman IC, Spangenberg A, Wright AL, DeVries A, et al. Maternal cytokine profiles during pregnancy predict asthma in children of nonasthmatic mothers. *Am J Respir Cell Mol Biol*. (2018) 59:592–600. doi: 10.1165/rcmb.2017-0410OC
59. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate immunity and asthma risk in amish and hutterite farm children. *N Engl J Med*. (2016) 375:411–21. doi: 10.1056/NEJMoa1508749
60. Carey MA, Card JW, Voltz JW, Arbes SJ Jr, Germolec DR, Korach KS, et al. It's all about sex: gender, lung development and lung disease. *Trends Endocrinol Metab*. (2007) 18:308–13. doi: 10.1016/j.tem.2007.08.003
61. Uekert SJ, Akan G, Evans M, Li Z, Roberg K, Tisler C, et al. Gender differences in cytokine immune response profiles and wheezing phenotypes during the first three years of life. *J Allergy Clin Immunol*. (2005) 115:S254. doi: 10.1016/j.jaci.2004.12.1024
62. AAAAI. Atopy Defined. Available online at https://www.aaaai.org/conditions-and-treatments/conditions-dictionary/atopy (Accessed August 28, 2018).
63. Mohammad HR, Belgrave D, Kopec Harding K, Murray CS, Simpson A, Custovic A. Age, sex and the association between skin test responses and IgE titres with asthma. *Pediatr Allergy Immunol*. (2016) 27:313–9. doi: 10.1111/pai.12534
64. Borish L, Chipps B, Deniz Y, Gujrathi S, Zheng B, Dolan CM. Total serum IgE levels in a large cohort of patients with severe or difficult-to-treat asthma. *Ann Allergy Asthma Immunol*. (2005) 95:247–53. doi: 10.1016/S1081-1206(10)61221-5
65. Papadopoulos NG, Gourgiotis D, Javadyan A, Bossios A, Kallergi K, Psarras S, et al. Does respiratory syncytial virus subtype influences the severity of acute bronchiolitis in hospitalized infants? *Respir Med*. (2004) 98:879–82. doi: 10.1016/j.rmed.2004.01.009
66. Vartiainen E. Changes in the clinical presentation of chronic otitis media from the 1970s to the 1990s. *J Laryngol Otol*. (1998) 112:1034–7. doi: 10.1017/S0022215100142409
67. Alho O-P, Oja H, Koivu M, Sorri M. Risk factors for chronic otitis media with effusion in infancy: each acute otitis media episode induces a high but transient risk. *Arch Otolaryngol Head Neck Surg*. (1995) 121:839–43. doi: 10.1001/archotol.1995.01890080011002
68. Hotomi M, Yamanaka N, Samukawa T, Suzumot M, Sakai A, Shimada J, et al. Treatment and outcome of severe and non-severe acute otitis media. *Eur J Pediatr*. (2005) 164:3–8. doi: 10.1007/s00431-004-1564-0
69. Bibi H, Shoseyov D, Feigenbaum D, Genis M, Friger M, Peled R, et al. The relationship between asthma and obesity in children: is it real or a case of over diagnosis? *J Asthma*. (2004) 41:403–10. doi: 10.1081/JAS-120026097
70. Ekström S, Magnusson J, Kull I, Andersson N, Bottai M, Besharat Pour M, et al. Body mass index development and asthma throughout childhood. *Am J Epidemiol*. (2017) 186:255–63. doi: 10.1093/aje/kwx081
71. Castro-Rodriguez JA, Holberg CJ, Morgan WJ, Wright AL, Martinez FD. Increased incidence of asthmalike symptoms in girls who become overweight or obese during the school years. *Am J Respir Crit Care Med*. (2001) 163:1344–9. doi: 10.1164/ajrccm.163.6.2006140
72. Motika CA, Papachristou C, Abney M, Lester LA, Ober C. Rising prevalence of asthma is sex-specific in a US farming population. *J Allergy Clin Immunol*. (2011) 128:774–9. doi: 10.1016/j.jaci.2011.06.046
73. Fuseini H, Newcomb DC. Mechanisms driving gender differences in asthma. *Curr Allergy Asthma Rep*. (2017) 17:19. doi: 10.1007/s11882-017-0686-1
74. Leuenberger P, Künzli N, Ackermann-Liebrich U, Schindler C, Bolognini G, Bongard JP, et al. [Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA)]. *Schweiz Med Wochenschr*. (1998) 128:150–61.
75. Murphy VE, Gibson PG, Talbot PI, Kessell CG, Clifton VL. Asthma self-management skills and the use of asthma education during pregnancy. *Eur Respir J*. (2005) 26:435–41. doi: 10.1183/09031936.05.00135604
76. Global Initiative for Asthma. *Global Strategy for Asthma Management and Prevention* (2018). Available online at: www.ginasthma.org
77. Balzano G, Fuschillo S, De Angelis E, Gaudiosi C, Mancini A, Caputi M. Persistent airway inflammation and high exacerbation rate in asthma that starts at menopause. *Monaldi Arch Chest Dis*. (2007) 67:135–41. doi: 10.4081/monaldi.2007.484
78. DeBoer MD, Phillips BR, Mauger DT, Zein J, Erzurum SC, Fitzpatrick AM, et al. Effects of endogenous sex hormones on lung function and symptom control in adolescents with asthma. *BMC Pulm Med*. (2018) 18:58. doi: 10.1186/s12890-018-0612-x
79. Mohan SS, Knuiman MW, Divitini ML, James AL, Musk AW, Handelsman DJ, et al. Higher serum testosterone and dihydrotestosterone, but not oestradiol, are independently associated with favourable indices of lung function in community-dwelling men. *Clin Endocrinol*. (2015) 83:268–76. doi: 10.1111/cen.12738
80. Cephus J-Y, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone attenuates group 2 innate lymphoid cell-mediated airway inflammation. *Cell Rep*. (2017) 21:2487–99. doi: 10.1016/j.celrep.2017.10.110
81. Chen Y, Dales R, Tang M, Krewski D. Obesity may increase the incidence of asthma in women but not in men: longitudinal observations from the Canadian national population health surveys. *Am J Epidemiol*. (2002) 155:191–7. doi: 10.1093/aje/155.3.191
82. Loerbroks A, Apfelbacher CJ, Amelang M, Stürmer T. Obesity and adult asthma: potential effect modification by gender, but not by hay fever. *Ann Epidemiol*. (2008) 18:283–9. doi: 10.1016/j.annepidem.2007.11.001
83. Sutherland ER, Goleva E, King TS, Lehman E, Stevens AD, Jackson LP, et al. Cluster analysis of obesity and asthma phenotypes. *PLoS ONE* (2012) 7:e36631. doi: 10.1371/journal.pone.0036631
84. Scott HA, Gibson PG, Garg ML, Upham JW, Wood LG. Sex hormones and systemic inflammation are modulators of the obese-asthma phenotype. *Allergy* (2016) 71:1037–47. doi: 10.1111/all.12891
85. Kennedy A, Gettys TW, Watson P, Wallace P, Ganaway E, Pan Q, et al. The metabolic significance of leptin in humans: gender-based differences in relationship to adiposity, insulin sensitivity, and energy expenditure. *J Clin Endocrinol Metab*. (1997) 82:1293–300. doi: 10.1210/jc.82.4.1293
86. Dixon AE, Pratley RE, Forgione PM, Kaminsky DA, Whittaker-Leclair LA, Griffes LA, et al. Effects of obesity and bariatric surgery on airway hyperresponsiveness, asthma control, and inflammation. *J Allergy Clin Immunol*. (2011) 128:508–15.e2. doi: 10.1016/j.jaci.2011.06.009
87. Laffont S, Blanquart E, Guéry J-C. Sex differences in asthma: a key role of androgen-signaling in group 2 innate lymphoid cells. *Front Immunol*. (2017) 8:1069. doi: 10.3389/fimmu.2017.01069
88. Chen W, Mempel M, Schober W, Behrendt H, Ring J. Gender difference, sex hormones, and immediate type hypersensitivity reactions. *Allergy Eur J Allergy Clin Immunol*. (2008). 63:1418–27. doi: 10.1111/j.1398-9995.2008.01880.x
89. Pinart M, Keller T, Reich A, Fröhlich M, Cabieses B, Hohmann C, et al. Sex-related allergic rhinitis prevalence switch from childhood to adulthood: a systematic review and meta-analysis. *Int Arch Allergy Immunol*. (2017) 172:224–35. doi: 10.1159/000464324

90. Kurukulaaratchy RJ, Karmaus W, Raza A, Matthews S, Roberts G, Arshad SH. The influence of gender and atopy on the natural history of rhinitis in the first 18 years of life. *Clin Exp Allergy* (2011) 41:851–9. doi: 10.1111/j.1365-2222.2011.03765.x
91. Moore MM, Rifas-Shiman SL, Rich-Edwards JW, Kleinman KP, Camargo CA Jr, Gold DR, et al. Perinatal predictors of atopic dermatitis occurring in the first six months of life. *Pediatrics* (2004) 113(3 Pt 1):468–74. doi: 10.1542/peds.113.3.468
92. Böhme M, Svensson A, Kull I, Nordvall SL, Wahlgren CF. Clinical features of atopic dermatitis at two years of age: a prospective, population-based case-control study. *Acta Derm Venereol*. 81:193–7. doi: 10.1080/00015501750376294
93. Smidesang I, Saunes M, Storrø O, Øien T, Holmen TL, Johnsen R, et al. Atopic dermatitis among 2-year olds; high prevalence, but predominantly mild disease—the pact study, Norway. *Pediatr Dermatol*. (2008) 25:13–8. doi: 10.1111/j.1525-1470.2007.00574.x
94. Kusel MMH, Holt PG, de Klerk N, Sly PD. Support for 2 variants of eczema. *J Allergy Clin Immunol*. (2005) 116:1067–72. doi: 10.1016/j.jaci.2005.06.038
95. Möhrenschlager M, Schäfer T, Huss-Marp J, Eberlein-König B, Weidinger S, Ring J, et al. The course of eczema in children aged 5-7 years and its relation to atopy: differences between boys and girls. *Br J Dermatol*. (2006) 154:505–13. doi: 10.1111/j.1365-2133.2005.07042.x
96. Wadonda-Kabondo N, Sterne JA, Golding J, Kennedy CT, Archer CB, Dunnill MG, et al. A prospective study of the prevalence and incidence of atopic dermatitis in children aged 0-42 months. *Br J Dermatol*. (2003) 149:1023–28. doi: 10.1111/j.1365-2133.2003.05605.x
97. Purvis DJ, Thompson JM, Clark PM, Robinson E, Black PN, Wild CJ, et al.Risk factors for atopic dermatitis in New Zealand children at 3.5 years of age. *Br J Dermatol*. (2005) 152:742–9. doi: 10.1111/j.1365-2133.2005.06540.x
98. Yang Y-C, Cheng Y-W, Lai C-S, Chen W. Prevalence of childhood acne, ephelides, warts, atopic dermatitis, psoriasis, alopecia areata and keloid in Kaohsiung County, Taiwan: a community-based clinical survey. *J Eur Acad Dermatology Venereol*. (2007) 21:643–9.doi: 10.1111/j.1468-3083.2006.02036.x
99. Zutavern A, Hirsch T, Leupold W, Weiland S, Keil U, Mutius E. Atopic dermatitis, extrinsic atopic dermatitis and the hygiene hypothesis: results from a cross-sectional study. *Clin Exp Allergy* (2005) 35:1301–8. doi: 10.1111/j.1365-2222.2005.02350.x
100. Harrop J, Chinn S, Verlato G, Olivieri M, Norbäck D, Wjst M, et al. Eczema, atopy and allergen exposure in adults: a population-based study. *Clin Exp Allergy* (2007) 37:526–35. doi: 10.1111/j.1365-2222.2007.02679.x
101. Saeki H, Tsunemi Y, Funita H, Kagami S, Sasaki K, Ohmatsu H, et al Prevalence of atopic dermatitis determined by clinical examination in Japanese adults. *J Dermatol*. (2006) 33:817–9. doi: 10.1111/j.1346-8138.2006.00187.x
102. Kelly C, Gangur V. Sex disparity in food allergy: evidence from the PubMed database. *J Allergy*. (2009) 2009:159845. doi: 10.1155/2009/159845
103. Bremond-Gignac D, Donadieu J, Leonardi A, Pouliquen P, Doan S, Chiambarretta F, et al. Prevalence of vernal keratoconjunctivitis: a rare disease? *Br J Ophthalmol*. (2008) 92:1097–102. doi: 10.1136/bjo.2007.117812
104. Light KP, Lovell AT, Butt H, Fauvel NJ, Holdcroft A. Adverse effects of neuromuscular blocking agents based on yellow card reporting in the U.K.: are there differences between males and females? *Pharmacoepidemiol Drug Saf*. (2006) 15:151–60. doi: 10.1002/pds.1196
105. Yung JA, Fuseini H, Newcomb DC. Hormones, sex, and asthma. *Ann Allergy Asthma Immunol*. (2018) 120:488–94. doi: 10.1016/j.anai.2018.01.016

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Gender Bias in Human Systemic Lupus Erythematosus: A Problem of Steroid Receptor Action?

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Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease resulting from abnormal interactions between T and B cells. The acquisition of SLE is linked to genetic susceptibility, and diverse environmental agents can trigger disease onset in genetically susceptible individuals. However, the strongest risk factor for developing SLE is being female (9:1 female to male ratio). The female sex steroid, estradiol, working through its receptors, contributes to the gender bias in SLE although the mechanisms remain enigmatic. In a small clinical trial, monthly administration of the estrogen receptor (ERα) antagonist, ICI182,780 (fulvestrant), significantly reduced disease indicators in SLE patients. In order to identify changes that could account for improved disease status, the present study utilized fulvestrant (Faslodex) to block ERα action in cultured SLE T cells that were purified from blood samples collected from SLE patients (n = 18, median age 42 years) and healthy control females (n = 25, median age 46 years). The effects of ERα antagonism on estradiol-dependent gene expression and canonical signaling pathways were analyzed. Pathways that were significantly altered by addition of Faslodex included T helper (Th) cell differentiation, steroid receptor signaling [glucocorticoid receptor (GR), ESR1 (ERα)], ubiquitination, and sumoylation pathways. ERα protein expression was significantly lower ($p < 0.018$) in freshly isolated, resting SLE T cells suggesting ERα turnover is inherently faster in SLE T cells. In contrast, ERα/ERβ mRNA and ERβ protein levels were not significantly different between SLE and normal control T cell samples. Plasma estradiol levels did not differ ($p > 0.05$) between SLE patients and controls. A previously undetected interaction between GR and ERα signaling pathways suggests posttranslational modification of steroid receptors in SLE T cells may alter ERα/GR actions and contribute to the strong gender bias of this autoimmune disorder.

Keywords: systemic lupus erythematosus, human T cells, estradiol, estrogen receptors, glucocorticoid receptors

INTRODUCTION

Systemic lupus erythematosus (SLE) is a strongly gender-biased autoimmune disease affecting women nine times more frequently than men (1, 2). Onset and progression of SLE involves abnormal T cell signaling, stimulation of autoantibody production, and abnormal cytokine synthesis (3–6). The acquisition of SLE is linked to genetic susceptibility and diverse environmental agents can serve as

triggers in genetically susceptible individuals to promote disease onset (4, 7, 8). However, the strongest risk factor for developing SLE is being female (1). The female sex steroid, estradiol, working through its receptors, contributes to the gender bias in SLE although the mechanisms are not well-understood (9–11).

Estradiol functions by binding to specific ERs, namely ERα and ERβ, which are members of a nuclear receptor ligand-regulated transcription factor family (12). Two independent genes that share a high degree of similarity in the DNA binding domain encode these receptors (13). In the classical mechanism of steroid hormone action, estradiol diffuses into target cells and binds to ERs in the nucleus (14). The ligand-activated receptors interact at specific DNA sites, termed estrogen response elements, along target genes and alter the rate of transcription (15, 16). Data from mice lacking ERα or ERβ suggest that each subtype performs specialized as well as overlapping functions to promote estradiol action *in vivo* (17). Male mice lacking functional ERα ($ER\alpha^{-/-}$) are resistant to developing a lupus phenotype in response to estradiol compared with their wild-type littermates suggesting ERα, rather than ERβ is responsible for inducing a lupus phenotype (18). This concept is supported by more recent data suggesting ERα promotes SLE in F1 females of a lupus mouse model (NZB × NZW) (19).

In female patients with SLE, T cell levels of ERα protein are lower after culture in estradiol, yet, T cells respond robustly to a ligand (ERα) selective agonist, 1, 3, 5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole by stimulating calcineurin and CD154 mRNA expression (20). Genomic analysis of ER binding in breast cancer cell lines (21, 22) indicates a substantial overlap in the chromatin binding sites for ERα and ERβ when a single receptor form is expressed. However, less overlap occurs, and, a greater number of unique binding sites are occupied, when both receptor subtypes are expressed in the same cells (21). Both receptor subtypes are expressed in human T cells (20), and the possibility exists that the receptors could form functional heterodimers when co-expressed (23, 24).

Steroid receptors are regulated by a large number of post-translational modifications including phosphorylation, acetylation sumoylation, and methylation (25–28). Conjugation of the small ubiquitin-like modifier SUMO (sumoylation) to acceptor lysine residues on substrate proteins occurs in a manner analogous to ubiquitination. Free SUMO is charged and transferred to an E2 ligase enzyme (UBC9), which acts in a catalytic manner to conjugate SUMO to an acceptor lysine. Once conjugated to SUMO, the substrate conformation changes with various functional consequences including alterations in protein-protein interactions, transcription, genomic stability and intracellular trafficking (28). Sumoylation and ubiquitin pathways are mechanistically similar but involve distinct enzymes and produce different cellular effects (28–31).

The hallmark of SLE is overproduction of autoantibodies that leads to irreversible, immune complex-mediated end-organ failure. Antibody responses depend on help from $CD4^+$ T cells that are required for the generation of germinal centers where selection of high-affinity B cells and B cell memory occurs (32). Studies *in vitro* indicated that Th2 cells are the major T cell subset engaged in helping B cells (33). Subsequently, T cells expressing the chemokine receptor, CXCR5, were identified as the major T cell subset that provides help to B cells (34). These follicular helper T (Tfh) cells are recognized as a distinct Th subset (35–37). Tfh cells secrete a unique combination of effector molecules that are critical for their development and function including high levels of ICOS, CD154, and IL-21 that promote growth, differentiation, and class-switching of B cells (38, 39). Humans with impaired germinal-center formation through a deficiency of CD154 or ICOS have fewer $CXCR5^+$ $CD4^+$ T cells (40). Targeted deletion of CD154/CD40, ICOS or IL-21 and its receptor compromises the generation of robust germinal-center reactions and impairs humoral responses (39, 40). Involvement of Tfh cells in shaping the effector function of B cells, and in particular, the final differentiation step in plasma cells, implicates Tfh cells as key players in immune disorders such as SLE.

In SLE T cells, signal transduction pathways are altered by estradiol compared with normal T cells (41). Previous studies in our laboratory showed that estradiol could activate and repress genes within the same signal transduction pathway (41). Of particular interest was an increase in calcineurin and CD154 expression in SLE T cell samples but not in T cell samples from control females (9, 10). Upregulation of these genes in SLE T cells was expected to enhance calcium–calcineurin–NFAT signaling, ultimately resulting in exaggerated help to B cells and hypersecretion of autoantibodies. Consistent with this postulate was improved disease activity, and, a reduction in the expression of these T cell activation markers (calcineurin and CD154) in female SLE patients treated with Faslodex, a selective ERα antagonist (42).

The present study investigates changes in signal transduction pathways that could underlie a significant reduction in disease activity in SLE patients treated with Faslodex that we reported previously (42). The results suggest that estradiol, working through ERα, affects the expression of genes involved in Th cell differentiation. An unexpected interaction between ERα and GR signaling points to an intrinsic mechanism(s) in SLE T cells that alters receptor ubiquitination and sumolyation pathways. Changes in these pathways are expected to modify steroid receptor function, influence T cell development and may underlie the strong gender bias of this autoimmune disease.

MATERIALS AND METHODS

Study Participants

This study was approved by the St. Luke's Hospital Institutional Review Board and the Committee for the Protection of Human Research Subjects at Pittsburg State University. All subjects provided written informed consent prior to participation. Eighteen female patients who met the American College of Rheumatology criteria for classification of SLE (43) were enrolled in the study (**Table 1**). The patient's systemic lupus erythematosus disease activity index (SLEDAI) ranged from mild to severe with a median SLEDAI value of 6 (range 2–18) at the time of blood draw. Ten of the patients were Caucasian, six were African American and two were of Asian descent. The age of the patients at the time of enrollment ranged from 24 to 51 years with a median age of 42 years. The SLE patients were taking various medications including

TABLE 1 | Systemic lupus erythematous (SLE) patient data.

Sample	SLEDAI	Medications	Disease duration (years)	Plasma estradiol (pg/ml)
SLE-1	4	MMF, Pret, HCQ	3	168.3
SLE-2	9	Pred	9	98.7
SLE-3	7	HCQ	5	90.3
SLE-4	2	Pred, HCQ	10	86.4
SLE-5	2	MMF, HCQ	12	128.7
SLE-6	4	Pred	3	84.5
SLE-7	12	MMF	11	98.4
SLE-8	5	Pred	32	123.7
SLE-9	4	HCQ, Pred	3	111.6
SLE-10	16	Pred, Cyclo	22	ND
SLE-11	6	Pred, HCQ	6	ND
SLE-12	10	Pred	7	ND
SLE-13	6	Pred, HCQ	19	188.8
SLE-14	4	Pred	11	98.6
SLE-15	11	Pred, HCQ	20	ND
SLE-16	18	Pred, HCQ	15	ND
SLE-17	4	Pred	9	ND
SLE-18	6	Pred, HCQ, Aza	20	95.6

Eighteen women with SLE volunteered for this study. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores ranged between 2 and 18 at the time of enrollment. The patients were taking medications including mycophenolate mofetil (MMF), prednisone (Pred), hydroxychloroquin (HCQ), cyclophosamide (Cyclo), and azathioprine (Aza) as indicated. The duration of disease ranged from 3 to 32 years. Enrolled patients had regular menstrual cycles and were not taking exogenous hormones. Plasma estradiol was determined at the time of blood draw. ND, not determined.

azathioprine, mycophenolate mofetil, hydroxychloroquine, and prednisone (Pred) (**Table 1**). Twenty-five healthy control females were enrolled in the study. The control volunteers were between the ages of 21 and 48 with a median age of 46 years. Seventeen of the controls were Caucasian, four were African American, and four were of Asian descent. Participants had regular menstrual cycles and none of the patients or control females were taking oral contraceptives or exogenous hormone therapy at the time of blood draw. The patients and control volunteers had no history of other collagen vascular diseases.

Measurement of Plasma Estradiol

Plasma samples were isolated at the time of blood collection for T cells. Estradiol levels were measured by duplicate using a commercial ELISA plate (Estradiol ELISA 11-ESTHU-E01, ALPCO Diagnostics, Salem, NH, USA). A standard curve was used to determine the amount of estradiol in circulation at a wavelength of 450 nm. The intra-assay coefficient of variation was 5.85%.

Collection of T Cell Enriched Peripheral Blood Mononuclear Cells

T cell enriched mononuclear cells were separated from blood samples (~90 ml) by density gradient (Histopaque, Sigma, St. Louis, MO, USA). Residual red blood cells were lysed (H-Lyse buffer, R&D Systems, Minneapolis, MN, USA). T cells were purified by negative selection through T cell isolation columns (Human T Cell Enrichment Columns, R&D Systems). The T cells were either used immediately (fresh T cell samples) or cultured overnight (18 h) at 37°C under 5% CO_2 in serum-free medium (Hybridoma, Sigma, St. Louis, MO, USA) supplemented with L-glutamine (200 mM). Some T cells were activated after 18 h of culture for 4 h with phorbol 12 myristate 13-acetate (PMA, Sigma, 10 ng/ml) and ionomycin (Sigma, 0.5 μg/ml). Estradiol-17β (10^{-7} M) was added (or not) to half of the replicate cultures for the entire culture period. We have previously shown that this dose, which is at the upper physiological range of estradiol, effectively upregulates calcineurin and CD154 expression in SLE T cells as described in detail elsewhere (9, 10).

T47D Cell Culture

T47D cells (ATCC, HTB-133, Manassas, VA, USA), a breast cancer cell line, which express ERα and ERβ were cultured at 37°C under 5% CO_2 to 80% confluence (75 mm flask) in T47D media [RPMI (Cellgro, Manassas, VA, USA)] with 200 mM L-glutamine, 10% fetal bovine serum (Harlan Bioproducts, Madison, WI, USA), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Hyclone, Logan, UT, USA). The cells were released from the flask with trypsin-EDTA (Fisher Scientific, Fair Lawn, NJ, USA).

RNA Isolation

RNA was isolated from T cells and T47D cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Phase Lock Gels Heavy (Eppendorf, Fisher Scientific). Total RNA was purified from T cells and treated with DNase I according to the manufacturer's protocol (DNA-free, Ambion, Austin, TX, USA).

Microarray Analysis

Gene profiling was carried out at the Kansas University School of Medicine Microarray Facility as described in detail elsewhere (41). The concentration and purity of total RNA was assessed with an Agilent Bioanalyzer and samples with RIN scores above 7.0 were used for complementary (cRNA) RNA synthesis. Biotinylated cRNA was hybridized to high density Affymetrix human GeneChips HG-U133_Plus_2, which contained 54,675 probe sets. The chips were scanned and analyzed using MAS5 type of data analysis with Affymetrix and Gene spring GX 7.3.1 (Agilent Technologies) software. Signal intensities of genes present in estradiol-treated activated T cell samples without and with Faslodex were compared to the non-treated activated T cell samples in order to generate a fold-change value. Differences greater than 1.5-fold were arbitrarily chosen for further study.

Pathway Analysis

Cell signaling pathways were identified using the Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) library of canonical pathways. The canonical pathways are manually curated algorithms that transform gene lists into relevant signal transduction networks. Gene lists comprising the data sets from SLE patient's T cells treated with and without estradiol and plus or minus Faslodex were entered into the IPA program and differences in gene expression among the treatments were matched to canonical pathways. Fischer's exact test calculated a *p*-value that determined the probability that the association between the genes in the data set (treatment) and the canonical pathway (network) were explained by chance alone.

The top canonical pathways are those with the largest number of gene matches within a signaling network.

Real-time Polymerase Chain Amplification

Selected target genes within differentially regulated pathways were independently investigated by examining expression levels using real-time PCR. Total T cell RNA was digested using DNase 1 and cDNA was synthesized from 4 μg of the resulting RNA using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR (Step-one, Applied Biosystems) was carried out according to the manufacturer's protocol. ERα and ERβ receptors were quantified from the same T cell template using a Taqman probe and ERα (Hs01046818, Applied Biosystems) and ERβ (Hs00230957, Applied Biosystems) primers. A Taqman probe and glyceraldehyde 3- phosphate dehydrogenase (Gapdh, Hs99999905, Applied Biosystems) specific gene primers were used for the internal control. The value of Ct was compared with a pooled T cell sample or T47D cell samples (ERα and ERβ) as positive controls. Samples without template were included in triplicate on each plate as a negative control. The relative expression levels were calculated by dividing the sample Ct values obtained from T cells cultured with and without estradiol from the same individual. The average Ct of Gapdh in untreated T cells was 21.8 ± SEM 1.8, whereas the average Ct of Gapdh in hormonally stimulated T cells was 22.3 ± SEM 1.5, indicating no change in response to treatment.

Isolation of T Cell Proteins

Total RNA and proteins were sequentially separated from the same freshly isolated T cell samples by column purification (Norgen Biotek, ON, Canada). Briefly, RNA was bound to the column and the proteins were collected in the flow through. RNA was treated with DNase I and eluted from the column. The pH of the flow through was adjusted, the proteins were bound to the column, and the columns were washed. The proteins were eluted and stored at −80°C.

Western Blot Analysis

Purified protein samples were heated at 95°C for 5 min. Total T cell proteins were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%). The T47D cell extract was used for a positive control for ERα and ERβ while the lysis solution served as a negative control. Proteins were transferred electrophoretically (18 h, 12 V) onto nitrocellulose membranes using Transblot buffer (25 mM Tris–HCL, pH 8.3, 192 mM glycine, and 20% methanol). After the protein transfer, non-specific protein binding sites were blocked with Superblock buffer (# 37515, Thermo Scientific, Rockford, IL, USA) for 1 h with gentle shaking. The membranes were incubated with an ERα rabbit polyclonal antibody (sc-542, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:1,000 dilution for 1 h, and washed four times (5 min each) with wash buffer (1× PBS containing 0.05% Tween-20). The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (10 μg/ml, 32460, Thermo Scientific, Rockford, IL, USA) at 1:4,000 dilution for 1 h. The blots were washed four times (5 min each) with wash buffer. The blot was incubated in a chemiluminescent reagent (Super Signal West Femto Maximum Sensitivity Substrate kit, 34096, Thermo Scientific, Rockford, IL, USA) for 5 min and exposed to chemiluminescent film (Kodak, BioMax) for 4–5 min. Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) for 15 min at 37°C to remove antibody. The membrane was exposed to chemiluminescent film for 5 min to ensure removal of the primary antibody. The membrane was reacted with ERβ antibody (sc-8974, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:250 dilution) for 1 h. The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (10 μg/ml, 32460, Thermo Scientific, Rockford, IL, USA, 1:4,000) for 1 h at 22°C with gentle shaking. The blot was incubated with Super Signal West Femto Maximum Sensitivity kit reagent for 5 min and the membrane was exposed to Biomax film for 4–5 min. The membrane was stripped and incubated with a β-actin antibody (2 mg/ml, A5441, Sigma, St. Louis, MO, USA, 1:6,000) for 1 h. The blots were washed and reacted with peroxidase-conjugated goat anti-mouse antibody (10 μg/ml, 32430, Thermo Scientific, Rockford, IL, USA) at a 1:4,000 dilution for 1 h. The blot was incubated with chemiluminescent substrate and exposed to Biomax film for approximately 10 s. The amount of receptor was determined using scanning densitometry (Kodak Gel Logic). The optical density of ERα and ERβ protein was divided by the optical density of β-actin on the same blot. Scanning densitometry of β-actin across assays did not vary more than 10% verifying its lack of response to treatment.

Statistical Analysis

Samples from each subject enrolled in this study were not tested in all assays because the entire blood draw was required for each assay. Gene chips were normalized and a Student's *t*-test was used to compare differences in T cell gene expression without estradiol and/or Faslodex and with estradiol and/or Faslodex. Comparison of differences in ERα/ERβ and CXCR5 expression were assessed using a nonparametric Mann–Whitney *U* test. A p-value < 0.05 (two-sided) was considered statistically significant.

RESULTS

Global changes in gene expression were compared between peripheral blood T cells of SLE patients ($n = 9$) cultured with and without estradiol in order to identify differential effects of estradiol on signaling pathways. The top five canonical pathways altered by estradiol treatment included Th cell differentiation, GR signaling, immune cell signaling in rheumatoid arthritis, cytokine communication between immune cells, and phospholipase C signaling (**Table 2**). The top five downstream genes altered by estradiol and shared among these canonical pathways are shown in **Table 2**. TNF and TGF-β1 were shared among four of the top five signaling pathways. IL-21 and NFATC3 were shared among two of the top five canonical pathways. Since the data were obtained from SLE T cells, we investigated if estradiol altered the canonical SLE signaling pathway. Estradiol changed gene expression in the SLE signaling pathway ($p = 4.8 \times 10^{-3}$), though this pathway was not among the top five significant pathways affected. Two candidate downstream genes, NFATC3 and TNF were shared between SLE and GR signaling pathways (**Table 2**).

TABLE 2 | The top five canonical signal transduction pathways affected by estradiol in activated systemic lupus erythematosus (SLE) T cells.

Pathway	SLE + E vs SLE − E	Shared downstream genes (pathway)
1- T helper cell differentiation	2.7×10^{-8}	TGF-β1 (1, 2, 3, 4)
2- Glucocorticoid Receptor Signaling	1.1×10^{-7}	TNF (1, 2, 3, 4, 6)
3- Role of macrophages, fibroblasts, and, endothelial cells in rheumatoid arthritis	1.5×10^{-7}	IL-21 (1, 3, 4) NFATC3 (2, 5, 6)
4- Role of cytokines in mediating commmnicalion between immune cells	1.9×10^{-6}	RAF1 (2, 5)
5- Phospholipase C Signaling	3.2×10^{-6}	
6- SLE signaling[a]	4.8×10^{-3}	

SLE T cell samples (n = 9) were cultured without and with estradiol. The T cells were activated as described in the text. Changes in global gene expression was profiled using microarray analysis. Gene lists were generated from the microarray data and pathways that were altered by estradiol were identified by Ingenuity Pathway Analysis. The p-value of overlap is shown for each pathway. Major downstream genes affected by estradiol treatment were variably shared among the pathways (shown in parentheses).

[a]Although the canonical SLE signal transduction pathway was affected by estradiol it was not within the top five.

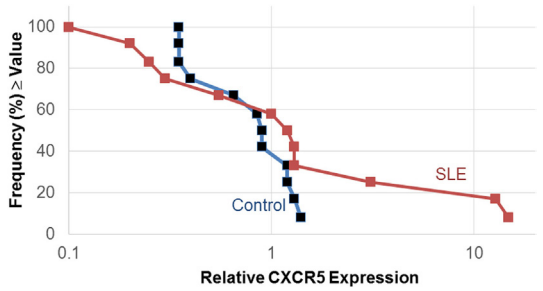

FIGURE 1 | Estradiol effects on CXCR5 expression in activated systemic lupus erythematosus (SLE) T cells. Human T cells were isolated as described in the text and cultured in serum-free medium without and with estradiol. The relative amount of CXCR5 expression following activation was measured by real-time PCR. Data shown are the frequency of T cell samples from controls (n = 12) and SLE patients (n = 12) that exhibited relative CXCR5 expression values that were at or above the value on the x-axis.

The expression of the chemokine receptor, CXCR5 was different between SLE T cells treated without and with estradiol only in the Th cell differentiation pathway. Owing to the importance of CXCR5 in T–B cell interactions, we further quantified CXCR5 expression in SLE patient T cell samples ($n = 12$) cultured without and with estradiol using real-time PCR (**Figure 1**). In 3 of the 12 SLE T cell samples investigated, CXCR5 expression increased robustly (samples 5, 9, 16) in response to estradiol. The SLEDAI scores for those patients 2 (mild), 4 (moderate), and 18 (active) ranged from mild to severe. There was no correlation between disease activity and CXCR5 expression in the patients. The relative median expression of CXCR5 in SLE T cells was 1.1. In the control T cell samples ($n = 12$), CXCR5 expression did not change in response to estradiol (**Figure 1**). CXCR5 expression in the T cells from control females was generally unaffected by estradiol with a median relative expression of 0.9, similar to that for T cell samples from the SLE patients (**Figure 1**). Interestingly, relative CXCR5 expression varied in the SLE T cell samples with both lower and higher expression values compared to those from the control T cell samples.

Administration of the ERα antagonist Faslodex to SLE patients in a small clinical trial significantly reduced their SLEDAI scores (42). It was of interest, therefore, to identify signaling pathways that could account for disease improvement when ERα action was blocked by Faslodex. We compared SLE T cell samples ($n = 9$) cultured with Faslodex to the same T cell samples cultured without Faslodex (**Table 3**). In a separate set of experiments, we added estradiol to the SLE T cell cultures ($n = 9$) without and with Faslodex (**Table 4**). In the absence of added estradiol, the top canonical pathways affected by Faslodex are shown in **Table 3**. Addition of estradiol to the T cells cultured without and with Faslodex changed some of the top pathways affected (**Table 4**). It is notable, that GR signaling was a top canonical pathway affected by estradiol without and with added Faslodex (compare **Tables 2**–**4**). A striking relationship emerged for the top upstream regulators in SLE T cells treated with Faslodex, regardless of estradiol addition or not. The principal regulators shared among Faslodex treated T cells included MYC, HNF4A, ESR1, and CST5 (**Tables 3** and **4**). Downstream genes affected by ERα antagonism and shared among the Faslodex treatments,

TABLE 3 | The top five canonical pathways affected by the estrogen receptor-alpha (ERα) antagonist, Faslodex, in activated systemic lupus erythematosus (SLE) T cells.

Pathway	SLE-E + F vs SLE-E	Tap upstream regulators
Glucocorticoid receptor signaling	2.8×10^{-6}	HNF4A
Sumoylation pathway	2.2×10^{-5}	MVC
Purine nucleotides biosynthesis II	8.6×10^{-5}	ESR1
Estrogen receptor signaling	1.1×10^{-4}	CSTS5
Cleavage and polyadenyladon of Pre-mRNA	1.8×10^{-4}	MMP3
SLE signaling[a]	7.7×10^{-4}	

SLE T cell samples (n = 9) were cultured without and with Faslodex and activated as described in the text. Gene lists were generated from microarray data and analyzed for canonical signaling pathways altered by Faslodex. The p-value of overlap is shown for each pathway.

[a]Although the canonical SLE signal transduction pathway was affected by Faslodex, it was not within the top five. MMP3 was a unique top upstream regulator in the T cells treated with Faslodex without estradiol addition.

TABLE 4 | The top five canonical pathways affected by the estrogen receptor-alpha (ERα) antagonist, Faslodex in activated systemic lupus erythematosus (SLE) T cells ($n = 9$) cultured with estradiol was determined.

Pathway	SLE + E + F vs SLE + E	Top Upstream Regulators
Glucocorticoid receptor signaling	1.1×10^{-16}	MYC
EIF2 signaling	3.8×10^{-15}	HNF4A
Hereditary breast cancer signaling	1.6×10^{-11}	ESR1
Protein ubiquitination pathway	9.6×10^{-10}	CSTS5
JAK/Stat Signaling	1.4×10^{-9}	TP53
SLE signaling[a]	3.4×10^{-8}	

Gene lists were generated from microarray data and analyzed for canonical signaling pathways altered by Faslodex. The p-value of overlap is shown for each pathway.

[a]Although the canonical SLE signal transduction pathway was affected by Faslodex plus estradiol, it was not within the top five. TP53 was a unique top upstream regulator in the T cell samples treated with Faslodex with estradiol addition.

regardless of added estradiol, included AKT3, CBL, cyclic AMP responsive element modulator (CREM), FOS, JUN, and NFAT5. In the absence of added estradiol, MMP3 was a unique upstream regulator (**Table 3**), while addition of estradiol to cultures revealed TP53 as a unique regulator (**Table 4**). The SLE canonical signaling pathway was significantly altered in SLE T cells cultured in Faslodex containing medium without (7.7×10^{-4}) and with (3.4×10^{-8}) estradiol, though it was not one of the top five canonical pathways.

ER antagonism altered the ubiquitination (SLE T cells + estradiol + Faslodex vs SLE T cells + estradiol) pathway in SLE T cells. This pathway was of particular interest because ubiquitin enzymes are essential for regulation of T cell, B cell, and TNF signaling cascades (44). Moreover, activated SLE T cells cultured in medium containing estradiol express less ERα protein than T cell samples from healthy women cultured under the same conditions (20). Investigation of the downstream genes that differed between SLE T cells cultured with estradiol and treated without and with Faslodex revealed changes in 57/255 genes in the ubiquitination pathway (data not shown). The affected genes included ubiquitin-activating enzymes (E1), conjugating enzymes (E2), ligases (E3 HECT), and deubiquitinases. Several factors within the immunoproteosome were altered and the transporter associated with antigen processing (TAP) differed in SLE T cells treated with Faslodex compared with untreated SLE T cells.

ERα antagonism altered sumolyation (SLE T cells − estradiol + Faslodex vs SLE T cells − estradiol) signaling in SLE T cells. Within the sumoylation pathway, the expression of 27/96 genes were affected by Faslodex treatment. Investigation of the genes involved, revealed expression of SUMO-1, FAS, RANBP2 and GR as candidates modified by Faslodex treatment. Since Faslodex altered GR signaling in all SLE T cells, we also compared the data sets to determine which key downstream genes were altered. Faslodex changed the expression of 62 genes (62/282) in the absence of added estradiol and 76 genes (76/287) when estradiol was added to the activated SLE T cell cultures (data not shown). Changes in key downstream genes revealed differences in SUMO-1 and UBE21 (UBC9) expression (data not shown). SUMO-1 and UBC9 target nuclear hormone receptors and their ability to modulate transcription.

To test if protein turnover of ERα was modified in SLE T cells, we compared mRNA and protein levels in freshly isolated T cells from SLE patients and control females. Receptor mRNA and protein were quantified in nine freshly isolated SLE T cell samples (**Table 5**) and 10 freshly isolated control T cell samples (**Table 6**). Comparison of the amount of ERα subtype mRNA revealed no significant differences ($p = 0.97$) between SLE patient and control T cell samples. The median relative value for ERα mRNA in SLE T cell samples was 0.027 (**Table 5**) while the median relative value in control T cell samples was 0.045 (**Table 6**). Comparison of the amount of ERβ subtype mRNA revealed no significant differences ($p = 0.18$) between SLE patient and control T cell samples. The median relative value for ERβ mRNA in the SLE T cell samples was 0.6 (**Table 5**) while the median value in the control T cell samples was 2.5 (**Table 6**). The difference in the ratio of ERα: ERβ mRNA between the control and SLE T cell samples approached significance ($p = 0.065$), even though the ratio was less than unity for all samples in both groups. The median concentration of estradiol in plasma was similar ($p = 0.28$) between the SLE patients (97 pg/ml, **Table 5**) and the control females (123 pg/ml, **Table 6**). Those values are within the normal range for women with regular menstrual cycles.

TABLE 5 | Measurements of ER subtype mRNA and protein in freshly isolated systemic lupus erythematosus (SLE) T cells.

Samples	ERα	ERβ	ERα	ERβ	Estradiol pg/ml
	(mRNA)		(Protein)		
SLE-1	0.036	0.811	0.62	0.61	166.3
SLE-2	0 026	0.170	0.35	0.85	98.7
SLE-3	0.018	0.443	0.93	0.80	90.3
SLE-4	0.027	0.442	0.29	0.67	86.4
SLE-5	0.083	1.365	0.40	0.87	128.7
SLE-6	0.071	0.370	1.00	0.90	84.5
SLE-7	0.015	0.660	0.31	0.65	98.4
SLE-8	0 024	0.600	0.80	0.98	123.7
SLE-9	0.238	18.15	0.76	0.98	111.6
Median	0.027	0.60	0.62	0.85	98.7

Blood samples were drawn from SLE patients (n = 9) and T cells were purified by negative selection. ER subtype mRNA was measured by real-time PCR and protein in the same T cell sample was quantified by Western blotting. Plasma estradiol was measured at the time of blood draw. The levels of hormone in circulation were within the normal range for women with regular menstrual cycles.
SLEDAI, systemic lupus disease activity index.

TABLE 6 | Measurements of ER subtype mRNA and protein in freshly isolated systemic lupus erythematosus T cells.

Samples	ERα	ERβ	ERα	ERβ	Estradiol (pg/ml)
	(mRNA)		(Protein)		
CTRL 1	0.068	7.40	1.10	0.88	99.5
CTRL 2	0.020	0.63	1.00	0.92	156.5
CTRL 3	0.004	0.08	1.60	1.60	88.9
CTRL 4	0.030	2.70	0.90	1.20	133.3
CTRL 5	0.008	0.30	1.10	1.40	99.6
CTRL 6	0.009	3.70	0.67	0.08	124.8
CTRL 7	0.060	4.70	0.83	1.20	143.9
CTRL 8	0.147	1.80	0.81	0.97	125.7
CTRL 9	0.090	2.20	0.63	0.46	121.5
CTRL 10	0.270	7.50	1.10	1.00	93.3
Median	0.045	2.5	0.97	0.99	123.1

Blood samples were drawn from healthy volunteers (n = 10) and T cells were purified by negative selection. ER subtype mRNA was measured by real-time PCR and protein in the same T cell sample was quantified by western blotting. Plasma estradiol was measured at the time of blood draw. The levels of hormone in circulation were within the normal range for women with regular menstrual cycles.
SLEDAI, systemic lupus disease activity index.

We next analyzed ER subtype protein expression in the same T cell samples. Incubation of western blots with ERα antibody revealed a single reactive protein that migrated at approximately 65 kDa, consistent with the size for ERα protein (**Figure 2**). After the membrane was stripped and reacted with ERβ antibody a single reactive protein was identified at approximately 56 kDa, consistent with the size of ERβ (**Figure 2**). The membrane was stripped and reacted with β-actin antibody and a single reactive protein was observed at a molecular size of approximately 42 kDa, consistent with the size for β-actin. In the absence of T cell extract, no reactive proteins were observed (data not shown).

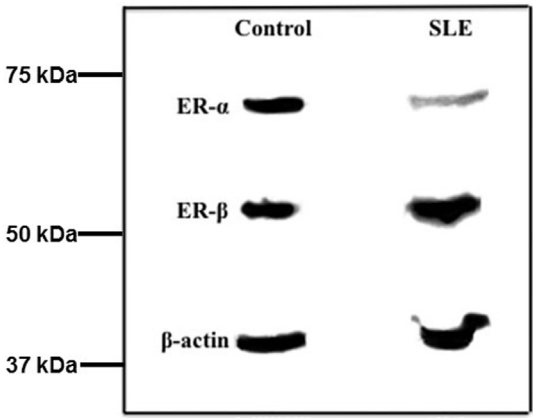

FIGURE 2 | Western blots of freshly isolated T cell proteins indicate the amount of ERα but not ERβ is less in systemic lupus erythematosus (SLE) patients compared with female controls. Fresh T cell extracts were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were sequentially reacted with antibodies to ERα, ERβ, and β-actin. The relative amount of receptor subtype was measured by scanning densitometry and values were adjusted to β-actin in the same T cell sample.

TABLE 7 | Comparison of the relative expression of estrogen receptor subtypes (ERα:ERβ) for protein:mRNA ratios indicates lower values in the systemic lupus erythematosus (SLE) T cell samples.

Parameter	Medlar control	Values SLE	*p*-Value (Mann–Whitney)
Ratio of protein:mRNA (ERα)	23	14	0.37
Ratio of protein:mRNA (ERβ)	0.3	1.5	0.14
Ratio of protein:mRNA (ERa:ERp)	34	21	0.018

These results suggest accelerated turnover of ERα in SLE T cells.

Comparison of the amount of ERα protein revealed a significant difference ($p = 0.010$) between SLE patient and control T cell samples. The median relative value in SLE T cell samples was 0.62 (**Table 5**) while that for ERα protein in control T cell samples was 0.97 (**Table 6**). There was no significant difference in the median values for ERα between patients with mild disease (median value 0.56, SLEDAI ≤ 4) and those with greater disease activity (median value 0.61, SLEDAI ≥ 5). Comparison of the amount of ERβ subtype protein revealed no significant differences ($p = 0.11$) between SLE patient and control T cell samples. The median relative value in SLE T cell samples was 0.85 (**Table 5**) while the median relative value for ERβ protein in control T cell samples was 0.99 (**Table 6**). The ratio of ERα: ERβ protein was a median of 0.78 in the SLE T cell samples and 1.05 in the control T cell samples, respectively. The difference in the ratio of ERα: ERβ protein between the SLE and the control T cell samples was not significant ($p = 0.079$).

Comparison of the ratio of protein to mRNA (designated as the relative productivity) showed all ratios > 1 for ERα while ratios for ERβ were < 1 in the majority (11/19) of samples. The relative productivity of ERα was always greater than for ERβ, but these did not differ between the two cohorts (**Table 7**). However, a comparison of the relative subtype expression (ERα:ERβ) for the protein:mRNA ratios between the SLE T cell samples and the normal T cell samples revealed lower values in the SLE T cell samples ($p = 0.018$, **Table 7**). The primary factor associated with ERα protein was ERβ protein ($p = 0.006$). The second factor associated with ERα protein was experimental group (cohort, i.e., SLE T cell samples vs control T cell samples).

DISCUSSION

The present study investigated global cell signaling changes in human SLE T cells treated with estradiol and the ERα antagonist, Faslodex. We compared the effects of blockading the action of ERα in order to identify signaling pathways that could contribute to improved disease activity in women with SLE we reported previously (42). Estradiol altered gene expression in pathways involved in Th cell differentiation, ERα/GR signaling and immune cell interactions. Antagonism of ERα by Faslodex revealed changes in protein ubiquitination and protein sumoylation pathways. We found that ERα protein but not mRNA was lower in SLE T cells compared with T cells from healthy individuals, suggesting more rapid turnover of ERα in SLE T cells. The results are consistent with the concept that turnover of ERα is accelerated in SLE T cells and may occur through alterations in the ubiquitination signaling pathway. Antagonism of ERα affected the sumoylation pathway and SUMO-1 and UBC9 expression were changed in GR signaling. Modification in these signaling pathways could account for the significant improvement of disease activity in SLE patients receiving monthly Faslodex treatments (42). Additional experiments are required to determine how changes of steroid receptors could alter signal transduction pathways in SLE T cells as suggested by the current results.

ERα protein levels are lower in SLE T cells compared with normal T cells although ERα mRNA and ERβ mRNA and protein are similar between SLE T cell samples and control T cell samples. The molecular basis for the difference in ERα protein levels remains to be established but the current results suggest ERα turnover accelerates owing to changes in the ubiquitination pathway. The half-life of ERα protein is short (~4 h) in primary uterine cells and breast cancer cell lines in culture (45). Consistent with other short-lived regulatory proteins, ERα turnover occurs through the 26S proteasome system (46–48). In spite of the shared structural similarities between the receptors, our results suggest that ERα and not ERβ is the target for accelerated turnover. A recent study in breast cancer cell lines revealed the S-phase kinase-associated protein 2 (*Skp2*), which is a substrate recognition component of the SC ubiquitin ligase complex, targets ERα but not ERβ for degradation (49). The basis of this difference resides in a serine residue (serine 294), which is phosphorylated in ERα by p38MAPK and is not present in ERβ. In SLE T cells treated with Faslodex, MAPK expression was altered in the ERα signaling pathway (data not shown). However, patterns of ERα phosphorylation, activation and turnover require additional analyses in T cells. Since appropriately timed destruction of ERα is essential for its function, it is now important to investigate potential post-translational modifications of ERα that may accelerate receptor turnover in SLE T cells and change the transcriptional activity of the receptor.

SUMO modification of ERα and GR alters protein–protein interactions and transcriptional profiles (25, 28, 50). The enzyme, UBC9, is the only known E2 SUMO-conjugating enzyme that is necessary for SUMO attachment to substrate proteins (28, 30, 31). Comparison of SLE T cells cultured with estradiol and Faslodex to the same T cell sample cultured with estradiol alone (**Table 4**), revealed changes in GR signaling. Downstream genes affected in the GR canonical signaling pathway included *Sumo1* and *Ubc9*. Estradiol increased UBC9 expression in MCF-7 breast cancer cells while ICI182,780 abrogated the response (51, 52). *Ubc9* deletion in T regulatory (Treg) cells results in early-onset lethal autoimmune disorders (53). Loss of *Ubc9* downregulated a variety of cytokine, chemokine and IL-1 receptor activity suggesting that sumoylation is required for proper immune function of Treg cells. Mice deficient in *Ubc9* show perturbations in early T cell maturation in the thymus and a reduction in the nuclear localization of NFAT in response to PMA-ionomycin activation (54). The results from our study suggest ERα antagonism changes the expression of genes involved in GR signal transduction in SLE T cells. To our knowledge, interaction between ER and GR signaling has not been studied in SLE T cells. However, in murine mammary cells chromatin accessibility is enhanced by activation of the opposite receptor (55), GR activation can displace ER from AP-1 sites (56), and ER and GR may act cooperatively at DNA regulatory sites (57). Disruption of GR signaling results in inflammation characterized by increased cytokines in the blood (58). The results from our study suggest that interaction between ERα and GR occurs in SLE T cells when ERα action is blocked. Increased understanding about the molecular basis of this interaction could explain the improvement in SLE patient's disease activity when Faslodex was administered monthly to SLE patients.

Antibody responses depend on help from $CD4^+$ T cells that are required for the generation of germinal centers where selection of high-affinity B cells and B cell memory occurs (32). Expression of CXCR5, when coupled with loss of the T cell zone-homing chemokine receptor CCR7, allows Tfh cells to relocate from the T cell zone to the B cell follicles, where they support B cell expansion and differentiation (59). In the present study, estradiol increased CXCR5 expression in 25% of the T cells from SLE patients. The difference in expression was primarily due to three T cell samples in which, expression levels robustly responded to estradiol. We did not find a correlation between CXCR5 expression and SLEDAI scores, but our study measured relative expression rather than the number of $CRCR5^+$ cells. Tfh cells in circulation constitute a small subset of total immune cells in the blood. Thus, it is possible that the median change in CXCR5 expression in circulating T cells is due to an increase in the number of cells expressing CXCR5 but additional experiments are necessary to resolve this question. Dysregulation of Tfh cells that promotes B cell activation is associated with SLE-like disease in the roquin san/san mouse (60, 61). This mouse model arose from a mutation in the ubiquitin ligase roquin that disrupts a repressor of ICOS, an essential stimulator of Tfh cells. Analysis of $CXCR5^+$ $CD4^+$ cells expressing high levels of Tfh-associated molecules, revealed a subset of SLE patients who showed increased Tfh cells in circulation. The increased Tfh cells correlated with the diversity and titers of autoantibodies and with the severity of end-organ involvement (62). Analysis of global gene expression in this study, indicate Th cell differentiation is affected by ERα antagonism. Since CXCR5 is a defining marker for Tfh cells, we explored changes in CXCR5 expression in SLE T cells. The results are equivocal because only 3 out of 12 SLE T cell samples were estradiol responsive. Moreover, Th responses can be mediated by Th1/Th2 and Th17 subsets (35, 37, 63). In order to define the role of ERα in Th differentiation, analysis of Th subsets and downstream effector functions are necessary.

Systemic lupus erythematosus is a multifactorial autoimmune disorder with numerous cellular abnormalities and clinical presentations. The unifying theme among SLE T cell dysfunction is a loss of the ability to sense antigenic signals and properly integrate these signals within the adaptive and innate immune systems. While progress has been made in understanding the molecular basis and genetic susceptibility for SLE, the strong gender bias in the disorder remains an enigma. The present study indicates a significant decline in the amount of ERα protein in resting SLE T cells relative to resting normal T cells. Alterations in the balance of ERα and ERβ will profoundly affect hormone-responsiveness of target cells as suggested from global analyses of ER subtype binding across the genome. Analysis of global changes in gene expression when we blocked ERα function with Faslodex in SLE T cells indicates both protein ubiquitination and sumoylation pathways are affected. Faslodex identified an unsuspected interaction between ERα and GR signaling. Steroid receptor function requires appropriately time destruction and sumoylation of receptors. The present results suggest that posttranslational modification of steroid receptors (ERα/GR) in SLE T cells may be aberrant. These alterations are likely to affect numerous pathways and lead to signaling dysfunction of SLE T cells.

In this study, we have focused on female SLE patients with regular menstrual cycles who were not taking exogenous estradiol. Although the study design controlled for exogenous estradiol effects, SLE is a heterogeneous autoimmune disorder with numerous clinical presentations. Current therapeutic agents used to treat SLE are based on patterns of end-organ appearance rather than from understanding the molecular basis of the disease. Since the goal of this study was to assess the importance of estradiol in SLE T cells, we did not select a homogeneous group of patients to study based on end-organ involvement. However, none of the patients, at the time of study, had active renal or central nervous system disease. Patients presented with polyarticular non-erosive arthritis ($n = 16$), proteinuria with normal renal function ($n = 7$), pleuritis (4), and lupus malar rash or discoid lupus rash ($n = 7$). Because the study population was heterogeneous, we cannot conclude that the results are representative of all SLE patients. Moreover, it is important to consider that immunosuppressant drugs, such as Pred, may have affected the results. Additional studies including SLE patients not taking medications are necessary to clarify this issue. Although SLE is a strongly gender-biased disorder, the disease occurs in males. The features of SLE in males is often more severe than in female patients (4). Future studies should include male SLE patients to determine if steroid receptor turnover is modified in male patients or accelerated turnover is gender specific. It will be interesting to investigate the interaction of steroid receptors, including the androgen receptor, with

various cofactors that may alter gene expression and lead to the onset or progression of SLE. A recent study in a breast cancer cell line reported that GR represses ERα action (64). The repression of ERα-dependent transcription appeared to be contingent on GR sumoylation, which caused the recruitment of GR and a corepressor complex to ERα occupied enhancers. Greater understanding of how posttranslational modifications of steroid receptors integrate immune-endocrine signaling will help in the molecular understanding of gender-biased autoimmunity. Ultimately, this knowledge will permit greater precision in diagnosis and treatment of patients with SLE and lead to better patient outcomes.

Datasets Are in a Publicly Accessible Repository

The datasets generated and analyzed in this study can be found on the KUMC public repository: (http://bioinformatics.kumc.edu/mdms/shares/data/VRider_exp1_raw_leixmWnf1mGhM/).

AUTHOR CONTRIBUTIONS

VR prepared the T cells, conducted the experiments and IPA analysis, and wrote the manuscript. NA enrolled the SLE patients, collected the blood samples, and provided patient data. BK carried out the statistical analyses and contributed to data interpretation. NL, SB, and BF generated the gene lists from the microarray data.

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REFERENCES

1. Cervera R, Khamashta MA, Font J, Sebastiani CD, Gil A, Lavilla P, et al. Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on systemic lupus erythematosus. *Medicine (Baltimore)* (1993) 72:113–24.
2. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita* (2016) 52:205–12. doi:10.4415/ANN_16_02_12
3. Moulton VR, Suarez-Fueyo A, Meidan E, Li H, Mizui M, Tsokos GC. Pathogenesis of human systemic lupus erythematosus: a cellular perspective. *Trends Mol Med* (2017) 23:615–35. doi:10.1016/j.molmed.2017.05.006
4. Krasselt M, Baerwald C. Sex, symptom severity, and quality of life in rheumatology. *Clin Rev Allergy Immunol* (2017) 53:1–16. doi:10.1007/s12016-017-8631-6
5. Kassi E, Moutsatsou P. Estrogen receptor signaling and its relationship to cytokines in systemic lupus erythematosus. *J Biomed Biotechnol* (2010) 2010:317452. doi:10.1155/2010/317452
6. Apostolidis SA, Leiberman LA, Kis-Toth K, Crispin J, Tsokos GC. The dysregulation of cytokine networks in systemic lupus erythematosus. *J. Interferon Cytokine Res* (2011) 31:769–79. doi:10.1089/jir.2011.0029
7. Karampetsou MP, Comte D, Kis-Toth K, Kyttaris VC, Tsokos GC. Expression patterns of signaling lymphocytic activation molecule family members in peripheral blood mononuclear cell subsets in patients with systemic lupus erythematosus. *PLoS One* (2017) 12:e0186073. doi:10.1371/journal.pone.0186073
8. Deng Y, Tsao BP. Updates in lupus genetics. *Curr Rheumatol Rep* (2017) 19:68. doi:10.1007/s11926-017-0695-z
9. Rider V, Jones SR, Evans M, Abdou NI. Molecular mechanisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus T cells. *Clin Immunol* (2000) 95:124–34. doi:10.1006/clim.2000.4844
10. Rider V, Jones S, Evans M, Bassiri H, Asfar Z, Abdou NI. Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. *J Rheumatol* (2001) 128:2644–9.
11. Moulton VR, Holcomb DR, Zajdel MC, Tsokos GC. Estrogen upregulates cyclic AMP response element modulator α expression and downregulates interleukin-2 production by human T lymphocytes. *Mol Med* (2012) 18:370–8. doi:10.2119/molmed.2011.00506
12. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* (2001) 276:36869–72. doi:10.1074/jbc.R100029200
13. Matthews J, Gustafsson J-A. Estrogen signaling: a subtle balance between ERα and ERβ. *Mol Interven* (2003) 3:281–92. doi:10.1124/mi.3.5.281
14. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. *Cell* (1995) 83:835–9. doi:10.1016/0092-8674(95)90199-X
15. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* (2001) 29:2905–19. doi:10.1093/nar/29.14.2905
16. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* (2002) 108:465–74. doi:10.1016/S0092-8674(02)00641-4
17. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* (1999) 20:358–417. doi:10.1210/edrv.20.3.0370
18. Feng F, Nylan J, Banyai M, Tatum A, Silverston AE, Gavalchin J. The induction of the lupus phenotype by estrogen is via an estrogen receptor-α-dependent pathway. *Clin Immunol* (2012) 134:226–36. doi:10.1016/j.clim.2009.10.004
19. Tabor DE, Gould KA. Estrogen receptor alpha promotes lupus in (NZB×NZW) F1 mice in a B cell intrinsic manner. *Clin Immunol* (2017) 174:41–52. doi:10.1016/j.clim.2016.10.011
20. Rider V, Li X, Peterson G, Dawson J, Kimler BF, Abdou NI. Differential expression of estrogen receptors in women with systemic lupus erythematosus. *J Rheumatol* (2006) 33:1093–101.
21. Charn TH, Liu ET, Change EC, Lee YK, Katzenellenboen JA, Katzenellenbogen BS. Genome-wide dynamics of chromatin binding of estrogen receptors alpha and beta: mutual restriction and competitive site selection. *Mol Endocrinol* (2012) 24:47–59. doi:10.1210/me.2009-0252
22. Grober OM, Mutarelli M, Giurato G, Ravo M, Cicatiello L, Rosaria De Filippo M, et al. Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for target gene regulation. *BMC Genomics* (2011) 12:36. doi:10.1186/1471-2164-12-36
23. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J-A, Kushner PJ, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* (1997) 277:1508–10. doi:10.1126/science.277.5331.1508
24. Gougelet A, Mueller SO, Korach KS, Renoir J-M. Oestrogen receptors pathways to oestrogen responsive elements: the transactivation function-1 acts as the keystone of oestrogen receptor (ER) β-mediated transcriptional repression of ERα. *Steroid Biochem Mol Biol* (2007) 104:110–22. doi:10.1016/j.jsbmb.2007.03.002

25. Sentis S, Le Romancer M, Bianchin C, Rostan MC, Corbo L. Sumoylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. *Mol Endocrinol* (2005) 19:2671–84. doi:10.1210/me.2005-0042
26. Faus H, Haendler B. Post-translational modifications of steroid receptors. *Biomed Pharmacother* (2006) 60:520–8. doi:10.1016/j.biopha.2006.07.082
27. Anbalagan M, Huderson B, Murphy L, Rowan BG. Post-translational modifications of nuclear receptors and human disease. *Nucl Recept Signal* (2012) 10:e001. doi:10.1621/nrs.10001
28. Knutson TP, Lange CA. Dynamic regulation of steroid hormone receptor transcriptional activity by reversible SUMOylation. *Vitam Horm* (2013) 93:227–61. doi:10.1016/B978-0-12-416673-8.00008-3
29. Varshavsky A. The ubiquitin system, autophagy, and regulated protein degradation. *Ann Rev Biochem* (2017) 86:123–8. doi:10.1146/annurev-biochem-061516-044859
30. Weissman AM. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* (2001) 2:169–78. doi:10.1038/35056563
31. Pichler A, Fatouros C, Lee H, Eisenhardt N. SUMO conjugation-a mechanistic view. *BioMol Concepts* (2017) 8:13–36. doi:10.1515/bmc-2016-0030
32. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* (2007) 27:190–202. doi:10.1016/j.immuni.2007.07.009
33. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* (1989) 7:145–73. doi:10.1146/annurev.iy.07.040189.001045
34. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
35. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol* (2015) 16:142–52. doi:10.1038/ni.3054
36. Mesquita D Jr., Cruvinel WM, Resende LS, Mesquita FV, Silva NP, Câmara NO, et al. Follicular helper T cell in immunity and autoimmunity. *Braz J Med Biol Res* (2016) 49:e5209. doi:10.1590/1414-431X20165209
37. Eivazi S, Bagheri S, Hashemzadeh MS, Ghalavand M, Qamsari ES, Dorostkar R, et al. Development of T follicular helper cells and their role in disease and immune system. *Biomed Pharmacother* (2016) 84:1668–78. doi:10.1016/j.biopha.2016.10.083
38. Ettinger RS, Kuchen S, Lipsky PE. The role of IL-21 in regulating B-cell function in health and disease. *Immunol Rev* (2008) 223:60–86. doi:10.1136/ard.2008.098400
39. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med* (2012) 209:1241–53. doi:10.1084/jem.20120994
40. Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* (2006) 77:4927–32. doi:10.4049/jimmunol.177.7.4927
41. Walters E, Rider V, Abdou NI, Greenwell C, Svojanovsky S, Smith P, et al. Estradiol targets T cell signaling pathways in human systemic lupus. *Clin Immunol* (2009) 133:428–36. doi:10.1016/j.clim.2009.09.002
42. Abdou NI, Rider V, Greenwell C, Li X, Kimler BF. Fulvestrant (Faslodex), an estrogen selective receptor downregulator, in therapy of women with systemic lupus erythematosus. Clinical, serologic, bone density, and T cell activation marker studies: a double-blind placebo-controlled trial. *J Rheumatol* (2008) 35:797–803.
43. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* (1982) 25:1271–7. doi:10.1002/art.1780251101
44. Ebner P, Versteeg GA, Ikeda F. Ubiquitin enzymes in the regulation of immune responses. *Crit Rev Biochem Mol Biol* (2017) 52:425–60. doi:10.1080/10409238.2017
45. Alarid ET. Lives and times of nuclear receptors. *Mol Endocrinol* (1981) 20:1972–81. doi:10.1210/me.2005-0481
46. Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW. Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci U S A* (1999) 96:1858–62. doi:10.1073/pnas.96.5.1858
47. Lonard DM, Nawaz Z, Smithe CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-α and coactivator turnover and for efficient estrogen receptor-α transactivation. *Mol Cell* (2000) 5:939–48. doi:10.1016/S1097-2765(00)80259-2
48. Laios I, Journé F, Nonclercq D, Salazar Vidal D, Toillon RA, Laurent G, et al. Role of the proteasome in the regulation of estrogen receptor alpha turnover and function in MCF-7 breast carcinoma cells. *J Steroid Biochem Mol Biol* (2005) 94:347–59. doi:10.1016/j.jsbmb.2005.02.005
49. Bhatt S, Xiao Z, Meng Z, Katzenellenbogen BS. Phosphorylation by p38 mitogen-activated protein kinase promotes estrogen receptor α turnover and functional activity via the SCFSkp2 proteasomal complex. *Mol Cell Biol* (2012) 32:1928–43. doi:10.1128/MCB.06561-11
50. Le Drean Y, Mincheneau N, Le Goff P, Michel D. Potentiation of glucocorticoid receptor transcriptional activity by sumoylation. *Endocrinology* (2002) 143:3482–9. doi:10.1210/en.2002-220135
51. Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, Kurihara I, et al. FHL2, UBC9, and PIAS1 are novel estrogen receptor α interacting proteins. *Endocr Res* (2004) 30:617–21. doi:10.1081/ERC-200043789
52. Ying S, Dunnebier T, Si J, Hamann U. Estrogen receptor alpha and nuclear factor Y coordinately regulate the transcription of the SUMO-conjugating UBC9 gene in MCF-7 breast cancer cells. *PLoS One* (2013) 13:e75695. doi:10.1371/journal.pone.0075695
53. Ding X, Wang A, Ma X, Demarque M, Jin W, Xin H, et al. Protein SUMOylation is required for regulatory T cell expansion and function. *Cell Rep* (2016) 16:1055–66. doi:10.1016/j.celrep.2016.06.056
54. Wang A, Ding X, Demarque M, Liu X, Pan D, Xin H, et al. Ubc9 is required for positive selection and late-stage maturation of thymocytes. *J Immunol* (2017) 198:3461–70. doi:10.4049/jimmunol.1600980
55. Miranda TB, Voss TC, Sung MH, Baek S, John S, Hawkins M, et al. Reprogramming the chromatin landscape: interplay of the estrogen and glucocorticoid receptors at the genomic level. *Cancer Res* (2013) 73:5130–9. doi:10.1158/0008-5472.CAN-13-0742
56. Karmakar S, Jin Y, Nagaich AK. Interaction of glucocorticoid receptor (GR) with estrogen receptor (ER) alpha and activator protein 1 (AP1) in dexamethasone-mediated interference of ERalpha activity. *J Biol Chem* (2013) 288:24020–34. doi:10.1074/jbc.M113
57. West D, Pan D, Tonsing-Carter EU, Hernandez KM, Pierce CF, Styke SC, et al. GR and ER coactivation alters the expression of differentiation genes and associates with improved ER+ breast cancer outcome. *Mol Cancer Res* (2016) 14:707–19. doi:10.1158/1541-7786.MCR-15-0433
58. Arango-Lievano M, Jeanneteau F. Timing and crosstalk of glucocorticoid signaling with cytokines, neurotransmitters and growth factors. *Pharmacol Res* (2016) 113:1–17. doi:10.1016/j.phrs.2016.08.005
59. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell eath gene-1high germinal center-associated subpopulation. *J Immunol* (2007) 179:5099–108. doi:10.4049/jimmunol.179.8.5099
60. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* (2005) 5:853–65. doi:10.1038/nri1714
61. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009) 206:561–76. doi:10.1084/jem.20081886
62. Le Coz C, Joublin A, Pasquali JL, Korganow AS, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* (2013) 19:e75319. doi:10.1371/journal.pone.0075319
63. Mackay CR. Follicular homing T helper (Th) cells and the Thy1/Th2 paradigm. *J Exp Med* (2000) 192:F31–4. doi:10.1084/jem.192.11.F31
64. Yang F, Ma Q, Liu Z, Li W, Tan Y, Jin C, et al. Glucocorticoid receptor: megatrans switching mediates the repression of an ERα-regulated transcriptional program. *Mol Cell* (2017) 66:321–31. doi:10.1016/j.molcel2017.03019

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The Natural Agonist of Estrogen Receptor β Silibinin Plays an Immunosuppressive Role Representing a Potential Therapeutic Tool in Rheumatoid Arthritis

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Estrogens, in particular 17β-estradiol (E2), have a strong influence on the immune system and also affect pathological conditions such as autoimmune diseases. The biological effects of E2 are mediated by two intracellular receptors, i.e., estrogen receptor (ER)α and ERβ, which function as ligand-activated nuclear transcription factors producing genomic effects. Immune cells express both ERα and ERβ that play a complex role in modulating inflammation. Phytoestrogens display estrogen-like effects. Among them, silibinin, the major active constituent of silymarin extracted by the milk thistle (*Silybum marianum*), has been suggested to have an ERβ selective binding. Silibinin is known to have anti-inflammatory, hepatoprotective, and anticarcinogenic effects; however, the role of silibinin in modulating human immune responses and its impact on autoimmunity remains unclear. Aim of this study was to dissect the ability of the ERβ natural ligand silibinin to modulate T cell immunity, taking into account possible differences between females and males, and to define its possible role as therapeutic tool in immune-mediated diseases. To this purpose, female and age-matched male healthy subjects and patients with active rheumatoid arthritis (RA) were recruited. We evaluated the ability of silibinin to modulate ERβ expression in T lymphocytes and its effects on T cell functions (i.e., apoptosis, proliferation, and cytokine production). We also analyzed whether silibinin was able to modulate the expression of microRNA-155 (miR-155), which strongly contributes to the pathogenesis of RA driving aberrant activation of the immune system. We demonstrated that silibinin upregulated ERβ expression, induced apoptosis, inhibited proliferation, and reduced expression of the pro-inflammatory cytokines IL-17 and TNF-α, through ERβ binding, in T lymphocytes from female and male healthy donors. We obtained similar results in T lymphocytes from patients with active RA in term of apoptosis, proliferation, and cytokine production. In addition, we found that silibinin acted as an epigenetic modifier, down-modulating the expression of miR-155. In conclusion, our data demonstrated

an immunosuppressive role of silibinin, supporting its application in the treatment of autoimmune diseases as drug, but also as dietary nutritional supplement, opening new perspective in the field of autoimmune disease management.

Keywords: estrogen receptor β, silibinin, T lymphocytes, immunity, sex, rheumatoid arthritis

INTRODUCTION

It is well known that estrogen (17β-estradiol—E2) influences different aspects of the immune system function and potentially affects the risk, activity, and progression of autoimmune diseases (1–4). In particular, E2 is able to modulate different aspects of immune responses, e.g., lymphocyte proliferation and apoptosis, cytokine, or antibody production (5–8). The immunomodulatory effects exerted by E2 are, at least partially, responsible for the existing differences between female and male immune systems, with females mounting stronger humoral and cellular immune responses than males (6). As a consequence, females are generally more resistant to infection but more susceptible to autoimmune diseases that are typically dominant in women in comparison to men (4, 9, 10). The most important factors responsible for this sex bias are sex hormones, genetic and epigenetic factors, as well as sociological differences between genders. Notably autoimmune diseases differ between males and females not only for their incidence but also for clinical outcome and response to therapy.

17β-estradiol effects are mediated by two intracellular estrogen receptors (ER), i.e., ERα and ERβ, which act as ligand-activated nuclear transcription factors generating genomic effects (8, 11). Our and other groups have demonstrated that immune cells express both ERα and ERβ (12–15) which have a complex role in modulating inflammation, thus representing potential therapeutic targets in autoimmune diseases (16–20). In particular, the low intracellular expression level of ERβ has been demonstrated to be associated with high disease activity in chronic inflammatory diseases such as systemic lupus erythematosus (21) and inflammatory bowel diseases (22). The downregulation of this receptor has been found to be dependent by a pro-inflammatory microenvironment (22). Accordingly, ERβ agonist ligands have been suggested to dampen inflammation in animal models of autoimmune diseases (17, 23).

Interestingly, some phytoestrogens, naturally occurring plant compounds, display ERβ selective binding with estrogen-like effects. Among them, silibinin (24), the major active constituent of silymarin extracted by the milk thistle (*Silybum marianum*), has been suggested by *in silico* studies to have an ERβ selective binding acting as agonist of this ER (25). Silibinin has been demonstrated to have anti-inflammatory, hepatoprotective, and anticarcinogenic properties interfering with multiple biochemical pathways (26); however, the role of silibinin in modulating human immune responses and its impact on autoimmunity remains unclear.

Hence, the aim of this study was to dissect the ability of the ERβ natural ligand silibinin to modulate T cell immunity, taking into account possible differences between females and males, and to define its possible role as a therapeutic tool in immune-mediated diseases. To this purpose, we first evaluated the ability of silibinin to modulate ERβ expression in T lymphocytes from female and age-matched male healthy subjects and its effects on T cell functions (i.e., apoptosis, proliferation, and cytokine production). Then we analyzed the effects played by silibinin on T lymphocytes from patients affected by rheumatoid arthritis (RA), a chronic autoimmune inflammatory disease, characterized by synovial inflammation and by cartilage and bone destruction (27), in which T lymphocytes play a key pathogenetic role (27). We also evaluated whether silibinin could modulate the expression of microRNA-155 (miR-155) which is involved in the modulation of T lymphocyte immunity (28) and strongly contributes to the pathogenesis of RA driving aberrant activation of the immune system (29–31).

MATERIALS AND METHODS

Study Population

Forty-four healthy subjects (23 postmenopausal females and 21 age-matched males, age range 55–75 years) as well as 10 postmenopausal female patients and 4 age-matched male patients with active RA, who had an inadequate response to drugs, followed at the Rheumatology outpatient Clinic (Arthritis Center, Policlinico Umberto I, Sapienza University of Rome, Italy), were included in the study. All RA patients fulfilled the 2010 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) classification criteria (32). Exclusion criteria were pregnancy, treatment with any kind of hormones. Clinical evaluation included the count of swollen and tender joints, patient and physician global disease assessment by VAS (0–100 mm). Disease activity was measured by Disease Activity Score 28 (DAS28) and clinical response was evaluated according to EULAR response criteria (33). The following laboratory tests were performed: complete blood count, erythrocyte sedimentation rate, C-reactive protein, antinuclear antibodies, rheumatoid factor, and anti-cyclic citrullinated peptide antibodies. Demographic and clinical features of RA patients are shown in **Table 1**.

This study was carried out in accordance with the recommendations of the Declaration of Helsinki. Written informed consent was obtained from all subjects, and the ethics committee of the Policlinico Umberto I (Rome, Italy) approved the study.

Abbreviations: APC, allophycocyanin; AV, annexin V; DAS28, disease activity score 28; E2, 17β-estradiol; ER, estrogen receptor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mAb, monoclonal antibody; miR-155, microRNA-155; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-*a*] pyrimidin-3-yl]phenol; PI, propidium iodide; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; SSNC, silencer select negative control.

TABLE 1 | Demographic and clinical features of rheumatoid arthritis patients (females, *N* = 10; males, *N* = 4).

Patients features	Females	Males
Median age (years), IQR	60 (18.5)	61.5 (9.0)
Median disease duration (years), IQR	17 (15.5)	9.5 (5.5)
Median DAS28, IQR	5.5 (1.3)	5.9 (1)
PDN, *N*/%	1/88.8	1/25
sDMARDs treatment, *N*/%	5/62.5	4/100
bDMARDs drugs, *N*/%	4/44.4	0

IQR, InterQuartile range; PDN, prednisone; DAS28, Disease Activity Score 28; sDMARDs, synthetic disease-modifying antirheumatic drugs; bDMARDs, biologic disease-modifying antirheumatic drugs; N, number.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Cell Culture Conditions

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation and cultured in RPMI-1640 medium without phenol red (Gibco BRL, Grand Island, NY, USA) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone Laboratories, South Logan, UT, USA), 2 mM glutamine (Sigma, St. Louis, MO, USA), and 50 µg/ml gentamycin (Sigma). Silibinin (Sigma) was dissolved in dimethyl sulfoxide and diluted in RPMI 1640. Preliminary dose response and time course experiments showed that silibinin should be used at a dose of 50 µM and at 24–72 h of culture (depending on the studied parameters) to obtain the highest detectable changes in the absence of toxic effects. For lymphocyte activation, PBMCs or sorted $CD4^+CD45RA^-CCR6^+CXCR3^-$ (see below for sorting) were cultured in the presence of plate-bound anti-CD3 monoclonal antibody (mAb, clone UCHT1, R&D Systems, Minneapolis, MN, USA) at 4 µg/ml for 72 h and treated with silibinin for the last 48 h of culture. In separate experiments, cells were pretreated with 100 nM 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) ERβ antagonist (Tocris Cookson, Ellisville, MO, USA) for 1 h before adding silibinin.

For cytokine production, untreated or treated PBMCs were stimulated as follows: (i) for IFN-γ, TNF-α, IL-2, and IL-4 analysis, 25 ng/ml phorbol myristate acetate (PMA, Sigma) and 1 µg/ml ionomycin (Sigma) for the last 16 h of culture; (ii) for IL-17 analysis, 50 ng/ml PMA (Sigma) and 1 µg/ml ionomycin (Sigma) for the last 4 h of culture; and (iii) for IL-10, 2.5 µg/ml phytohemagglutinin (Sigma) for the last 16 h of culture. To inhibit cytokine secretion, 10 µg/ml brefeldin A (Sigma) was added to each condition at the beginning of stimulation.

Flow Cytometry

Cell surface phenotyping was performed by flow cytometry as previously described (13). Allophycocyanin (APC)-conjugated anti-CD3, APC- or phycoerythrin (PE)-conjugated anti-CD4, peridinin chlorophyll protein-conjugated anti-CD8 mAbs (all from BD Biosciences, San Jose, CA, USA) were used. Equal amount of mouse IgG isotype control was run in parallel. Analysis of cytokine production at the single cell level was performed as previously described with minor changes (34). Briefly, treated cells (see above for details) were either fixed with 4% paraformaldehyde and permeabilized with FACS permeabilizing solution (BD Biosciences) for IFN-γ, TNF-α, IL-2, IL-4, and IL-10 detection or fixed and permeabilized with intracellular fixation and permeabilization buffer (eBioscience, San Diego, CA, USA) for IL-17 detection. The following cytokine-specific mAbs were used: fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ, FITC-labeled anti-IL-2, PE-labeled anti-TNF-α, PE-labeled anti-IL-4, PE-labeled anti-IL-10 (all from BD Biosciences), and FITC-labeled anti-IL-17A (eBioscience). Appropriate isotypic negative controls were run in parallel. Apoptosis was quantified using FITC- or PE-conjugated annexin V (AV) and propidium iodide (PI) detection kit (Marine Biological Laboratory, Woods Hole, MA, USA) according to the manufacturer's protocol. Proliferation was evaluated by measuring Ki-67 nuclear antigen expression using FITC-labeled anti-human Ki-67 mAb according to the manufacturer's protocol (BD Biosciences). For ERβ intracellular staining of sorted $CD4^+CD45RA^-CCR6^+CXCR3^-$ Th17 lymphocytes, cells were fixed and permeabilized as described above, and stained with the anti-ERβ mAb (clone CWK-F12 from DSHB, Iowa City, IA, USA). Equal amount of mouse IgG isotype control was run in parallel. The primary antibody was visualized by FITC-conjugated F(ab′)2 fragment secondary antibody (Abcam, Cambridge, UK).

To determine the frequency of T cell subsets, total lymphocytes were first gated by forward and side scatter and then additionally gated for CD3 and CD4 or CD3 and CD8 molecule expression. Acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences) and at least 50,000 events per sample were run. Data were analyzed using the Cell Quest Pro software (BD Biosciences).

MACS and FACS Cell Sorting

For Western blot and quantitative real-time PCR (qRT-PCR) analyses, untouched T cells were separated using the Pan T Cell isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of recovered cells, assessed by flow cytometer, was ≥97%.

For apoptosis and ERβ analyses of Th17 cells (i.e., $CD4^+CD45RA^-CCR6^+CXCR3^-$), $CD4^+$ T cells were separated from PBMC by positive selection using CD4 MicroBeads (Miltenyi Biotec), with a purity ≥97%, as determined by flow cytometer. Then, $CD4^+CD45RA^-CCR6^+CXCR3^-$ T cell subset was sorted by FACS (BD FACSAria; BD Biosciences) upon staining with the following mixture of mAb: CD4 PE/Cy7 (BD Biosciences), CD45RA FITC (BD Biosciences), CCR6 PE (Miltenyi Biotec), and CXCR3 APC (BD Biosciences). Sorted T cell subset was on average >95% pure as determined by postsorting flow cytometry analysis.

SDS-PAGE and Western Blot

SDS-PAGE and Western blot were performed as previously described (13). Briefly, cells were lysed in RIPA buffer [100 mM tris(hydroxymethyl)aminomethane (Tris)–HCl pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$] in the presence of a complete protease-inhibitor mixture. Protein content was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA). Cell lysates (30 µg/ml) were loaded onto SDS-PAGE and, after electrophoresis, proteins were transferred onto nitrocellulose

membrane (GE Healthcare, Pittsburgh, PA, USA) by means of a Trans-Blot transfer cell (Bio-Rad Laboratories). The membranes were then blocked in 5% nonfat milk and incubated with the appropriate antibodies in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk. Anti-ERβ mAb (clone CWK-F12 from DSHB) was used as primary Ab. Peroxidase-conjugated goat anti-mouse IgG was used as secondary Ab (Bio-Rad Laboratories) and the reactions were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). To ensure the presence of equal amounts of protein, the membranes were reprobed with a rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase Ab (Sigma). Quantification of protein expression was performed by densitometry analysis of the autoradiograms (GS-700 Imaging Densitometer, Bio-Rad Laboratories).

qRT-PCR Analysis of miR-155 Expression Level

Total RNA, including short RNA, was isolated from T lymphocytes of female RA patients, treated and untreated with silibinin for 48 h, using the Total RNA Purification Plus Kit (Norgen Biotek Corp., Thorold, ON, Canada), according to the manufacturer's instructions. RNA samples, after quantity evaluation using a NanoDrop ND-1000 spectrophotometer, were used for qRT-PCR analysis. miR-155 and RNU6B, as normalizator, expression levels were quantitated using specific inventoried TaqMan MicroRNA Assays (Thermo Fisher Scientific, Waltham, MA USA), according to the manufacturer's instructions, and all samples were run in triplicate. Briefly, 15 ng of each RNA sample were reverse transcribed by the Taq-Man® MicroRNA Reverse Transcription (RT) Kit (Thermo Fisher Scientific) using individual miR-specific RT primers, and 1.3 µl of RT product were analyzed by qRT-PCR on the ABI7000 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). The relative expression level of miR-155 was determined by the $2-\Delta\Delta Ct$ method, after normalization to the RNU6B Ct. 1.5 miR fold changes between RA patients treated or untreated with silibinin were considered significant.

qRT-PCR Analysis of ERβ mRNA Expression Level

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Milan, Italy). RNA was DNase-I digested (Roche) and reverse transcribed as previously described (35). Quantitative PCR was performed in duplicate by the real-time fluorescence detection method with the fluorescent DNA binding dye SYBR green (Power SYBR Green PCR master kit; Applied Biosystems) by using an ABI PRISM 7900 (Applied Biosystems). The relative expression levels were calculated by the comparative cycle threshold (ΔΔCt) method and were normalized by hypoxanthine-guanine phosphoribosyl transferase expression. Homo sapiens estrogen receptor 2 (ESR2, ERβ1) primers used for RT-PCR were designed by using the Primer3Plus software, crossing exon-intron junctions and checking for secondary structures; sequences are 5′-GCTCCTGTCCCACGTCAG-3′, 5′-CACATAATCCCATCCCAAGC-3′.

ERβ Silencing by Small Interfering RNA (siRNA)

The silencing of ERβ was performed with the following Silencer Select siRNA for ESR2, sense, AGUGUACAAUCGAUAAAA ATT, antisense, UUUUUAUCGAUUGUACACUGA (Ambion, Milan, Italy). Silencer select negative control siRNA (SSNC, Ambion) was also used as negative control. For transfection of human T lymphocyte, the Amaxa Human T cell Nucleofector® kit was used (Lonza, Walkersville, MD, USA) according to the manufacturer's protocol. In brief, 6×10^6 cells per condition were resuspended in 100 µl of the Nucleofactor kit solution, combined with 300 nM of the indicated siRNA or pmaxGFP vector (2 µg), and electroporated using the U-014 program of the Nucleofector (Amaxa Biosystems, Köln, Germany). Transfection efficiency was monitored in all samples by FACS analysis of GFP fluorescence and was about 50%. Cell apoptosis, measured by AV/PI detection kit was <20% (data not shown). After 6 h, siRNA-transfected PBMCs were treated with silibinin (Sigma) for 48 h and analyzed for IL-17 and TNF-α expression after stimulation with PMA and ionomycin (both from Sigma) in the presence of brefeldin A (Sigma). See above for methodological details.

Statistical Analysis

Statistical analysis was performed by the Mann–Whitney *U* test using GraphPad Prism, version 7.0 software (GraphPad Software, San Diego, CA, USA). A P value <0.05 was considered statistically significant.

RESULTS

The Natural ERβ Agonist Silibinin Modulates ERβ Expression in Peripheral Blood T Lymphocytes From Female and Male Healthy Subjects

As stated above, we and other groups previously demonstrated that immune cells have detectable levels of intracellular ERβ (12–14). As literature data indicate for phytoestrogens the ability to upregulate ERβ in different cell types (36, 37), we first evaluated if this effect could be also evident in silibinin-treated T lymphocytes, taking into account possible differences between females and males. To this aim, both mRNA and protein expression level of ERβ was determined by qRT-PCR and Western blot analysis in peripheral T lymphocytes (**Figures 1A–C**) from healthy subjects after 16 h treatment with silibinin. A significant increase of ERβ mRNA expression level was detectable after treatment with silibinin in T cells (treated versus untreated cells, $P = 0.01$ and $P = 0.0274$, in females and males, respectively, **Figure 1A**). Hence, we analyzed by Western blot the protein expression level of ERβ in T lymphocytes and we found significantly higher levels of ERβ in silibinin-treated cells when compared with untreated cells (treated versus untreated cells, $P < 0.0001$ in both females and males, **Figures 1B,C**). T lymphocytes from female and male subjects showed comparable susceptibility to silibinin treatment.

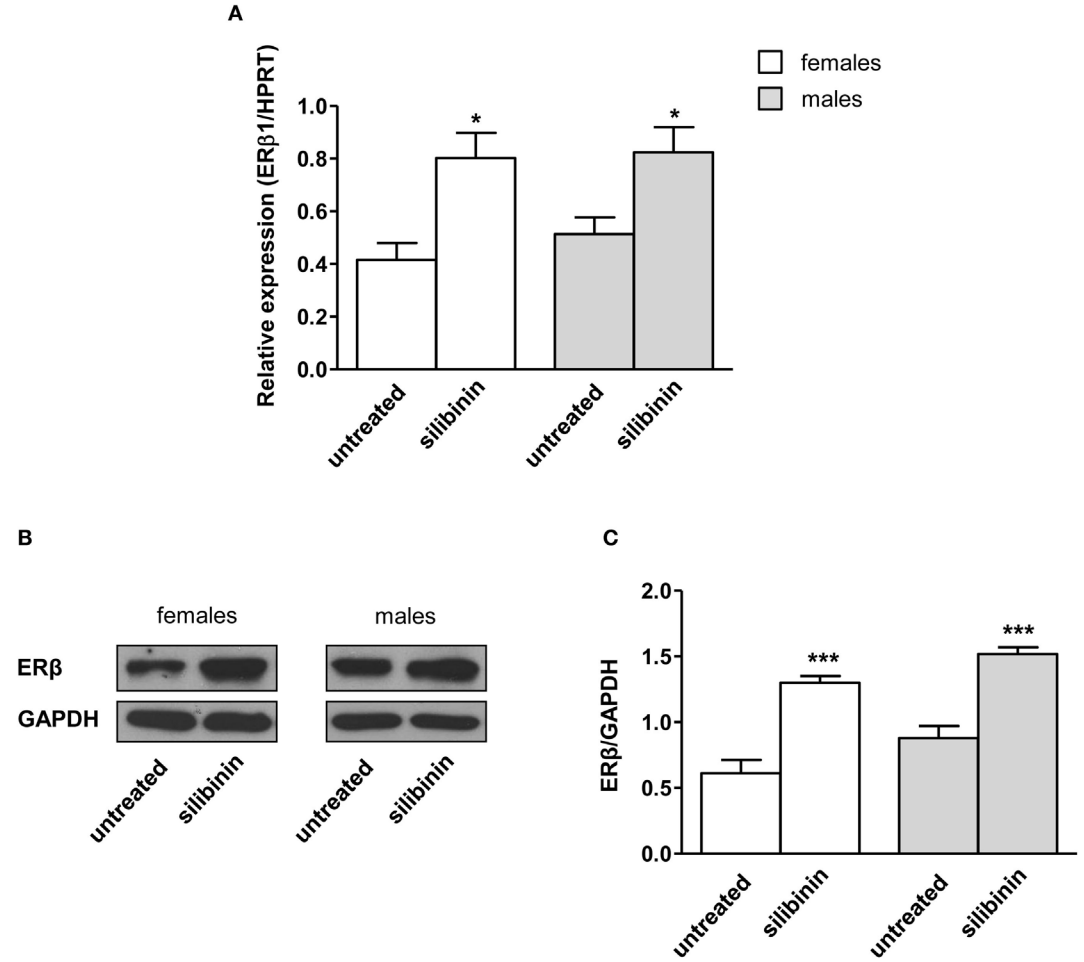

FIGURE 1 | Silibinin increased the expression of estrogen receptor (ER) β in peripheral blood T lymphocytes from female and male healthy subjects. **(A)** ERβ1 mRNA levels were evaluated by quantitative real-time PCR after 24 h silibinin treatment. Data are expressed as ratios of the expression of ERβ1 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene. Results are shown as mean ± SD from 10 randomly selected female and male healthy subjects. **(B,C)** ERβ protein levels were also evaluated by Western blot analysis of T-cell lysates after 24 h silibinin treatment. Blots shown are representative of experiments performed in T cells from 10 randomly selected female and male healthy subjects **(B)**. Densitometry analysis of ERβ levels relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also shown. Values are expressed as mean ± SD **(C)**. $^{*}P < 0.05$, $^{***}P < 0.001$ versus untreated cells.

Silibinin-Dependent Effects on Cell Apoptosis, Cell Proliferation, and Cytokine Production in Peripheral Blood T Lymphocytes From Female and Male Healthy Subjects

As a second step, we evaluated the ability of silibinin to impact T lymphocyte homeostasis in term of cell apoptosis, proliferation, and cytokine production. Silibinin was able to induce a significant increase in resting T lymphocyte apoptosis (treated versus untreated cells, $P = 0.0002$ and $P = 0.0074$, in females and males, respectively, **Figures 2A,B**). Similarly, activated T cells treated with silibinin showed a significant increase of apoptotic levels (treated versus untreated cells, $P = 0.0029$ and $P = 0.0013$, in females and males, respectively, **Figures 2C,D**).

In parallel, a significant reduction of proliferation of activated T lymphocytes, measured by the analysis of nuclear antigen Ki-67 expression, was observed after treatment with silibinin (treated versus untreated cells, $P = 0.0024$ and $P = 0.0081$, in females and males, respectively, **Figures 2E,F**).

A panel of pro-inflammatory (IFNγ, TNF-α, IL-2, and IL-17) and anti-inflammatory (IL-4 and IL-10) cytokines was also studied. Notably, silibinin significantly reduced the intracellular expression level of the pro-inflammatory cytokines IL-17 and TNF-α in $CD4^+$ T lymphocytes (for IL-17, treated versus untreated cells, $P = 0.0078$ and $P = 0.0077$, in females and males, respectively, **Figures 3A,B**; for TNF-α, treated versus untreated cells, $P = 0.0028$ and $P = 0.0272$, in females and males, respectively, **Figures 4A,B**). No changes were induced by silibinin in the percentage of $CD8^+$/TNF-α^+, $CD4^+$/and $CD8^+$/IL-2^+, IFNγ^+, IL-4^+, and IL-10^+ T lymphocytes (**Table 2**). In order to clarify the

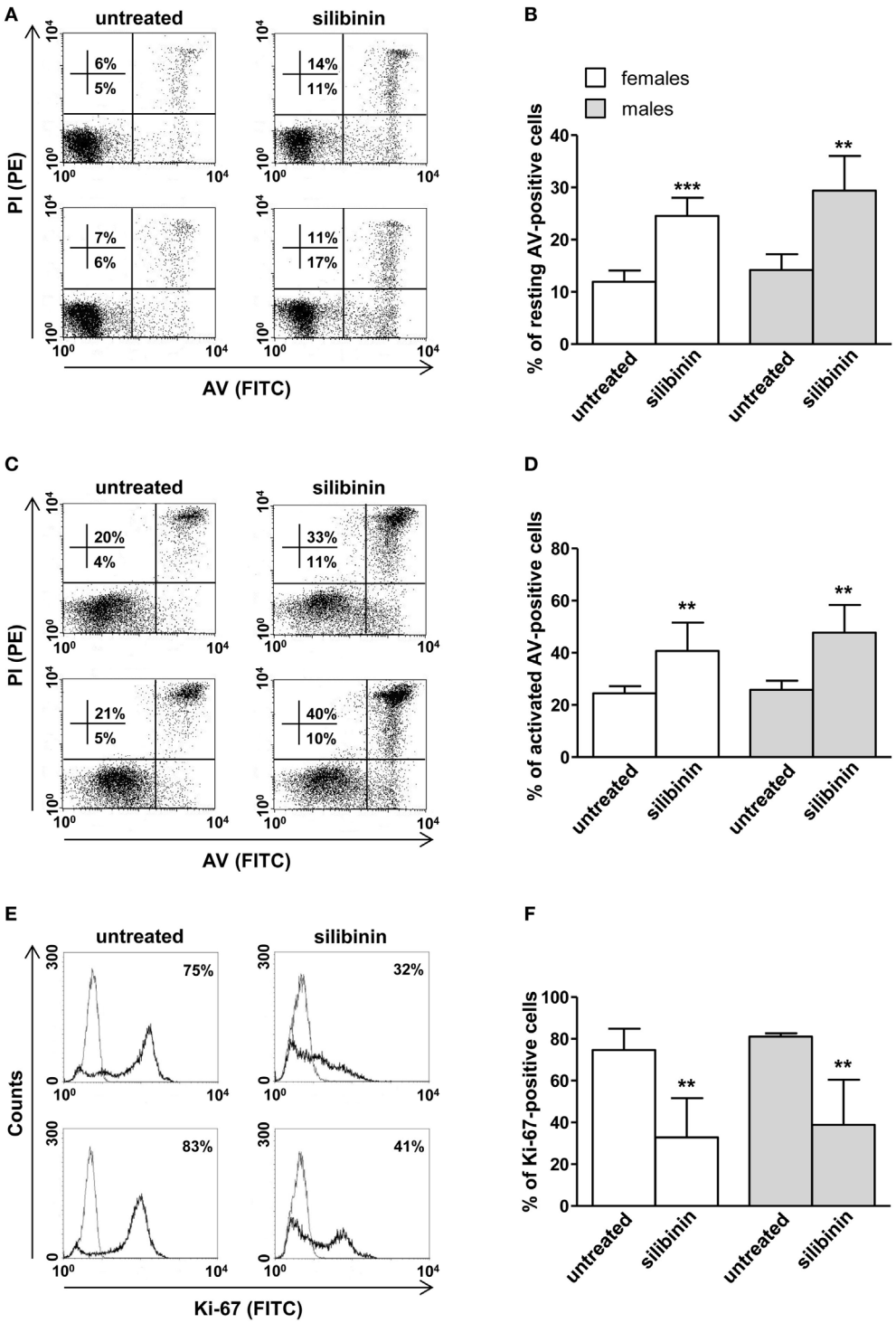

FIGURE 2 | Silibinin-dependent effects on apoptosis and proliferation levels of peripheral blood T lymphocytes from female and male healthy subjects. All experiments were performed in 23 female and 21 male healthy subjects. **(A–D)** Apoptosis assay involving dual staining with annexin V (AV) and propidium iodide (PI) was carried out using flow cytometry in resting T cells treated or not with silibinin for 48 h **(A,B)** and in T cells activated by anti-CD3 monoclonal antibody (mAb) for 72 h and treated or not with silibinin for the last 48 h of culture **(C,D)**. Results from representative female (upper panels) and male (lower panels) healthy donors are shown **(A,C)**. Numbers reported represent the percentages of AV positive/PI negative (early apoptotic, bottom right quadrant) and AV positive/PI positive (late apoptotic or necrotic cells, top right quadrant). Data referred to both AV positive/PI negative and AV positive/PI positive cells are also reported as mean ± SD **(B,D)**. **(E,F)** Cell proliferation was evaluated by flow cytometry measuring Ki-67 nuclear antigen expression in T lymphocytes after activation with anti-CD3 mAb for 72 h and treatment with silibinin for the last 48 h of culture. Results from representative female (upper panels) and male (lower panels) healthy donors are shown **(E)**. Data are also reported as mean ± SD **(F)**. $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated cells.

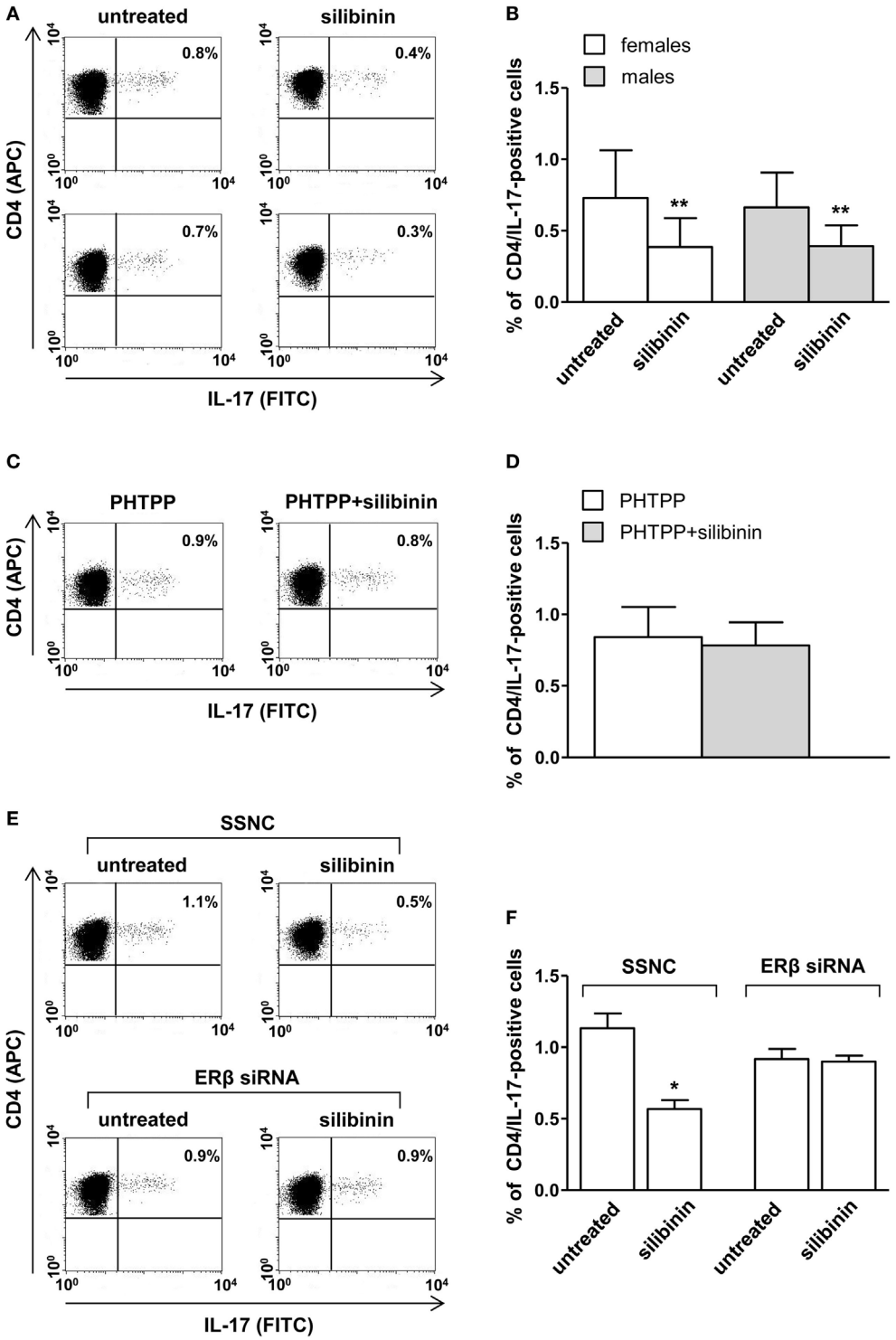

FIGURE 3 | Silibinin decreased IL-17 expression in peripheral blood T lymphocytes from female and male healthy subjects acting through estrogen receptor (ER) β binding. **(A,B)** Cytokine expression was analyzed in 23 female and 21 male healthy subjects by flow cytometry after 48 h of culture with silibinin and stimulation with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin for the last 4 h of culture as detailed in Section "Materials and Methods." Results from representative female (upper panels) and male (lower panels) healthy donors are shown **(A)**. Data are also reported as mean ± SD **(B)**. **(C,D)** IL-17 expression was analyzed in T lymphocytes from three randomly selected female healthy donors pretreated with the ERβ antagonist PHTTP for 1 h before adding silibinin. Results from a representative female healthy donor are shown **(C)**. Data are also reported as mean ± SD **(D)**. **(E,F)** IL-17 expression was analyzed in T lymphocytes from three randomly selected female healthy donors after silencing ERβ expression. Results from a representative female healthy donor are shown **(E)**. Data are also reported as mean ± SD **(F)**. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells. Abbreviation: SSNC, silencer select negative control siRNA.

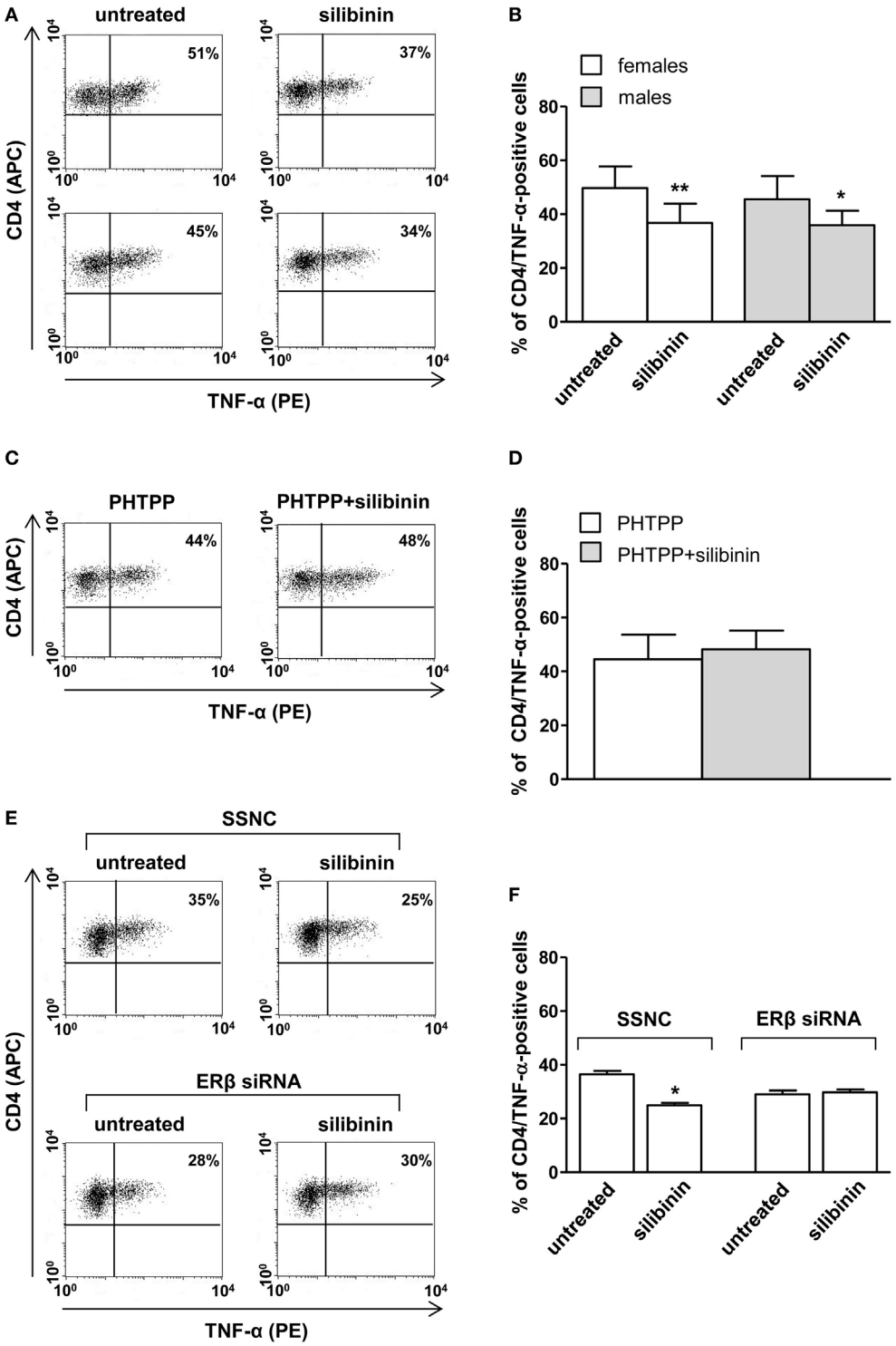

FIGURE 4 | Silibinin decreased TNF-α expression in peripheral blood CD4+ T lymphocytes from female and male healthy subjects acting through ERβ binding. **(A,B)** Cytokine expression were analyzed in 23 female and 21 male healthy subjects by flow cytometry after 48 h of culture with silibinin and stimulation with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin for the last 16 h of culture as detailed in Section "Materials and Methods." Results from representative female (upper panels) and male (lower panels) healthy donors are shown **(A)**. Data are also reported as mean ± SD **(B)**. **(C,D)** TNF-α expression was analyzed in T lymphocytes from three randomly selected female healthy donors pretreated with the ERβ antagonist PHTTP for 1 h before adding silibinin. Results from a representative female healthy donor are shown **(C)**. Data are also reported as mean ± SD **(D)**. **(E,F)** TNF-α expression was analyzed in T lymphocytes from three randomly selected female healthy donors after silencing ERβ expression. Results from a representative female healthy donor are shown **(E)**. Data are also reported as mean ± SD **(F)**. *$P < 0.05$, **$P < 0.01$ versus untreated cells. Abbreviation: SSNC, silencer select negative control siRNA.

TABLE 2 | Cytokine expression at the single cell level by flow cytometry analysis of $CD4^+$ and $CD8^+$ T lymphocytes from female and male healthy donors.

Cytokines	Females			Males		
	Untreated	Silibinin	*P*	Untreated	Silibinin	*P*
% $CD4^+/IL\text{-}17^+$	0.7 ± 0.3	0.4 ± 0.2	0.0078	0.7 ± 0.2	0.4 ± 0.1	0.0077
% $CD4^+/TNF\text{-}\alpha^+$	50 ± 8	37 ± 7	0.0028	45 ± 9	36 ± 5	0.0272
% $CD8^+/TNF\text{-}\alpha^+$	30 ± 12	23 ± 11	0.1022	44 ± 23	36 ± 26	0.0943
% $CD4^+/IL\text{-}2^+$	43 ± 12	39 ± 12	0.1141	49 ± 10	38 ± 19	0.0622
% $CD8^+/IL\text{-}2^+$	18 ± 6	16 ± 9	0.0562	23 ± 6	17 ± 10	0.0703
% $CD4^+/IFN\gamma^+$	30 ± 11	26 ± 6	0.4936	33 ± 21	42 ± 22	0.1236
% $CD8^+/IFN\gamma^+$	56 ± 14	48 ± 18	0.0937	65 ± 33	70 ± 28	0.7238
% $CD4^+/IL\text{-}4^+$	0.3 ± 0.2	0.2 ± 0.1	0.0979	0.7 ± 0.7	1.4 ± 1.5	0.2364
% $CD8^+/IL\text{-}4^+$	0.3 ± 0.2	0.1 ± 0.1	0.0655	0.1 ± 0.1	0.1 ± 0.1	0.9892
% $CD4^+/IL\text{-}10^+$	1.1 ± 0.6	1.1 ± 0.7	0.9644	0.9 ± 0.7	1 ± 1	0.5081

For $CD4^+$ and $CD8^+$ T lymphocyte subsets, data were expressed as the percentage of each subset within the $CD3^+CD4^+$ or $CD3^+CD8^+$ population considered as 100%. Data are represented as mean ± SD from 23 female and 21 male healthy subjects. P values were calculated using the Mann–Whitney U test.

role of ERβ in the anti-inflammatory effects induced by silibinin, we used two different approaches: (i) the pretreatment of PBMC with the ERβ antagonist PHTTP and (ii) the silencing of ERβ with specific siRNA to knockdown ERβ gene. Notably, in both experimental conditions, silibinin lost the ability to inhibit IL-17 (**Figures 3C–F**) and TNF-α expression (**Figures 4C–F**), confirming that the observed effects were mediated by ERβ binding.

Also in this set of experiments, no significant difference was observed between cells from male and female subjects after silibinin treatment (**Figures 2**–**4**).

To investigate whether the silibinin-mediated IL-17 inhibition could be due to elimination of memory Th17 cells or by a block of IL-17 production, we first evaluated intracellular ERβ expression in sorted Th17 cells (i.e., $CD4^+CD45RA^-CCR6^+CXCR3^-$, Figures S1A,B in Supplementary Material). As expected, the Th17 cell subset expressed intracellular ERβ. Then, we evaluated apoptosis level of this cell subset, treated or not with silibinin (Figure S1C in Supplementary Material). An increase of apoptotic level after silibinin treatment was detected, suggesting that the effect of silibinin on IL-17 production was, at least partially, due to apoptosis induction in this cell subset. Also in this case, the ERβ antagonist PHTTP was able to inhibit silibin-induced apoptosis.

Silibinin Effects on Peripheral Blood T Lymphocytes From Patients With Active RA

Based on the results obtained in healthy donors, we decided to test the anti-inflammatory potential of silibinin on T lymphocytes from female and male patients with active RA, analyzing its ability to modulate apoptosis, proliferation, and cytokine expression. Similarly to that observed in healthy donors, silibinin induced a significant increase of apoptosis in T lymphocytes from RA patients in both resting ($P = 0.0161$ for females and $P = 0.0286$ for males, **Figures 5A,B**) and activated state ($P = 0.0403$ for females and $P = 0.0421$ for males, **Figures 5C,D**). In addition, a significant reduction of proliferation level was detected after cell treatment with silibinin ($P = 0.0160$ for females and $P = 0.0286$ for males, **Figures 5E,F**). Notably, silibinin was able to significantly reduce IL-17 and TNF-α expression levels in $CD4^+$ T lymphocytes (IL-17: $P = 0.0235$ for females and $P = 0.0294$ for males; TNF-α: $P = 0.0032$ for females and $P = 0.0421$ for males, versus untreated cells, respectively, **Figures 6A–D**). Similarly to that observed in healthy subject, no significant difference was observed between cells from male and female subjects after silibinin treatment.

Silibinin Effect on miR-155 Expression in Peripheral T Lymphocytes From Patients With Active RA

Finally, we asked whether silibinin could act as an epigenetic modifier modulating miRNA expression. We focused on miR-155 that plays a crucial role in the pathogenesis of RA (29–31) and which expression has been demonstrated to be modulated by estrogen through ERβ (38). Thus, we quantitatively analyzed miR-155 expression in T lymphocytes from RA patients, upon treatment with silibinin, by qRT-PCR assay. The results, shown in **Figure 7**, indicated an average 54 and 50% (for females and males respectively) decreased expression of miR-155 in T lymphocytes after silibinin treatment, thus suggesting that this phytoestrogen acted as a downregulator of miR-155.

DISCUSSION

Our study provides new insights regarding the anti-inflammatory effects of the phytoestrogen silibinin in T cell immunity. First, we demonstrated that silibinin upregulates ERβ expression, induces apoptosis, inhibits proliferation, and reduces expression of the pro-inflammatory cytokines IL-17 and TNF-α, through ERβ binding, in T lymphocytes from both female and male healthy subjects. Then, we confirmed these results in T lymphocytes from patients with RA in term of apoptosis, proliferation, and cytokine production. Finally, we found that silibinin acts as an epigenetic modifier, down-modulating the expression of miR-155 which plays a key role in the pathogenesis of RA.

Growing evidence suggests that ERα and ERβ subtypes mediate distinct transcriptional activities when they are co-expressed in the same cells and that the quantity and distribution of these receptors are crucial for their biological effects (8). In particular, ERβ displays an anti-inflammatory effect and the upregulation of this receptor in immune cells may provide a useful tool in creating an anti-inflammatory milieu. Here, we observed for the

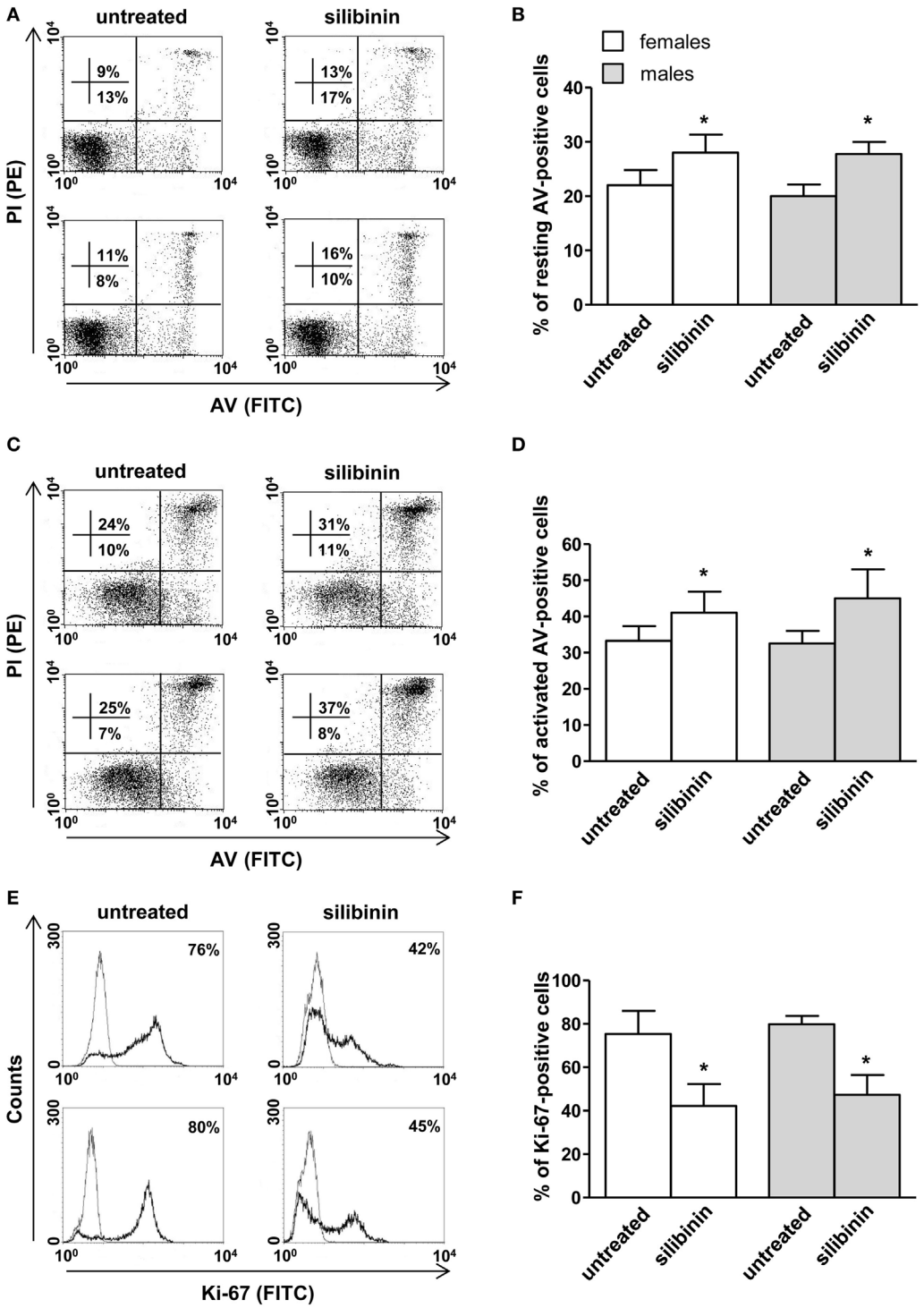

FIGURE 5 | Silibinin-dependent effects on apoptosis and proliferation levels of peripheral blood T lymphocytes from patients with active rheumatoid arthritis (RA). All experiments were performed in 10 female and 4 male RA patients. **(A–D)** Apoptosis assay involving dual staining with annexin V (AV) and propidium iodide (PI) was carried out using flow cytometry in resting T cells treated or not with silibinin for 48 h **(A,B)** and in T cells activated by anti-CD3 monoclonal antibody (mAb) for 72 h and treated or not with silibinin for the last 48 h of culture **(C,D)**. Results from representative female (upper panels) and male (lower panels) RA patients are shown **(A,C)**. Numbers reported represent the percentages of AV positive/PI negative (early apoptotic, bottom right quadrant) and AV positive/PI positive (late apoptotic or necrotic cells, top right quadrant). Data referred to both AV positive/PI negative and AV positive/PI positive cells are also reported as mean ± SD **(B,D)**. **(E,F)** Cell proliferation was evaluated by flow cytometry measuring Ki-67 nuclear antigen expression in T lymphocytes after activation with anti-CD3 mAb for 72 h and treatment with silibinin for the last 48 h of culture. Results from representative female (upper panels) and male (lower panels) RA patients are shown **(E)**. Data are also reported as mean ± SD **(F)**. $^*P < 0.05$ versus untreated cells.

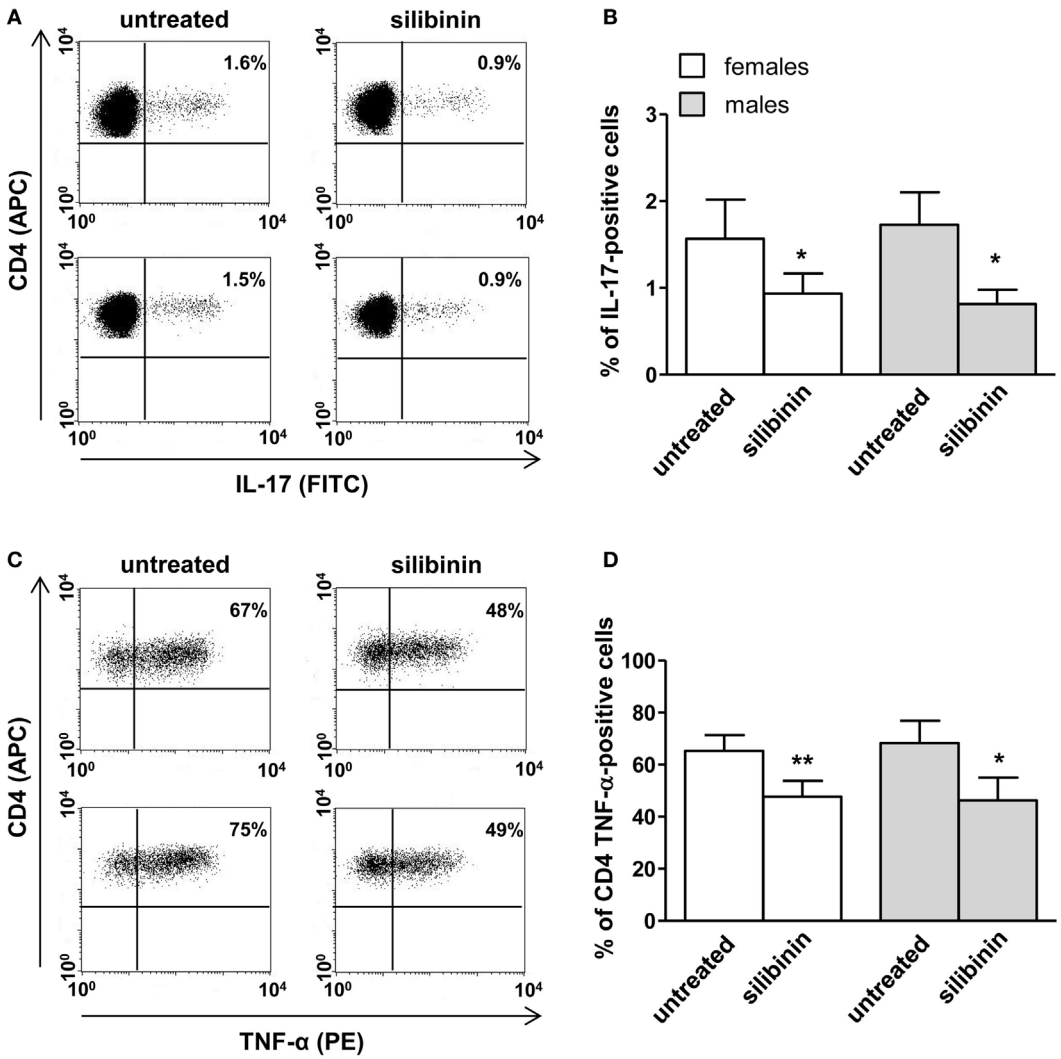

FIGURE 6 | Silibinin-dependent effects on IL-17 and TNF-α expression in peripheral blood T lymphocytes from rheumatoid arthritis (RA) patients. T lymphocytes from 10 female and 4 male RA patients were evaluated by flow cytometry for cytokine production after 48 h of culture with silibinin and stimulation with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin as detailed in Section "Materials and Methods." Results from representative female (upper panels) and male (lower panels) RA patients are shown **(A,C)**. Data are also reported as mean ± SD **(B,D)**. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells.

first time that silibinin was able to upregulate ERβ expression in T lymphocytes from both women and men.

Ligation of this receptor by silibinin induced an antiproliferative and a proapoptotic effect in T lymphocytes. These results are partially in accordance with those previously reported by other groups who observed that silymarin plays an antiproliferative activity (39–41) but, unlike what we have seen, it also has a proapoptotic effect (39, 40) in human T lymphocytes. The use by these authors of silymarin, which consists of a family of flavolignans including silybinin, isosilybinin, silychristin, isosilychristin, silydianin, and the flavonoid taxifoline, instead of the pure silibinin, might account for this discordant result. Interestingly, silibinin was able to strongly decrease the expression of the pro-inflammatory cytokines IL-17 and TNF-α through a mechanism that foresees its binding to ERβ, as demonstrated by the lack of silibinin effect when this receptor was knocked down. To note, all these experiments revealed that T lymphocytes from both sexes had the same susceptibility to silibinin. This is in line with previous observations that T lymphocytes from females and males subjects express similar basal levels of ERβ (12) and with our finding that silibinin upregulated ERβ in both sexes, assigning to silibinin a role for the treatment of inflammatory diseases both in women and in men. To note that the selective activation of ERβ could be of clinical value since it does not induce the classic side effects, mediated by ERα, observed after estrogen administration (e.g., cerebro- and cardiovascular events, higher occurrence of endometrial and breast cancer).

Both IL-17 and TNF-α are known to play a critical role in the pathogenesis of RA as demonstrated by the success of treatments based on their inhibition by biological disease-modifying

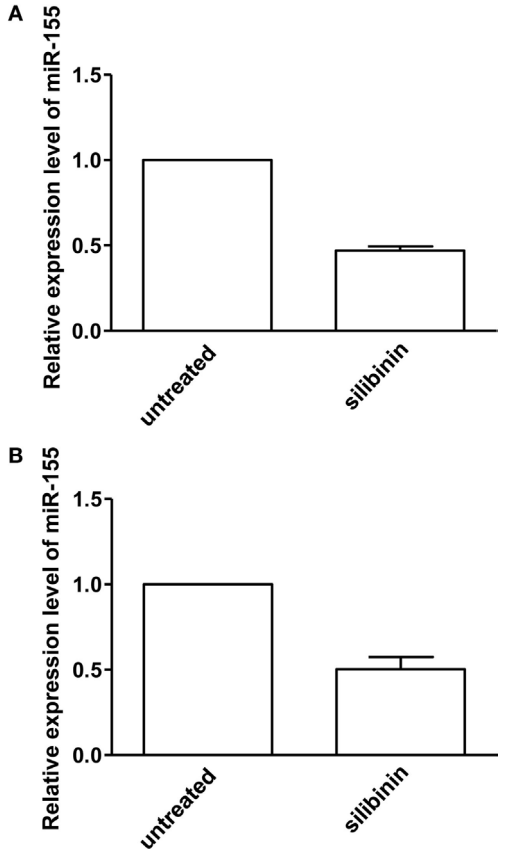

FIGURE 7 | Quantitative real-time PCR (qRT-PCR) analysis of microRNA-155 (miR-155) expression level in peripheral blood T lymphocytes from rheumatoid arthritis (RA) patients. qRT-PCR analysis of miR-155-3p expression level in T lymphocytes isolated from five female **(A)** and two male **(B)** RA patients, treated or not with silibinin for 48 h. The values of fold change were calculated by the $2-\Delta\Delta Ct$ method relative to untreated RA patient. The mean ± SD values, referred to the fold of change between treated and untreated patients, were shown.

antirheumatic drugs (42, 43). Interleukin-17 and TNF-α modulate the migration of inflammatory cells into the joints and display additive or synergistic effects on human synovial fibroblasts (44). Accordingly, alterations in peripheral T lymphocyte homeostasis and unbalanced Th1 and Th17 cytokine profiles have been repeatedly demonstrated in RA patients (27). Thus, a drug able to block these two cytokines at the same time would be very useful for an effective therapeutic approach in inflammatory arthritis. Hence, with in mind the goal to propose silibinin as therapeutic tool in RA, we evaluated whether this phytoestrogen could exert its anti-inflammatory effects on T lymphocytes from patients with active RA who had a poor response to disease-modifying antirheumatic drugs. In agreement with the results obtained in healthy donors, silibinin appeared to have immunosuppressive/anti-inflammatory effects on T lymphocytes from RA patients inducing apoptosis, inhibiting proliferation and both IL-17 and TNF-α expression, thus assigning to this molecule a potential value as therapeutic tool in this disease. In support of this assumption, a recent study by Tong et al. (45) showed that silibinin alleviated inflammation and induced apoptosis in human RA fibroblast-like synoviocytes and had a beneficial effect on arthritis in rats.

Interestingly, Th1 and Th17 polarization have been recently associated with an overexpression of miR-155 whose deregulation plays a crucial role in the pathogenesis of RA contributing to the progress of inflammation (29, 30). miR-155 has been reported to be abnormally expressed in arthritis models and miR-155-deficient mice do not develop collagen-induced arthritis and show significant reduced Th17 cells and autoantibody production (46). Furthermore, miR-155 expression in RA patients has been positively related to TNF-α, C-reactive protein, erythrocyte sedimentation rate levels, and DAS28 (47). Noteworthy, in this study, we observed a downregulation of miR-155 expression in T lymphocytes from RA patients treated with silibinin. Accordingly, He et al. (38) demonstrated that miR-155 is downregulated by estrogen through ERβ, further supporting the crucial role of this receptor as potential therapeutic target in RA.

In conclusion, our *in vitro* study provided new insights regarding the anti-inflammatory activities of silibinin. However, *in vivo* assays (e.g., collagen-induced arthritis model) are needed to confirm the potential role of this compound as therapeutic tool in RA, paving the way for clinical trials in this disease. In particular, the use of silibinin in combination with synthetic drugs might reduce their standard dosage and their related side effects. To note, clinical trials on silibinin effectiveness in the treatment of patients with hepatitis, cirrhosis, or biliary disorders demonstrated its safety with adverse events comparable to placebo (24, 48).

ETHICS STATEMENT

Investigation has been conducted in accordance with the ethical standards and with the Declaration of Helsinki, and according to national and international guidelines. It was approved by the institutional review board of Policlinico Umberto I (Rome, Italy). All enrolled subjects were provided with complete information about the study and asked to sign an informed consent.

AUTHOR CONTRIBUTIONS

MD, MG, AM, AR, SA, AF, and MTP designed and performed *in vitro* experiments and analyzed data. MS performed flow cytometry and cell sorting analysis. CA and FCe contributed to patient enrollment and sample collection. FCo, LG, and GV provided intellectual input throughout the study. MP and EO provided important contribution to the conception of the work as well as interpretation of data and manuscript writing. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01903/full#supplementary-material.

REFERENCES

1. Cutolo M, Brizzolara R, Atzeni F, Capellino S, Straub RH, Puttini PC. The immunomodulatory effects of estrogens: clinical relevance in immune-mediated rheumatic diseases. *Ann N Y Acad Sci* (2010) 1193:36–42. doi:10.1111/j.1749-6632.2009.05383.x
2. Cunningham M, Gilkeson G. Estrogen receptors in immunity and autoimmunity. *Clin Rev Allergy Immunol* (2011) 40:66–73. doi:10.1007/s12016-010-8203-5
3. Karpuzoglu E, Zouali M. The multi-faceted influences of estrogen on lymphocytes: toward novel immuno-interventions strategies for autoimmunity management. *Clin Rev Allergy Immunol* (2011) 40:16–26. doi:10.1007/s12016-009-8188-0
4. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. *Ann Ist Super Sanita* (2016) 52:205–12. doi:10.4415/ANN_16_02_12
5. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Hum Reprod Update* (2005) 11:411–23. doi:10.1093/humupd/dmi008
6. Oertelt-Prigione S. The influence of sex and gender on the immune response. *Autoimmun Rev* (2012) 11:A479–85. doi:10.1016/j.autrev.2011.11.022
7. Pernis AB. Estrogen and CD4+ T cells. *Curr Opin Rheumatol* (2007) 19:414–20. doi:10.1097/BOR.0b013e328277ef2a
8. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* (2007) 28:521–74. doi:10.1210/er.2007-0001
9. Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. *Front Neuroendocrinol* (2014) 35:347–69. doi:10.1016/j.yfrne.2014.04.004
10. Rider V, Foster RT, Evans M, Suenaga R, Abdou NI. Gender differences in autoimmune diseases: estrogen increases calcineurin expression in systemic lupus erythematosus. *Clin Immunol Immunopathol* (1998) 89:171–80. doi:10.1006/clin.1998.4604
11. Ascenzi P, Bocedi A, Marino M. Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol Aspects Med* (2006) 27:299–402. doi:10.1016/j.mam.2006.07.001
12. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunol Lett* (2005) 97:107–13. doi:10.1016/j.imlet.2004.10.007
13. Pierdominici M, Maselli A, Colasanti T, Giammarioli AM, Delunardo F, Vacirca D, et al. Estrogen receptor profiles in human peripheral blood lymphocytes. *Immunol Lett* (2010) 132:79–85. doi:10.1016/j.imlet.2010.06.003
14. Rider V, Li X, Peterson G, Dawson J, Kimler BF, Abdou NI. Differential expression of estrogen receptors in women with systemic lupus erythematosus. *J Rheumatol* (2006) 33:1093–101.
15. Yakimchuk K, Jondal M, Okret S. Estrogen receptor alpha and beta in the normal immune system and in lymphoid malignancies. *Mol Cell Endocrinol* (2013) 375:121–9. doi:10.1016/j.mce.2013.05.016
16. Itoh N, Kim R, Peng M, DiFilippo E, Johnsonbaugh H, MacKenzie-Graham A, et al. Bedside to bench to bedside research: estrogen receptor beta ligand as a candidate neuroprotective treatment for multiple sclerosis. *J Neuroimmunol* (2017) 304:63–71. doi:10.1016/j.jneuroim.2016.09.017
17. Wisdom AJ, Cao Y, Itoh N, Spence RD, Voskuhl RR. Estrogen receptor-beta ligand treatment after disease onset is neuroprotective in the multiple sclerosis model. *J Neurosci Res* (2013) 91:901–8. doi:10.1002/jnr.23219
18. Tiwari-Woodruff S, Morales LB, Lee R, Voskuhl RR. Differential neuroprotective and antiinflammatory effects of estrogen receptor (ER)alpha and ERbeta ligand treatment. *Proc Natl Acad Sci U S A* (2007) 104:14813–8. doi:10.1073/pnas.0703783104
19. Saijo K, Collier JG, Li AC, Katzenellenbogen JA, Glass CK. An ADIOL-ERbeta-CtBP transrepression pathway negatively regulates microglia-mediated inflammation. *Cell* (2011) 145:584–95. doi:10.1016/j.cell.2011.03.050
20. Mohammad I, Starskaia I, Nagy T, Guo J, Yatkin E, Vaananen K, et al. Estrogen receptor alpha contributes to T cell-mediated autoimmune inflammation by promoting T cell activation and proliferation. *Sci Signal* (2018) 11:eaa9415. doi:10.1126/scisignal.aap9415
21. Maselli A, Conti F, Alessandri C, Colasanti T, Barbati C, Vomero M, et al. Low expression of estrogen receptor beta in T lymphocytes and high serum levels of anti-estrogen receptor alpha antibodies impact disease activity in female patients with systemic lupus erythematosus. *Biol Sex Differ* (2016) 7:3. doi:10.1186/s13293-016-0057-y
22. Pierdominici M, Maselli A, Varano B, Barbati C, Cesaro P, Spada C, et al. Linking estrogen receptor beta expression with inflammatory bowel disease activity. *Oncotarget* (2015) 6:40443–51. doi:10.18632/oncotarget.6217
23. Aggelakopoulou M, Kourepini E, Paschalidis N, Panoutsakopoulou V. ERbeta in CD4+ T cells is crucial for ligand-mediated suppression of central nervous system autoimmunity. *J Immunol* (2016) 196:4947–56. doi:10.4049/jimmunol.1600246
24. Comelli MC, Mengs U, Schneider C, Prosdocimi M. Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy. *Integr Cancer Ther* (2007) 6:120–9. doi:10.1177/1534735407302349
25. El-Shitany NA, Hegazy S, El-Desoky K. Evidences for antiosteoporotic and selective estrogen receptor modulator activity of silymarin compared with ethinylestradiol in ovariectomized rats. *Phytomedicine* (2010) 17:116–25. doi:10.1016/j.phymed.2009.05.012
26. Recalde G, Moreno-Sosa T, Yudica F, Quintero CA, Sanchez B, Jahn GA, et al. Contribution of sex steroids and prolactin to the modulation of T and B cells during autoimmunity. *Autoimmun Rev* (2018) 17(5):504–12. doi:10.1016/j.autrev.2018.03.006
27. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet* (2016) 388:2023–38. doi:10.1016/S0140-6736(16)30173-8
28. Podshivalova K, Salomon DR. MicroRNA regulation of T-lymphocyte immunity: modulation of molecular networks responsible for T-cell activation, differentiation, and development. *Crit Rev Immunol* (2013) 33:435–76. doi:10.1615/CritRevImmunol.2013006858
29. Su LC, Huang AF, Jia H, Liu Y, Xu WD. Role of microRNA-155 in rheumatoid arthritis. *Int J Rheum Dis* (2017) 20:1631–7. doi:10.1111/1756-185X.13202
30. Alivernini S, Gremese E, McSharry C, Tolusso B, Ferraccioli G, McInnes IB, et al. MicroRNA-155-at the critical interface of innate and adaptive immunity in arthritis. *Front Immunol* (2017) 8:1932. doi:10.3389/fimmu.2017.01932
31. Alivernini S, Kurowska-Stolarska M, Tolusso B, Benvenuto R, Elmesmari A, Canestri S, et al. MicroRNA-155 influences B-cell function through PU.1 in rheumatoid arthritis. *Nat Commun* (2016) 7:12970. doi:10.1038/ncomms12970
32. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62:2569–81. doi:10.1002/art.27584
33. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* (1995) 38:44–8. doi:10.1002/art.1780380107
34. Pierdominici M, Maselli A, Cecchetti S, Tinari A, Mastrofrancesco A, Alfe M, et al. Diesel exhaust particle exposure in vitro impacts T lymphocyte phenotype and function. *Part Fibre Toxicol* (2014) 11:74. doi:10.1186/s12989-014-0074-0
35. Fragale A, Romagnoli G, Licursi V, Buoncervello M, Del Vecchio G, Giuliani C, et al. Antitumor effects of Epidrug/IFNalpha combination driven by modulated gene signatures in both colorectal cancer and dendritic cells. *Cancer Immunol Res* (2017) 5:604–16. doi:10.1158/2326-6066.CIR-17-0080
36. Cappelletti V, Miodini P, Di Fronzo G, Daidone MG. Modulation of estrogen receptor-beta isoforms by phytoestrogens in breast cancer cells. *Int J Oncol* (2006) 28:1185–91. doi:10.3892/ijo.28.5.1185
37. Zheng N, Liu L, Liu W, Zhang P, Huang H, Zang L, et al. ERbeta up-regulation was involved in silibinin-induced growth inhibition of human breast cancer MCF-7 cells. *Arch Biochem Biophys* (2016) 591:141–9. doi:10.1016/j.abb.2016.01.002
38. He YQ, Sheng JQ, Ling XL, Fu L, Jin P, Yen L, et al. Estradiol regulates miR-135b and mismatch repair gene expressions via estrogen receptor-beta in colorectal cells. *Exp Mol Med* (2012) 44:723–32. doi:10.3858/emm.2012.44.12.079
39. Gharagozloo M, Jafari S, Esmaeil N, Javid EN, Bagherpour B, Rezaei A. Immunosuppressive effect of silymarin on mitogen-activated protein kinase

signalling pathway: the impact on T cell proliferation and cytokine production. *Basic Clin Pharmacol Toxicol* (2013) 113:209–14. doi:10.1111/bcpt.12088
40. Gharagozloo M, Javid EN, Rezaei A, Mousavizadeh K. Silymarin inhibits cell cycle progression and mTOR activity in activated human T cells: therapeutic implications for autoimmune diseases. *Basic Clin Pharmacol Toxicol* (2013) 112:251–6. doi:10.1111/bcpt.12032
41. Morishima C, Shuhart MC, Wang CC, Paschal DM, Apodaca MC, Liu Y, et al. Silymarin inhibits in vitro T-cell proliferation and cytokine production in hepatitis C virus infection. *Gastroenterology* (2010) 138:.e1–2. doi:10.1053/j.gastro.2009.09.021
42. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. *Nat Rev Rheumatol* (2015) 11:415–29. doi:10.1038/nrrheum.2015.53
43. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* (2007) 7:429–42. doi:10.1038/nri2094
44. Fischer JA, Hueber AJ, Wilson S, Galm M, Baum W, Kitson C, et al. Combined inhibition of tumor necrosis factor alpha and interleukin-17 as a therapeutic opportunity in rheumatoid arthritis: development and characterization of a novel bispecific antibody. *Arthritis Rheumatol* (2015) 67:51–62. doi:10.1002/art.38896
45. Tong WW, Zhang C, Hong T, Liu DH, Wang C, Li J, et al. Silibinin alleviates inflammation and induces apoptosis in human rheumatoid arthritis fibroblast-like synoviocytes and has a therapeutic effect on arthritis in rats. *Sci Rep* (2018) 8:3241. doi:10.1038/s41598-018-21674-6
46. Bluml S, Bonelli M, Niederreiter B, Puchner A, Mayr G, Hayer S, et al. Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. *Arthritis Rheum* (2011) 63:1281–8. doi:10.1002/art.30281
47. Long L, Yu P, Liu Y, Wang S, Li R, Shi J, et al. Upregulated microRNA-155 expression in peripheral blood mononuclear cells and fibroblast-like synoviocytes in rheumatoid arthritis. *Clin Dev Immunol* (2013) 2013:296139. doi:10.1155/2013/296139
48. Post-White J, Ladas EJ, Kelly KM. Advances in the use of milk thistle (*Silybum marianum*). *Integr Cancer Ther* (2007) 6:104–9. doi:10.1177/1534735407301632

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Risk Factors for Adverse Maternal and Fetal Outcomes in Women With Confirmed aPL Positivity: Results From a Multicenter Study of 283 Pregnancies

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Objective: Antiphospholipid antibodies positivity (aPL) is considered as a risk factor for adverse pregnancy outcome (APO). The aim of this study was to determine the risk factors for APO in patients with confirmed aPL positivity, isolated (aPL carriers) or associated with a definite primary antiphospholipid syndrome (PAPS).

Methods: The clinical and laboratory features of 283 pregnancies occurring between 2000 and 2014 in 200 women were collected in three institutions.

Results: The rate of live birth was 87.9% and APO was observed in 50 cases (17.7%). Multivariate analysis showed that the independent variables related to APO were the concomitant diagnosis of an organ-specific autoimmune disease ($p = 0.012$, odds ratio (OR) 3.29, confidence interval (CI) 95% 1.29–8.38) and the presence of low complement levels during the first trimester ($p = 0.02$, OR 2.3, CI 95% 1.17–9.15). No statistical differences were found in APO occurrence among patients treated with low-dose aspirin (LDA) versus those treated with LDA plus heparin (LMWH), but LDA + LMWH was more frequently administered in patients with triple aPL positivity ($p = 0.001$, OR 3.21, CI 95% 1.48–7.11) and with PAPS ($p < 0.001$, OR 8.08, CI 95% 4.3–15.4). Based on clinical history, the patients were divided into four groups: obstetric, thrombotic, non-criteria antiphospholipid syndrome (clinical non-criteria), and aPL carriers. APOs were more frequent in the thrombotic group (24%). Seven patients had a thrombotic event during pregnancy or puerperium (2.4%).

Conclusion: Maternal and fetal complications were observed in some aPL-positive patients despite their efficient management according to the current recommendations. A higher risk of APO was observed in patients with a previous thrombosis and/or more complex autoimmune phenotype.

Keywords: pregnancy, adverse pregnancy outcome, antiphospholipid antibodies, autoimmune thyroiditis, risk factors, therapy

INTRODUCTION

The antiphospholipid syndrome (APS) is an acquired systemic autoimmune disease characterized by the presence of obstetrical morbidity and recurrent thrombotic vascular events associated with antiphospholipid antibodies (aPL). aPL is a heterogeneous group of autoantibodies reacting against phospholipids, phospholipid–protein complexes, and phospholipid-binding proteins (1). The clinical classification "criteria" include arterial/venous thrombosis and obstetric morbidity (more than three consecutive early pregnancy loss, fetal death (FD) at or beyond 10 week of gestation, and early severe preeclampsia or placental insufficiency necessitating delivery before 34 weeks of gestation). The laboratory criteria include the persistent positivity for at least one test among lupus anticoagulant (LA), anticardiolipin (aCL), and anti beta2glycoprotein I antibodies (anti-B2GPI). According to the criteria, both aCL IgG/IgM or/and anti-B2GPI IgG/IgM should be at medium or high titer.

Furthermore, aPL may also be associated with less specific clinical features, defined as "non-criteria"(1). These include heart valve disease, livedo reticularis, thrombocytopenia, aPL nephropathy, neurological manifestations such as epilepsy and cognitive dysfunction as well as previous pregnancy morbidity which do not fulfill the formal "criteria" for APS (two consecutive early pregnancy losses, late-onset preeclampsia, etc.). The presence of aPL antibodies has also been detected in "aPL carriers," subjects without any clinical features of APS (with or without systemic autoimmune diseases). Primary APS was defined as the absence of associated systemic connective tissue disease (CTD).

The clinical management of pregnant patients with aPL aims at preventing obstetric complications and maternal thrombotic events. Combination therapy of low-dose aspirin (LDA) and heparin is regarded as conventional treatment for patients with an established diagnosis of obstetric APS (2, 3), generally resulting in over 70% successful pregnancies. However, a significant number of pregnancies are still complicated or unsuccessful in women with APS. In patients not fulfilling the criteria for definite APS, the management is still debated and different protocols are applied during pregnancy with contrasting results. In clinical practice, LDA is usually administered to patients with aPL (4). However, a recent systematic review, including three studies of aPL-positive patients not fulfilling the clinical criteria for APS (154 pregnancies), did not find clear evidence of LDA superiority in the prevention of pregnancy loss and complications (5). The discrepancy between the published literature and the "real life" emphasizes the need to better classify the patients according to the stratification of obstetric risk. In fact, the definition of the risk factors for pregnancy failure will provide an objective tool for tailoring the management of patients on their individual risk profile. Therefore, the aim of this collaborative work was to assess the risk factor of obstetric complications in patients with confirmed aPL positivity with or without a diagnosis of primary antiphospholipid syndrome.

PATIENTS AND METHODS

Study Cohort

Medical records of pregnant women with confirmed positivity for aPL antibodies attending three referral centers (Rheumatology or Internal Medicine Departments with consolidate experience on APS) from January 2000 to December 2014 were retrospectively evaluated. Patients with a diagnosis of systemic CTD (according to the international classification criteria) at the beginning of the follow-up were excluded. The presence of other autoantibodies and/or low complement levels was not considered an exclusion criterion if not associated with clinical manifestations specific for CTDs.

This study was performed according to the principles of the Declaration of Helsinki with written informed consent from all subjects and was approved by the Ethic Committee of the Promoting Centre (approval number 1088) and it has been approved by the other centers.

Autoantibodies Detection

Lupus anticoagulant was detected by coagulation assay according to the guidelines of the International Society on Thrombosis and Haemostasis (6), while aCL and anti-B2GPI IgG and IgM by ELISA according to the current recommendations (7). Antinuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA) antibodies, antibodies to extractable nuclear antigens (anti-ENA), and complement C3 and C4 fractions were detected as for clinical practice. Antiphospholipid antibodies were considered positive if confirmed at least 12 weeks apart, according to the classification "criteria." In each center, the tests were performed in a referral laboratory certified for diagnosis. Data concerning the prevalence of other antiphospholipid antibodies not included in the classification criteria were not collected because they were not routinely performed.

Adverse Pregnancy Outcome (APO) Definition

In this study, we considered the following events as aPL-related APO: spontaneous abortions (SAs) (<10 weeks of gestation), FD (≥10 weeks of gestation), neonatal death before hospital discharge due to complications of prematurity, preterm delivery before 34 weeks of gestation with or without preeclampsia, hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome (concomitant presence of thrombocytopenia, evidence of hepatic dysfunction, and hemolysis), and small for gestational age babies (SGA) associated with abnormal Doppler flow velocimetry. Pregnancies with an identifiable other cause for APO (i.e., anatomical abnormalities, cervix dilatation) were excluded from statistical analysis.

Statistical Evaluation

Categorical variables were reported as proportion and/or percentage, continuous variables as mean (±SD) values. Fisher's exact test or chi-squared test for categorical variables and Student's *t*-test or Wilcoxon–Mann–Whitney test for continuous variables

were applied as appropriate. For the multivariate analyses, we included the features associated to APO at the univariate analysis. Multivariate analysis was conducted by logistical regression model (Statview). *P*-values of <0.05 were considered significant and Odds Ratio (OR) with 95% confidence interval (95% CI) was indicated.

RESULTS

Patients

The 200 patients included were Caucasian (n = 177, 88.5%), African (n = 8, 4%), and others (n = 15, 7.5%). An organ-specific autoimmune disease was diagnosed in 28 women: autoimmune thyroiditis (n = 20, 10%), celiac disease (n = 2, 1%), cutaneous psoriasis (n = 2, 1%), autoimmune hepatitis (n = 2, 1%), autoimmune thyroiditis and celiac disease (n = 1, 0.5%), and primary biliary cirrhosis (n = 1, 0.5%).

Sixty patients (30%) had at least one modifiable cardiovascular risk factor (cigarette smoke, obesity, arterial hypertension) at pregnancy onset. Inherited thrombophilic factors (factor II and factor V mutation, protein C/protein S/antithrombin deficiency) were available for 168 pregnancies and abnormalities were found in 16 patients (9.5%).

According to the classification "criteria" for APS, the patients had obstetric morbidity only (O-APS; n = 85, 42.5%), thrombotic events (with or without pregnancy morbidity) (T-APS; n = 42, 21%), clinical "non-criteria" manifestations (NC-APS; n = 39, 19.5%) or aPL positivity without any clinical manifestations (aPL carriers; n = 34, 17%). The details about the clinical criteria manifestations at study onset are reported in **Table 1**. One hundred and fifty patients had 344 previous pregnancies not followed in one of the three centers, with a live birth in 98 cases (28%).

TABLE 1 | Clinical criteria manifestations at study onset.[a]

Clinical manifestation patients with primary antiphospholipid syndrome (APS)	
Arterial/venous thrombotic manifestation[a]	**Number of patients 42 (%)**
Deep venous thrombosis	32 (76.2)
Pulmonary embolism	8 (19.4)
Stroke	7 (16.6)
Myocardial infarction	3 (7.1)
Peripheral arterial thrombosis	2 (4.8)
Gastrointestinal venous tract thrombosis	1 (2.3)
Obstetrical manifestation[a]	**Number of patients 99 (%)[b]**
Fetal deaths (≥1 event)	57 (57.5)
Premature births before 34 weeks	20 (20.2)
Spontaneous abortion (SA) (≥3 consecutive events)	17 (17.2)
Preeclampsia[c]/Eclampsia[d]/Hemolysis, elevated liver enzymes, and low platelets (HELLP syndrome)[e]	16 (16.2)
Clinical manifestation of "non-criteria APS" patients 39 (%)	
Obstetrical non-criteria (i.e., <3 consecutive or not consecutive SA)	28 (71.8)
Premature births >34 weeks but <37 weeks	20 (51.3)
Preeclampsia/Eclampsia/HELLP syndrome/IUGR[f] after 34 weeks	5 (12.8)
Livedo reticularis	5 (12.8)
Thrombocytopenia	3 (7.7)
Chorea	2 (5.2)
Valvulopathy	2 (5.2)
Hemolytic anemia	1 (2.6)

[a]The same patient can be included in more than one category. The obstetrical manifestations were previously described (see Patients and Methods).
[b]We considered the patients included in the O-APS category (85 patients) and 14 patients in the T-APS who also pregnancy morbidity.
[c]Preeclampsia, increased blood pressure associated with proteinuria in pregnancy; proteinuria is defined as the excretion of 300 mg of protein or greater in a 24-h specimen.
[d]Eclampsia, onset of seizures during preeclampsia.
[e]HELLP syndrome, concomitant presence of severe thrombocytopenia (platelets ≤50,000/μl), evidence of hepatic dysfunction (liver enzymes ≥70 IU/l), and evidence suggestive of hemolysis (total serum lactate dehydrogenase ≥600 IU/l).
[f]IUGR, intrauterine growth restriction (assessed by ultrasound as a fetal abdominal circumference in the <5th percentile).

Frequency of positive autoantibodies

The results of the three aPL assays were available for all the patients. LA was detectable in 80 patients (40%). aCL antibodies were positive in 131 patients (65.5%), 101 IgG and 60 IgM (50.5 and 30%); anti-B2GPI in 124 (62%), 84 IgG and 71 IgM (42 and 35.5%). A triple aPL positivity was observed in 46 women (23%) while double in 43 (21.5%) and single in 111 (55.5%). ANAs were persistently positive in 75 patients (37.5%), anti-dsDNA in 9 (4.5%), and anti-ENA in 8 (4%) (anti-Ro/SSA in 6 cases, anti-Sm/RNP in 2). Complement levels were tested at the beginning of the pregnancy in 134 patients and were found to be low in 16 (12%).

Pregnancy Outcome

During the study period, data of 283 pregnancies were collected. The mean maternal age at conception was 32.4 ± 5.1 years. The outcome of the pregnancies was as follows: 248 live births (88%) with 251 babies (3 twin pregnancies) at a mean gestational age of 37.6 ± 3.4 weeks (range 25.6–41.5), SA before the 10 weeks of gestation (n = 20), FD (n = 12), voluntary (n = 1), and medical terminations (n = 2: one Steinert's syndrome and one trisomy 18).

At least one complication occurred in 110 pregnancies (38.9%) (see **Table 2**) including the neonatal death of a child born at 27 weeks to a patient with HELLP syndrome.

Fifty out of 110 (45.4%) complicated pregnancies were defined as APO (17.7% of all pregnancies) (**Table 3**). **Tables 4** and **5** report the univariate statistical comparison of clinical and laboratory features between APO and uneventful pregnancies. In multivariate analysis, the independent features related to APO were either the concomitant diagnosis of an organ-specific autoimmune disease (p = 0.012, OR 3.29, CI 95% 1.29–8.38) or the presence of low complement levels at conception or during the first trimester (p = 0.02, OR 2.3, CI 95% 1.17–9.15). The positivity of other autoantibodies (i.e., ANA medium titer, anti-ENA, anti-dsDNA) almost reached the significant threshold (p = 0.06, OR 2.34, CI 95% 0.96–5.72).

The most prevalent autoimmune disease was thyroiditis (22 patients with 31 pregnancies) which resulted in a significantly higher risk of SA (p = 0.014, OR 4.1; CI 95% 1.26–12.73). The

TABLE 2 | Outcome of 283 pregnancies in 200 patients during the follow-up.

Gestational outcome and obstetrical complications*	283 Pregnancies (%)
Spontaneous abortion	20 (7.1)
Fetal death	12 (4.2)
Voluntary/medically induced interruption of pregnancy	3 (1)
Deliveries	**248 (87.6)**
Live births	247 (87.3)
Neonatal death[a]	1 (0.3)
Preterm deliveries <37 weeks	41 (14.4)
Preterm deliveries <34 weeks	16 (5.7)
Preeclampsia	22 (7.8)
Small for gestational age (SGA)[b]	17 (6)
Intrauterine growth restriction	11 (3.9)
Preterm premature rupture of membranes[c]	7 (2.4)
HELLP syndrome[d]	7 (2.4)
Gestational diabetes	13 (4.6)
Gestational hypertension[e]	4 (1.4)

**The same patient can be included in more than one category.*
[a]Neonatal death, death of a formed fetus alive at birth in the first 28 days of life.
[b]SGA, small for gestational age was defined as a birth weight in the <10th percentile for gestational age.
[c]Preterm premature rupture of membranes was defined as rupture of the membranes before 37 weeks of gestation.
[d]HELLP syndrome concomitant presence of severe thrombocytopenia (platelets ≤50,000/μl), evidence of hepatic dysfunction (liver enzymes ≥70 IU/l), and evidence suggestive of hemolysis (total serum lactate dehydrogenase ≥600 IU/l).
[e]Gestational hypertension, onset of hypertension after 20 weeks of gestation, without proteinuria.

TABLE 3 | Lists of adverse pregnancy outcome (APO) related to aPL presence.

APO related to aPL*	Pregnancies ($n = 50$)
Spontaneous abortion	20 (40)
Fetal death	12 (24)
Neonatal death due to prematurity	1 (2)
Preterm deliveries <34 weeks	16 (23)
Small for gestational age (SGA) associated with abnormal Doppler flow velocimetry	4 (8)
Hemolysis, elevated liver enzymes, and low platelet	7 (14)

**The same patient can be included in more than one category.*

only other association was found to be with low levels of C3 and/or C4 and fetal losses ($p = 0.008$, OR 9.56, CI 95% 1.66–56.4).

Pregnancy Outcome in Patients With or Without a Formal Diagnosis of APS

During the follow-up, 137 patients classified as T-APS or O-APS had 190 pregnancies, whereas 73 I-APS or aPL carrier patients had 93. APO in these two groups was not significantly different (20 versus 13%; $p = 0.19$). The frequency distribution of APO in four subgroups (T-APS, O-APS, NC-APS, and aPL carrier) was 24.2, 18.7, 9.2, and 17.9%, respectively. In **Tables 6** and **7**, we describe the serological profile, the different APO, and the risk factor associated with APO in the four subgroups.

Therapy

All patients were treated with LDA ($n = 278$; 98.2%) and/or low-molecular-weight heparin (LMWH; $n = 216$; 76.3%). In 85% of the cases, LDA was used before the eighth week of gestation. LMWH was introduced before the eighth week of gestation in 164 pregnancies (76% of all pregnancies treated with heparin). Combination therapy was more frequent in patients with triple aPL positivity compared to single/double positivity ($p = 0.001$, OR 3.21, CI 95% 1.48–7.11). In addition, immunomodulatory or immunosuppressive therapy was recorded in 42 pregnancies with hydroxychloroquine and corticosteroids in 32 (11.3%) and 16 (5.7%) cases, respectively. Moreover, combination therapy was used more frequently in patients satisfying the criteria for primary APS compared to the others ($p < 0.001$, OR 8.086, CI 95% 4.3–15.4). **Table 6** outlines the distribution of treatments in the four subgroups of patients. There were no significant differences in the rate of APO among the patients treated with LDA only or the combination therapy (9/47 in LDA versus 58/231 in LDA + LMWH) (**Table 8**). Moreover, the number of APO was neither related to the time of introduction of the combination therapy nor to the heparin dosage. Interestingly, three out of five

TABLE 4 | Univariate analysis of clinical features in successful and adverse pregnancy outcome (APO) pregnancies.

Clinical/serological features	APO ($n = 50$) (17.7%)	Successful pregnancies ($n = 233$) (82.3%)	*p*-Value	OR (CI 95%)
Age at the onset ≥35 years ($n = 107$)	20/50 (40)	87/233 (37.3)	NS	–
Formal diagnosis of antiphospholipid syndrome (APS) ($n = 190$)	38/50 (76)	152/233 (65.2)	NS	–
Organ-specific autoimmune disease[a] ($n = 38$)	12/50 (24)	26/233 (11.2)	**0.016**[e]	**2.51 (1.09–5.75)**
Previous thrombosis ($n = 66$)	16/50 (32)	50/233 (21.5)	NS	–
Previous pregnancy morbidity ($n = 151$)	27/50 (54)	124/233 (53.2)	NS	–
Previous premature birth ($n = 32$)	10/50 (20)	22/233 (9.4)	**0.032**[f]	**2.39 (0.99–5.82)**
Previous ≥3 spontaneous abortion ($n = 29$)	4/50 (8)	25/233 (10.7)	NS	–
Prior pregnancy morbidity and thrombosis ($n = 27$)	5/50 (10)	22/233 (9.4)	NS	–
Other APS-related manifestations[b] ($n = 48$)	16/50 (32)	32/233 (13.7)	**0.002**[f]	**2.95 (1.38–6.29)**
Acquired risk factors for thrombosis[c] ($n = 90$)	21/50 (42)	69/233 (29.6)	NS	–
Inherited thrombophilia (data available for 237 pregnancies)[d] ($n = 23$)	5/40 (12.5)	18/197 (9.1)	NS	–

[a]Associated autoimmune organ disease, thyroiditis, autoimmune hepatitis, primary biliary cirrhosis, psoriasis, celiac disease.
[b]Other APS-related manifestations, thrombocytopenia, epilepsy, headache, livedo reticularis, heart valve lesions, and hemolytic anemia.
[c]Acquired risk factors for thrombosis, hypertension, BMI >30 kg/m², and smoke.
[d]Inherited thrombophilia, factor II and factor V mutation, protein C and S and antithrombin III deficiency.
NS, not statistically significant.
[e]Significant at multivariate analysis, p = 0.012 (OR 3.29, CI 95% 1.29–8.38).
[f]Not significant at multivariate analysis.

TABLE 5 | Univariate analysis of serological features in successful and adverse pregnancy outcome (APO) pregnancies.

Clinical/serological features	APO ($n = 50$) (17.7%)	Successful pregnancies ($n = 233$) (82.3%)	p-Value	OR (CI 95%)
Lupus anticoagulant (LA) positivity ($n = 125$)	29/50 (58)	96/233 (41.2)	**0.03**[c]	**1.97 (1.01–3.83)**
LA single positivity ($n = 20$)	2/50 (4)	18/233 (7)	NS	**–**
IgG anticardiolipin (aCL) ($n = 153$)	36/50 (72)	117/233 (50.2)	**0.005**[c]	**2.54 (1.25–5.26)**
IgG aCL single positivity ($n = 37$)	8/50 (16)	29/233 (12.4)	NS	
IgM aCL ($n = 53$)	13/50 (26)	40/233 (17.2)	NS	–
IgM aCL single positivity ($n = 6$)	1/50 (2)	5/233 (2.1)	NS	–
IgG anti-B2GPI ($n = 134$)	22/50 (44)	112/233 (48.1)	NS	–
IgG anti-B2GPI single positivity ($n = 36$)	4/50 (8)	32/233 (13.7)	NS	
IgM anti-β2GPI positivity ($n = 107$)	17/50 (34)	90/233 (38.6)	NS	–
IgM anti-B2GPI single positivity ($n = 24$)	2/50 (4)	22/233 (9.4)	NS	–
Single aPL positivity ($n = 144$)	20/50 (40)	124/233 (53.2)	NS	–
Double aPL positivity ($n = 55$)	8/50 (16)	47/233 (21)	NS	–
Triple aPL positivity ($n = 84$)	22/50 (44)	62/233 (26.6)	**0.015**[c]	**2.16 (1.10–4.25)**
Other autoantibodies[a] ($n = 78$)	21/50 (42)	57/233 (24.5)	**0.012**[c]	**2.23 (1.12–4.42)**
Low complement levels[b] (data available for 195 pregnancies) ($n = 27$)	11/34 (32.3)	16/161 (9.9)	**0.001**[d]	**4.33 (1.63–11.46)**

[a]*Other autoantibodies, antinuclear antibody ≥1:320, anti-double-stranded DNA, antibodies to extractable nuclear antigens.*

[b]*Low complement levels, decrease in C3 and/or C4 at conception or during first trimester.*

NS, not statistically significant.

[c]*Not significant at multivariate analysis.*

[d]*Significant at multivariate analysis, p = 0.02 (OR 2.3, CI 95% 1.17–9.15).*

TABLE 6 | Description of serological profile, treatment, and prevalence of adverse pregnancy outcome (APO) in the subgroups (all pregnancies included in the study).

	Serological profile			
	O-APS, $n = 124$ (%)	**T-APS, $n = 66$ (%)**	**NC-APS, $n = 54$ (%)**	**aPL carrier, $n = 39$ (%)**
Single aPL positivity	75 (60.4)	16 (24.2)	32 (59.2)	21(53.8)
Double aPL positivity	20 (16.1)	14 (21.2)	11(20.3)	10 (25.6)
Triple aPL positivity	29 (23.3)	36 (54.5)	11(20.3)	8 (20.6)
	Treatment			
LDA monotherapy	17 (13.7)	1 (1.5)	23 (42.6)	26 (66.6)
LMWH monotherapy	0 (0)	5 (7.5)	0 (0)	0 (0)
Combination treatment (LDA + LMWH) prophylactic dose	97 (78.2)	21 (31.2)	31 (57.4)	12 (30.8)
Combination treatment (LDA + LMWH) therapeutic dose	10 (8)	39 (59.1)	0 (0)	1 (2.6)
Hydroxychloroquine	8 (6.4)	19 (28.8)	3 (5.5)	2 (5.1)
Steroids	4 (3.2)	9 (13.6)	3 (5.5)	4 (10.2)
	APO[a]			
	O-APS, $n = 22$ (17.7%)	**T-APS, $n = 16$ (24.2%)**	**NC-APS, $n = 5$ (9.2%)**	**aPL carrier, $n = 7$ (17.9%)**
Spontaneous abortion	9 (41)	8 (50)	2 (40)	1 (14.2)
Fetal death	4 (18.2)	4 (25)	1 (20)	3 (42.8)
Neonatal death due to prematurity	0 (0)	0 (0)	0 (0)	1 (14.2)
Preterm deliveries <34 weeks	7 (31.8)	4 (25)	2 (40)	3 (42.8)
Small for gestational age associated with abnormal Doppler flow velocimetry	2 (9)	0 (0)	1(20)	1 (14.2)
Hemolysis, elevated liver enzymes, and low platelets	2 (9)	3 (18.7)	0 (0)	2 (28.5)

LDA, low-dose aspirin; LMWH, low-molecular-weight heparin.

[a]*The same patient can be included in more than one category.*

pregnancies treated only with LMWH experienced an adverse outcome ($p = 0.04$, OR 7.37, CI 95% 0.96–65).

Maternal Outcome

Seven patients (2.5%) experienced a thrombotic event during pregnancies or puerperium: three during pregnancies (all during the first trimester) and four during puerperium (in three cases within 1 week after delivery and in one case 1 month after birth). No patient received ovulation induction therapy. Of these thrombotic events, four were venous (three deep venous thrombosis and one pulmonary embolism) and three arterial [one myocardial infarction and two catastrophic antiphospholipid syndrome (CAPS)]. Five of these events occurred in the T-APS subgroup, one in O-APS, and one in aPL carrier. In addition, the

TABLE 7 | Laboratory and clinical features associated with adverse pregnancy outcome (APO) in the four subgroups.

	APO *n*(%)	Features associated with APO	*p*-Value	OR (95% CI)
T-APS, 42 patients, 66 pregnancies	16 (24)	Prior venous thrombotic event	0.028	>20 (0.09–150)
O-APS, 85 patients, 124 pregnancies	22 (18)	Previous premature birth	0.037	2.85 (0.92–8.78)
		Other autoantibodies[a]	0.023	3.02 (1.01–9.02)
		Low complement levels[b]	0.04	3.63 (0.83–15.6)
NC-APS, 39 patients, 54 pregnancies	5 (9)	LA positive (any combination)	0.03	1.97 (1.01–3.83)
		Anti-B2GPI IgG positive (any combination)	0.017	6.91 (1.28–49)
aPL carrier, 34 patients, 39 pregnancies	7 (18)	Triple aPL-positive	0.022	9.33 (1.13–90.3)

[a]*Other autoantibodies, antinuclear antibody ≥1:320, anti-double-stranded DNA, antibodies to extractable nuclear antigens.*
[b]*Low complement levels, decrease in C3 and/or C4 at conception or during first trimester.*

TABLE 8 | Comparison of treatment in successful and complicated pregnancy.

Treatment	Adverse pregnancy outcome (*n* = 50) (17.7%)	Successful pregnancies (*n* = 233) (82.3%)	*p*-Value
LDA monotherapy (*n* = 67)	9/50 (18)	58/233 (24.9)	NS
LMWH monotherapy (*n* = 5)	3/50 (6)	2/233 (0.8)	**0.040***
LDA + LMWH (*n* = 211)	38/50 (76)	173/233 (74.2)	NS
LDA + LMWH prophylactic dosage (*n* = 161)	28/50 (56)	133/233 (57.1)	NS
LDA + LMWH therapeutic dosage (*n* = 50)	10/50 (20)	40/233 (17.2)	NS
LDA + LMWH, start at positive pregnancy test (*n* = 143)	28/50 (56)	115/233 (49.3)	NS
LDA + LMWH, start between 6 and 8 weeks of gestation (*n* = 16)	0/50 (0)	16/233 (6.8)	NS
LDA + LMWH, Start between 9 and 12 weeks of gestation (*n* = 22)	6/50 (12)	16/233 (6.8)	NS
LDA + LMWH, start after 12 weeks of gestation (*n* = 30)	4/50 (8)	26/233 (11.1)	NS
Hydroxychloroquine (*n* = 32)	6/50 (12)	26/233 (11.2)	NS
Steroids (*n* = 16)	4/50 (8)	12/233 (5.1)	NS

LDA, low-dose aspirin; LMWH, low-molecular-weight heparin.
**OR 7.37; CI 95% 0.96–65.*

serological profile of these patients showed that four (53%) were triple positive, one double positive (LA and aCL), and two single positive (aCL). Six out of these seven patients (85%) were already receiving antithrombotic prophylaxis at the time of the event. The two patients who experienced CAPS have been previously described (8).

DISCUSSION

The aim of the present study was to analyze the gestational and maternal outcome in patients with confirmed positivity for antiphospholipid antibodies (aPL) followed up during their pregnancies. The most important exclusion criteria were the concomitant presence of another defined CTD, primarily SLE, a condition recognized as an independent risk factor for pregnancy failure in previous multicenter studies (9, 10).

The gestational outcome of these pregnancies significantly improved as compared to historic ones, with a live birth rate of 87.9%. In fact, a collaborative European study (EUROAPS) reported a live birth rate of 77.7% in patients with pure obstetric APS (11), while a recent retrospective Italian Study (PREGNANT) (12) reported 54.3% in patients with primary APS. Our results are difficult to compare with previously published cohorts as they mainly analyzed pregnancy outcome in patients with established APS but also with the incomplete form only.

Despite the high rate of live births in our study, maternal–fetal complications still occurred, and APOs related to aPL were identified in 17.7% of the pregnancies. Several studies have previously attempted to identify the clinical and laboratory variables predictive of APO (9, 10), and a concomitant SLE diagnosis and prior thrombotic events were found to be associated with poor pregnancy outcome. Moreover, previous works have identified the triple aPL positivity as an independent risk factor (9), while a prospective multicenter study (13, 14) supported the key role of LA as the main predictor of APO.

In this study, we show by means of multivariate analysis that the presence of a concomitant organ-specific autoimmune disease and/or low levels of C3 and/or C4 at conception are the only two independent factors associated with APO, although it seems to be associated with several clinical and serological factors from the univariate analysis. Autoimmune thyroiditis accounted for 71% of organ-specific autoimmune disease in our cohort and was associated with SA as previously reported (15). Particularly, we did not find any association between APO and any peculiar aPL positivity. This may be due to the small number of complicated pregnancies collected and/or to the fact that patients with LA or triple positivity were more frequently treated with the combination therapy. This observation could also account for the lack of difference in pregnancy outcome between women treated with combination therapy and those receiving LDA alone, which is generally considered as a less effective treatment (2).

Low complement levels at the beginning of pregnancy were also observed as an independent risk factor for APO. The role

of complement activation in the pathogenesis of APS pregnancy morbidity is an intriguing question. Previous studies have demonstrated that the activation of complement components C3, C4, and C5 increases the risk of injury or death in animal models injected with aPL (16, 17). Our group has also investigated this relationship in a multicenter study (18). Utilizing pregnancy C3 and C4 normality ranges (in each trimester), we did not show an association between hypocomplementemia and obstetric complications in primary APS. Conversely, a prospective cohort study (19) that included both primary and secondary APS has recognized hypocomplementemia as an independent predictor of low birth weight and premature delivery.

As expected, T-APS group had the highest rate of complicated pregnancies (24.2%), confirming the previous reports (9). We did not find any statistical difference in the rate of APO among patients with or without a full blown picture of primary APS (O + T vs NC + carriers).

Till date, there are no generalized recommendations on how to treat women not fulfilling the APS criteria or if a prophylactic treatment is required during pregnancy and puerperium. A recent systematic review reported that LDA is comparable to the usual care or placebo in the prevention of pregnancy complications. However, the authors were able to include only a limited number of studies in their analysis (5). The majority of the patients included in that review belonged to a large retrospective observational Italian study on aPL patients that also included patients with organ or systemic autoimmune diseases (20). The authors concluded that, in their cohort, the most important factor related to pregnancy outcome was the antibody profile (medium–high titers of aPL) and not the treatment or the previous pregnancy morbidity. Hence, these results cannot be compared with those obtained in the present study in which all the patients received antithrombotic therapy. However, in the aPL carrier group, we observed a significant association between APO and triple aPL positivity, a feature included in the definition of the "high-risk" aPL profile (9).

Beside pregnancy morbidity, we reported the occurrence of a moderate number of thrombotic events during pregnancy or puerperium. The majority of the thrombotic events occurred during puerperium, despite the use of adequate antithrombotic treatments. This pattern is consistent to the well-known risk of postpartum thrombosis in the general obstetric population. The two cases of CAPS onset during the puerperium were already described in a retrospective series of 13 patients (8). In more than 90% of their cases (as well as in our two women), the occurrence of a HELLP syndrome can be considered as a predictive factor for CAPS.

This study has several limitations: the retrospective design, even if data were prospectively collected during each pregnancy; the lack of a centralized laboratory, although all the laboratories were referral centers; the wide temporal range of the pregnancies (15 years, 2000–2014); and the multicenter nature, possible source of heterogeneity. Nevertheless, APS is a rare disease, and a collaborative study including several pregnancy clinics was required in order to achieve a significant number of cases.

In conclusion, maternal and fetal complications were observed in nearly 18% of aPL-positive patients despite the conventional treatment according to the current recommendations. Additional immunomodulatory treatment might be required for these difficult patients (21, 22).

In the last two decades, a great improvement was certainly achieved in the outcome of APS pregnancies. This success is probably due to multi-specialistic teams devoted to the tight control of aPL-positive women. A preconception risk stratification is recognized as crucial. The results obtained in this study confirmed the role of some of the parameters lately included in the recently revised risk factors for APO in APS patients (23). In addition, we underline the influence of a nonsystemic autoimmune phenotype even in patients with aPL without a formal diagnosis of APS.

In the absence of controlled trials and with very limited prospective studies available, the present work, collecting a very large number of pregnancies from experienced centers, might contribute to a better definition of the clinical and laboratory features associated with a poor prognosis that deserve attention by the clinicians in the everyday practice.

ETHICS STATEMENT

This study was performed according to the principles of the Declaration of Helsinki with written informed consent from all subjects and was approved by the Ethic Committee of the Promoting Centre (Brescia) (approval number 1088) and it has been approved by the other centers.

AUTHOR CONTRIBUTIONS

MF, LA, NC-C, and AT designed the study. MF, LA, ML, VL, NM, JP, AL, and SZ evaluated the patients. MF, EA, and EB recruited the patients. MF, LA, NC-C, and AT wrote the manuscript. All the coauthors reviewed the manuscript.

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REFERENCES

1. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* (2006) 4:295–306. doi:10.1111/j.1538-7836.2006.01753.x
2. Mak A, Cheung MW, Cheak AA, Ho RC. Combination of heparin and aspirin is superior to aspirin alone in enhancing livebirths in patients with recurrent pregnancy loss and positive anti-phospholipid antibodies: a meta-analysis of randomized controlled trials and meta-regression. *Rheumatology* (2010) 49:281–8. doi:10.1093/rheumatology/kep373
3. Empson M, Lassere M, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. *Cochrane Database Syst Rev* (2005) 8(2):CD002859. doi:10.1002/14651858.CD002859.pub2
4. Erkan D, Patel S, Nuzzo M, Gerosa M, Meroni PL, Tincani A, et al. Management of the controversial aspects of the antiphospholipid syndrome

pregnancies: a guide for clinicians and researchers. *Rheumatology* (2008) 47:23–7. doi:10.1093/rheumatology/ken181
5. Amengual O, Fujita D, Ota E, Carmona L, Oku K, Sugiura-Ogasawara M, et al. Primary prophylaxis to prevent obstetric complications in asymptomatic women with antiphospholipid antibodies: a systematic review. *Lupus* (2015) 24(11):1135–42. doi:10.1177/0961203315578765
6. Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the subcommittee on lupus anticoagulant/antiphospholipid antibody of the scientific and standardisation committee of the ISTH. *Thromb Haemost* (1995) 74:1185–90.
7. Bertolaccini ML, Amengual O, Andreoli L, Atsuni T, Chighizola CB, Forastiero R, et al. 14th international congress on antiphospholipid antibodies task force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmun Rev* (2014) 13(9):917–30. doi:10.1016/j.autrev.2014.05.001
8. Hanouna G, Morel N, Le Thi Huong D, Josselin L, Vauthier-Brouzes D, Saadoun D, et al. Catastrophic antiphospholipid syndrome and pregnancy: an experience of 13 cases. *Rheumatology (Oxford)* (2013) 52(9):1635–41. doi:10.1093/rheumatology/ket167
9. Ruffatti A, Tonello M, Visentin MS, Bontadi A, Hoxha A, De Carolis S, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre case–control study. *Rheumatology* (2011) 50:1684–9. doi:10.1093/rheumatology/ker139
10. Lockshin MD, Kim M, Laskin CA, Guerra M, Branch WD, Merrill J, et al. Prediction of adverse pregnancy outcome by the presence of lupus anticoagulant, but not anticardiolipin antibody, in patients with antiphospholipid antibodies. *Arthritis Rheum* (2012) 64:2311–8. doi:10.1002/art.34402
11. Alijotas-Reig J, Ferrer-Oliveras R, Ruffatti A, Tincani A, Lefkou E, Bertero MT, et al. The European Registry on Obstetric Antiphospholipid Syndrome (EUROAPS): a survey of 247 consecutive cases. *Autoimmun Rev* (2015) 14(5):387–95. doi:10.1016/j.autrev.2014.12.010
12. Saccone G, Berghella V, Maruotti GM, Ghi T, Rizzo G, Simonazzi G, et al. Antiphospholipid antibody profile based obstetric outcomes of primary antiphospholipid syndrome: the PREGNANTS study. *Am J Obstet Gynecol* (2017) 216:.e1–525. doi:10.1016/j.ajog.2017.01.026
13. Bouvier S, Cochery-Nouvellon E, Lavigne-Lissalde G, Mercier E, Marchetti T, Balducchi JP, et al. Comparative incidence of pregnancy outcomes in treated obstetric antiphospholipid syndrome: the NOH-APS observational study. *Blood* (2014) 123(3):404–13. doi:10.1182/blood-2013-08-522623
14. Yelnik CM, Laskin CA, Porter TF, Branch DW, Buyon JP, Guerra MM, et al. Lupus anticoagulant is the main predictor of adverse pregnancy outcomes in aPL-positive patients: validation of PROMISSE study results. *Lupus Sci Med* (2016) 3(1):e000131. doi:10.1136/lupus-2015-000131
15. De Carolis C, Greco E, Guarino MD, Perricone C, Dal Lago A, Giacomelli R, et al. Anti-thyroid antibodies and antiphospholipid syndrome: evidence of reduced fecundity. *Immunol* (2004) 52:263–6. doi:10.1111/j.1600-0897.2004.00215.x
16. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* (2002) 195:211–20. doi:10.1084/jem.200116116
17. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* (2003) 112:1644–54. doi:10.1172/JCI200318817
18. Reggia R, Ziglioli T, Andreoli L, Bellisai F, Iuliano A, Gerosa M, et al. Primary anti-phospholipid syndrome: any role for serum complement levels in predicting pregnancy complications? *Rheumatology (Oxford)* (2012) 51(12):2186–90. doi:10.1093/rheumatology/kes225
19. De Carolis S, Botta A, Santucci S, Garofalo S, Martino C, Perrelli A, et al. Predictors of pregnancy outcome in antiphospholipid syndrome: a review. *Clin Rev Allergy Immunol* (2010) 38:116–24. doi:10.1007/s12016-009-8144-z
20. Del Ross T, Ruffatti A, Visentin MS, Tonello M, Calligaro A, Favaro M, et al. Treatment of 139 pregnancies in antiphospholipid-positive women not fulfilling criteria for antiphospholipid syndrome: a retrospective study. *J Rheumatol* (2013) 40(4):425–9. doi:10.3899/jrheum.120576
21. Sciascia S, Hunt BJ, Talavera-Garcia E, Lliso G, Khamashta MA, Cuadrado MJ. The impact of hydroxychloroquine treatment on pregnancy outcome in women with antiphospholipid antibodies. *Am J Obstet Gynecol* (2016) 214(2):.e1–8. doi:10.1016/j.ajog.2015.09.078
22. Mekinian A, Lazzaroni MG, Kuzenko A, Alijotas-Reig J, Ruffatti A, Levy P, et al. The efficacy of hydroxychloroquine for obstetrical outcome in antiphospholipid syndrome: data from a European Multicenter Retrospective Study. *Autoimmun Rev* (2015) 14(6):498–502. doi:10.1016/j.autrev.2015.01.012
23. Andreoli L, Bertsias GK, Agmon-Levin N, Brown S, Cervera R, Costedoat-Chalumeau N, et al. EULAR recommendations for women's health and the management of family planning, assisted reproduction, pregnancy and menopause in patients with systemic lupus erythematosus and/or antiphospholipid syndrome. *Ann Rheum Dis* (2017) 76(3):476–85. doi:10.1136/annrheumdis-2016-209770

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A Monocentric Cohort of Obstetric Seronegative Anti-Phospholipid Syndrome

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The present study was conducted to diagnose obstetric anti-phospholipid syndrome (OAPS) in patients with clinical signs suggestive of anti-phospholipid syndrome (APS), but persistently negative for conventional anti-phospholipid antibodies (aPL). Sera from 61 obstetrical seronegative APS (SN-APS) patients were analyzed for anti-cardiolipin antibodies (aCL) using thin-layer chromatography (TLC)-immunostaining, for anti-cardiolipin/vimentin antibodies (aCL/Vim), anti-phosphatidylserine/prothrombin antibodies, IgA anti-$\beta_2$glycoprotein I antibodies (aβ_2GPI), and IgA aCL antibodies by enzyme-linked immunosorbent assay. Taken together, our findings show that in 50 out of 61 SN-APS (81.9%) at least one aPL/cofactor antibody was detected using the assays under test. Results revealed that 76% of SN-APS patients resulted positive for aCL by TLC-immunostaining, 54% for aCL/Vim, 12% for aPS/PT, 4% for IgA aβ_2GPI, and 2% for IgA aCL. Thirty-five out of 61 patients were followed up and the tests were repeated on two occasions, at least 12 weeks apart. Twenty-six out of 35 SN-APS (74.3%) were positive at least one non-conventional test; only 2 patients (5.7%) did not confirm the positivity to the second test. These findings suggest that non-conventional tests, mainly aCL/Vim and aCL detected by TLC-immunostaining, seem to be the most sensitive approaches for finding out aPL in patients with obstetric SN-APS. The use of these tests can be useful for accurate and timely diagnosis of patients with obstetrical APS who are negative for conventional laboratory criteria markers.

Keywords: anti-phospholipid syndrome, seronegative anti-phospholipid syndrome, anti-vimentin/cardiolipin, thin-layer chromatography, anti-phosphatidylserine/prothrombin

Abbreviations: APS, anti-phospholipid syndrome; aPL, anti-phospholipid antibodies; aCL, anti-cardiolipin; aPS/PT, anti-phosphatidylserine/prothrombin; aCL/Vim, anti-vimentin/cardiolipin; aPTT, activated partial thromboplastin time; aβ_2GPI, anti-$\beta_2$glycoprotein I; BSA, bovine serum albumin; LA, lupus anticoagulant; dRVVT, dilute Russell's viper venom time; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; HPTLC, high-performance thin-layer chromatography; TLC, thin-layer chromatography; OD, optical density; OAPS, obstetrical APS; OSN-APS, obstetrical seronegative APS; PBS, phosphate buffered saline; SLE, systemic lupus erythematosus; SN-APS, "seronegative APS"; SP-APS, seropositive APS; FGR, fetal growth restriction; RSA, recurrent spontaneous abortion.

INTRODUCTION

Anti-phospholipid syndrome (APS) is a systemic autoimmune disease characterized by arterial and venous thrombosis and pregnancy morbidity associated with circulating anti-phospholipid antibodies (aPL) (1). Obstetrical APS (OAPS) is characterized by early recurrent miscarriage, unexplained fetal loss, and/or premature birth due to eclampsia, preeclampsia, or placental insufficiency as stated in the classification criteria for definite APS (2). APS is the most frequently acquired risk factor for a treatable cause of recurrent pregnancy loss (3) and it increases the risk for pregnancy complications associated with placental dysfunction, such as stillbirth, placental abruption, and fetal growth restriction (4). Classification of OAPS requires the combination of at least one clinical and one laboratory criterion (2), including anti-cardiolipin (aCL) and anti-$\beta_2$glycoprotein I (aβ_2GPI) antibodies detected by enzyme-linked immunosorbent assay (ELISA) and the lupus anticoagulant (LA) detected by clotting assays (1). In clinical practice there are individuals with clinical signs highly suggestive of APS who are persistently negative for conventional aPL laboratory tests; therefore, physicians proposed for this population the term of "seronegative APS" (SN-APS) (5, 6). Since APS is the commonest treatable cause of recurrent miscarriage, for women with a history of recurrent early abortions or fetal loss, a diagnosis of APS significantly improves the rate of live births (7). New antigenic targets or methodological approaches to detect aPL in SN-APS have been investigated and several non-conventional anti-phospholipid antibodies have been described (8, 9). Recently, with a proteomic approach, we identified cardiolipin/vimentin (CL/Vim) as a "new" target for APS, also detectable in SN-APS patients (10). In addition, it has demonstrated the possibility of detecting aPL in SN-APS patients by immunostaining on thin-layer chromatography (TLC) plates (11, 12).

The aim of the present study was to investigate the potential clinical usefulness of "new" antigenic targets and methodological approaches in detecting serum aPL in patients with obstetrical SN-APS (OSN-APS).

MATERIALS AND METHODS

Patients

The study includes all consecutive patients, presenting clinical features consistent with a diagnosis of obstetrical APS but tested persistently negative for conventional aCL, aβ_2GPI, and LA tests referred to the Lupus Clinic, Rheumatology Unit of the Sapienza University of Rome from 2012 to 2017. Clinical manifestations consisted of pregnancy morbidity in women with and without history of thrombosis as stated in the classification criteria for definite APS (2).

Sera were collected at several times and stored at −20°C until use. Moreover, all patients were tested for common inherited thrombophilic defects, such as protein C and protein S deficiency, hyperhomocysteinemia, factor V Leiden, MTHFR, and prothrombin mutations. This study was approved by the local ethic committees and participants gave written informed consent.

ELISA for Anti-Cardiolipin and Anti-β_2Glycoprotein I Antibodies

Antibodies specific for aCL and aβ_2GPI (IgG, IgM, and IgA) were detected by ELISA, using QUANTA Lite™ detection kit (INOVA Diagnostic Inc., San Diego, CA, USA) assay. ELISA was performed for all the patients' sera according to manufacturer's instructions; a positive control and several normal human sera were run in the same assay to confirm the specificity of the results.

Chemiluminescence Assay

IgG, IgM, and IgA for aCL and aβ_2GPI were also tested by chemiluminescence assay using Zenit RA Immunoanalyzer (A. Menarini Diagnostics, Florence, Italy).

LA Test

Lupus anticoagulant was studied in two coagulation systems, a dilute sensitized activated partial thromboplastin time and a dilute Russell's viper venom time, followed by confirm test, using reagents and instrumentation by Hemoliance Instrumentation Laboratory, Lexington, MA, USA.

Detection of aCL by TLC-Immunostaining

Thin-layer chromatography-immunostaining was performed as previously described, with slight modification (11, 12). Briefly, 2 μg (in chloroform/methanol, 2:1 v/v) of cardiolipin (CL, Sigma-Aldrich) were run on aluminum-backed silica gel 60 (20 × 20) high-performance thin-layer chromatography plates (Merck Co., Darmastdt, Germany). Chromatography was performed in chloroform:methanol:CH_3COOH:water (100:75:7:4) (v:v:v:v) as eluent system. The dried chromatograms were soaked for 90 s in a 0.5% (w:v) solution of poly(isobutyl methacrylate) beads (Polysciences, Eppelheim, Germany) dissolved in hexane. After air-drying for 5 min, the chromatograms were incubated at room temperature for 1 h with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (blocking buffer) to eliminate non-specific binding. After washing by gentle shaking 3 times for 10 min in PBS containing 0.1% Tween 20 (PBS-T), the chromatograms were incubated with sera diluted 1:100 in the blocking solution, for 1 h at room temperature. Sera were removed and chromatograms were washed as above. Bound antibodies were visualized with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma-Aldrich) diluted 1:1,000 in blocking buffer and incubated at room temperature for 1 h. After washing, immunoreactivity was assessed by chemiluminescence reaction using the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Detection of Anti-Cardiolipin/Vimentin Complex (aCL/Vim) Antibodies by ELISA

Anti-cardiolipin/vimentin complex antibodies were detected by ELISA with slight modification of previously reported method (13). Ninety-six-well polystyrene plate (Thermo Fisher Scientific, Waltham, MA, USA) were coated and incubated overnight at 4°C with 100 μl/well of CL (from bovine heart, Sigma-Aldrich, Milan, Italy) (50 μg/ml in methanol), and then with 100 μl/well of human recombinant vimentin (5 μg/ml in 0.05 mM $NaHCO_3$

buffer, pH 9.5) (R&D System, Minneapolis, MN, USA). Coated plates were incubated overnight at 4°C and then washed three times with PBS-T. Plates were blocked with 100 µl of 1% BSA in PBS (blocking buffer) for 2 h at room temperature. After three washes with PBS-T the wells were incubated with 100 µl of patients sera (diluted 1:100 in the blocking buffer) for 1 h at room temperature. Goat polyclonal anti-vimentin (R&D Systems) was used as positive control. After washing, as above, the plates were incubated for 1 h at room temperature with HRP-conjugated antibodies, either goat anti-human IgG or rabbit anti-goat IgG (Sigma-Aldrich), diluted in blocking buffer. The plates were washed three times with PBS-T, the bound peroxidase was then revealed with *O*-phenylenediamine dihydrochloride development buffer (100 µl/well) and stopped with 50 µl/well of H_2SO_4 0.2 M for 5 min. Absorbance was measured at 492 nm in a microplate reader. ELISA assay was also performed without coated vimentin/cardiolipin complex. Virtually no reactivity was detected in all the samples (data not shown).

Data were analyzed as the mean optical density (OD) corrected for background (wells without coated antigen). Thirty-two normal human sera were also tested and a cutoff value was established as mean of OD ± 3 SDs of normal human sera. Each serum was analyzed in triplicate.

Detection of Anti-Phosphatidylserine/Prothrombin (aPS/PT) Antibodies by ELISA

Antibodies specific for aPS/PT were detected by ELISA using a QUANTA Lite™ detection kit (INOVA Diagnostic Inc.). All patient samples including those from healthy donors were tested and ELISA was performed according to manufacturer's instructions.

Statistical Analysis

All the statistical analyses were performed by GraphPad Prism software Inc. (San Diego, CA, USA). Kolmogorov–Smirnov test was used to assess the normal distribution of the data. Differences between numerical variables were tested with the Wilcoxon test. Statistical coefficient Cohen's kappa was used to analyze agreement between first and second test. For comparison of categorical variables or percentages we used Fisher's exact and X^2 tests when appropriate. *P*-values less than 0.05 were considered as significant.

RESULTS

We enrolled 61 women with clinical features consistent with a diagnosis of obstetrical APS, but tested persistently negative for conventional aCL, aβ_2GPI (detected by both ELISA and Chemiluminescence assay), and LA tests. All OSN-APS patients were Caucasian with a median age of 39 years (IQR 8). The clinical characteristics of patients are reported in **Table 1**. Mixed thrombotic and obstetrical features were present in 9 patients out of 61 (14.6%) and isolated obstetrical features in remaining patients (85.4%). All patients were tested for common inherited thrombophilic defects: 18/61 (29.5%) patients presented mutation of MTHFR in heterozygosity, with normal levels of homocysteine; 6/61 (9.8%) mutation of MTHFR in homozygosity, with normal levels of homocysteine; 1/61 (1.6%) mutation of V factor of Leiden in homozygosity; 1/61 (1.6%) protein S deficiency. Because of normal level of homocysteine, these patients cannot be considered at increased risk for inherited thrombophilia.

TABLE 1 | Clinical characteristics of patients studied.

Characteristics *n* (%)	Obstetric seronegative anti-phospholipid syndrome (OSN-APS) (total) (*n* = 61)	OSN-APS (followed up) (*n* = 35)
Other autoimmune diseases		
Systemic lupus erythematosus	7 (11.5)	5 (14.3)
Discoid lupus erythematosus	5 (8.2)	5 (14.3)
Autoimmune thyroiditis	11 (18.0)	10 (28.6)
Mixed connective tissue disease	3 (4.9)	1 (2.8)
Undifferentiated connective tissue disease	2 (3.3)	1 (28)
Pregnancy morbidity		
Spontaneous abortions[a]	41 (67.2)	25 (71.4)
Intrauterine death of a normal fetus[b]	27 (44.3)	15 (42.9)
Premature births[c]	4 (6.6)	2 (5.7)
Vascular thrombosis[d]	9 (14.6)	6 (17.1)
Arterial thrombosis	4 (6.6)	3 (8.6)
Venous thrombosis	6 (9.8)	4 (11.4)
Recurrent thrombosis	4 (6.6)	3 (8.6)
Non-criteria APS features		
Livedo reticularis	11 (18.0)	8 (22.9)
Thrombocytopenia	4 (6.6)	4 (11.4)
Migraine	9 (14.6)	5 (14.3)
Seizures	1 (1.6)	1 (2.9)
Thrombotic risk factors		
Hypercholesterolemia	3 (4.9)	3 (8.5)
Smoking	10 (16.4)	7 (20.0)
Hypertension	7 (11.5)	6 (17.1)
Oral contraceptive/hormone replacement therapy	1 (1.6)	0 (0)

[a] *3 or + losses <10 weeks of gestation.*
[b] *1 or + losses ≥10 weeks of gestation.*
[c] *Preterm birth <34 weeks due to eclampsia, pre-eclampsia, or placental insufficiency.*
[d] *Thrombosis (arterial, venous, or in small vessels) in any tissue, confirmed by imaging or histopathology (thrombosis without significant inflammation).*

Taken together, our findings show that in 50 out of 61 OSN-APS (81.9%) at least one aPL/cofactor antibody was detected using the assays under test; in particular, 50 out of 61 patients (82%) were positive for at least one of the tests used. Thirty-eight out of 50 patients (76%) showed the presence of anti-cardiolipin (aCL) antibodies detected by TLC-immunostaining, 27/50 (54%) were positive for aCL/Vim antibodies, 6/50 (12%) for aPS/PT antibodies, 2/50 (4%) for IgA aCL, and 1/50 (2%) for IgA aβ_2GPI (**Figure 1**). **Figure 2** shows the percentage of patients displaying multiple positivity for different antibodies. The combination of TLC-immunostaining and aCL/Vim approaches detected autoantibodies in the majority of the patients. No patients were contemporary positive for aCL (by TLC-immunostaining) and aβ_2GPI IgA antibodies. **Table 2** shows autoantibody prevalence in OSN-APS patients according to the clinical manifestations.

Thirty five out of 61 patients were followed up and the tests were repeated on two occasions, at least 12 weeks apart (**Table 1**). To concern inherited thrombophilic defects: 12/35 (34.3%) patients presented mutation of MTHFR in heterozygosity, with

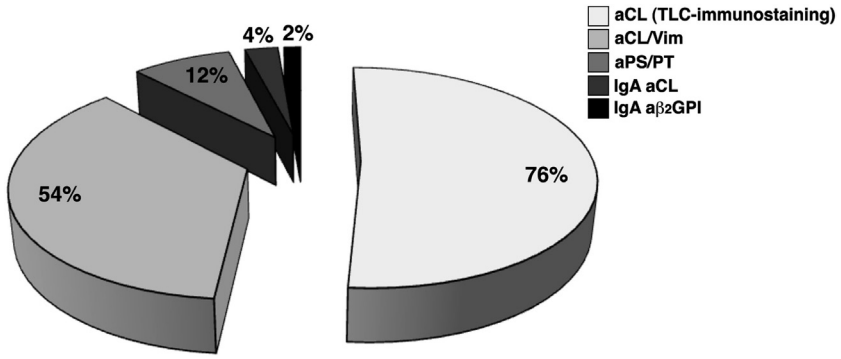

FIGURE 1 | The prevalence of autoantibodies in obstetrical seronegative anti-phospholipid syndrome patients. Fifty out of 61 patients (82%) were positive for at least one of the tests used. Thirty-eight out of 50 patients (76%) showed the presence of anti-cardiolipin (aCL) antibodies detected by thin-layer chromatography-immunostaining, 27/50 (54%) were positive for anti-cardiolipin/vimentin (aCL/Vim) antibodies, 6/50 (12%) for anti-phosphatidylserine/prothrombin antibodies, 2/50 (4%) for IgA aCL, and 1/50 (2%) for IgA aβ_2GPI.

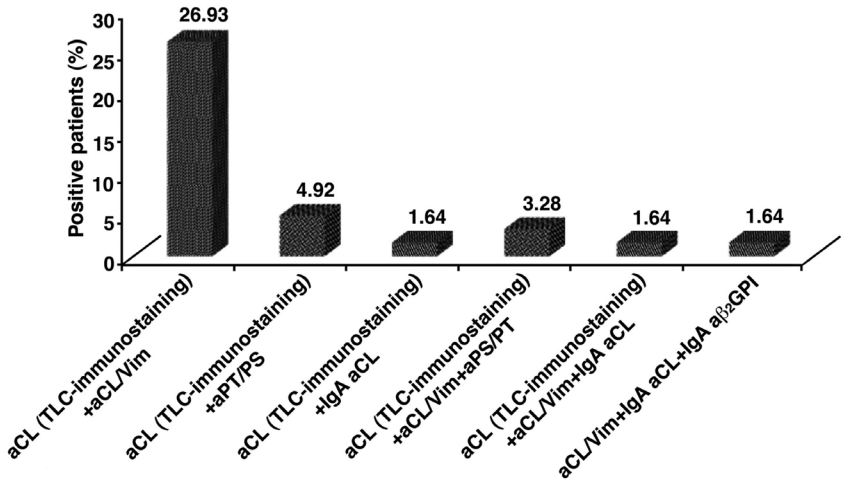

FIGURE 2 | The percentage of obstetrical seronegative anti-phospholipid syndrome patients positive for more than one test used to detect the presence of autoantibodies. The combination of thin-layer chromatography (TLC)-immunostaining and anti-cardiolipin/vimentin (aCL/Vim) approaches detected autoantibodies in the majority of the patients.

TABLE 2 | Autoantibody prevalence according to the clinical manifestations.

Autoantibodies (assay) *n* (%)	Arterial or venous thrombosis (*n* = 9)	Pregnancy morbidity (*n* = 61)	Spontaneous abortions (*n* = 41)	Intrauterine death of a normal fetus (*n* = 27)	Premature births (*n* = 4)
Anti-anti-CL [by thin-layer chromatography (TLC)-immunostaining]	7 (77.8)	38 (62.2)	25 (41.0)	18 (29.5)	3 (4.9)
Anti-CL/Vim	4 (44.4)	27 (44.3)	15 (24.6)	13 (21.3)	1 (1.6)
Anti-PS/PT	1 (11.1)	6 (9.8)	3 (4.9)	3 (4.9)	0
Anti-CL IgA	0	2 (3.3)	2 (3.3)	0	0
Anti-β_2GPI IgA	0	1 (1.6)	1 (1.6)	0	0
Anti-CL (by TLC-immunostaining) + anti-CL/Vim	3 (33.3)	16 (26.2)	11 (18.0)	8 (13.1)	1 (1.6)
Anti-CL (by TLC-immunostaining) + anti-CL/Vim + anti-PS/PT	0	2 (3.3)	2 (3.3)	0	0
No autoantibodies	1 (11.1)	11 (18.0)	10 (16.4)	3 (4.9)	1 (1.6)

CL, cardiolipin; CL/Vim, cardiolipin/vimentin; PS/PT, phosphatidylserine/prothrombin; β_2GPI, beta2Glycoprotein I.

normal levels of homocysteine; 2/35 (0.6%) mutation of MTHFR in homozygosity, with normal levels of homocysteine; 1/35 (0.3%) protein S deficiency. In 26 out of 35 SN-APS (74.3%) at least one aPL/cofactor antibody was detected using the assays under test; only 2 patients (5.7%) did not confirm the positivity in the second test. **Figure 3** shows the concordance of the antibodies positivity

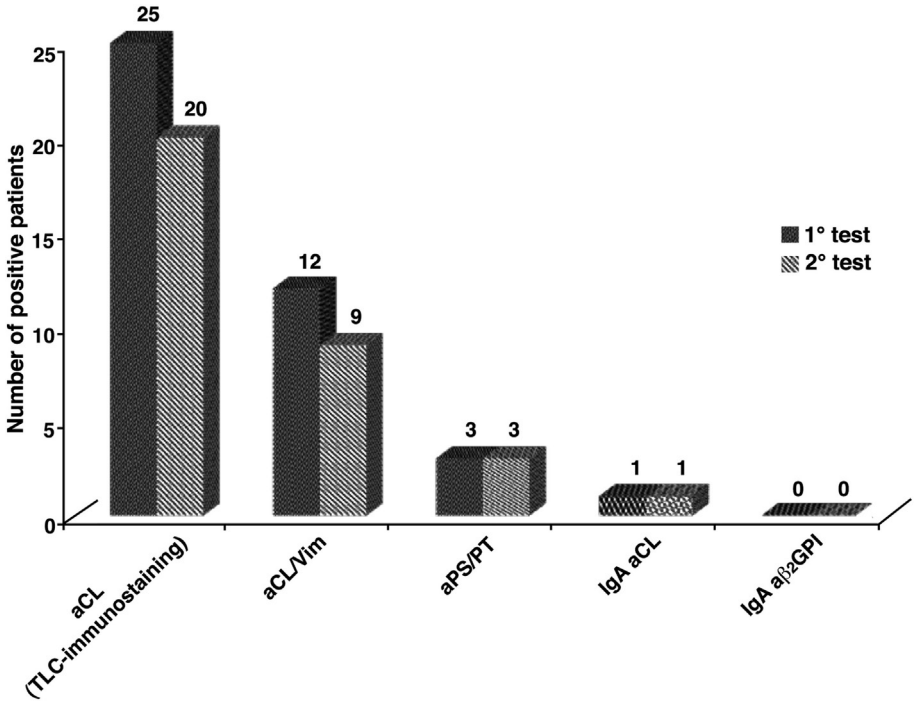

FIGURE 3 | The concordance of the autoantibodies positivity, between first and second test, in obstetrical seronegative anti-phospholipid syndrome followed up where the tests were repeated on two occasions. For thin-layer chromatography (TLC)-immunostaining and anti-cardiolipin/vimentin (aCL/Vim) a "substantial agreement" was found between first and second test, revealed by Cohen's kappa test ($K = 0.696$ and $K = 0.789$).

between first and second test. To concern TLC-immunostaining and aCL/Vim, Cohen's kappa test resulted respectively of $K = 0.696$ and $K = 0.789$, an agreement that can be described as "substantial agreement" between first and second test.

In this group of patients, a statistically significant correlation was found between anti-PS/PT and anti-CL/Vim ($p = 0.01$), between positivity for aCL (by TLC-immunostaining) and mutation of MTHFR in heterozygosis ($p = 0.026$) and finally between arterial thrombosis and premature births ($p = 0.039$).

The combination of two of the used methodological approaches, TLC-immunostaining for aCL and ELISA for anti-CL/Vim complex antibodies, was able to detect aPL/cofactors in about two-thirds of OSN-APS patients with a small additional gain when also performing ELISA for aPS/PT or aCL and aβ_2GPI IgA.

DISCUSSION

In this study new antigenic targets and methodological approaches were used to detect anti-phospholipid antibodies in a monocentric cohort of patients with suspected "seronegative" obstetric APS. Using these approaches, it was possible to demonstrate the presence of aPL in about two-thirds of the enrolled patients.

Differences were not observed in the prevalence of obstetric events, including early spontaneous abortions, fetal deaths, prematurity, or pre-eclampsia, between women with SN-APS and seropositive APS (SP-APS) (14). Likewise, since APS is now recognized as the most common treatable cause of recurrent miscarriage, for women with a history of recurrent early abortions or fetal loss, a diagnosis of APS addresses them toward treatments, which significantly improve the rate of live births (15).

Furthermore, it is mandatory to identify among the so-called SN-APS patients who need long-term secondary thromboprophylaxis (16).

In this regard, we judge not sufficiently satisfying the current panel test to detect antibodies in patients for whom there is a clinical suspicion. This may depend on the limitations of traditional technical approaches or on the existence of antigenic targets other than those known. In order to overcome the limits imposed by conventional tests, we employed a different methodological approach for detection of aCL, TLC-immunostaining, showing the presence of aCL in more than three-quarters (76%) of patients with obstetric SN-APS; these data confirmed the previous results obtained in patients with different records of SN-APS, but also in a case of catastrophic APS (15, 17). This test takes advantage from the different characteristics of binding of phospholipid to solid phase which involves both electrostatic and hydrophobic interactions. Thus, antigen exposure is quite different as compared to that on the surface of microtiter wells, where phospholipids are coated in a layer of immobilized lamellar phospholipids. Our results suggest that this test may represent a very useful tool to detect aPL in the majority of obstetric SN-APS, according to previous papers (11, 18).

Moreover, aPL represents a very heterogeneous family of antibodies and more than 30 different antibodies have been reported in APS patients, the so-called autoantibody explosion

in APS (19). Several studies reported various non-conventional aPL in patients with thrombosis and pregnancy morbidity, but relatively few data are available yet. In a study of Zohoury et al., non-criteria tests were used in a cohort of SN-APS and SP-APS showing that anti-CL/Vim antibodies together with aPS/PT were the most sensitive of the non-criteria biomarker in the SN-APS group (20). The present study confirms and extends these data revealing that aCL/Vim antibodies are present in 54% of OSN-APS patients, with a prevalence significantly higher as compared to aPS/PT (12%) in this specific group of SN-APS patients. However, this finding is not surprising, since Žigon et al., who studied the prevalence and clinical association of aPS/PT in patients with a history of pregnancy complications relevant to APS, showed positive of aPS/PT in about 13% of OAPS patients; aPS/PT were the only antibodies associated with early recurrent pregnancy loss, as well as with late pregnancy morbidity and prematurity (21). Indeed, aPS/PT IgG and IgM were shown more frequent in SP-APS than in SN-APS (63 and 37% versus 4 and 5%, respectively) (22).

Taken together, these findings indicate that the execution of all these tests (TLC-immunostaining, aCL/Vim, and aPS/PT) can be very useful for identification of autoantibodies in obstetric SN-APS. The analysis of multiple positivity for different antibodies revealed that the combination of positivity by TLC-immunostaining and aCL/Vim IgG detects aPL in a large proportion of patients. As expected, the contribution of IgA aCL and/or aβ_2GPI is virtually negligible. Although IgA aCL antibodies have been associated with poliabortivity and fetal deaths in women with primary APS, systemic lupus erythematosus, and unexplained recurrent spontaneous abortion (23), in the present study we showed that the IgA isotypes of the aCL is detectable only in 4% of SN-APS patients. Moreover, in our cohort we found that only 2% of SN-APS patients resulted positive for IgA aβ_2GPI antibodies. Only few studies showed that IgA aβ_2GPI antibodies were significantly increased in women with pregnancy morbidity (24, 25).

Thus, the data of the present study suggest that TLC-immunostaining and aCL/Vim seem to be the most sensitive tests able to reveal hidden positivity and, therefore, reducing the risk of a missed diagnosis. The use of new diagnostic tests and new biomarkers will be helpful for clinicians in the accurate and timely diagnosis of patients with obstetrical APS who are negative for conventional laboratory criteria markers. At the end, testing for these antibodies may contribute to the evaluation of the stratification of risk for thrombotic events and/or pregnancy morbidity. In particular, we suggest that in subjects with obstetric APS, the presence of these antibodies may represent an alarm signal. This is important because patients with obstetric SN-APS as well as obstetric SP-APS should be closely monitored by a multidisciplinary team to receive full treatment to have a successful pregnancy outcome.

However, a percentage of obstetric SN-APS remains seronegative to all these tests, indicating that other unidentified cofactors may be involved in sera reactivity. Further studies will shed light on possible "new" antigenic specificities in patients with obstetric SN-APS.

ETHICS STATEMENT

Ethics committee, Sapienza Università di Roma-Policlinico Umberto I. All the patients signed an informed consent prior to enter in the study.

AUTHOR CONTRIBUTIONS

FC, MS, GV, and CA conceived and designed the study. AC, SR, VM, and GR performed the experiment. ST, SM, FS, and CP analyzed the data. ST, AC, RM, AL, and TG wrote the original manuscript. FC, MS, RM, CC, and SC read and approved the final version of the manuscript.

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REFERENCES

1. Hughes GRV. The anticardiolipin syndrome. *Clin Exp Rheumatol* (1985) 3(4):285–6.
2. Miyakis S, Lockshin MD, Atsumi T, Branch W, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* (2006) 4(2):295–6. doi:10.1111/j.1538-7836.2006.01753.x
3. Branch DW, Silver RM, Porter TF. Obstetric antiphospholipid syndrome: current uncertainties should guide our way. *Lupus* (2010) 19(4):446–52. doi:10.1177/0961203310361490
4. Danza A, Ruiz-Irastorza G, Khamashta MA. Antiphospholipid syndrome in obstetrics. *Best Pract Res Clin Obstet Gynaecol* (2012) 26(1):65–76. doi:10.1016/j.bpobgyn.2011.10.006
5. Hughes GRV, Khamashta MA. Seronegative antiphospholipid syndrome. *Ann Rheum Dis* (2003) 62(12):1127. doi:10.1136/ard.2003.006163
6. Nayfe R, Uthman I, Aoun J, Saad Aldin E, Merashli Munther M, Khamashta MA. Seronegative antiphospholipid syndrome. *Rheumatology (Oxford)* (2013) 52(8):1358–67. doi:10.1093/rheumatology/ket126
7. Misasi R, Capozzi A, Longo A, Recalchi S, Lococo E, Alessandri C, et al. "New" antigenic targets and methodological approaches for refining laboratory diagnosis of antiphospholipid syndrome. *J Immunol Res* (2015) 2015:858542. doi:10.1155/2015/858542
8. Valesini G, Alessandri C. New facet of antiphospholipid antibodies. *Ann N Y Acad Sci* (2005) 1051(1):487–97. doi:10.1196/annals.1361.089
9. Sciascia S, Amigo MC, Roccatello D, Khamashta M. Diagnosing antiphospholipid syndrome: 'extra-criteria' manifestations and technical advances. *Nat Rev Rheumatol* (2017) 13(9):548–60. doi:10.1038/nrrheum.2017.124
10. Ortona E, Capozzi A, Colasanti T, Conti F, Alessandri C, Longo A, et al. Vimentin/cardiolipin complex as a new antigenic target of the antiphospholipid syndrome. *Blood* (2010) 116(16):2960–7. doi:10.1182/blood-2010-04-279208
11. Sorice M, Griggi T, Circella A, Lenti L, Arcieri P, di Nucci GD, et al. Protein S antibodies in acquired proteinS deficiencies. *Blood* (1994) 83(8):2383–4.
12. Conti F, Alessandri C, Sorice S, Capozzi A, Longo A, Garofalo T, et al. Thin-layer chromatography immunostaining in detecting anti-phospholipid antibodies in seronegative. *Clin Exp Immunol* (2012) 167(3):429–37. doi:10.1111/j.1365-2249.2011.04532.x
13. Harris EN. The second international anti-cardiolipin standardization workshop/The Kaps Group. *Am J Clin Pathol* (1990) 94(4):476–84. doi:10.1093/ajcp/94.4.476
14. Rodriguez-Garcia JL, Bertolaccini ML, Cuadrado MJ, Sanna G, Ateka-Barrutia O, Khamashta MA. Clinical manifestations of antiphospholipid

syndrome (APS) with and without antiphospholipid antibodies (the so-called 'seronegative APS'). *Ann Rheum Dis* (2012) 71(2):242–4. doi:10.1136/annrheumdis-2011-200614
15. Conti F, Capozzi A, Truglia S, Lococo E, Longo A, Misasi R, et al. The Mosaic of "Seronegative" Antiphospholipid Syndrome. *J Immunol Res* (2014) 2014:389601. doi:10.1155/2014/389601
16. Derksen RHWM, De Groot PG. Towards evidence-based treatment of thrombotic antiphospholipid syndrome. *Lupus* (2010) 19(4):470–4. doi:10.1177/0961203309361483
17. Conti F, Priori R, Alessandri C, Misasi R, Capozzi A, Pendolino M, et al. Diagnosis of catastrophic anti-phospholipid syndrome in a patient tested negative for conventional tests. *Clin Exp Rheumatol* (2017) 35(4):678–80.
18. Conti F, Alessandri C, Spinelli FR, Capozzi A, Martinelli F, Recalchi S, et al. TLC immunostaining for detection of "antiphospholipid" antibodies. *Methods Mol Biol* (2014) 1134:95–101. doi:10.1007/978-1-4939-0326-9_8
19. Schoenfeld Y, Twig G, Katz U, Sherer Y. Autoantibody explosion in antiphospholipid syndrome. *J Autoimmun* (2008) 30(1–2):74–83. doi:10.1016/j.jaut.2007.11.011
20. Zohoury N, Bertolaccini ML, Rodriguez-Garcia JL, Shums Z, Ateka-Barrutia O, Sorice M, et al. Closing the serological gap in the antiphospholipid syndrome: the value of "Non-criteria" antiphospholipid antibodies. *J Rheumatol* (2017) 44(11):1597–602. doi:10.3899/jrheum.170044
21. Žigon P, Perdan Pirkmajer K, Tomšič M, Kveder T, Božič B, Sodin Šemrl S, et al. Anti-phosphatidylserine/prothrombin antibodies are associated with adverse pregnancy outcomes. *J Immunol Res* (2015) 2015:975704. doi:10.1155/2015/975704
22. Mekinian A, Bourrienne M, Carbillon L, Benbara A, Noémie A, Cholllet-Martin S, et al. Non-conventional antiphospholipid antibodies in patients with clinical obstetrical APS: prevalence and treatment efficacy in pregnancies. *Semin Arthritis Rheum* (2016) 46(2):232–7. doi:10.1016/j.semarthrit.2016.05.006
23. Carmo-Pereira S, Bertolaccini ML, Escudero-Contreras A, Khamashta MA, Hughes GRV. Value of IgA anticardiolipin and anti-β2-glycoprotein I antibody testing in patients with pregnancy morbidity. *Ann Rheum Dis* (2003) 62(6):540–3. doi:10.1136/ard.62.6.540
24. Lee RM, Branch DW, Silver RM. Immunoglobulin A anti-β2-glycoprotein antibodies in women who experience unexplained recurrent spontaneous abortion and unexplained fetal death. *Am J Obstet Gynecol* (2001) 185(3):748–53.
25. Yamada H, Tsutsumi A, Ichikawa K, Kato EH, Koike T, Fujimoto S. IgA-class anti-beta2-glycoprotein I in women with unexplained recurrent spontaneous abortion. *Arthritis Rheum* (1999) 42(12):2727–8. doi:10.1002/1529-0131(199912)42:12<2727::AID-ANR33>3.0.CO;2-Q

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Inefficient N2-Like Neutrophils Are Promoted by Androgens During Infection

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Neutrophils are major effectors of acute inflammation against infection and tissue damage, with ability to adapt their phenotype according to the microenvironment. Although sex hormones regulate adaptive immune cells, which explains sex differences in immunity and infection, little information is available about the effects of androgens on neutrophils. We therefore aimed to examine neutrophil recruitment and plasticity in androgen–dependent and –independent sites under androgen manipulation. By using a bacterial model of prostate inflammation, we showed that neutrophil recruitment was higher in testosterone-treated rats, with neutrophil accumulation being positively correlated to serum levels of testosterone and associated to stronger inflammatory signs and tissue damage. Testosterone also promoted LPS-induced neutrophil recruitment to the prostate, peritoneum, and liver sinusoids, as revealed by histopathology, flow cytometry, and intravital microscopy. Strikingly, neutrophils in presence of testosterone exhibited an impaired bactericidal ability and a reduced myeloperoxidase activity. This inefficient cellular profile was accompanied by high expression of the anti-inflammatory cytokines IL10 and TGFβ1, which is compatible with the "N2-like" neutrophil phenotype previously reported in the tumor microenvironment. These data reveal an intriguing role for testosterone promoting inefficient, anti-inflammatory neutrophils that prolong bacterial inflammation, generating a pathogenic environment for several conditions. However, these immunomodulatory properties might be beneficially exploited in autoimmune and other non-bacterial diseases.

Keywords: neutrophils, testosterone, androgens, bacterial prostatitis, infection

INTRODUCTION

Neutrophil granulocytes lead the initial leukocyte influx to sites of injury in order to eliminate invading pathogens or damaged tissues. Their response is mediated by phagocytosis and NETosis, as well as by releasing defensins, enzymes, and cytokines to active the immune response (1). Subsequently, once the inflammatory stimuli has been eradicated, neutrophils die by apoptosis

and the elimination of apoptotic bodies by macrophages ensures the correct resolution of the inflammatory process and tissue repair (2). However, if the inflammatory process is not controlled, the products generated by neutrophils can induce multiple tissue alterations and loss of cellular function (3, 4). This is particularly important in endotoxemia- and burn-induced multiple organ dysfunction and in unresolved infections of the reproductive tract, where neutrophil activation could be harmful causing degradation of the extracellular matrix and additional gamete damage beyond that associated with the initial injury (5–7).

Although for decades it was thought that neutrophils constitute a homogeneous cell population, reports of neutrophils showing different behaviors in front of diverse steady state and pathological conditions are shifting this notion (8–10). For instance, neutrophils are able to shape and regulate immune and inflammatory responses against tumor cells by exerting either pro-tumor or anti-tumor effects on tumor development (9, 11). Given these varied effects, the concept of neutrophil plasticity and diversity has emerged, leading to the paradigm of anti-tumoral "N1 neutrophils" vs. pro-tumoral "N2 neutrophils" proposed by Fridlender and coworkers (9), in neoplastic scenarios. However, little is known about different neutrophil phenotypes promoted by non-tumoral environments as metabolic or hormonal imbalance.

The prostate gland is the main target of infectious and inflammatory diseases in the male reproductive tract, with prostatic inflammation representing a worldwide health issue. Moreover, a strong relationship between prostatitis and other conditions with high impact on human health such as male infertility (12), benign prostatic hyperplasia (13), and prostate cancer (14) has been reported. Unlike many organs in the body, the prostate is under the strict control of testicular male hormones for its development and function. Hence, it is not surprising that testosterone might influence the expression of pro- and anti-inflammatory mechanisms, as well as the local production of cytokines and chemokines, the recruitment and activation of immune cells, and the outcome of infectious diseases of the prostate gland. In this sense, we previously reported that testosterone negatively modulates the Toll-like receptor 4 (TLR4) pathway and downregulates antimicrobial substances in prostatic cells, which correlates with a decreased inhibition of bacterial growth in the presence of testosterone in both *in vivo* and *in vitro* models (15).

It has long been recognized that androgens dampen host defenses through multiple mechanisms of the adaptive immunity, explaining the sex-specific biases reported in immunocompetence, autoimmunity, and cancer incidence (16). Androgenic effects include apoptosis of T and B cells and the induction of T regulatory cells and CD8+ suppressive cells (16, 17). Additionally, in monocytes/macrophages, androgens reduce proinflammatory signaling (TLR4, TNFα, IL1β, and IL6) (18) but enhance anti-inflammatory (IL10) cytokine production (19). Regarding innate immunity, a few published articles have suggested that testosterone maintains a reduced expression of key elements such as TLRs and modulates the activity of different professional cells (18, 20). Nevertheless, the influence of androgen levels on neutrophil activity and plasticity in the initial inflammatory response remains to be investigated. Therefore, the aim of this work was to determine whether testosterone is able to modulate neutrophil recruitment and behavior in androgen dependent- and independent-sites.

MATERIALS AND METHODS

Animals

Wistar strain male rats, 12 weeks old, and weighing 250–350 g, were housed at the Animal Research Facility of the Universidad Nacional de Cordoba, in air-conditioned quarters, under a controlled photoperiod (14 h light/10 h darkness) with free access to rodent food and tap water. C57BL/6 mice were from Centro de Bioterismo in Universidade Federal de Minas Gerais (CEBIO, UFMG, Brazil). Animal care and experiments were conducted following the recommendations of the International Guiding Principles for Biomedical Research Involving Animals and approved by the local CICUAL (FCM-UNC, Argentina) Ethical Committee.

Androgen Manipulation and Prostatitis Models

Rats were orchidectomized via the scrotal route under ketamine (80 mg/kg)/ xylazine (8 mg/kg) and divided into three groups, receiving immediately testosterone s.c. at physiological (2 mg/kg/day; T group; Sustanon, Organon, Argentina) or supraphysiological doses (10 mg/kg/day; TT group), or vehicle alone (sunflower oil; OX group). To confirm the androgen status, serum total testosterone levels of individual rats were determined by electrochemiluminescence immunoassay using a Roche Elecsys E170 immunoassay analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

An acute bacterial prostatitis model was performed according to a protocol from our laboratory (21, 22). Briefly, two days after castration, OX, T, and TT rats were anesthetized and subjected to a laparotomy to expose the ventral prostate; infection was induced by an intraprostatic injection of 200 μL of *Uropathogenic E. coli* (10^8 CFU/ml, isolated from a patient with complicated urinary tract infection), with a 30-gauge needle. Animals were killed at 1, 3, and 5 days after infection, with the ventral prostate being processed for morphological, biochemical, and microbiological analyses. As controls, rats subjected to the same surgical procedures were used, replacing the bacterial suspension with sterile PBS.

On the other hand, a lipopolysaccharide (LPS)-induced model for prostatic inflammation was carried out by inoculating 50 μl of a solution of LPS from *E. coli* 055:B5 (20 mg/ml, Sigma–Aldrich, St. Louis, MO) in OX and T groups using the same surgical procedure described for bacterial prostatitis. Control animals received the vehicles. Ventral prostates were harvested and processed at 24 h after inoculation.

Neutrophil Recruitment to the Peritoneum and Microbial Killing Assay

Neutrophil recruitment was induced by a single i.p. injection of thioglycollate (3 ml of a 3% solution; Sigma–Aldrich, St. Louis, MO) or LPS from *E. coli* 055:B5 (1 mg/kg) in rats of OX

and T groups at day 3 post-castration. Sterile saline-injected animals were used as controls. Animals were anesthetized 4 h after thioglycollate or 12 h after LPS injection and the peritoneal lavage was harvested for analysis by injection of 10 mL of sterile PBS containing 0.835 UI/mL sodium heparin. The abdomen was gently massaged and the blood-free cell suspension was carefully aspirated with a syringe. The cell suspension was then spun for 6 min, 300×g, at 20°C. After removal of the supernatant, residual red blood cells were removed by hypotonic lysis and the cells were spun again for 6 min, 300×g, at 20°C. The cell pellet was washed and resuspended in 2 ml HBSS and total leukocyte counts were performed immediately in peritoneal lavage samples using a Neubauer chamber. To evaluate the neutrophil percentage, the differential leukocyte population was analyzed in cytospins after May-Grünwald-Giemsa staining (Biopur, Rosario, Argentina); a minimum of 500 leukocytes were counted, containing >85% pure populations of neutrophils. Additionally, peritoneal cells were stained with a FITC anti-rat granulocyte (Gr) antibody that recognizes rat neutrophils (BD Biosciences, San Jose, CA) and analyzed by flow cytometry using a FACSCanto II cytometer (Becton Dickinson, San Jose, CA).

For assessing *ex vivo* neutrophil bactericidal activity, thioglycollate-recruited neutrophils from OX and T groups were counted manually using a standard hemocytometer. Cytospins confirmed >90% neutrophils. *Uropathogenic E. coli* (the same used for prostatitis model) were pre-opsonized in 10% mouse serum on ice for 15 min. Neutrophils in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) were plated onto 24 well plates at 1×10^6 neutrophils/well and infected with equal volume of *E. coli* in serum at a multiplicity of infection of 1 bacterium:1 neutrophil. Following 10 and 40 min incubation times, 50 µl of the suspension were taken for bacterial colony-forming units (CFU) quantification by serial agar plating.

Intravital Liver Imaging

Mice were treated with testosterone (i.p. 10 mg/kg/day) or flutamide (s.c 7 mg/kg/day; Sigma–Aldrich, St. Louis, MO) for 3 days. Then, neutrophil recruitment was induced by a single i.p. injection of LPS from *E. coli* O111:B4 (0.5 mg/kg, Sigma–Aldrich, St. Louis, MO). Six hours after, mice received a single i.v. dose of a FITC anti-mouse Ly-6G antibody (4 µg/mouse; BioLegend, San Diego, CA), diluted in sterile saline (in a total volume of 100 µl), and confocal intravital imaging was performed as described (23, 24). In brief, mice were anesthetized (i.p.) with a mixture of ketamine (60 mg/kg) and xylazine (15 mg/kg) and a midline laparotomy was performed to expose the liver for imaging. Mice were imaged using Nikon Eclipse Ti (Nikon, Tokyo, Japan) with a C2 confocal head equipped with three different lasers (excitation at three wavelengths: 405, 488, and 543 nm) and emission bandpass filters at 450/50, 515/30, and 584/50 nm. The z-position is controlled by an automated device and 10X objective was used on the required resolution. Ten-minute movies were taken from each mouse and Ly-6G (+) neutrophil quantification was performed using Volocity 6.3 software (PerkinElmer, Waltham, MA).

Histopathological Analysis and Immunocytochemistry

Tissue samples of ventral prostates were formalin-fixed and paraffin-embedded for routine hematoxylin–eosin (H&E) staining (Biopur, Rosario, Argentina). An Axiostar Plus microscope equipped with a digital camera (Zeiss, Oberkochen, Germany) was used to acquire 60X photographs, which were examined using Fiji software (NIH, Bethesda, MD). For prostate neutrophil quantification, 20 fields of 2 different sections from the same gland were analyzed, with at least three animals per experimental group.

Immunocytochemistry was performed on slides from paraffin-embedded prostates, which were cleared with xylene and rehydrated in a descending concentration series of ethanol. Samples were then incubated in EDTA pH 9.0 to perform antigen retrieval using microwave pre-treatment (except for detection of *E. coli*). To block the endogenous peroxidase activity, slides were treated with H_2O_2 in methanol for 15 min. Sections were treated with PBS-BSA 5% to block non-specific binding for 30 min, followed by an overnight incubation with the primary antibody (diluted in 1% PBS-BSA) at 4°C in a humidified chamber. Afterwards, slides were incubated for 1 h with a specific biotinylated secondary antibody (at 1:180; Amersham Pharmacia, Buckinghamshire, UK) followed by 30 min in ABC complex (Vector, Burlingame, CA). Diaminobenzidine (Sigma–Aldrich, St. Louis, MO) was used as a chromogen substrate for 10 min and Harris hematoxylin as a counterstaining solution. Primary antibodies used for this study were: anti-PBP (rabbit polyclonal at 1:2000, developed by Dr. Maccioni (22), anti-ACTA2 (mouse monoclonal at 1:50; Novocastra, Newcastle, UK), and anti-*E. coli* antigen (rabbit polyclonal at 1:250, Affinity BioReagents, Golden, CO). For negative controls, antibodies were pre-absorbed with specific blocking peptides or replaced by rabbit or mouse normal serum.

Transmission Electron Microscopy

Tissue blocks (1 mm^3) from ventral prostate and pellets of peritoneal neutrophils were fixed in Karnovsky's mixture containing 2% (v/v) glutaraldehyde (EMS, Hatfield, PA) and 4% (w/v) formaldehyde in 0.1 M cacodylate buffer, pH 7.3, at 4°C for 24 h. Samples were post-fixed in 1% osmium tetroxide (EMS, Hatfield, PA) for 2 h and washed in 0.1 M cacodylate buffer, before being dehydrated through a graded series of cold acetone and embedded in Araldite epoxy resin (EMS, Hatfield, PA) as previously published (21, 22, 25). Ultrathin sections were cut in a JUM-7 ultramicrotome (Jeol, Tokyo, Japan) and examined in a Zeiss LEO 906E electron microscope (Zeiss, Oberkochen, Germany) with digital acquisition of images.

Sorting of Prostate Infiltrating Neutrophils by Flow Cytometry

Ventral prostates from rats with LPS-induced prostatitis were quickly excised, rinsed in fresh saline and weighted. Cell dissociation and neutrophil purification were performed using an adaptation of a published protocol (26). Briefly, tissues were minced into small fragments and treated with a digestion solution

containing 200 U/ml collagenase type IA (Sigma Aldrich, St. Louis, MO) and 0.05% deoxyribonuclease type I (Sigma Aldrich, St. Louis, MO) in HBSS without calcium, magnesium or phenol red, pH 7.4 for 30 min, at 37°C, and rocking at 60 rpm. The digested tissue was then passed through a 70-μm pore cell strainer with fresh sterile PBS. The cell suspension was then spun for 6 min, 300×g at 20°C. After removal of the supernatant, residual red blood cells were removed by hypotonic lysis and cells were spun again for 6 min, 300×g, at 20°C, washed, and resuspended in 1 ml of PBS. Cells were counted by collecting events for a fixed time (90 s) on a FACSCanto II cytometer. Neutrophil percentage was calculated by using a FITC anti-rat Gr monoclonal antibody (1/500 for 1 h at 4°C).

To purify prostate-infiltrating neutrophils, Gr (+) cells were sorted in a FACSAria II cell sorter (BD Biosciences, San Jose, CA), with a purity of >97% being achieved and confirmed by electron microscopy.

Measurement of Myeloperoxidase (MPO) Activity

Whole ventral prostate glands were removed, frozen in liquid nitrogen, and stored at −80°C. After thawing, tissue was weighed and homogenized on ice in 50 mM potassium phosphate buffer (1 g in 10 ml, pH 6.0). Homogenates were centrifuged (30.000×g, 15 min, 4°C) and pellets were resuspended in hexadecyltrimethylammonium bromide (HTAB; Sigma Aldrich, St. Louis, MO) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). Lysates were sonicated twice for 10 s each and freeze-thawed three times, after which sonication was repeated. Suspensions were then centrifuged (20.000×g, 15 min, 4°C) and the resulting supernatants were assayed for MPO activity. Supernatants (30 μl) were added to 970 μl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride (Sigma Aldrich, St. Louis, MO) and 0.0005% H_2O_2 and the change in absorbance at 460 nm (A460) was measured. One unit (U) of MPO activity was defined as that degrading 1 μmol of H_2O_2 per minute at 25°C and results were expressed as U MPO activity/g prostate and U MPO activity/mg protein, determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

To assess MPO activity in peritoneal cells, cellular MPO was extracted with 0.5% HTAB buffer from 4×10^6 cells obtained after hypotonic lysis of residual red blood cells. Lysates were then sonicated twice for 10 s each and spun for 15 min, 40.000×g, at 4°C. Resulting supernatant was assayed for MPO activity as described, with results being expressed as U MPO activity/mg protein and μU MPO activity/cells.

Microbiological Studies

To evaluate *in vivo* the growth of *E. coli*, pieces of ventral prostate from each rat were weighed, minced and gently homogenized in tryptic soy broth (1 g of tissue in 20 ml) in sterile conditions. Then, serial dilutions, i.e., 1/5 to 1/50, were made and 100 μl of these solutions were spread on Mueller Hinton agar, with the plates being incubated overnight at 37°C. Finally, bacterial counting was expressed as CFU per mg of prostatic tissue.

Ex vivo Determination of IL10 Production

Wistar rats were treated with flutamide (s.c 7.5 mg/kg/day) or its vehicle for 5 days. Peritoneal cells were harvested after 4 h of thyoglycollate i.p injection, washed, and resuspended in HBSS as described above. Total cells were then plated onto 24 well plates (1×10^6 neutrophils/well in RPMI 1640) and pulsed *ex vivo* with LPS O55:B5 1 μg/ml for 24 h to elicit cytokine secretion. *In vitro* assays maintained the same conditions as *in vivo* (i.e., flutamide stimulation). Immunofluorescence for IL10 in neutrophils was carried out on methanol-fixed, permeabilized cells on coverslides using PE anti-rat IL10 (1/50; BD Biosciences, San Jose, CA) and FITC anti-rat Gr (1/50) antibodies.

For flow cytometry, cells were treated with cycloheximide 100 μM (Sigma Aldrich, St. Louis, MO) 3 h before the analysis. The cell concentration was adjusted to 5×10^5 cells/ml and labeled for 30 min with the following antibodies: PE anti-rat IL10 (1/60), APC anti-rat CD11b (1/100; BD Biosciences, San Jose, CA), and FITC anti-rat Gr (1/150). Signals were acquired in a FACSCanto II cytometer and analyzed using FlowJo X software (Tree Star, Ashland, OR).

Immunoblotting

Prostate tissues were minced and homogenized on ice with a teflon-glass tissue grinder in 2 ml cold PBS containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM PMSF, 10 ug/ml leupeptin, and 10 ug/ml aprotinin. The lysate was centrifuged at 14,000×g for 20 min at 4°C and the supernatant was withdrawn and stored in aliquots frozen at −70°C until required. Prostatic lysates from triplicate experimental conditions were pooled before loading into electrophoresis gels. Total protein concentration was measured with a Bio-Rad Protein Assay kit. For western blot, denatured protein samples (45 μg/lane) were separated on 12% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amersham Pharmacia, Freiburg, Germany). Incubation steps were performed in 5% defatted dry milk in PBS/0.1% Tween 20. Blots were incubated for the detection of SP-D (rabbit polyclonal antibody at 1:1000; Chemicon, Temecula, CA) during 3 h. After that, membranes were treated with peroxidase-conjugated goat anti-rabbit antibodies (Jackson, West Grove, PA) and visualized applying the chemiluminescence technique. The expression of β-actin (mouse monoclonal antibody at 1:5000, Sigma Aldrich, St. Louis, MO) was used as an internal control to confirm the equivalent total protein loading.

The peptide βdefensin-1 (HBD-1) was tested in homogenates of ventral prostates by dot blot. For that purpose, prostatic lysates were matched at a concentration of 100 μg/ml, and 4 μl of each sample was spotted onto a Hybond C Super membrane (Amersham-Pharmacia, Freiburg, Germany). The membrane was then treated as explained above for western blot using an anti-HBD-1 (at 1:250, Santa Cruz Biotech, Santa Cruz, CA) as primary antibody.

Semiquantitative signals were derived by densitometric analysis from western and dot blots using Fiji software and data were displayed as area units.

ELISA

In order to quantify TNFα in prostate homogenates, tissues were minced and homogenized on ice in cold PBS, as described for immunoblotting, centrifuged at 4°C, 1.400 rpm for 15 min and stored at −20°C until the day of the assay. TNFα amount was measured by a commercially available sandwich ELISA kit (eBioscience, San Diego, CA), following the manufacturer's instructions.

Measurement of Gene Expression by Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA samples were extracted from peritoneal neutrophils and prostate Gr (+)- and Gr (–)-sorted cells using TRIzol (Thermo Fisher, Carlsbad, CA). RNA was subsequently purified using Direct-zol RNA miniprep kit (Zymo Res., Irvine, CA) according to manufacturer's instructions. Then, 0.5 μg of RNA was used as a template for reverse transcription using EpiScript™ Reverse Transcriptase (Epicentre, Madison, WI) with random hexamer primers (Fermentas, Thermo Fisher, Carlsbad, CA). qPCR was performed with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in a ABI Prism 7500 detection system (Thermo Fisher, Carlsbad, CA). The expression of ACTB was chosen as housekeeping gene. Data analysis was based on the $2^{-\Delta\Delta Ct}$ method for normalization of raw data. All primers used are described in the **Supplementary Table 1.**

Statistical Analysis

The characterization of data was accomplished by comparing their mean values ± standard error of the mean (SEM) from at least four independent protocols. Statistical differences between means were analyzed by unpaired Student's *t*-test. Data from more than two groups were analyzed using analysis of variance (ANOVA) with Tukey as the post-test to compare all pairs of columns. Significant differences were considered at $p < 0.05$. Statistical analyses and graphics were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6 (La Jolla, CA).

RESULTS

Androgen Withdrawal Modifies the Progression of the Inflammatory Response in the Prostate Gland

In order to study the effects of testosterone on prostatitis progression, castrated animals treated with different doses of testosterone were subjected to intraprostatic bacterial inoculation and analyzed at days 1, 3, and 5 after infection. As shown in **Figure 1A**, the prostate from rats supplemented with testosterone exhibited a massive neutrophil infiltration and invasion into the prostatic acini; this effect was more evident at day 5 post-infection. Moreover, serum androgen levels positively correlated to the number of neutrophils infiltrating the gland (**Figure 1B**). In contrast, rats with low testosterone levels displayed reduced inflammatory signs, with few focal infiltrates in the prostatic interstitium. Accordingly, the prostatic levels of TNF-α, SP-D, and HBD-1 were higher in rats treated with high doses of testosterone (**Figure 1C**), demonstrating a stronger inflammatory reaction in these animals. Considering the deleterious effects of neutrophils on tissue function, we assessed the histoarchitecture as well as the expression of prostatic binding protein (PBP) and α-smooth muscle actin (ACTA2, markers of epithelial secretory function and stromal integrity respectively) of the prostate gland. At day 5 post-infection, both the secretory and the stromal compartments of the ventral prostate were conserved in the castrated animals as judged by PBP and ACTA2 expressions (**Figure 1D**). This was consistent with the conservation of ultrastructural features, including a prominent Golgi complex, developed endoplasmic reticulum, and secretory vesicles (**Figure 1E**). Conversely, the epithelial layer of the testosterone-treated rats exhibited atrophy, desquamation, and invasion of neutrophils, suggesting that the exacerbated presence of neutrophils is associated to more inflammatory signs and tissue damage in the prostate gland of animals with high levels of testosterone. Taking together, these results evidence adverse effects of testosterone in a dose dependent manner during bacterial acute prostatitis.

Testosterone Increases Neutrophil Recruitment Independently of the Stimulus Nature or the Site of Injury

The higher amount of neutrophils observed in testosterone-treated animals could be attributed to an androgen-related neutrophil malfunction which, unable to eradicate bacteria, maintains a constant recruitment of inflammatory cells. In order to further understand this, we used bacterial LPS instead of the live bacterium as stimulus. In line with the bacterial-induced prostatitis model, LPS promoted an intense inflammatory infiltration in testosterone-treated animals, resulting in a higher number of neutrophils within the prostate, assessed in histological slides as well as by flow cytometry using a specific antibody (anti-Gr) for rat neutrophils (**Figures 2A,B**).

Testosterone-treated rats also displayed a higher thioglycollate-induced neutrophil recruitment to the peritoneum (not shown), with this effect being even stronger when neutrophil recruitment was elicited by LPS for 12 h (**Figure 2C**), indicating that testosterone is associated to a higher recruitment of neutrophils not only in androgen-dependent but also in androgen-independent sites. Indeed, to corroborate this notion, a widely validated intravital mouse model (23, 24, 27) for visualization of LPS-induced neutrophil recruitment to the liver was performed. In mice treated with testosterone, LPS injection caused a massive neutrophil adhesion to hepatic sinusoids, which was reduced by the testosterone inhibitor flutamide (**Figure 2D** and **Supplementary Movie 1**).

Taking in mind that neutrophil recruitment from peripheral blood is a consequence of local chemokine production, mainly CXCL1 and CXCL2 (28), their mRNA expressions were analyzed in cells from prostates and peritoneal cavity after LPS challenge. As shown in **Figure 3A**, both chemokines increased after testosterone restoration in resident prostatic

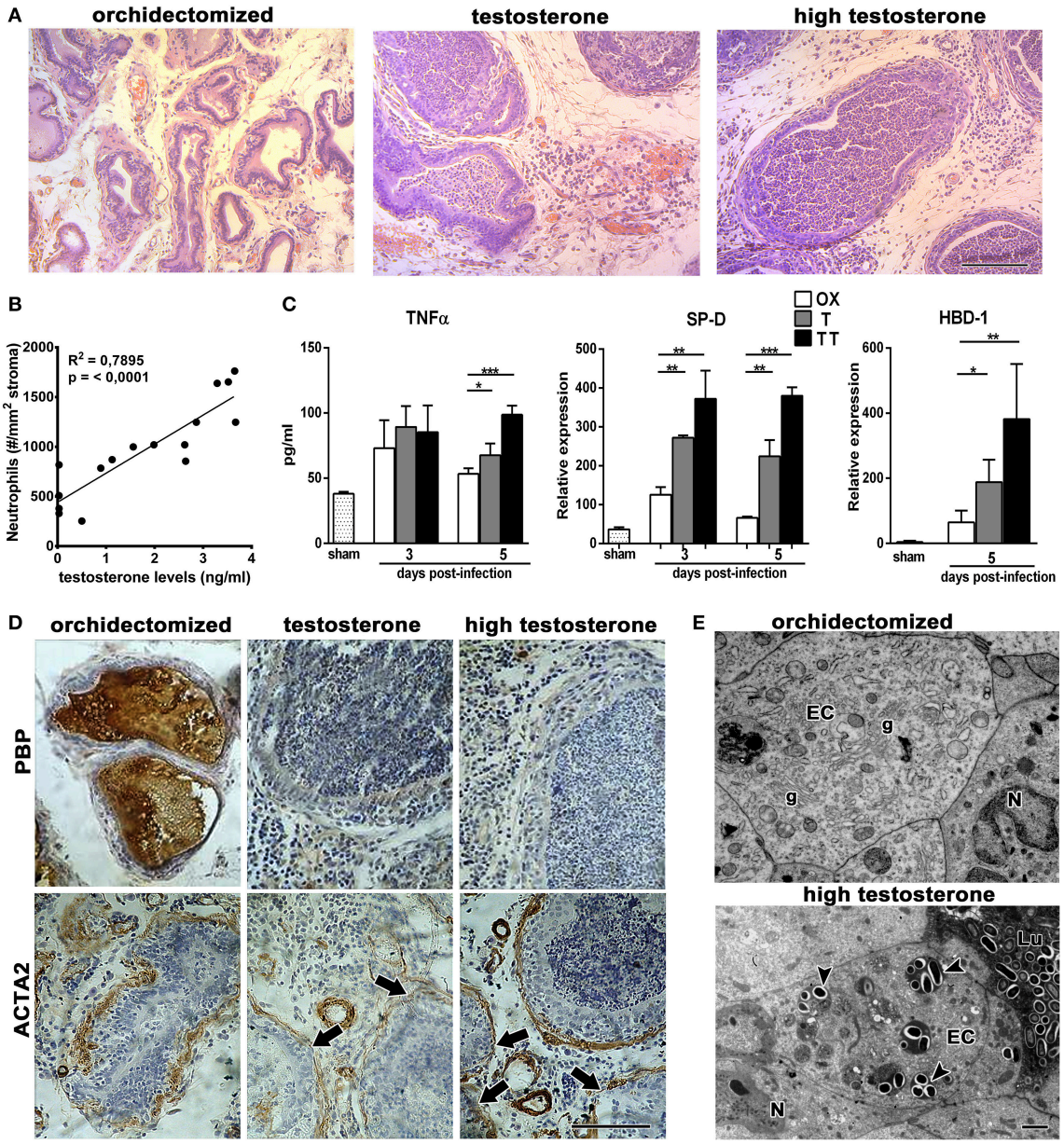

FIGURE 1 | Androgens increase neutrophil infiltration and tissue damage in bacterial infection of the prostate gland. Rats were orchidectomized (OX) and treated with testosterone 2 mg/kg/day (T) or 10 mg/kg/day (high testosterone-TT) before being inoculated with *E. coli* intraprostatically. **(A)** H&E staining at day 5 after infection shows not only interstitial inflammatory infiltrates but also a massive neutrophil invasion to prostatic acini in testosterone-treated animals. Bar = 100 μm. **(B)** Correlative analysis showing serum testosterone levels and prostatic neutrophil counts ($n = 16$, Pearson's correlation test). Neutrophils counts were calculated on H&E sections at day 5 post-infection. **(C)** The inflammatory parameters TNFα, SP-D, and HBD-1 are strongly increased in the prostate from testosterone-treated animals, with the TT group showing the highest levels (mean ± SEM; $n = 4$ per group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). **(D)** Representative images of immunocytochemistry for PBP and ACTA2, at day 5 post-infection, displaying a loss in epithelial secretory function when testosterone levels remain high (top images). ACTA2, as a marker for stromal organization, shows a weak expression and disruptions in the periacinar layer (arrows in bottom) of these animals. Bar = 100 μm. **(E)** At ultrastructural level, prostatic damage was related to the presence of numerous bacteria in the lumen (Lu) and invading epithelial cells (arrowheads, bottom). Orchidectomized rats show conserved morphology, with the conservation of secretory organelles such as Golgi complexes (g). EC, epithelial cell; N, neutrophil. Bar = 2 μm.

cells. Moreover, CXCL1 and CXCL2 were enhanced in Gr(+)-sorted neutrophils infiltrating the gland in the LPS-induced prostatitis model (**Figure 3B**) as well as in LPS-elicited peritoneal cells from animals treated with testosterone (**Figure 3C**). Interestingly, the expression levels of MCP-1 did not change significantly between groups (**Figure 3B**). Altogether, these data indicate that androgens promote neutrophil recruitment in both androgen-dependent and –independent sites by increasing the

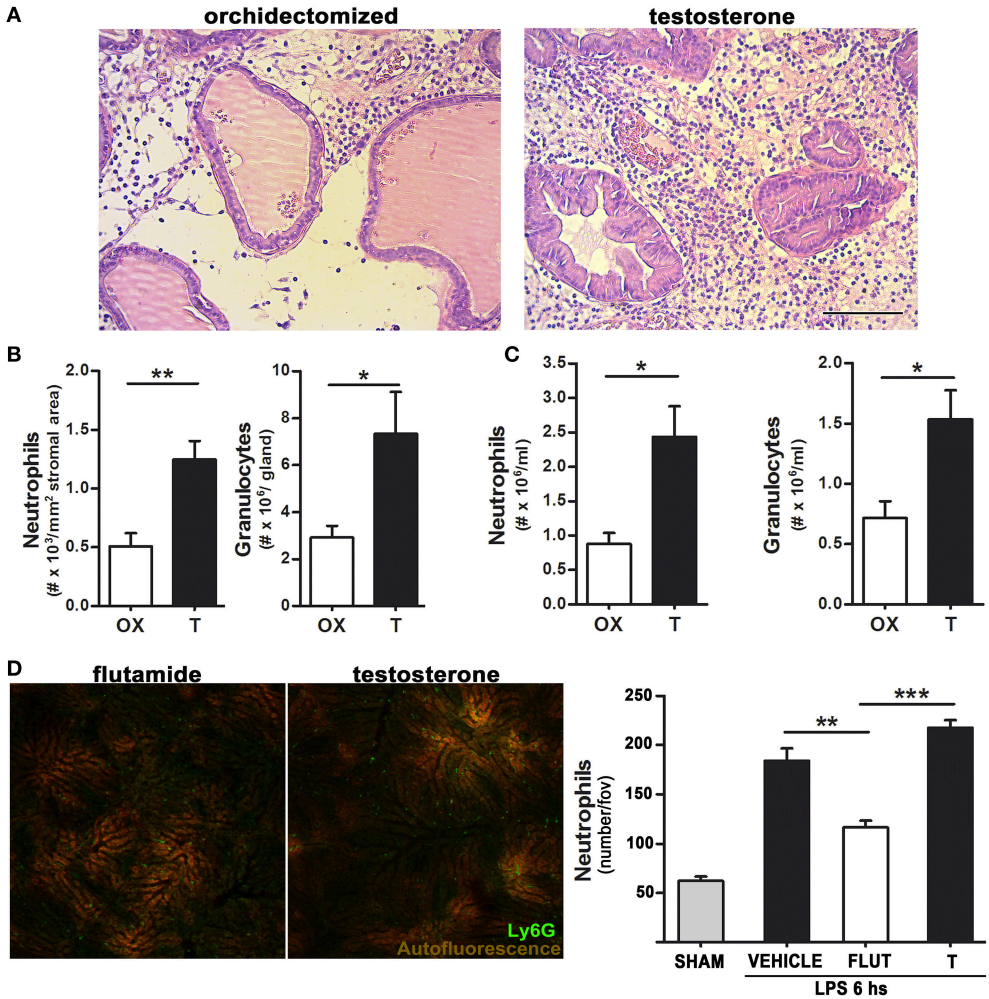

FIGURE 2 | Testosterone signaling favors LPS-induced neutrophil recruitment in both androgen-dependent and –independent sites.**(A)**–**(B)** Rats were orchidectomized (OX) and treated with testosterone 2 mg/kg/day (T) before being inoculated with 1 mg of LPS intraprostatically for 24 h. **(A)** Representative H&E staining showing an intense neutrophil infiltration in testosterone-treated animals. Bar = 100 μm. **(B)** Quantification of neutrophil recruitment to the prostate in H&E-stained slides (left) and by flow cytometry using a FITC anti-Gr antibody (right). **(C)** Peritoneal neutrophil recruitment was achieved by injecting LPS 1 mg/kg for 12 h, with the quantification of neutrophils being performed by hemocytometer (left) and flow cytometry (right). **(D)** Neutrophil recruitment in the liver observed by intravital microscopy after an i.p. LPS 0.5 mg/kg injection. Mice were previously treated with testosterone (T; 10 mg/kg/day) or with the inhibitor of androgen signaling flutamide (FLUT; 7 mg/kg/day). The presence of testosterone increases neutrophil recruitment to the liver sinosoids. Left: representative images (see **Supplementary Movie 1**). Right: quantification of Ly6G (+) neutrophils per field of view. Data represent the mean ± SEM from at least three independent animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

expression of specific chemokines in local cells as well as in neutrophils.

Androgens Decrease the Bactericidal Ability of Neutrophils

Ultrastructural analysis of the prostate revealed frequent undigested bacteria within neutrophils and invading the epithelial layer in animals treated with testosterone after 5 days of bacterial infection (**Figure 4A**). This was consistent with an intense immunostaining for *E. coli* in prostates from testosterone-treated rats compared to those from castrated animals (**Figure 4B**); this was also confirmed by bacterial cultures (data not shown), indicating an abnormal clearance of bacteria in presence of androgens. In contrast, most of neutrophils from castrated animals exhibited apoptotic features and absence of phagocytosed bacteria (**Figure 4A**), along with a weak *E. coli* immunostaining, all signs compatible with an accurate resolution phase of inflammation. In addition, thioglycollate-elicited peritoneal neutrophils from castrated or testosterone-treated rats were challenged *ex vivo* to *E. coli* and bacterial counting and electron microscopy was performed at 10 and 40 min after coincubation. As shown in **Figure 4C**, neutrophils from testosterone-treated rats decreased their bactericidal ability. Moreover, the ultrastructural analysis of these cells revealed

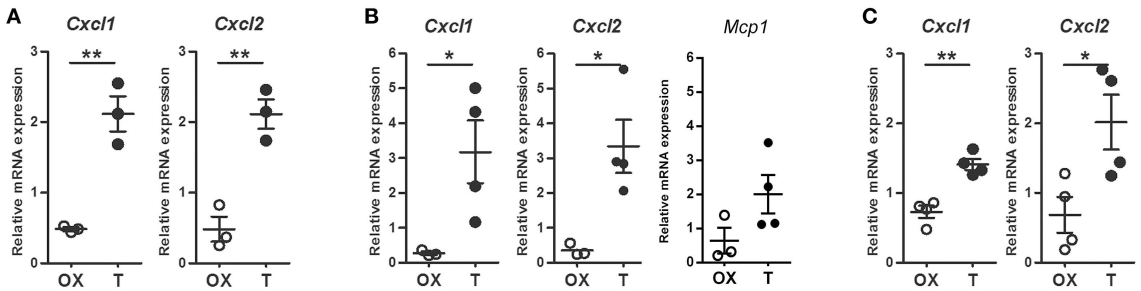

FIGURE 3 | Androgens augment LPS-induced, neutrophil-specific chemokine mRNA expression. **(A–B)** Rats were orchidectomized (OX) and treated with testosterone 2 mg/kg/day (T) before being inoculated with 1 mg of LPS intraprostatically for 24 h. Cell sorting was carried out to isolate Gr (+) neutrophils from prostatic cells and mRNA expression was evaluated by qPCR. **(A)** Testosterone treatment promotes an increase in CXCL1 and CXCL2 in prostatic cells. **(B)** Neutrophils exhibit a similar behavior, with no changes in Monocyte Chemoattractant Protein-1 (MCP1), the key chemokine regulating migration of monocytes/macrophages. **(C)** LPS-induced peritoneal neutrophils from testosterone-treated animals (T) showing an increase in CXCL1 and CXCL2. In all cases, the mRNA levels are relative to those of ACTB. Mean ± SEM, each dot represents one animal. $^{*}p < 0.05$; $^{**}p < 0.01$.

frequent undigested live bacteria in both the extracellular and intracellular compartments (**Figure 4D**), confirming that androgens regulate negatively neutrophil activity even in androgen-independent sites.

MPO is packaged in the azurophilic granules of neutrophils and released into phagosomes when they uptake and kill bacteria (1). We wondered whether the decreased bactericidal effect of neutrophils associated to testosterone could be related to an alteration in the activity of MPO. Prostatic tissues from rats with LPS-induced prostatitis and low level of testosterone showed a higher MPO activity compared to those with normal androgen status (**Figure 5A**). Consistently, a lower MPO activity per neutrophil was observed in LPS-induced peritoneal neutrophils of animals treated with testosterone (**Figure 5B**). Interestingly, when the MPO mRNA expression was assessed in Gr (+)-sorted prostate infiltrating neutrophils, no differences were found between groups (**Figure 5C**), suggesting that androgens regulate MPO at post-transcriptional level.

Testosterone Favors a "N2-Like" Neutrophil Phenotype, With High Expression of Anti-inflammatory Cytokines

Considering the existence of different neutrophil phenotypes, particularly in the tumor microenvironment (9), we wondered if testosterone manipulation could result in a shift of cytokine expression by neutrophils in the context of acute inflammation. By using the LPS-induced prostatitis model, performed in castrated or testosterone-treated rats, the mRNA expression for "N1-" and "N2-like" neutrophils was assessed by qPCR in Gr (+)-sorted cells. Prostatic neutrophils from animals supplemented with testosterone displayed a higher expression of IL10 and TGFβ1 along with a lower IL12 expression compared to those with low testosterone (**Figure 5D**). When analyzing LPS-recruited peritoneal neutrophils, similar findings were observed, with high testosterone levels being related to high IL10 and TGFβ expressions and to a decrease in the pro-inflammatory cytokines IL12, IL1β, and TNFα (**Figure 5E**). On the other hand, the morphological evaluation at ultrastructural level showed the existence of clear alterations in LPS-recruited prostatic neutrophils from testosterone-treated animals, including cellular swelling and the occurrence of vacuoles (**Figure 5F**). These findings suggest that in acute inflammatory scenarios, androgens promote "N2-like" anti-inflammatory and dysfunctional neutrophils, which extend the inflammatory process.

Together, these data reveal a stimulatory effect of testosterone on neutrophil-produced anti-inflammatory cytokines. To further corroborate these results, rats were treated with flutamide (an antiandrogen widely used in therapy) for 5 days; peritoneal cells were harvested after 4 h of thioglycollate and pulsed *ex vivo* with LPS for 24 h to elicit cytokine secretion. As expected, flow cytometry analysis demonstrated that flutamide decreased the expression of IL10, not only in the granulocytic population but also in mononuclear cells (whose frequency was increased by the anti-androgen, as shown in **Figure 6**).

DISCUSSION

Sex differences in mortality and immunocompetence are well documented in humans and other vertebrates (29). Males are at higher risk of developing acute respiratory distress, sepsis, and multiorgan failure after traumatic hemorrhagic shock and thermal injury, in part due to abnormal activation of neutrophils (5). Although morphological differences in granulocytes from men and women have been known since 1954 (30), the need to expose factors explaining functional differences has resurfaced recently in order to adapt new treatments depending on the characteristics of the pathophysiological process in each sex. In this sense, various studies have uncovered important immune regulatory functions for androgens, including effects on neutrophil accumulation (31–33), maturation, activation (34), and survival (35). In line with this evidence, we here report that testosterone increases local chemokine expression, leading to a higher recruitment of neutrophils to the site of infection, but at the same time, these cells exhibit a "N2-like" phenotype with a reduced efficiency in killing bacteria and high expression of immunomodulatory molecules such as IL10 and TGFβ1.

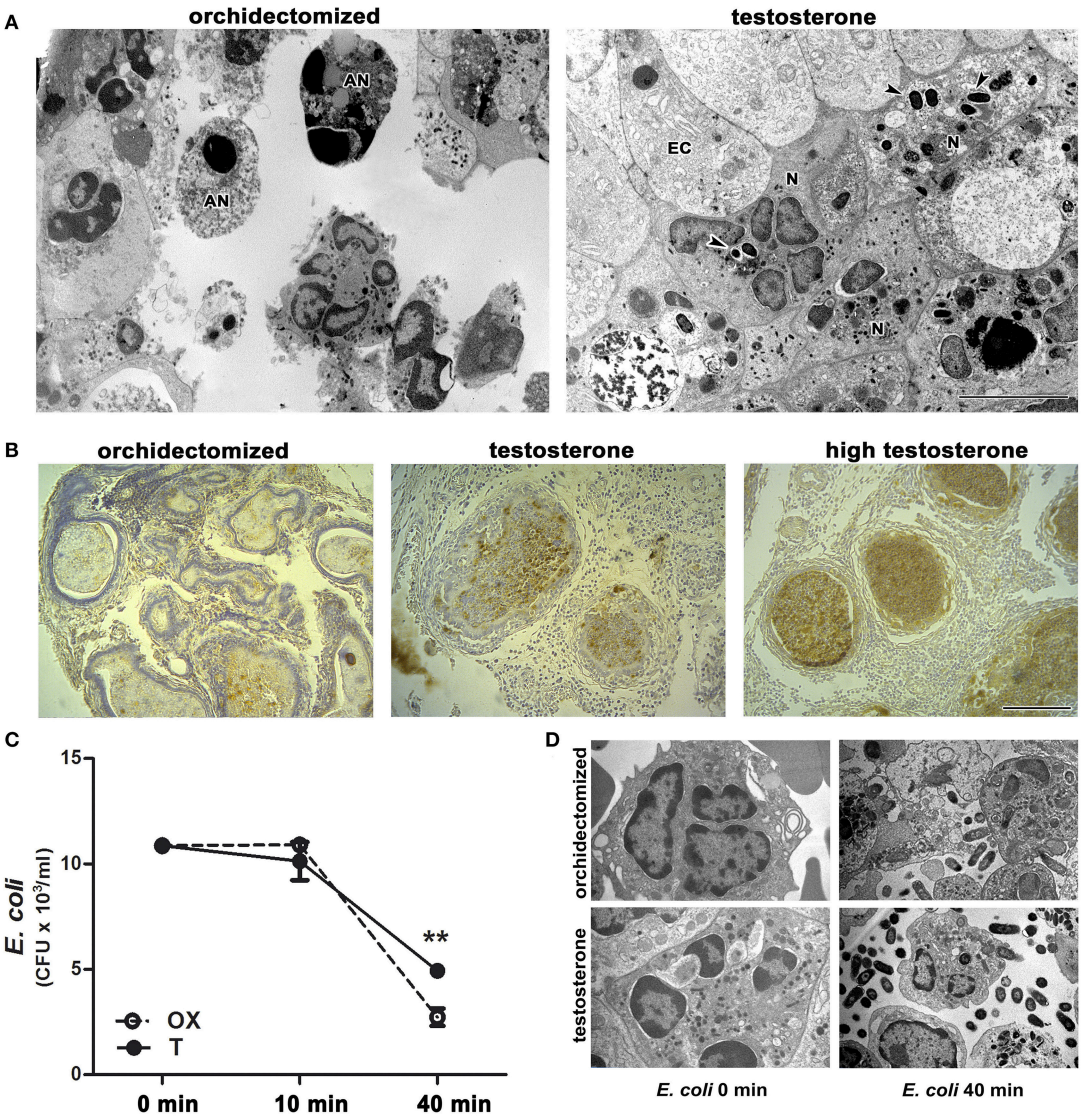

FIGURE 4 | Testosterone treatment impairs neutrophil ability to kill bacteria. **(A-B)** Rats were orchidectomized and treated with testosterone at 2 mg/kg/day or 10 mg/kg/day (high testosterone) before being inoculated with *E. coli* intraprostatically. **(A)** Representative electron microscopy images showing apoptotic neutrophils (AN) free of bacteria (left), while prostate infiltrating neutrophils (N) in testosterone-treated rats display intact phagocytosed bacteria (right, arrowheads) after 5 days of infection. EC: epithelial cell. Bar = 5 μm. **(B)** This is consistent with the intense *E. coli* immunostaining, localized in intracinar neutrophils in testosterone- and high testosterone-treated rats. Bar = 100 μm. **(C–D)** Bacterial growth after being co-incubated *ex vivo* with peritoneal neutrophils from orchidectomized (OX) and testosterone-treated (T) rats. The reduced bactericidal ability of neutrophils from T rats depicting in (**C**) is also seen at ultrastructural level **(D)** where abundant intact free bacteria are observed in presence of testosterone. Data are representative from at least 3 independent experiments. $^{**}p < 0.01$.

Neutrophil recruitment is an important early step in inflammatory response against pathogenic invasion or sterile tissue damage. The recruitment of neutrophils into the tissue is initiated by neutrophil-active chemoattractants, mainly CXCR2 ligands CXCL1 and CXCL2, released from danger signal-activated professional tissue-resident sentinel cells or stromal cells (28). Recruited neutrophils into injury site can also deliver active chemokines directly contributing to their own recruitment (36). In the present study, we observed that the presence of testosterone leads to an increased bacterial-induced mRNA expression of CXCL1 and CXCL2, which was associated to a higher neutrophil recruitment. In agreement, androgen supplementation has been shown to augment neutrophil infiltration in penile urethroplasty (33) and after myocardial infarction in both male and females (31). Human studies demonstrated that decreasing testosterone levels results in an attenuation of exercise-induced neutrophil accumulation in muscles (37), indicating a strong positive correlation between androgen levels and neutrophil infiltration. Furthermore, hyperandrogenemia is associated to a higher neutrophil count in steady state conditions in women with polycystic ovary syndrome (38). The excessive or aberrant

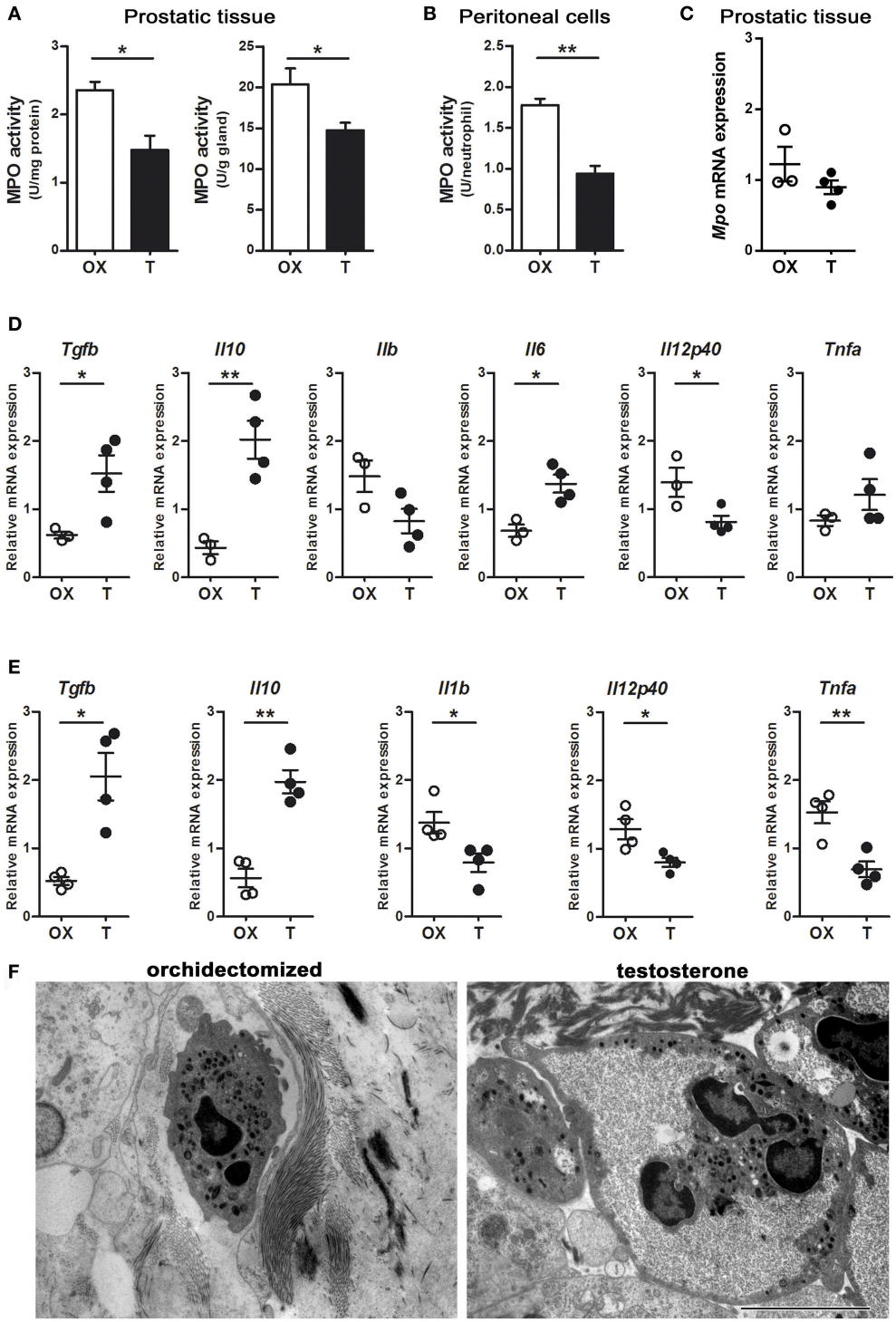

FIGURE 5 | Androgens modulate neutrophil phenotype. Rats were orchidectomized (OX) and treated with testosterone 2 mg/kg/day (T) before being inoculated with 1 mg of LPS intraprostatically for 24 h **(A, C, D, F)**. To elicit peritoneal neutrophils, a single i.p. injection of LPS 1 mg/kg was applied **(B, E)**. **(A)** Myeloperoxidase (MPO) activity in prostatic tissue is impaired in testosterone-treated animals, referred per mg of proteins (left) as well as per g of tissue (right). **(B)** Peritoneal neutrophils also show a decrease in MPO activity in animals treated with T. Data are mean ± SEM, from $n = 4$ per group. $^{*}p < 0.05$; $^{**}p < 0.01$. **(C)** The mRNA expression for MPO show no changes between groups. **(D)** Cytokine profiling of Gr (+)-sorted prostatic neutrophils by qPCR, depicting that cells from testosterone-treated animals express high levels of anti-inflammatory TGFβ and IL10, while pro-inflammatory cytokines are reduced. **(E)** Peritoneal neutrophils from testosterone-treated rats also have an anti-inflammatory/ immunomodulatory/"N2-like" phenotype, compatible to that reported by Fridlender et al. (9) and characterized by high expression of TGFβ and IL10 along with low levels of IL1b, IL12p40, and TNFα. ACTB was used as reference mRNA. Mean ± SEM, each dot represents one animal. $^{*}p < 0.05$; $^{**}p < 0.01$. **(F)** Representative images of prostatic neutrophils showing cellular edema and vacuolization in testosterone-treated animals. Bar = 5 μm.

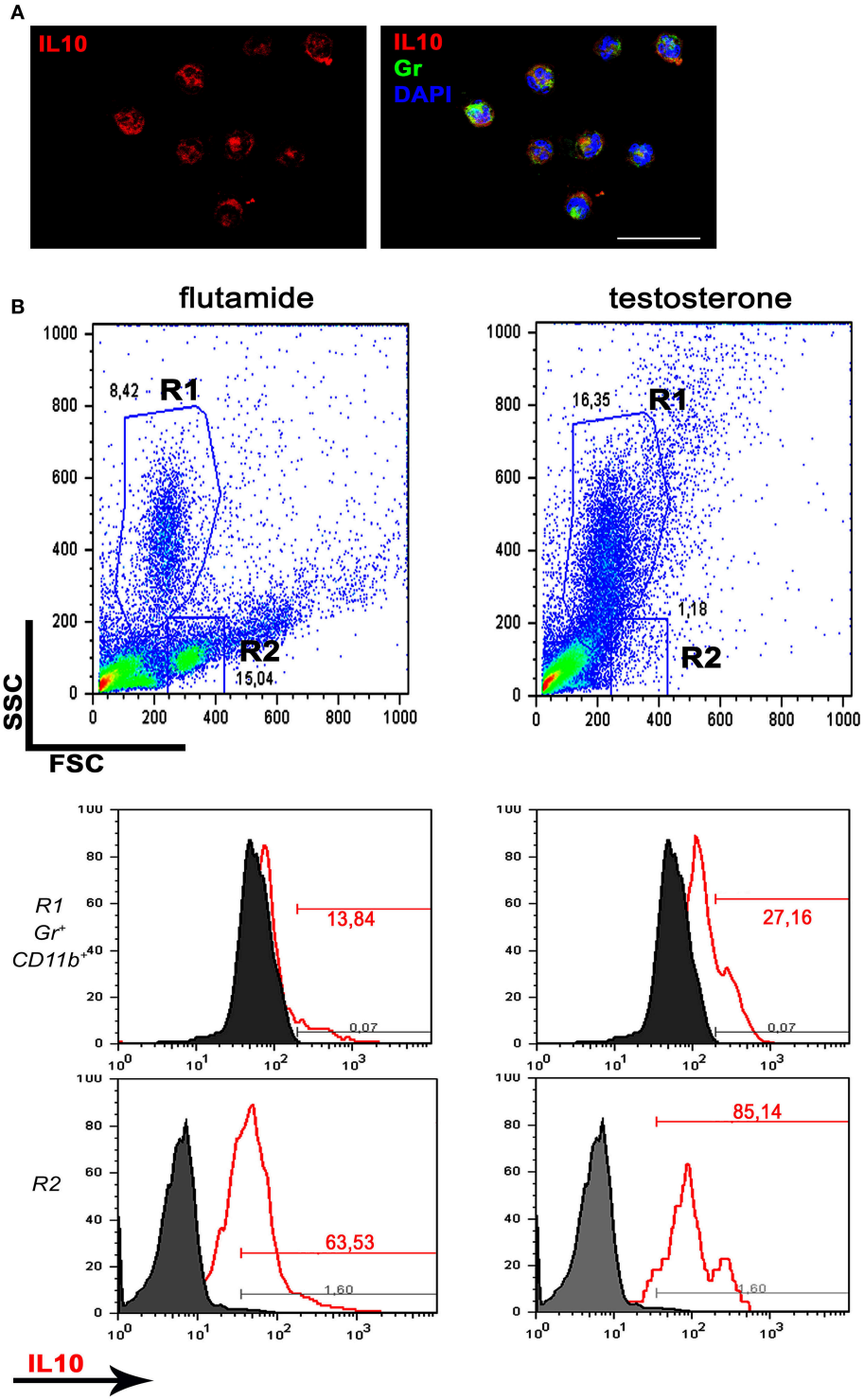

FIGURE 6 | Antiandrogen therapy inhibits IL10 production. Rats were treated with flutamide (7.5 mg/kg/day) for 5 days and thioglycollate-elicited peritoneal cells were pulsed *ex vivo* with LPS 1 μg/ml for 24 h. **(A)** Immunofluorescence for IL10 in absence of antiandrogens, analyzed by confocal microscopy. Most of Gr (+) peritoneal neutrophils express IL10. Bar = 50 μm. **(B)** Flow cytometry showing a decrease in the granulocytic population (R1) along with an increase in the frequency of mononuclear cells (R2) after flutamide treatment (representative dot plots; $n = 3$; top panels). The Gr (+) CD11b (+) R1 neutrophil population shows a decrease in IL10 expression after flutamide treatment (center panels, left) while the monocytic R2 population also displays low expression of IL10 in the same group (bottom panels). Data are representative of $n = 3$.

neutrophil infiltration in the presence of high androgen levels seems to favor tissue damage and organ dysfunction not only after infection but also in non-infectious conditions (31, 33). However, novel evidence suggests that neutrophil accumulation could also play a positive role in organ homeostasis by resolving inflammation (39–42), with a phenotypic characterization (i.e., pro-inflammatory vs. pro-resolving neutrophils) being necessary in order to predict the final effect of these cells on damage progression and tissue function.

Unlike other immune cells, existence of clearly defined neutrophils subtypes remains unclear. Accumulating evidence suggests that neutrophils may exhibit certain plasticity according to the microenvironment (8, 9, 43, 44). For instance, different subtypes of neutrophils were identified during infection with methicillin-resistant *S. aureus* (MRSA), associated either to resistance or to susceptibility to infection in mice (8). Neutrophils from MRSA-resistant hosts show a pro-inflammatory phenotype, with IL12 and CCL3 production, whereas those from MRSA- susceptible mice are anti-inflammatory in nature (IL10+/CCL2+), inducing M2 macrophages (8). Neutrophil polarization to an IL10-producing anti-inflammatory phenotype has also been reported by different pathogens (43, 45) as well as by serum amyloid A1 (46). Nevertheless, neutrophil subsets have mainly been characterized in tumoral conditions, where constitutively produced cytokines and growth factors can promote polarization of cells recruited into the tumor (9, 47). Of note, IFNβ and TGFβ1 induce neutrophils to acquire anti-tumoral (N1) or pro-tumoral (N2) phenotypes respectively (9, 47). N2 neutrophils are postulated to have a main role in promoting tumoral growth by increasing extracellular matrix deposition and by dampening a proper immune response (9). In this context, TGFβ1 appears as a central player in the tumor microenvironment orchestrating diverse pro-tumoral, anti-inflammatory, and immunomodulatory actions, including the induction and maintenance of an N2 phenotype. The induction of "N2-like" neutrophils in the presence of testosterone in our study can also be explained by overexpression of TGFβ1 since its promoter activity is directly regulated by androgens through the androgen receptor (AR) (48, 49). However, whether TGFβ1 acts directly or by other mechanisms to induce N2 neutrophil maturation by androgens deserves further research.

The ability of androgens to promote anti-inflammatory/immunomodulatory phenotypes has been previously recognized for professional immune cells (16, 20). In general, testosterone tends to inhibit pro-inflammatory molecules such as TNFα, iNOS, and NO whereas induces IL10 and TGFβ1 anti-inflammatory signaling (19, 50–52). Accordingly, we found that androgens favor a higher IL10 and TGFβ1 expression along with a lower IL12 expression on recruited neutrophils. Strikingly, this phenotype was accompanied by a reduction in both MPO activity and bactericidal ability *in vivo* as well as *ex vivo*, resulting in an inadequate bacterial clearance. In accordance, it has been reported that, *in vitro*, testosterone decreases the microbicidal activity of human neutrophils by dampening the production of reactive oxygen species (53, 54). In contrast, after trauma or burn injury, androgens have been reported to enhance neutrophil activation in blood, as judged by CD11b expression and respiratory burst activity (32), which might explain why males are more susceptible to acute shock aggressiveness than females. These ambivalent results could be attributed to the type of stimuli used and the pathological conditions, with scarce data available on the bactericidal ability of neutrophils after androgen manipulation. In any case, the promotion of "N2-like" neutrophils by androgens may be deleterious as well as favorable, depending on the particular context; albeit being inefficient in killing bacteria, their ability to generate TGFβ1, as described before (39, 40), could be beneficial in repairing tissues and resolving inflammation (39, 40).

Our results indicate that testosterone modulates neutrophil activity within the prostate and in androgen-independent sites as well. In a bacterial milieu, testosterone promotes, in a dose dependent manner, a recruitment of malfunctioning neutrophil that amplifies and prolongs the inflammatory response, with the persistence of their toxic products destroying cellular components and generating a favorable environment for the development of pathologies (14, 55). On the other hand, the testosterone-induced anti-inflammatory profile displayed by these neutrophils could be beneficial in some non-bacterial types of inflammation.

AUTHOR CONTRIBUTIONS

MS and AQ performed most protocols. MS, NP, MC, CL, and AQ analyzed data. JN carried out and analyzed qPCR experiments. GM performed, analyzed, and supervised intravital imaging. MS, CM, and AQ wrote the manuscript. CM and AQ designed the study and supervised the research work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01980/full#supplementary-material

Supplementary Movie 1 | Testosterone increases LPS-induced neutrophil accumulation in the liver. Intravital microscopy after an i.p. LPS 0.5 mg/kg injection for the visualization of neutrophil recruitment to liver sinusoids. Mice were previously treated with the antiandrogen flutamide (7 mg/kg/day) or with testosterone (10 mg/kg/day) for 3 days. Quantification of Ly6G (+) neutrophils (green) is shown in **Figure 2D**.

Supplementary Table 1 | List of primers *(Rattus norvegicus)* used for different target mRNAs.

REFERENCES

1. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annu Rev Pathol.* (2014) 9:181–218. doi: 10.1146/annurev-pathol-020712-164023
2. Savill J. Apoptosis in resolution of inflammation. *J Leukoc Biol.* (1997) 61:375–80. doi: 10.1002/jlb.61.4.375
3. Snelgrove RJ, Jackson PL, Hardison MT, Noerager BD, Kinloch A, Gaggar A, et al. A critical role for LTA4H in limiting chronic pulmonary neutrophilic inflammation. *Science* (2010) 330:90–4. doi: 10.1126/science.1190594
4. Schloss MJ, Horckmans M, Nitz K, Duchene J, Drechsler M, Bidzhekov K, et al. The time-of-day of myocardial infarction onset affects healing through oscillations in cardiac neutrophil recruitment. *EMBO Mol Med.* (2016) 8:937–48. doi: 10.15252/emmm.201506083
5. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg.* (1992) 216:117–34. doi: 10.1097/00000658-199208000-00002
6. Shi TY, Chen G, Huang X, Yuan Y, Wu X, Wu B, et al. Effects of reactive oxygen species from activated leucocytes on human sperm motility, viability and morphology. *Andrologia* (2012) 44(Suppl. 1):696–703. doi: 10.1111/j.1439-0272.2011.01252.x
7. Sonego F, Castanheira FV, Ferreira RG, Kanashiro A, Leite CA, Nascimento DC, et al. Paradoxical roles of the neutrophil in sepsis: protective and deleterious. *Front Immunol.* (2016) 7:155. doi: 10.3389/fimmu.2016.00155
8. Tsuda Y, Takahashi H, Kobayashi M, Hanafusa T, Herndon DN, Suzuki F. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity* (2004) 21:215–26. doi: 10.1016/j.immuni.2004.07.006
9. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
10. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature* (2015) 525:528–32. doi: 10.1038/nature15367
11. Brandau S. The dichotomy of neutrophil granulocytes in cancer. *Semin Cancer Biol.* (2013) 23:139–40. doi: 10.1016/j.semcancer.2013.02.008
12. Motrich RD, Mackern-Oberti JP, Maccioni M, Rivero VE. Effects of autoimmunity to the prostate on the fertility of the male rat. *Fertil Steril.* (2009) 91:2273–80. doi: 10.1016/j.fertnstert.2008.06.046
13. Kramer G, Mitteregger D, Marberger M. Is benign prostatic hyperplasia (BPH) an immune inflammatory disease? *Eur Urol.* (2007) 51:1202–16. doi: 10.1016/j.eururo.2006.12.011
14. De Marzo AM, Nakai Y, Nelson WG. Inflammation, atrophy, and prostate carcinogenesis. *Urol Oncol.* (2007) 25:398–400. doi: 10.1016/j.urolonc.2007.05.007
15. Quintar AA, Leimgruber C, Pessah OA, Doll A, Maldonado CA. Androgen depletion augments antibacterial prostate host defences in rats. *Int J Androl.* (2012) 35:845–59. doi: 10.1111/j.1365-2605.2012.01288.x
16. Gubbels Bupp MR, Jorgensen TN. Androgen-induced immunosuppression. *Front Immunol.* (2018) 9:794. doi: 10.3389/fimmu.2018.00794
17. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Hum Reprod Update* (2005) 11:411–23. doi: 10.1093/humupd/dmi008
18. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biol Reprod.* (2008) 78:432–7. doi: 10.1095/biolreprod.107.063545
19. D'agostino P, Milano S, Barbera C, Di Bella G, La Rosa M, Ferlazzo V, et al. Sex hormones modulate inflammatory mediators produced by macrophages. *Ann N Y Acad Sci.* (1999) 876:426–9. doi: 10.1111/j.1749-6632.1999.tb07667.x
20. Quintar AA, Maldonado CA. Androgen regulation of host defenses and response to inflammatory stimuli in the prostate gland. *Cell Biol Int.* (2017) 41:1223–33. doi: 10.1002/cbin.10755
21. Quintar AA, Roth FD, De Paul AL, Aoki A, Maldonado CA. Toll-like receptor 4 in rat prostate: modulation by testosterone and acute bacterial infection in epithelial and stromal cells. *Biol Reprod.* (2006) 75:664–72. doi: 10.1095/biolreprod.106.053967
22. Quintar AA, Doll A, Leimgruber C, Palmeri CM, Roth FD, Maccioni M, et al. Acute inflammation promotes early cellular stimulation of the epithelial and stromal compartments of the rat prostate. *Prostate* (2010) 70:1153–65. doi: 10.1002/pros.21150
23. Marques PE, Oliveira AG, Pereira RV, David BA, Gomides LF, Saraiva AM, et al. Hepatic DNA deposition drives drug-induced liver injury and inflammation in mice. *Hepatology* (2015) 61:348–60. doi: 10.1002/hep.27216
24. Antunes MM, Carvalho E, Menezes GB. DIY: "Do Imaging Yourself" - Conventional microscopes as powerful tools for *in vivo* investigation. *Int J Biochem Cell Biol.* (2018) 94:1–5. doi: 10.1016/j.biocel.2017.11.004
25. Quintar A, Mcardle S, Wolf D, Marki A, Ehinger E, Vassallo M, et al. Endothelial protective monocyte patrolling in large arteries intensified by western diet and Atherosclerosis. *Circ Res.* (2017) 120:1789–99. doi: 10.1161/CIRCRESAHA.117.310739
26. Cotter MJ, Muruve DA. Isolation of neutrophils from mouse liver: a novel method to study effector leukocytes during inflammation. *J Immunol Methods* (2006) 312:68–78. doi: 10.1016/j.jim.2006.02.019
27. Mcdonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* (2010) 330:362–6. doi: 10.1126/science.1195491
28. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol.* (2011) 32:452–60. doi: 10.1016/j.it.2011.06.008
29. Nunn CL, Lindenfors P, Pursall ER, Rolff J. On sexual dimorphism in immune function. *Philos Trans R Soc Lond B Biol Sci.* (2009) 364:61–9. doi: 10.1098/rstb.2008.0148
30. Davidson WM, Smith DR. A morphological sex difference in the polymorphonuclear neutrophil leucocytes. *Br Med J.* (1954) 2:6–7. doi: 10.1136/bmj.2.4878.6
31. Cavasin MA, Tao ZY, Yu AL, Yang XP. Testosterone enhances early cardiac remodeling after myocardial infarction, causing rupture and degrading cardiac function. *Am J Physiol Heart Circ Physiol.* (2006) 290:H2043–50. doi: 10.1152/ajpheart.01121.2005
32. Deitch EA, Ananthakrishnan P, Cohen DB, Xu DZ, Feketeova E, Hauser CJ. Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries. *Am J Physiol Heart Circ Physiol.* (2006) 291:H1456–65. doi: 10.1152/ajpheart.00694.2005
33. Hofer MD, Cheng EY, Bury MI, Xu W, Hong SJ, Kaplan WE, et al. Androgen supplementation in rats increases the inflammatory response and prolongs urethral healing. *Urology* (2015) 85:691–7. doi: 10.1016/j.urology.2014.11.025
34. Chuang KH, Altuwaijri S, Li G, Lai JJ, Chu CY, Lai KP, et al. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *J Exp Med.* (2009) 206:1181–99. doi: 10.1084/jem.20082521
35. Lai JJ, Lai KP, Zeng W, Chuang KH, Altuwaijri S, Chang C. Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol.* (2012) 181:1504–12. doi: 10.1016/j.ajpath.2012.07.008
36. Chou RC, Kim ND, Sadik CD, Seung E, Lan Y, Byrne MH, et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity* (2010) 33:266–78. doi: 10.1016/j.immuni.2010.07.018
37. Macneil LG, Baker SK, Stevic I, Tarnopolsky MA. 17beta-estradiol attenuates exercise-induced neutrophil infiltration in men. *Am J Physiol Regul Integr Comp Physiol.* (2011) 300:R1443–51. doi: 10.1152/ajpregu.00689.2009
38. Alexiou E, Hatziagelaki E, Pergialiotis V, Chrelias C, Kassanos D, Siristatidis C, et al. Hyperandrogenemia in women with polycystic ovary syndrome: prevalence, characteristics and association with body mass index. *Horm Mol Biol Clin Investig.* (2017) 29:105–11. doi: 10.1515/hmbci-2016-0047
39. Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, et al. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. *Sci Transl Med.* (2015) 7:315ra190. doi: 10.1126/scitranslmed.aac5608
40. Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. *Science* (2017) 358:111–6. doi: 10.1126/science.aam9690
41. Kulkarni U, Goldstein DR. Divergent roles for neutrophils: promoting and resolving inflammation? *Transplantation* (2018) 102:542–3. doi: 10.1097/TP.0000000000002062

42. Snelgrove RJ, Patel DF, Patel T, Lloyd CM. The enigmatic role of the neutrophil in asthma: Friend, foe or indifferent? *Clin Exp Allergy* (2018). doi: 10.1111/cea.13191. [Epub ahead of print].
43. Tosello Boari J, Amezcua Vesely MC, Bermejo DA, Ramello MC, Montes CL, Cejas H, et al. IL-17RA signaling reduces inflammation and mortality during *Trypanosoma cruzi* infection by recruiting suppressive IL-10-producing neutrophils. *PLoS Pathog.* (2012) 8:e1002658. doi: 10.1371/journal.ppat.1002658
44. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood* (2016) 127:2173–81. doi: 10.1182/blood-2016-01-688887
45. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* (2009) 31:761–71. doi: 10.1016/j.immuni.2009.09.016
46. De Santo C, Arscott R, Booth S, Karydis I, Jones M, Asher R, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol.* (2010) 11:1039–46. doi: 10.1038/ni.1942
47. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest.* (2010) 120:1151–64. doi: 10.1172/JCI37223
48. Yoon G, Kim JY, Choi YK, Won YS, Lim IK. Direct activation of TGF-beta1 transcription by androgen and androgen receptor complex in Huh7 human hepatoma cells and its tumor in nude mice. *J Cell Biochem.* (2006) 97:393–411. doi: 10.1002/jcb.20638
49. Qi W, Gao S, Wang Z. Transcriptional regulation of the TGF-beta1 promoter by androgen receptor. *Biochem J.* (2008) 416:453–62. doi: 10.1042/BJ20080651
50. Ahmed SA, Talal N. Sex hormones and the immune system–part 2. Animal data. *Baillieres Clin Rheumatol.* (1990) 4:13–31. doi: 10.1016/S0950-3579(05)80241-9
51. Kanda N, Tsuchida T, Tamaki K. Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. *Clin Exp Immunol.* (1996) 106:410–5. doi: 10.1046/j.1365-2249.1996.d01-842.x
52. Friedl R, Brunner M, Moeslinger T, Spieckermann PG. Testosterone inhibits expression of inducible nitric oxide synthase in murine macrophages. *Life Sci.* (2000) 68:417–29. doi: 10.1016/S0024-3205(00)00953-X
53. Bekesi G, Kakucs R, Varbiro S, Racz K, Sprintz D, Feher J, et al. *In vitro* effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. *Steroids* (2000) 65:889–94. doi: 10.1016/S0039-128X(00)00183-5
54. Marin DP, Bolin AP, Dos Santos Rde C, Curi R, Otton R. Testosterone suppresses oxidative stress in human neutrophils. *Cell Biochem Funct.* (2010) 28:394–402. doi: 10.1002/cbf.1669
55. Gu X, Gao X, Li X, Qi X, Ma M, Qin S, et al. Prognostic significance of neutrophil-to-lymphocyte ratio in prostate cancer: evidence from 16,266 patients. *Sci Rep.* (2016) 6:22089. doi: 10.1038/srep22089

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Differential Redox State Contributes to Sex Disparities in the Response to Influenza Virus Infection in Male and Female Mice

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Influenza virus replicates intracellularly exploiting several pathways involved in the regulation of host responses. The outcome and the severity of the infection are thus strongly conditioned by multiple host factors, including age, sex, metabolic, and redox conditions of the target cells. Hormones are also important determinants of host immune responses to influenza and are recently proposed in the prophylaxis and treatment. This study shows that female mice are less susceptible than males to mouse-adapted influenza virus (A/PR8/H1N1). Compared with males, PR8-infected females display higher survival rate (+36%), milder clinical disease, and less weight loss. They also have milder histopathological signs, especially free alveolar area is higher than that in males, even if pro-inflammatory cytokine production shows slight differences between sexes; hormone levels, moreover, do not vary significantly with infection in our model. Importantly, viral loads (both in terms of viral M1 RNA copies and tissue culture infectious dose 50%) are lower in PR8-infected females. An analysis of the mechanisms contributing to sex disparities observed during infection reveals that the female animals have higher total antioxidant power in serum and their lungs are characterized by increase in (i) the content and biosynthesis of glutathione, (ii) the expression and activity of antioxidant enzymes (peroxiredoxin 1, catalase, and glutathione peroxidase), and (iii) the expression of the anti-apoptotic protein Bcl-2. By contrast, infected males are characterized by high expression of NADPH oxidase 4 oxidase and phosphorylation of p38 MAPK, both enzymes promoting viral replication. All these factors are critical for cell homeostasis and susceptibility to infection. Reappraisal of the importance of the host cell redox state and sex-related effects may be useful in the attempt to develop more tailored therapeutic interventions in the fight against influenza.

Keywords: sex differences, gender, hormones, influenza virus, redox state, glutathione

INTRODUCTION

Viruses replicate in the living cells of their hosts and use many intracellular pathways for their own advantage. Consequently, host factors like age, general health, metabolic, and redox conditions of the cells can have important repercussions on different steps of the virus life cycle (1–4). Furthermore, general redox state may also affect host immune response to viral replication (5–8). Cells containing high levels of thiols, e.g., glutathione (GSH) and cysteine content, or characterized by higher antioxidant defenses, as well as abundant expression of Bcl-2 proteins family, are less permissive to viral replication, including influenza (2). Moreover, our and other groups previously demonstrated that cells infected with influenza virus were characterized by low levels of GSH (2, 9–12) and by an increase of reactive oxygen species (ROS) production (10, 11, 13). During influenza virus infection, there is also a depletion of key antioxidant enzymes, due to their secretion or because of virus-induced loss of lung cells (14, 15). The oxidative stress is useful for the virus since many pathways involved in the regulation of viral replication and host responses to viral infection are highly responsive to even transient changes in the redox state of the cytoplasmic environment (16). In fact, some enzymes like protein disulfide isomerase (PDI) or NADPH oxidase 4 (NOX4) regulate specific steps of virus life cycle, including the folding and maturation of viral glycoprotein hemagglutinin (10) and the nuclear-cytoplasmic export of viral nucleoprotein (NP) (11, 17, 18).

Sex and gender, that refer to biology and behavior, respectively (19), also impact viral infections. Analysis of several epidemiological studies has highlighted that disease severity and fatality following exposure to influenza A viruses are generally higher in women than men (19, 20). The mechanisms underlying this sex/gender difference are several and tightly interconnected; behavioral, immunological, hormonal, and genetic factors are all included (20). Focusing on biological factors, it is known that females mount a higher immune response than males, which can accelerate virus clearance and reduce virus load, but can also make females more prone to immunopathology and to development of autoimmune disease (21). Klein et al. (20, 22, 23) reported that the exaggerated immunity and consequent immunopathology lead females to greater morbidity and mortality with respect to males. Such a response can be modulated by hormone concentrations, and so age may also affect the sex-related variability (24, 25). Epidemiological studies in which results were stratified by age in fact, report that hospitalization and morbidity rates due to influenza A viruses are higher in males than in females from birth to 15–19 years (26–31). Non-endocrine factors, as genetic ones, could prevail in the latter case. It has been shown that genetic variation in chromosome Y regulates susceptibility to influenza A virus, making specific variants in males mice more susceptible to infection (32).

Interesting parameters that also differently characterize cells isolated from male and female animals were the redox ones (33, 34). Malorni et al. (35) reported differences between vascular smooth muscle cells (VSMC) from male and female rats in terms of "basal" redox balance. Either H_2O_2 or O_2^- levels were significantly lower in VSMC from females than those from male rats. Moreover, the intracellular GSH content was higher in female than in male rats. The same authors found that antioxidant enzyme activity was significantly higher in VSMC from female than in male, independently from the stimuli that induced stress (35, 36). Many redox-sensitive cell-signaling pathways are differently activated in both sexes (37).

On the basis of this evidence, in this study, we verified the hypothesis that host redox state plays a role in sex disparities in the outcome of influenza virus infection. To evaluate viral replication in male and female mice, we chose the Balb/c strain, which is considered a Th2-type strain (38), to better highlight the effect of the virus (as opposed to the immune response). Female and male mice were infected with a mouse-adapted strain of influenza A (H1N1) and the progression of disease was monitored by measuring some redox parameters usually altered during infection. We found that in terms of both survival and clinicopathological parameters of disease, the female mice displayed higher resistance to the infection, due to significant differences in the systemic and pulmonary "redox profiles" between female and male mice.

MATERIALS AND METHODS

In accordance with national law, the experiments described in this manuscript were approved by the Italian Ministry of Health, which verified the ethical and scientific appropriateness of the research. All animals received humane treatment, and every effort was made to minimize their suffering. Unless otherwise stated, all commercial products cited were used in accordance with the manufacturers' instructions.

Mice and Virus Infection

Balb/c 6-week-old mice [400 females, body weight (bw) range = 15–19 g; 400 males, bw = 19–23 g] were purchased from Harlan Laboratories (Milan, Italy). Animals were housed under specific pathogen-free conditions (5/cage, SmartFlow IVC Rack, Tecniplast, Varese, Italy) at 12:12 h light:dark cycle, and *ad libitum* access to food and water. After 1 week, each mouse was individually weighed and randomly assigned to an experimental group.

A mouse-adapted strain of influenza A/Puerto Rico/8/34 (H1N1; PR8) was used. In our experiments, 1 plaque-forming unit (PFU) of PR8 stock was equivalent to 2.9×10^3 genome copies, approximately 2.0×10^3 genome copies/tissue culture infectious dose 50% ($TCID_{50}$) according to the relationship between $TCID_{50}$ and PFU provided by the American Type Culture Collection. The 50% mouse lethal dose (MLD_{50}) was determined in female and male mice that had been lightly anesthetized by isofluorane

Abbreviations: BALF, broncho-alveolar lavage fluid; CAT, catalase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gusb, glucuronidase beta; GCL, glutamate cysteine ligase; GR, glutathione reductase; GSS, glutathione synthase; GSHPx, glutathione peroxidase; H&E, hematoxylin & eosin-stained; MLD_{50}, 50% mouse lethal dose; NOX4, NADPH oxidase 4; NP, nucleoprotein; GSSG, oxidized glutathione; PRDX, peroxiredoxin; PFU, plaque-forming unit; PDI, protein disulfide isomerase; ROS, reactive oxygen species; GSH, reduced glutathione; Rpl13a, ribosomal protein L13A; SOD, superoxide dismutase; $TCID_{50}$, tissue culture infectious dose 50%; TAC, total antioxidant capacity; VSMC, vascular smooth muscle cells.

(Esteve, Milan, Italy) inhalation and intranasally inoculated with PR8 at different doses (0.01–10 PFU/animal).

For assessment of morbidity and survival related to seasonal-like influenza infections, the *inoculum* consisted of 50 µl of sterile phosphate-buffered saline (PBS), alone (mock-infected controls) or containing 0.5 PFU/mouse of PR8 (infected animals).

Infected and control animals were daily monitored up to 21 days post-infection (p.i.). Each animal was weighed, its rectal temperature was measured (Temp Thermocouple Meter, Oakton, USA), and the clinical severity of disease was scored using the following scale (39, 40): 0 = no visible signs of disease; 1 = slight ruffling of fur; 2 = ruffled fur, reduced mobility; 3 = ruffled fur, reduced mobility, rapid breathing; 4 = ruffled fur, minimal mobility, huddled appearance, rapid and/or labored breathing indicative of pneumonia.

At the end of the experiments, the mice were euthanized with an overdose of tiletamine/zolazepam (Virbac, Milan, Italy) (800 mg/kg bw) and xylazine (Bayer, Milan, Italy) (100 mg/kg bw). Specimens for analysis [blood, broncho-alveolar lavage fluid (BALF), and lungs] were then collected as described below.

Blood

Serum Total Antioxidant Capacity (TAC) Assay

On p.i. days 3, 6, 9, and 21, blood was collected from the retro-orbital venous sinuses of control and PR8-infected mice. The recovery was made with a Pasteur pipette after ocular instillation of oxybuprocaine (1 drop/eye) (Novartis, Siena, Italy). The sample was allowed to clot for 45 min (to facilitate removal of all platelets and precipitates) and then centrifuged at 10,000 × *g* for 15 min at +4°C. The serum was stored at −80°C prior to assay with the TAC Kit (JaICA, Florence, Italy), which measures the sample's capacity to convert Cu^{+2} to Cu^{+1}.

Sex Hormone Quantification

Testosterone and estradiol quantification was performed using a colorimetric competitive enzyme immunoassay kit purchased from Enzo Life Sciences (3V Chimica, Rome, Italy), according to the manufacturers' instructions.

Broncho-Alveolar Lavage Fluid (BALF)

Mice were euthanized, and a sterile 23-G catheter was inserted into the exposed tracheal lumen. Two instillations of sterile PBS (0.8 ml) containing protease inhibitors (Sigma-Aldrich, Milan, Italy) were injected through the catheter and aspirated as previously described (41). The BALF samples were centrifuged at 1,000 × *g* for 15 min at +4°C and the supernatant stored at −80°C prior to analysis.

Total Protein Content

For assessment of lung damage, the total protein content of each BALF specimen was measured with a standard Micro BCA Kit (Pierce, Monza, Italy). BALF samples (150 µl) were pipetted into a microplate well, working reagent (150 µl) was added, and the plate was incubated at 37°C for 2 h and cooled to room temperature. The optical density of each solution was measured at 570 nm with a Multiskan Ex Reader (Thermo Fisher Scientific, Monza, Italy).

Cytokine Quantification

A multiplex assay was used to measure cytokine (IL-1, IL-6, TNF-α, IL-10, IFN-γ, CCL2-MCP1, and CCL3-MIP1) levels in each BALF sample. Plates were read on a Bio-Plex MAGPIX instrument, and data were analyzed with Bio-Prosoftware (Bio-Rad, Milan, Italy).

Lungs

Assay of Viral Titers

Whole lungs isolated from infected female and male mice were removed, weighed, frozen, and stored at −80°C. For the quantification of viral M1 RNA copies, total RNA was extracted from thawed lungs that had been homogenized in TRI Reagent (Sigma-Aldrich, Milan, Italy) (1 ml/75 mg of tissue) with a Polytron homogenizer. The RNA pellet was washed with 1 ml of 75% ethanol (7,500 × *g* for 5 min at +4°C) and air-dried for 30 min. Diethylpyrocarbonate water (100 µl) was added, and tube was heated to 55°C for 15 min to facilitate dissolution. The isolated RNA was treated with DNase I (Invitrogen, Life Technologies, Monza, Italy), and its quality and quantity were verified spectrophotometrically (Pearl Nanophotometer, IMPLEN, Munich, Germany). The number of viral M1 RNA copies was determined by quantitative real time RT-PCR using the One Step Influenza A/B r-gene and Quanti FluA kits (BioMérieux, Florence, Italy). For the evaluation of $TCID_{50}$, lungs were homogenized in RPMI 1640 medium, and homogenates were subjected to $TCID_{50}$ assay on MDCK cells. The number of wells showing positive cytopathic effects was scored, and the titer was calculated as previously described (42).

Histologic Examination

Lung histology was evaluated in female and male infected mice ($n = 25$/group). Mock-infected mice were used as controls. Mice were sacrificed at 3, 6, 9, and 21 days p.i. Each sacrifice was followed by complete necroscopy with macroscopic and microscopic examinations of the lungs.

For the histopathological and morphological examination, each lung was fixed in buffered formalin at room temperature for 48 h and embedded in paraffin with a melting point of 55–57°C. Sections (3-µm thick) were stained with hematoxylin and eosin and Masson's trichrome.

The samples were evaluated independently and blindly by three investigators (Caterina Loredana Mammola, Antonio Franchitto, and Romina Mancinelli), and necroinflammatory changes were scored as follows (43, 44): 0 = no lesions; 1 = mild focal inflammation; 2 = moderate–severe inflammation or necrosis affecting less than 25% of lung tissue examined; 3 = severe inflammation with necrosis or severe inflammation affecting 25–50% of lung tissue examined; 4 = severe inflammation with necrosis affecting more than 50% of the lung tissue examined. For each lung, at least five slides were analyzed. Briefly, serial paraffin sections were obtained per animal. For each sample, 10 fields were analyzed per section. Alveoli were identified and bordered to calculate the corresponding areas. All ambiguous structures, airways, and vascular structures were excluded. The tissue and airspace areas were tabulated using the IAS Delta Sistemi software (Rome, Italy) (10, 45–47).

Assays of Thiols Levels and Antioxidant Enzyme Activities

A sterile 23-G butterfly needle was inserted into the euthanized mouse's right ventricle and connected to a peristaltic pump (Generalcontrol, Milan, Italy). The lungs were then perfused with PBS containing 50 U/ml heparin (Sigma-Aldrich, Milan, Italy) to remove erythrocytes and clots. Cuts were made in the liver to facilitate perfusate outflow. The lungs were then removed, weighed, frozen in liquid nitrogen, and stored at −80°C until assayed.

Intracellular glutathione (GSH) and oxidized forms [oxidized glutathione (GSSG)] were measured in lung homogenates with the Glutathione Assay Kit (Cayman Chemical, Florence, Italy) following the manufacturer's instructions, after deproteinization with metaphosphoric acid of the samples. For GSSG quantification, an aliquot of deproteinized samples was first incubated with 2-vinylpyridine to derivatize GSH. Reduced GSH levels were obtained by differences between total GSH and GSSG.

The total amount of free thiols in deproteinized samples from lung homogenates and in serum were measured by a standard colorimetric assay using Ellman's reagent (48).

Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSHPx) activities were also measured with specific kits (Cayman Chemical, Florence, Italy). Calculation of enzymatic activity was determined following the manufacturer's instructions.

RT-PCR Analysis of Pulmonary mRNA Levels

Total RNA was isolated from the lungs as described above and used as a template for generating cDNA (iScript cDNA Synthesis Kit, Bio-Rad, Milan, Italy). An aliquot of the cDNA was subjected to 40 cycles of RT-PCR amplification (95°C, 10 s; 60°C, 30 s) using iQ SYBR Green Supermix and a LightCycler iQ 5 (Bio-Rad, Milan, Italy). To ensure that the primers produced a single and specific PCR amplification product, a melting curve analysis was carried out at the end of the PCR cycle. The housekeeping genes glucuronidase beta (Gusb), ribosomal protein L13A (Rpl13a), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used for normalization. Relative quantitative evaluation was performed by the comparative ΔΔCt method.

The following forward and reverse primers were used: glutathione reductase (GR) (TTCAGTTGGCATGTCATC forward; CCGTGGATAATTTCTATGTGA reverse), glutathione synthase (GSS) (GTGCTACTGATTGCTCAA forward; ACATG GATCTTCCTGTCT reverse), glutamate cysteine ligase (GCL) (AA GTCCCTCTTCTTTCCA forward; CCTTGAATATTGGCAC ATTG reverse), Bcl-2 (CCTACGGATTGACATTCTC forward; AT ACATAAGGCAACCACAC reverse), Rpl13a (ATGGGATGAAT CAGTTGAG forward; ATAGGGTACTTGGTCAGG reverse), Gapdh (TGCGACTTCAACAGCAACTC forward; ATGTAG GCCATGAGGTCCAC reverse), Gusb (GTACTCCTTGGAG GTGAA forward; TGAATCCTCGTGCTTATTG reverse). The results are presented as fold increases relative to levels observed in mock-infected control mice.

Western Blot Analysis

Whole lungs of female and male infected mice ($n = 9$/group) were homogenized in RIPA lysis buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGDA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS)] supplemented with phenylmethylsulfonyl fluoride, protease inhibitor mixture, and phosphatase inhibitor (Sigma-Aldrich, Milan, Italy). Lung lysates were incubated for 30 min on ice and then centrifuged at 13,000 × *g* for 30 min. The protein concentration of the supernatants was determined with the Micro BCA Protein Assay Kit (Pierce, Monza, Italy). Samples were separated by SDS-PAGE, blotted onto nitrocellulose membranes, blocked with 10% non-fat dry milk, and stained with primary (see below) and secondary antibodies peroxidase-conjugated (Jackson ImmunoResearch, Milan, Italy). Blots were developed with the ECL-Plus Detection System (GE Healthcare, Milan, Italy) and subjected to densitometry with the Quantity One Program (Bio-Rad, Milan, Italy).

Primary antibodies included rabbit polyclonal anti-NOX4, anti-phospho-p38, anti-Bcl-2 (Santa Cruz Biotechnology, Dallas, TX, USA); rabbit polyclonal anti-PRDX1 (Abcam); and mouse monoclonal anti-actin (Sigma-Aldrich).

Statistical Analyses

The long-rank test was used to assess the difference in the overall Kaplan–Meier survival curves. Variations on bw and temperature were assigned by using a linear mixed model for repeated measures adjusted by baseline value followed by *post hoc* analysis (Bonferroni's correction). The Wilcoxon test was performed to compare the values of Glutathione, viral M1 RNA copies, and protein concentrations in BALF in the two groups (all statistical analyses were performed using R version 3.3).

Unpaired two-tailed Student's *t*-test or one-way ANOVA test were used for antioxidant enzyme activity; gene and protein expression; cytokine levels; alveolar area in both sexes (statistical analysis was performed using GraphPad Prism™ software version 6.0).

p-Values of less than 0.05 ($p \leq 0.05$) were considered statistically significant.

RESULTS

Female Mice Are More Resistant to Influenza Virus Infection Than Males

Female and male Balb/c mice were infected intranasally with 0.5 PFU/mouse and clinical signs of infection, bw, body temperature, and survival were monitored daily until 21 days after infection. The clinical responses and survival rates observed in female and male mice up to 21 days p.i are shown in **Figure 1**. The first symptoms of disease (piloerection, reduced food intake, and lethargy) appeared in both sexes 4 days p.i. and increased rapidly in intensity. The males exhibited more pronounced horripilation (as the first sign of pain) than the females and higher clinical scores at peak disease intensity (on p.i. days 6–12) (**Figure 1A**). In addition, the percentage of bw decreased rapidly in each male, whereas for some females the bw did not decrease considerably. The overall trend of bw loss at day 9 p.i. was higher in males than in females (26.6 and 23.2%, respectively), even if no statistically

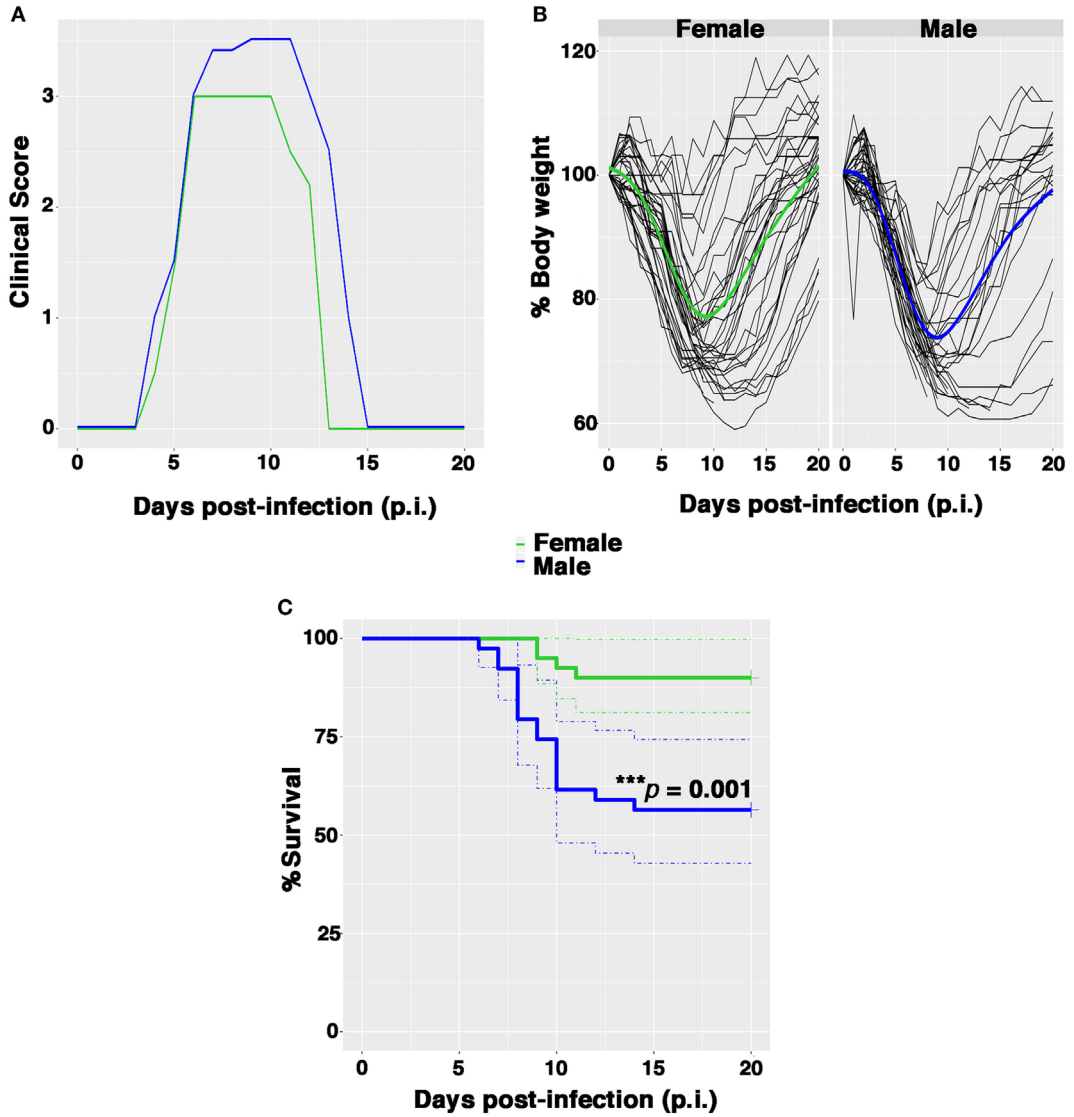


FIGURE 1 | Female mice are more resistant than males to PR8 infection. Female and male mice were monitored for 21 days after intranasal inoculation with 0.5 plaque-forming unit of mouse-adapted influenza A virus (PR8). **(A)** Clinical scores: the graph represents the combined results of two separate experiments, each performed with 10 male and 10 female animals. Scores ranged from 0 (no disease) to 4 (signs and symptoms that are indicative of pneumonia). See Section "Materials and Methods" for details. **(B)** Spaghetti plot of the daily body weight (expressed as percentage respect to day 0); the bold lines represent the overall trend. **(C)** Kaplan–Meier overall survival curves. Results represent data pooled from four independent experiments, each performed with 10 males and 10 females (n = 40/sex), ***p-value = 0.001.

substantial differences were detected (**Figure 1B**; Figure S1A in Supplementary Material). In terms of body temperature, no differences were observed in the two groups as well (Figure S1B in Supplementary Material).

Nevertheless, the percentage of survival following infection was significantly lower among males in comparison to females (53.8 and 90%, respectively, log rank ***p-value = 0.001) (**Figure 1C**). Furthermore, the average day of death occurred earlier in male than in female group (on p.i. day 7 vs. on p.i. day 10).

Influenza Virus Causes More Severe Lung Damage in Male Mice

To look at the damage caused by PR8 infection in the lungs of mice, the animals were euthanized and lungs fixed in 10% buffered formalin prior to sectioning at 3 μm and staining with hematoxylin & eosin-stained (H&E) and Masson's trichrome as described in Section "Materials and Methods" (**Figure 2**).

The observation of lung tissues from uninfected control mice (n = 5/sex) did not highlight lesions in any of the sacrificed animals. No changes were found in the normal architecture of pulmonary parenchyma, as well as in the normal morphology of airways, alveolus, and alveolar septa. The free alveolar area for these animals (Ctr) compared with that measured in infected mice (I) is reported in **Figures 2** and **3A**. Results are shown for 3, 6, and 9 days p.i., since on day 21 p.i. both female and male mice that survived did not show significant differences. As reported on the table under the graph (**Figure 3A**), the percentage of reduction of free alveolar area in males was higher than in females.

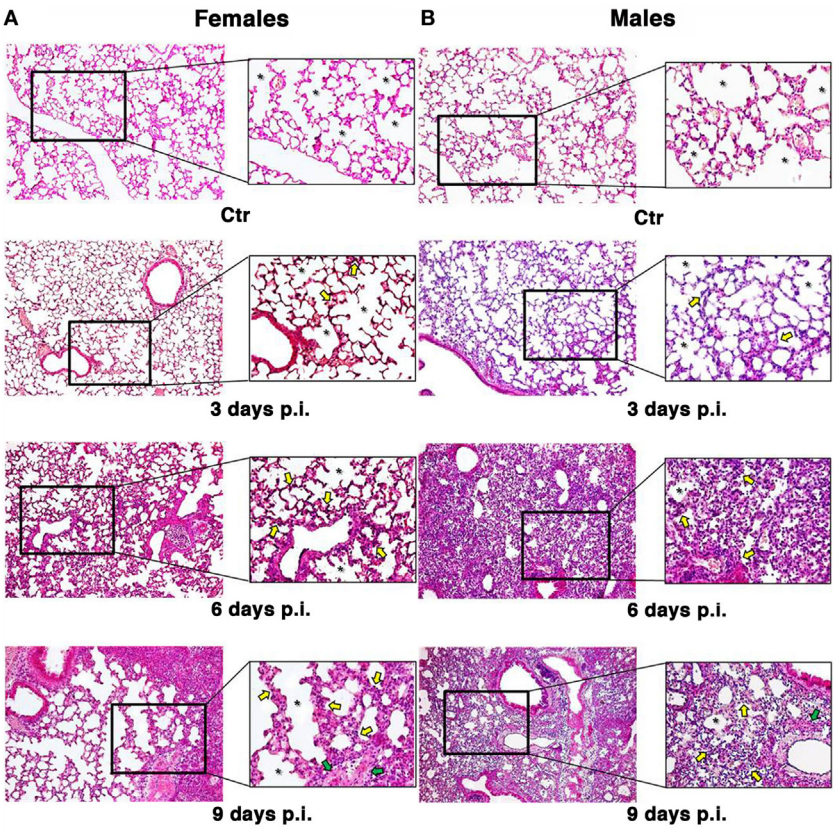
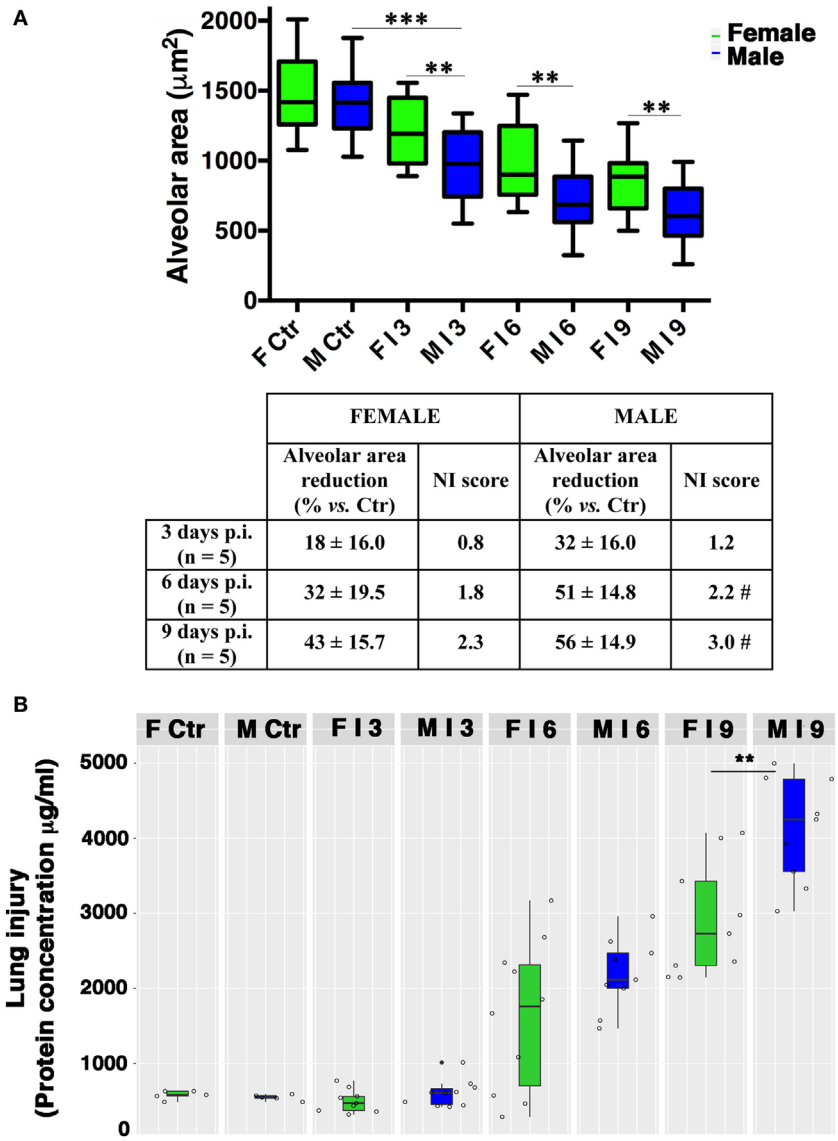


FIGURE 2 | Influenza A virus produces more severe lung damage in male mice. Hematoxylin & eosin-stained section of the female **(A)** and male **(B)** pulmonary tissues from mock-infected (Ctr) or infected mice with influenza A virus and sacrificed at different times (3, 6, and 9 days). Early structural changes caused by influenza virus in the epithelium of the lower airway are variable, with cytonecrosis involving shrinkage, decreasing in alveolar surface, followed by desquamation of these cells into the luminal space. In addition, there is necrosis of the bronchiolar wall, with submucosal edema and vascular congestion. These structural changes are irregularly distributed among male and female mice. In fact, the female mice **(A)** sacrificed after 3 days still show an higher amount of alveolar surface (see the asterisks) with some initial alterations, such as thickening of the alveolar septa and inflammatory infiltration (see yellow arrows) compared to the control and the corresponding male samples **(B)**. After 6 days, we found an increase in inflammation both in male and female mice, the epithelial layer is desquamating, and necrotic epithelial cells are present in the lumen (see green arrows). But, in male **(B)**, massive pulmonary edema and hemorrhage with the alveolar air spaces fill of edema fluid and erythrocytes are also present. After 9 days, the male tissue presents a slight worsening of the previous features, whereas the female lungs start to display the same aspects, maintaining a greater alveolar area (original magnification 10×).

In details, in PR8-infected male mice ($n = 5$) sacrificed after 3 days p.i. (**Figures 2B** and **3A**), we described initial alterations of lungs parenchyma; alveolar area resulted slightly reduced compared with control lungs with thickened alveolar septa. In addition, all samples showed peripheral edema and alteration of epithelium with inflammatory cells adhering to the surface of bronchioles. The mean ± SD of free alveolar area was 975 ± 235 with a % reduction respect to Ctr of 32 ± 16. Female mice lungs ($n = 5$) at 3 days p.i. (**Figures 2A** and **3A**) displayed a similar histopathological damage and the mean ± SD of alveolar area was 1,204 ± 238 with a % reduction of 18 ± 16.

In infected male mice ($n = 5$) sacrificed after 6 days p.i. (**Figures 2B** and **3A**), we found widespread impairment of pulmonary parenchyma; the pictures of interstitial pneumonia were characterized by the presence of higher inflammatory exudate (interstitial and alveolar) with inflammatory cells, fibrin, cellular debris, and obvious vascular congestion and areas of necrosis. The alveolar area is greatly decreased if compared with the control lungs and strikingly, the lungs of the male mice displayed signs of more severe damage than those of the females consisting of bronchiolitis, peri-bronchiolitis, interstitial edema, alveolar wall thickening, dense interstitial granulocyte, and lymphocyte infiltrates, and the alveolar area was 708 ± 212 with a % reduction of 51 ± 14.8. By p.i. day 6, these lesions already involved over 25% of the considered parenchyma, and similar involvement was observed in survivors sacrificed 9 days p.i. Female mice lungs ($n = 5$) at 6 days p.i. (**Figures 2A** and **3A**) showed similar histopathological alterations from a qualitative point of view, but larger preserved parenchymal areas; therefore, the alveolar area was significantly higher than male mice and this difference persisted for the duration of the experiment, indicating that the virally induced inflammation had a lower impact on lung's female (alveolar area: mean ± SD 996 ± 286 with a % reduction of 32 ± 19.5).



	FEMALE		MALE	
	Alveolar area reduction (% *vs.* Ctr)	NI score	Alveolar area reduction (% *vs.* Ctr)	NI score
3 days p.i. (n = 5)	18 ± 16.0	0.8	32 ± 16.0	1.2
6 days p.i. (n = 5)	32 ± 19.5	1.8	51 ± 14.8	2.2 #
9 days p.i. (n = 5)	43 ± 15.7	2.3	56 ± 14.9	3.0 #

FIGURE 3 | Male mice display reduced alveolar area compared with females. **(A)** Morphometric analysis at different days post-infection in female (F) and male (M) mice infected as described in **Figure 2**. The graph shows box-plots of alveolar area (μm²) of PR8-infected (I) and mock-infected mice (Ctr). ***p*-Value <0.01 females vs. males (unpaired *t*-test); ****p*-value <0.001 Infected vs. Ctr (One-way ANOVA Bonferroni multiple comparisons test). On table below the graph, the percentage of reduction of free to air exchange vs. mock-infected (considered 100%), and the score of inflammation and necrosis (NI score) are shown. #Relevant reduction of the alveolar area, thickening of the alveolar septa, vascular congestion. **(B)** Box-plots of protein concentrations in the BALF from mock-infected (Ctr) and PR8-infected female and male mice at the time points indicated. Results represent data pooled from three separate experiments. In details, mock-infected mice were 10 (5/sex), infected mice on p.i. day 3 were 19 (9 females and 10 males), on p.i. day 6 were 19 (10 females and 9 males), on p.i. day 9 were 18 (9/sex), ***p*-value = 0.006.

In infected male mice lungs ($n = 5$) at 9 days p.i. (**Figures 2B** and **3A**), diffuse impairment of pulmonary parenchyma was observed. The "alveolar area" was greatly reduced compared with the lung of the controls but lightly reduced compared with the animals after 6 days p.i. (free alveolar area 625 ± 213 with a % reduction of 56 ± 14.9). Mice female lungs ($n = 5$) at 9 days p.i. (**Figures 2A** and **3A**) presented chronic flogistic infiltrate with prevalent interstitial localization activated by epithelial/endothelial lesions: the picture is similar to that of male animals, but there are a lower incidence of collagen and exudative deposition and necrosis; moreover, reconstitution areas of the alveolar epithelium is observed in female lungs (free alveolar area 837 ± 230 with a % reduction of 43 ± 15.7).

Finally, as an indirect measure of the diffuse alveolar damage, protein concentrations in BALF samples from PR8-infected mice and mock-infected controls (Ctr) were assessed. As shown in **Figure 3B**, increases were observed on p.i. day 6 in infected animals of both sexes. By p.i. day 9 (when maximal lung damage was

noted in lung sections), BALF protein levels were significantly higher in the male group (**p-value = 0.006).

From a molecular point of view, a panel of different inflammatory cytokines and chemokines (IL-1, IL-6, TNF-α, IL-10, IFN-γ, CCL2-MCP1, and CCL3-MIP1) was evaluated in BALF from males and females mice. As shown in **Figure 4**, both sexes produced all the cytokines and their levels were higher than those measured in mock-infected mice. In fact, the pro-inflammatory cytokines IL-6, TNF-α, and IL-1 were increased in both sexes, the latter particularly in males. Instead, IFN-γ cytokine levels resulted more pronounced in females. Regarding chemokines CCL2-MCP1 and CCL3-MIP1, an increase was observed in both sexes, with MIP-1 higher in males. The immunosuppressive cytokine (IL-10) was increased in males on day 6 p.i.

These results apparently contradicted most of the literature that report that adult female mice experienced a greater morbidity and mortality after influenza virus infection than males, and this was correlated to immunopathology (24, 49); because hormones affect the immune response to viral infection, we wondered what are the hormonal levels in our model. We found plasma estradiol levels of 39.75 ± 18.6 pg/ml in control female

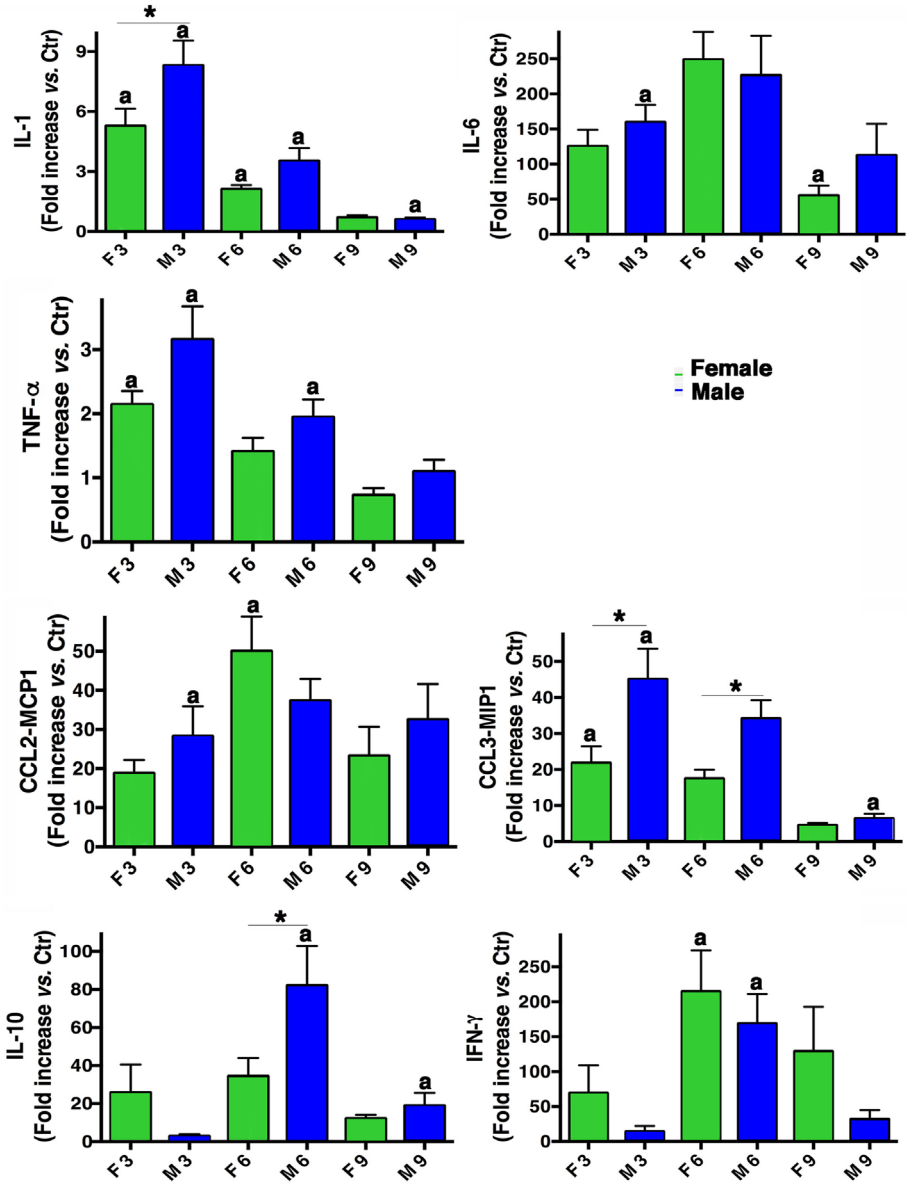


FIGURE 4 | Inflammatory cytokine production in infected mice. BALF concentrations of IL-1, IL-6, TNF-α, IL-10, IFN-γ, CCL2-MCP1, and CCL3-MIP1 were measured in male (M) and female (F) mice from the mock-infected (Ctr) and PR8-infected groups. The data (mean ± SEM) are represented as the concentration of cytokines at days 3, 6, and 9 p.i. relative to Ctr. Results are obtained from five different experiments, each performed with seven male and seven female mice. *p-Value <0.05 (female vs. male group); $^a p$-value <0.05 (differences within a sex across time-points p.i.).

mice and 30.3 ± 9.5 pg/ml in infected females (p.i. day 6); regarding testosterone, we measured 18.74 ± 6.2 ng/ml in control males and 15.12 ± 1.6 ng/ml in infected males (p.i. day 6); therefore, no significant differences in hormone level between uninfected and infected mice were detectable.

As the lung damage appeared less severe in females and on the basis of the results from hormone quantification, which seemed not to change during infection, we finally looked at the viral replication. As displayed in **Figure 5A**, viral M1 RNA copies in lung homogenates were consistently higher in the male group, and this difference was statistically significant on p.i. day 6 (**p-value = 0.004). Similarly, viral M1 RNA copies measured in BALF samples were also considerably higher in males than in females, during peak illness (**Figure 5B**). Accordingly, on p.i. day 6, the $TCID_{50}$ measured on lung homogenates obtained from infected male mice was higher than in female mice (1,582 ± 457 and 654 ± 32 U/ml, respectively).

Therefore, collectively these data suggest that the higher morbidity and, consequently, the lower survival, as well as the more severe and extended lung damage exhibited by PR8-infected male mice, may be the result of a higher replication of influenza virus in the lungs of the male mice.

Enhanced Systemic Antioxidant Power Protects Female Mice During Viral Infection

Influenza virus infection is known to be strongly conditioned by host redox environment, including the intracellular GSH content, antioxidant defense, and expression of redox-regulated

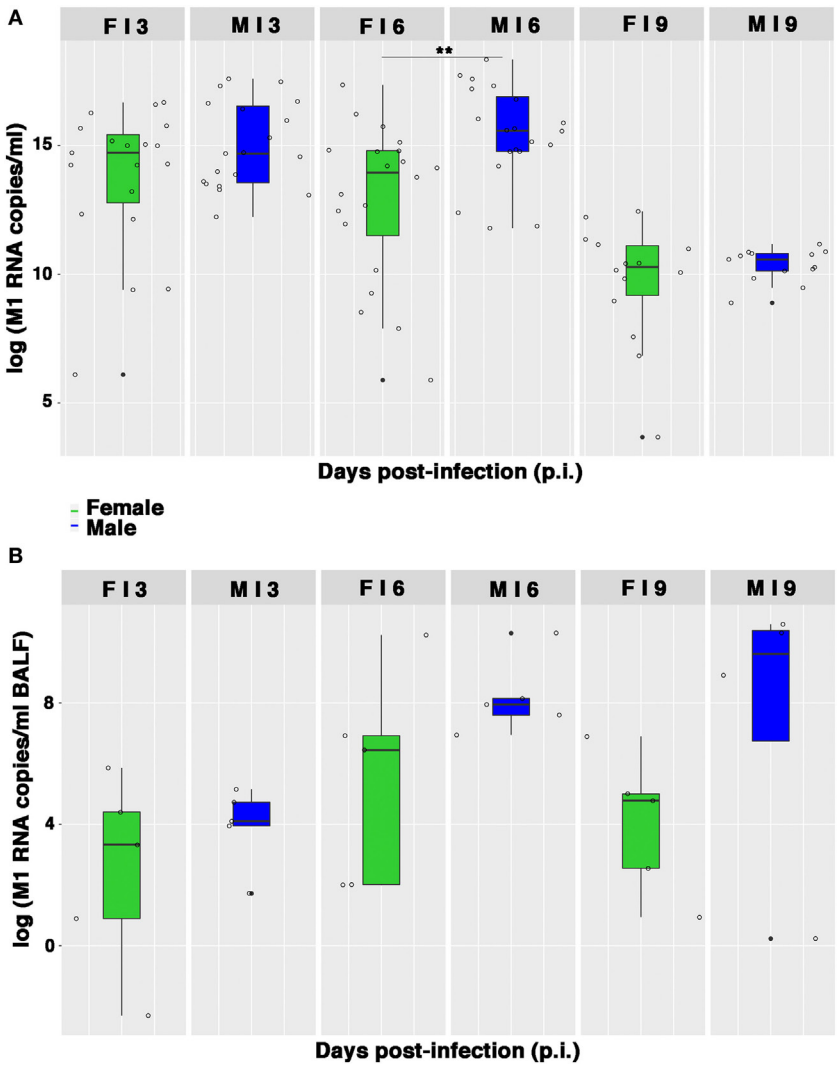


FIGURE 5 | Viral M1 RNA copies are highly produced by infected males. **(A)** Box-plots of the viral M1 RNA copies measured by quantitative RT-PCR in homogenates of lungs collected on p.i. days 3, 6, and 9. Results represent data pooled from four independent experiments, each performed with five females and five males for each time-point (n = 20/sex), **p-value = 0.004. **(B)** Box-plots of the viral M1 RNA copies in BALF measured by RT-PCR on p.i. days 3, 6, and 9. Data shown are from one of the three experiments performed (each with five male and five female mice).

cell pathways (2, 9–13, 17, 18). To determine whether these factors contributed to the sexual disparities in susceptibility to PR8 infection observed in our model, we first compared the TAC of female and male mice. The TAC reflects the abundance of antioxidant molecules and enzymes available in the blood to counteract the effects of ROS/reactive nitrogen species, such as those produced during viral infection. As shown in **Figure 6A**, mock-infected female mice displayed appreciably higher TAC than their male counterparts. More striking sex-related differences were seen in PR8-infected mice. The reduction potential of serum from female mice remained high (near baseline levels) throughout the viral infection, whereas that of the males dropped significantly. On p.i. days 3 and 6, the TAC recorded for the males was significantly lower than those of the females (unpaired *t*-test ***p*-value <0.01). By p.i. day 21, TAC of surviving animals had returned to their respective baseline levels, which were once again lower in males. Accordingly, the analysis of free thiols in serum and lung homogenates from infected and mock-infected mice showed a slight reduction in infected males compared with mock infected, while no differences were detectable between infected and non-infected females (data not shown).

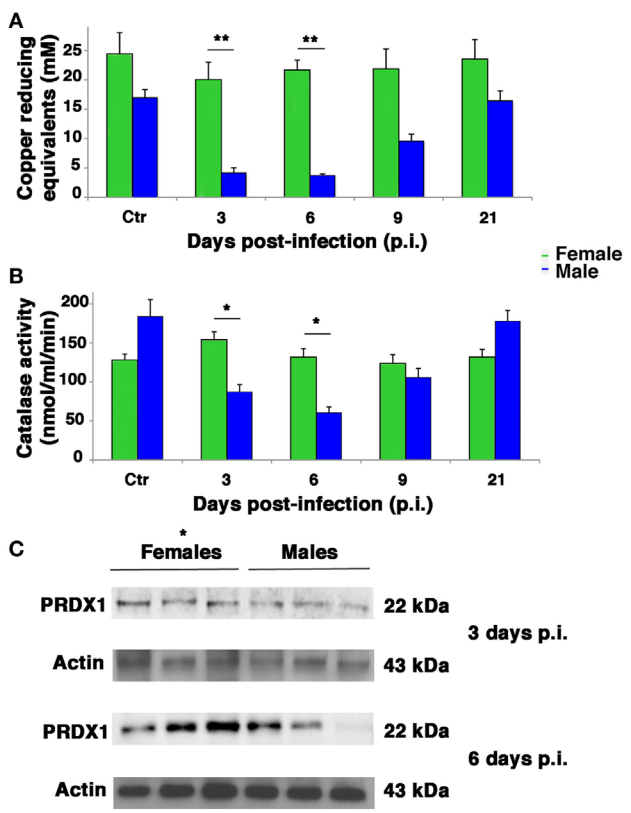


FIGURE 6 | Antioxidant defenses are higher in infected female mice. **(A)** Serum from mock-infected (Ctr) and PR8-infected mice was collected on p.i. days 3, 6, 9, and 21 and assayed with the potential antioxidant test, which measures the total antioxidant power in terms of the sample's ability to reduce copper. **(B)** Lung homogenates from Ctr and PR8-infected mice were assayed for CAT activity at the same time points than in panel **(A)**. Each value reported represents the mean ± SD of results from two separate experiments, each performed in duplicate ($n = 4$), **p*-value <0.05 and ***p*-value <0.01. **(C)** Peroxiredoxin (PRDX)1 protein expression was analyzed by western blotting in the lungs of infected female and male mice. Actin was used as loading control. Blot shows three animals for each sex (**p*-value ≤0.05) and is representative of three independent experiments performed.

Next, we assessed antioxidant enzyme activities in lung homogenates. As shown in **Figure 6B** in mock-infected controls, CAT activity did not significantly differ among males and females. After PR8 infection, however, CAT activity in the lungs of male mice dropped substantially, reaching levels on p.i. days 3 and 6 that were significantly lower than those of the female group, which remained stable throughout the viral infection (unpaired *t*-test **p*-value <0.05).

Activity of SOD increased appreciably in both sexes on p.i. day 3, but this change was not statistically significant. Essentially, PR8 infection was not associated with any significant change in pulmonary SOD activity in either the female or male mice, and no significant sex-related differences were observed at any of the time points (Figure S2 in Supplementary Material). Furthermore, we measured GSHPx activity, finding that it decreased significantly in both sexes but in greater extent in infected male mice than female. To note that female mock-infected mice showed significantly higher basal activity of this enzyme (Figure S3 in Supplementary Material). Finally, the expression of another antioxidant enzyme, peroxiredoxin (PRDX)1, was analyzed by western blot in lung of infected female and male mice at p.i. days 3 and 6 (time when the maximal difference in redox conditions was observed). As shown in **Figure 6C**, the expression of this enzyme was higher in females than in males. The densitometric analysis of ratio PRDX1/actin of three animals for each sex at 3 and 6 days p.i. was 1.5- and 3-fold higher, respectively, unpaired *t*-test **p*-value ≤0.05, suggesting that females are more protected by influenza for the presence of reducing conditions.

The Intracellular Content and Biosynthesis of GSH Are Preserved in Infected Female Mice

Influenza virus infection is associated with reductions in the GSH content of infected cells, which facilitate viral replication (2, 9–12). As shown in **Figure 7A**, basal levels of GSH in lung homogenates from the mock-infected control group were slightly higher and less variable in females than in males. As expected, levels decreased in both sexes after infection, but on p.i. day 6, there was a sharp drop in the GSH content of male lungs, which resulted in significantly lower levels than those found in females (**p*-value = 0.034). Interestingly, this drop coincided with the time of viral loads peak in the lungs of the male mice.

Reduced glutathione depletion may be due to its buffering role against ROS that, during viral infection, essentially derive from NOX4 (11). Therefore, we evaluated the expression of this enzyme in the lung homogenate of females and males. Densitometric analysis of three animals for both sexes demonstrated that NOX4 was less expressed in females than in males (3 and 6 days p.i., 1.5-fold lower, unpaired *t*-test **p*-value ≤0.05; **Figure 7B**).

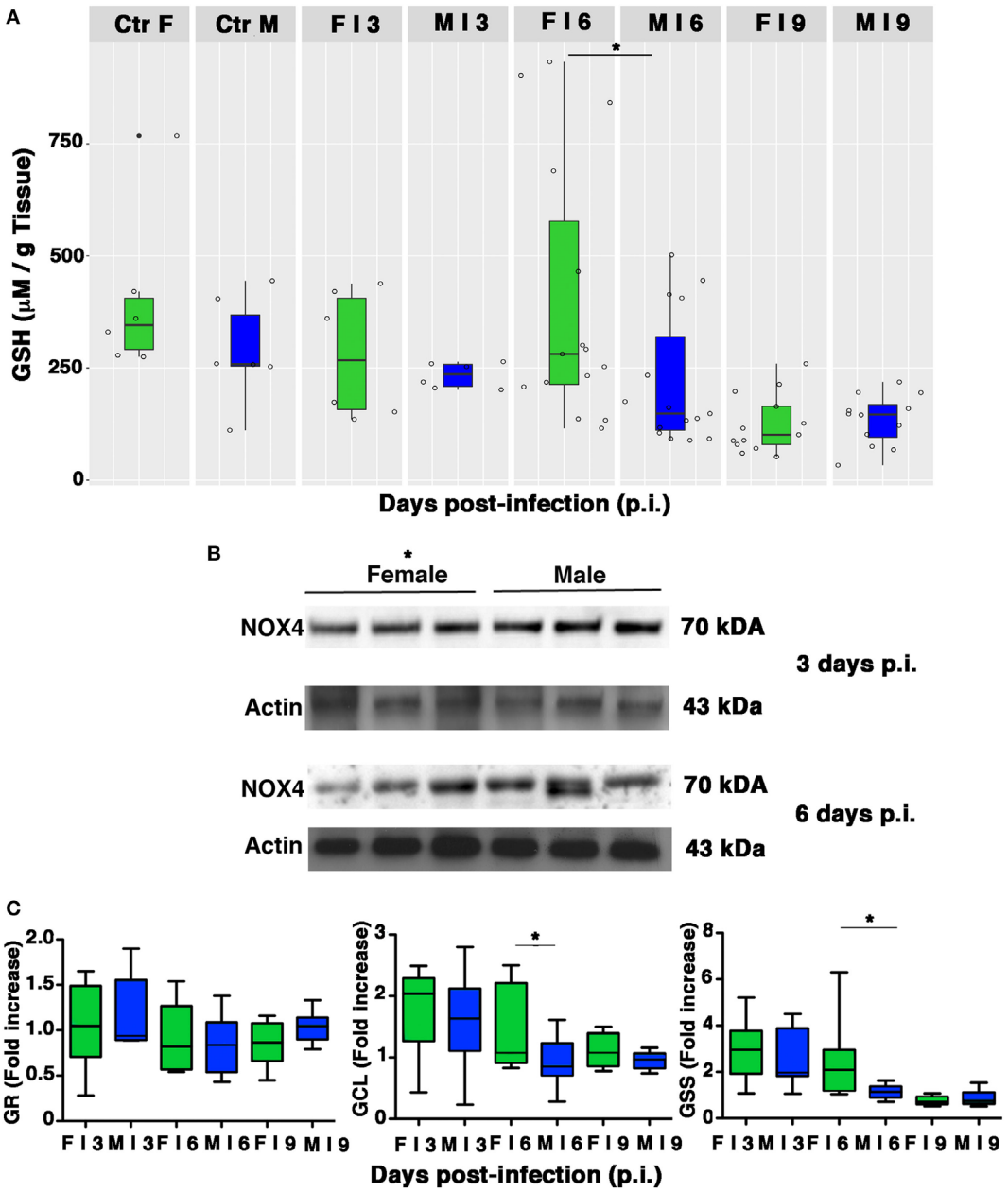


FIGURE 7 | Pulmonary intracellular reduced glutathione (GSH) levels are higher in females than in males. **(A)** GSH content was measured in the lungs of mock-infected (Ctr) or infected (I) female and male mice sacrificed at indicated time points. Results represent data pooled from four separate experiments. In details, mock-infected mice were 12 (6/sex), infected mice on p.i. day 3 were 12 (6/sex), on p.i. day 6 were 30 (15/sex), on p.i. day 9 were 25 (13 females and 12 males) **p*-value = 0.034. **(B)** NADPH oxidase 4 (NOX4) expression was analyzed by western blotting in the lungs of infected female and male mice. Actin was used as loading control. Blots shown are one representative experiment of three performed (three animals for each sex, **p*-value ≤0.05). **(C)** RT-PCR quantification of enzymes responsible for recycling and biosynthesis of GSH [glutathione reductase (GR), glutamate cysteine ligase (GCL), and glutathione synthase (GSS)]. Gene expression was measured in lung homogenates of animals sacrificed on p.i. days 3, 6, and 9. Box-plots represent the fold increases relative to levels observed in mock-infected controls (n = 8/sex). Unpaired t-test **p*-value ≤0.05.

Intracellular GSH is regenerated from the oxidized form (GSSG) by GR or synthesized *ex novo* by the consecutive actions of GCL and GSS (50). Our next step was thus aimed at determining whether the sex-related differences in pulmonary GSH levels were also associated with differences in transcriptional expression of these three enzymes. As shown in **Figure 7C**, compared with their male counterparts, female PR8-infected mice showed a greater upregulation of GCL and GSS expression, suggesting more efficiency in counteracting PR8-induced GSH depletion (unpaired t-test **p*-value <0.05). Collectively, these results indicate that female mice have an intrinsically higher antioxidant capacity, and during PR8 infection they are also capable of more

efficient restoration of the physiological redox milieu in terms of GSH content that could be due to the upregulation of its synthesis.

Lung Homogenates From Females Contain Higher Levels of the Anti-Apoptotic Bcl-2 Protein

Several intracellular redox-regulated pathways are involved in regulation of influenza virus replication, particularly the kinase p38MAP that is activated by NOX4-derived ROS (11). In cells that are highly permissive to viral infection, activated p38MAPK is entirely addressed to the nucleus, in which it participates efficiently in vRNP phosphorylation. In cells that are characterized by high levels of GSH and abundant expression of the anti-apoptotic protein Bcl-2, influenza virus replication is reduced (2). The inhibition is due to co-localization of activated p38MAPK with its cytosolic substrate (Bcl-2) and block of its translocation to the nucleus. As a consequence, NP is retained in the nucleus and viral replication is inhibited (18). Thus, we decided to evaluate whether the differences in viral load observed between the two sexes were also related to differences in p38MAPK activation and in Bcl-2 expression in the lungs. We found that p38MAPK was early activated in both groups on p.i. day 3 (**Figure 8A**). However, densitometric analysis of three different animals revealed that the kinase was more activated (almost twofold) in males compared to three homogenates of females (unpaired *t*-test **p*-value = 0.02), thereby indicating more efficiency of p38MAPK in males. Afterward, we evaluated the expression of Bcl-2 (both mRNA and protein) in the lungs of females and males on p.i. days 3 and 6. We found that during viral infection, female mice exhibited more substantial *bcl-2* gene upregulation compared with males (**Figure 8B** unpaired *t*-test: ***p*-value = 0.0018; ****p*-value = 0.0002). Specifically, *bcl-2* transcript levels in females were approximately two times as high as those found from mock-infected controls. On the contrary, there was no significant upregulation in the male mice. At the same time, densitometric analysis three different animals revealed that Bcl-2 protein levels found in the lung homogenates were also clearly higher in the female group (**Figure 8C**, 3 and 6 days p.i., unpaired *t*-test **p*-value ≤0.05).

All these results indicate that during PR8 viral infection, females activate transcriptional processes to maintain high levels of Bcl-2 protein. This event might contribute to keep p38MAPK in the cytosol and to inhibit NP traffic and viral replication.

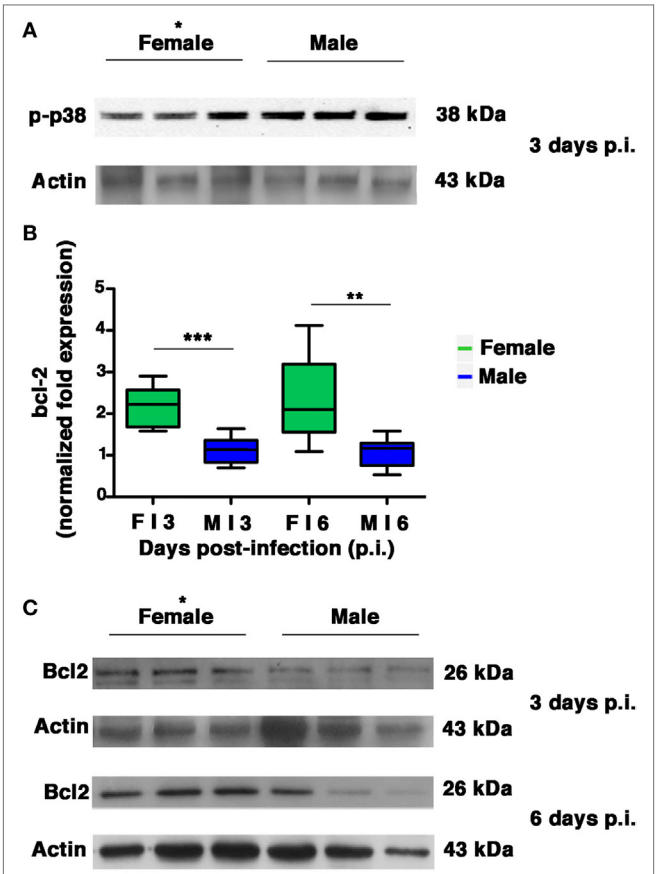


FIGURE 8 | Female mice respond to PR8 infection with marked upregulation of Bcl-2 expression. **(A)** p-p38MAPK expression was analyzed by western blotting in the lungs of infected female and male mice at 3 days p.i. (three animals for each sex, **p*-value = 0.02). **(B)** RT-PCR quantification of *bcl-2* gene expression in the lung homogenates of infected mice euthanized on p.i. days 3 and 6. Box-plots represent the fold increases relative to levels observed in mock-infected controls (n = 8/sex), unpaired *t*-test ***p*-value = 0.0018; ****p*-value = 0.0002. **(C)** Bcl-2 protein expression was analyzed by western blotting in the lungs of infected female and male mice. Actin was used as loading control. Blots shown are one representative experiment of three performed (three animals for each sex, **p*-value ≤0.05).

DISCUSSION

In this article, we focused on one of the *in vivo* mechanisms contributing to sex-related disparities in influenza virus infection. In particular, we pointed at systemic and organ redox state as critical determinant for influenza virus replication. We found that female mice infected with PR8 displayed a higher survival rate, milder clinical disease, and lower pulmonary viral loads than their male counterparts. These sex-based disparities correlate largely on differences between the redox conditions in the female and male animals. Mock-infected female mice have an intrinsically higher antioxidant capacity, measured as total serum antioxidant power and GSH content in lung homogenates. These better physiological conditions persist during viral infection when we observed: upregulation of enzymes responsible for GSH biosynthesis, higher level of PRDX1, maintenance of CAT activity, and a less decrease of GSHPx activity. Infected females display also higher expression (at the mRNA and protein levels) of the anti-apoptotic protein Bcl-2, which is involved in the regulation of specific steps of influenza virus replication (2, 18). On the other hand, infected male mice displayed high expression of NOX4 enzyme, and increased levels of phosphorylated p38MAPK.

The impact of sex on susceptibility to viral infections has been hypothesized several years ago (51). Generally, females and males of various species respond differently to many DNA and RNA viruses. The mechanisms underpinning sex differences in response to viral infections are controversial, and roles for immunological, hormonal, behavioral, epigenetic, and genetic factors have all been proposed (20, 52).

It has been shown that females generate stronger innate and adaptive immune responses than males, with immune cells higher in number and activity, as well as with higher antibodies levels than males (21–23). This immunological advantage contributes to virus clearance, but on the other hand it makes females more prone to autoimmune diseases and to infectious disease-derived immunopathology (21–23). In fact, infectious diseases pathogenesis derives both from the pathogen and from the host immune response (22, 23). Influenza viruses can cause severe disease as interstitial pneumonia and bronchiolitis, characterized by typical inflammatory anatomical–pathological lesions and sometimes, massive hemorrhage, with interstitial, bronchiolar, and alveolar localization (53).

In addition to a massive cell infiltrates in the infected lungs, there is an overproduction of several pro-inflammatory cytokines and chemokines (54, 55). In fact, in this study, we observed histopathological alterations relative to interstitial pneumonia and in line with this observation, we found high levels of IL-1, IL-6, TNF-α, IL-10, IFN-γ, CCL2-MCP1, and CCL3-MIP1 in infected mice of both sexes.

However, the picture in female mice appeared different because of larger preserved parenchymal areas and a consequently total alveolar area significantly higher than in male mice. This difference persisted during the infection, suggesting that the virally induced inflammation had a lower impact on lung's female. Hormones exert a complex role in inflammation, in particular, estradiol enhances inflammation at low doses, but reduces it at higher concentrations (56), while low concentration of testosterone has reported to have a negative impact on the outcome of influenza disease (57). In our model, we found basal levels of estradiol, which did not significantly change with the infection, similar to that reported by Robinson et al. (24). Although a slight decrease, testosterone as well did not significantly vary with the infection. Therefore, in the attempt to explain the less severe lung damage that we observed in females, especially between 6 and 9 days p.i., we looked at the pathogen: we found lower viral titer in lung homogenates and BALF from female mice, with a lower infectivity, as shown by $TCID_{50}$. So, these results lead us to argue that the lower impact observed in females is related to a less extent of virus replication and spread, more than a host immune effect.

With this hypothesis our attention focused on viral replication and the possible redox-related mechanisms underlying sex disparity. Female and male mice differed remarkably in terms of their basal redox state and their ability to counteract virus-associated oxidative imbalance. Prior to inoculation, more reducing conditions were found in the female animals in terms of GSH levels in the lungs and the TAC. These differences are in line with those reported in VSMC isolated from the aortas of male and female rats (35). *In vitro* data suggest that this sexual dimorphism can be maintained after the induction of oxidative stress, which results in females displaying greater resistance to oxidative injury and an increased capacity to counteract it (35). For example, some authors report different sex-dependent susceptibility to cytotoxic agents and treatments that is related to the incapacity of XY neurons to maintain GSH intracellular levels (58). Our *in vivo* findings support this view: during the course of influenza virus infection, the intrinsic redox balance, i.e., reducing conditions, was more effectively maintained in the female mice. In these animals, inoculation was promptly followed by the activation of enzymes involved in biosynthesis of GSH aimed at counteracting the GSH depletion induced by the virus. The period of upregulated GSH synthesis and higher levels of GSH in the females coincided with the period characterized by peak viral loads in males.

Thiols are key players in conditions of oxidative stress. Most non-protein antioxidants as well as antioxidant enzymes are thiol based (59). GSH acts as radical scavenger by directly neutralizing a variety of reactive molecules, like superoxide anion and hydroxylradicals (60), and indirectly through enzymatic reactions being a cofactor of GSHPx (61). Here, we found in male mice, higher levels of NOX4, one of the major enzymes producing ROS, thus suggesting that GSH depletion in males might be due to its consumption for its ROS buffering function. In fact, we have previously demonstrated that inhibition of NOX4 activity through chemical inhibitors or RNA silencing blocks the influenza virus-induced ROS increase, restores the content of GSH, and inhibits viral replication (11). Interestingly, several studies demonstrate that estrogens inhibit ROS production (56) by modulating antioxidant enzyme activities (62); estrogen levels have been shown also to be positively correlated to GSHPx activity in women, while no significant correlation was observed with SOD (63, 64) that in our model did not change between sexes. Moreover, estradiol has been shown to increase expression of GCL (65), that is the rate-limiting enzyme for the synthesis of GSH (60) and therefore, together with GSS and GSHPx, closely linked to the GSH levels. On the contrary, testosterone has been shown to have pro-oxidant effect (66, 67) and so we cannot exclude that it could contribute to viral replication in males by activating redox-sensitive pathways. We also found a drop in CAT activity in infected males, especially when the viral replication peaked at 6 days p.i. Accordingly, a time-dependent decrease in CAT activity has been observed in parallel to increase in influenza NS1-protein expression (13).

Several authors report that by restoring reducing conditions, viral replication and virus-induced host damage are inhibited, suggesting antioxidant therapy as a potential antiviral strategy (8, 9, 68–70). Indeed, various synthesized or natural compounds characterized by antioxidant activity have been proposed as anti-influenza agents (17, 71–76). For example, our group has shown that GSH treatment strongly inhibits viral replication by impairing glycoprotein folding (10); on the other hand, we have recently shown that GSH depletion increased influenza virus replication by preventing activation of innate antiviral response (7). Indeed, the role of GSH in modulating immune response is well known (8, 77–79). For example, in antigen-presenting cells, GSH depletion correlates with defective antigen processing and reduced secretion of T helper 1 (Th1) cytokines, thus favoring polarization from the typical Th1 profile toward a Th2 response (8). Furthermore, in T lymphocytes, intracellular GSH content is critical for their proliferation as well as extracellular thiols for their activation and function. Angelini et al. (80) demonstrated that exogenous thiols, i.e., free cysteine and thioredoxin, were released by monocyte-derived human dendritic cells (DCs) in the extracellular space to provide a reducing microenvironment required for T lymphocyte activation and an efficient immune

response. In our study, we found a slight decrease of free thiols in the lung homogenates and serum of infected males, while no changes were observed in infected females compared with control. It would be interesting to investigate whether the observed decrement in males is due to a dysfunction of DCs and to impairment in T lymphocyte activation.

The imbalance in the redox state is fundamental for the activation of many cell factors, involved in the regulation of host response and in the control of influenza virus life cycle (16). Among them, MAPKs and Bcl-2 protein regulate the intracellular trafficking of the viral NP (18). In this study, we found that phosphorylation of p38MAPK is highly expressed in lung homogenate of males, suggesting that this phenomenon could explain in part the high viral load measured in males. Conversely, we found Bcl-2 to be highly expressed in the lungs of infected female mice, at both transcriptional and translational level. Furthermore, overexpression of Bcl-2 protein has been hypothesized to be associated with increased GSH levels (81, 82), and these characteristics have been found in lung homogenates of female infected mice. Based on this evidence, we can hypothesize that in females the more resistance to oxidative damage during PR8 infection may impair virus replication probably by blocking viral protein maturation and vRNP complex formation.

A final point to be considered in this scenario concerns the hypothesized role of autophagy, a cytoprotective host process that is subverted by the influenza virus to ensure its own replication (83). Metabolic stress appears to bolster a stronger, more sustained autophagic response in cells from females than in those collected from males (84). Therefore, we cannot exclude the possibility that more effective autophagic cytoprotection in lung cells from female mice led to a "weaker" cytopathological cascade.

In conclusion, our data suggest that the mechanisms underlying the sexual disparities observed in the host response to influenza can be ascribed in part to differences in their capacity to maintain redox homeostasis. In fact, in our model, we have found that females are more resistant to the influenza virus due to their ability to maintain reduced conditions during infection, thereby hindering completion of the virus life cycle and inhibiting viral replication. Therefore, although further studies are needed to fine characterize redox mechanisms underlying sex disparities in infections, i.e., the use of different antioxidants like *N*-acetylcysteine, GSH, or natural polyphenols, as well as the silencing of antioxidant enzymes that regulate viral replication, our findings may contribute to the identification of new targets for sex-based antiviral therapies. Indeed, generally, sex-related differences are not considered in current strategies for the prevention, management, and treatment of many diseases (85). Instead, a more detailed knowledge of the metabolic conditions that characterize the two sexes could ultimately improve our ability to provide patients with individualized therapies and cost-effective solutions.

ETHICS STATEMENT

In accordance with national law, the experiments described in this manuscript were approved by the Italian Ministry of Health, which verified the ethical and scientific appropriateness of the research. All animals received humane treatment, and every effort was made to minimize their suffering.

AUTHOR CONTRIBUTIONS

IC, PChecconi, DA, MA, PColuccio, RD, and PM performed experiments. DF, AC, MT, RM, CM, AV, WM, and LN analyzed data. IC, PChecconi, EG, ATP, and LN designed and supervised experiments. IC, PChecconi, ATP, and LN wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01747/full#supplementary-material.

FIGURE S1 | Body weight (bw) and temperature in female and male infected mice. **(A)** Spaghetti plot of the daily bw (expressed in grams); the bold lines represent the overall trend. **(B)** Spaghetti plot of the daily body temperature, the bold lines represent the overall trend. Results represent data pooled from 4 independent experiments, each performed with 10 males and 10 females ($n = 40$/sex).

FIGURE S2 | No differences in superoxide dismutase (SOD) activity were observed in infected female and male mice. Lung homogenates from Ctr and PR8-infected mice were assayed for SOD activity on p.i. days 3, 6, 9, and 21. Each value reported represents the mean ± SD of results from two separate experiments, each performed in duplicate ($n = 4$).

FIGURE S3 | Glutathione peroxidase (GSHPx) activity is less reduced in infected female mice. Lung homogenates from Ctr and PR8-infected mice were assayed for GSHPx activity on p.i. day 6. Each value reported represents the mean ± SD of results from 3 mice/sex, each performed in triplicate ($n = 9$). One-way ANOVA test **p-value <0.01; ***p-value <0.001.

REFERENCES

1. Nencioni L, Sgarbanti R, Amatore D, Checconi P, Celestino I, Limongi D, et al. Intracellular redox signaling as therapeutic target for novel antiviral strategy. *Curr Pharm Des* (2011) 17:3898–904. doi:10.2174/138161211798357728
2. Nencioni L, Iuvara A, Aquilano K, Ciriolo MR, Cozzolino F, Rotilio G, et al. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. *FASEB J* (2003) 17:758–60. doi:10.1096/fj.02-0508fje
3. Checconi P, Sgarbanti R, Celestino I, Limongi D, Amatore D, Iuvara A, et al. The environmental pollutant cadmium promotes influenza virus replication in MDCK cells by altering their redox state. *Int J Mol Sci* (2013) 14:4148–62. doi:10.3390/ijms14024148
4. Ehrhardt C, Seyer R, Hrincius ER, Eierhoff T, Wolff T, Ludwig S. Interplay between influenza A virus and the innate immune signaling. *Microbes Infect* (2010) 12:81–7. doi:10.1016/j.micinf.2009.09.007
5. Vlahos R, Stambas J, Bozinovski S, Broughton BR, Drummond GR, Selemidis S. Inhibition of Nox2 oxidase activity ameliorates influenza A virus-induced

lung inflammation. *PLoS Pathog* (2011) 7:e1001271. doi:10.1371/journal.ppat.1001271
6. Olagnier D, Peri S, Steel C, van Montfoort N, Chiang C, Beljanski V, et al. Cellular oxidative stress response controls the antiviral and apoptotic programs in dengue virus-infected dendritic cells. *PLoS Pathog* (2014) 10:e1004566. doi:10.1371/journal.ppat.1004566
7. Diotallevi M, Checconi P, Palamara AT, Celestino I, Coppo L, Holmgren A, et al. Glutathione fine-tunes the innate immune response toward antiviral pathways in a macrophage cell line independently of its antioxidant properties. *Front Immunol* (2017) 8:1239. doi:10.3389/fimmu.2017.01239
8. Fraternale A, Brundu S, Magnani M. Glutathione and glutathione derivatives in immunotherapy. *Biol Chem* (2017) 398:261–75. doi:10.1515/hsz-2016-0202
9. Cai J, Chen Y, Seth S, Furukawa S, Compans RW, Jones DP. Inhibition of influenza infection by glutathione. *Free Radic Biol Med* (2003) 34:928–36. doi:10.1016/S0891-5849(03)00023-6
10. Sgarbanti R, Nencioni L, Amatore D, Coluccio P, Fraternale A, Sale P, et al. Redox-regulation of the influenza hemagglutinin maturation process: a new cell-mediated strategy for anti-influenza therapy. *Antioxid Redox Signal* (2011) 15:593–606. doi:10.1089/ars.2010.3512
11. Amatore D, Sgarbanti R, Aquilano K, Baldelli S, Limongi D, Civitelli L, et al. Influenza virus replication in lung epithelial cells depends on redox-sensitive pathways activated by NOX4-derived ROS. *Cell Microbiol* (2015) 17:131–45. doi:10.1111/cmi.12343
12. Kumar P, Khanna M, Srivastava V, Tyagi YK, Raj HG, Ravi K. Effect of quercetin supplementation on lung antioxidants after experimental influenza virus infection. *Exp Lung Res* (2005) 5:449–59. doi:10.1080/019021490927088
13. Qi X, Zhang H, Wang Q, Wang J. The NS1 protein of avian influenza virus H9N2 induces oxidative-stress-mediated chicken oviduct epithelial cells apoptosis. *J Gen Virol* (2016) 97:3183–92. doi:10.1099/jgv.0.000625
14. Checconi P, Salzano S, Bowler L, Mullen L, Mengozzi M, Hanschmann EM, et al. Redox proteomics of the inflammatory secretome identifies a common set of redoxins and other glutathionylated proteins released in inflammation, influenza virus infection and oxidative stress. *PLoS One* (2015) 10:e0127086. doi:10.1371/journal.pone.0127086
15. Yamada Y, Limmon GV, Zheng D, Li N, Li L, Yin L, et al. Major shifts in the spatio-temporal distribution of lung antioxidant enzymes during influenza pneumonia. *PLoS One* (2012) 7:e31494. doi:10.1371/journal.pone.0031494
16. Nencioni L, Sgarbanti R, De Chiara G, Garaci E, Palamara AT. Influenza virus and redox mediated cell signaling: a complex network of virus/host interaction. *New Microbiol* (2007) 30:367–75.
17. Palamara AT, Nencioni L, Aquilano K, De Chiara G, Hernandez L, Cozzolino F, et al. Resveratrol inhibits influenza A virus replication in vitro and in vivo. *J Infect Dis* (2005) 191:1719–29. doi:10.1086/429694
18. Nencioni L, De Chiara G, Sgarbanti R, Amatore D, Aquilano K, Marcocci ME, et al. Bcl-2 expression and p38MAPK activity in cells infected with influenza A virus: impact on virally induced apoptosis and viral replication. *J Biol Chem* (2009) 284:16004–15. doi:10.1074/jbc.M900146200
19. WHO, Department of Gender, Women and Health. *Sex, Gender and Influenza*. Geneva, Switzerland: WHO press (2010).
20. Klein SL, Hodgson A, Robinson DP. Mechanisms of sex disparities in influenza pathogenesis. *J Leukoc Biol* (2012) 92:67–73. doi:10.1189/jlb.0811427
21. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. *Nat Rev Immunol* (2010) 10:594–604. doi:10.1038/nri2815
22. Vom Steeg LG, Klein SL. SeXX matters in infection disease pathogenesis. *PLoS Pathog* (2016) 12(2):e1005374. doi:10.1371/journal.ppat.1005374
23. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* (2016) 16:626–38. doi:10.1038/nri.2016.90
24. Robinson DP, Lorenzo ME, Jian W, Klein SL. Elevated 17β-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses. *PLoS Pathog* (2011) 7:e1002149. doi:10.1371/journal.ppat.1002149
25. Hall OJ, Nachbagauer R, Vermillion MS, Fink AL, Phuong V, Krammer F, et al. Progesterone-based contraceptives reduce adaptive immune responses and protection against sequential influenza A virus infections. *J Virol* (2017) 91(8):e02160-16. doi:10.1128/JVI.02160-16
26. Crighton EJ, Elliott SJ, Moineddin R, Kanaroglou P, Upshur RE. An exploratory spatial analysis of pneumonia and influenza hospitalizations in Ontario by age and gender. *Epidemiol Infect* (2007) 135:253–61. doi:10.1017/S095026880600690X
27. Jensen-Fangel S, Mohey R, Johnsen SP, Andersen PL, Sørensen HT, Ostergaard L. Gender differences in hospitalization rates for respiratory tract infections in Danish youth. *J Infect Dis* (2004) 36:31–6. doi:10.1080/00365540310017618
28. Wang XL, Yang L, Chan KH, Chan KP, Cao PH, Lau EH, et al. Age and sex differences in rates of influenza-associated hospitalizations in Hong Kong. *Am J Epidemiol* (2015) 182:335–44. doi:10.1093/aje/kwv068
29. Bonmarin I, Belchior E, Bergounioux J, Brun-Buisson C, Mégarbane B, Chappert JL, et al. Intensive care unit surveillance of influenza infection in France: the 2009/10 pandemic and the three subsequent seasons. *Euro Surveill* (2015) 20:46. doi:10.2807/1560-7917
30. Kawado M, Hashimoto S, Murakami Y, Izumida M, Ohta A, Tada Y, et al. Annual and weekly incidence rates of influenza and pediatric diseases estimated from infectious disease surveillance data in Japan, 2002–2005. *J Epidemiol* (2007) 17(Suppl):S32–41. doi:10.2188/jea.17.S32
31. Eshima N, Tokumaru O, Hara S, Bacal K, Korematsu S, Tabata M, et al. Sex- and age-related differences in morbidity rates of 2009 pandemic influenza A H1N1 virus of swine origin in Japan. *PLoS One* (2011) 6(4):e19409. doi:10.1371/journal.pone.0019409
32. Krementsov DN, Case LK, Dienz O, Raza A, Fang Q, Ather JL, et al. Genetic variation in chromosome Y regulates susceptibility to influenza A virus infection. *Proc Natl Acad Sci U S A* (2017) 114:3491–6. doi:10.1073/pnas.1620889114
33. Straface E, Gambardella L, Brandani M, Malorni W. Sex differences at cellular level: "cells have a sex". *Handb Exp Pharmacol* (2012) 214:49–65. doi:10.1007/978-3-642-30726-3_3
34. Matarrese P, Colasanti T, Ascione B, Margutti P, Franconi F, Alessandri C, et al. Gender disparity in susceptibility to oxidative stress and apoptosis induced by autoantibodies specific to RLIP76 in vascular cells. *Antioxid Redox Signal* (2011) 15:2825–36. doi:10.1089/ars.2011.3942
35. Malorni W, Straface E, Matarrese P, Ascione B, Coinu R, Canu S, et al. Redox state and gender differences in vascular smooth muscle cells. *FEBS Lett* (2008) 582:635–42. doi:10.1016/j.febslet.2008.01.034
36. Straface E, Vona R, Campesi I, Franconi F. Mitochondria can orchestrate sex differences in cell fate of vascular smooth muscle cells from rats. *Biol Sex Differ* (2015) 16(6):34. doi:10.1186/s13293-015-0051-9
37. Straface E, Malorni W, Pietraforte D. Sex differences in redox biology: a mandatory new point of view approaching human inflammatory diseases. *Antioxid Redox Signal* (2017) 26:44–5. doi:10.1089/ars.2016.6931
38. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock* (2004) 22:460–6. doi:10.1097/01.shk.0000142249.08135.e9
39. Tate MD, Brooks AG, Reading PC. The role of neutrophils in the upper and lower respiratory tract during influenza virus infection of mice. *Respir Res* (2008) 9:57. doi:10.1186/1465-9921-9-57
40. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* (2013) 497:498–502. doi:10.1038/nature12118
41. Maxeiner JH, Karwot R, Hausding M, Sauer KA, Scholtes P, Finotto S. A method to enable the investigation of murine bronchial immune cells, their cytokines and mediators. *Nat Protoc* (2007) 2:105–12. doi:10.1038/nprot.2007.8
42. Conti G, Magliani W, Conti S, Nencioni L, Sgarbanti R, Palamara AT, et al. Therapeutic activity of an anti-idiotypic antibody-derived killer peptide against influenza A virus experimental infection. *Antimicrob Agents Chemother* (2008) 52:4331–7. doi:10.1128/AAC.00506-08
43. Hu Q, Zuo P, Shao B, Yang S, Xu G, Lan F, et al. Administration of nonviral gene vector encoding rat *β*-defensin-2 ameliorates chronic *Pseudomonas aeruginosa* lung infection in rats. *J Gene Med* (2010) 12:276–86. doi:10.1002/jgm.1435
44. Lee-Lewis H, Anderson DM. Absence of inflammationand pneumonia during infection with nonpigmented *Yersinia pestis* reveals a new role for the pgm locus in pathogenesis. *Infect Immun* (2010) 78:220–30. doi:10.1128/IAI.00559-09
45. Litzlbauer HD, Neuhaeuser C, Moell A, Greschus S, Breithecker A, Franke FE, et al. Three-dimensional imaging and morphometry of alveolar tissue from microfocal x-ray-computed tomography. *Am J Physiol Lung Cell Mol Physiol* (2006) 291:L535–45. doi:10.1152/ajplung.00088.2005

46. Carpino G, Morini S, Ginanni Corradini S, Franchitto A, Merli M, Siciliano M, et al. Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Dig Liver Dis* (2005) 37:349–56. doi:10.1016/j.dld.2004.11.009
47. Mouded M, Egea EE, Brown MJ, Hanlon SM, Houghton AM, Tsai LW, et al. Epithelial cell apoptosis causes acute lung injury masquerading as emphysema. *Am J Respir Cell Mol Biol* (2009) 41:407–14. doi:10.1165/rcmb.2008-0137OC
48. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* (1959) 82:70–7. doi:10.1016/0003-9861(59)90090-6
49. Larcombe AN, Foong RE, Bozanich EM, Berry LJ, Garratt LW, Gualano RC, et al. Sexual dimorphism in lung function responses to acute influenza A infection. *Influenza Other Respir Viruses* (2011) 5:334–42. doi:10.1111/j.1750-2659.2011.00236
50. Meister A. Glutathione biosynthesis and its inhibition. *Methods Enzymol* (1995) 252:26–30. doi:10.1016/0076-6879(95)52005-8
51. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev* (2000) 24:627–38. doi:10.1016/S0149-7634(00)00027-0
52. Torcia MG, Nencioni L, Clemente AM, Civitelli L, Celestino I, Limongi D, et al. Sex differences in the response to viral infections: TLR8 and TLR9 ligand stimulation induce higher IL10 production in males. *PLoS One* (2012) 7:e39853. doi:10.1371/journal.pone.0039853
53. Fukushi M, Ito T, Oka T, Kitazawa T, Miyoshi-Akiyama T, Kirikae T, et al. Serial histopathological examination of the lungs of mice infected with influenza A virus PR8 strain. *PLoS One* (2011) 6:e21207. doi:10.1371/journal.pone.0021207
54. Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* (2007) 445:319–23. doi:10.1038/nature05495
55. Wareing MD, Lyon AB, Lu B, Gerard C, Sarawar SR. Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. *J Leukoc Biol* (2004) 76:886–95. doi:10.1189/jlb.1203644
56. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* (2007) 28:521–74. doi:10.1210/er.2007-0001
57. Vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, et al. Age and testosterone mediate influenza pathogenesis in male mice. *Am J Physiol Lung Cell Mol Physiol* (2016) 311:L1234–44. doi:10.1152/ajplung.00352.2016
58. Du L, Bayir H, Lai Y, Zhang X, Kochanek PM, Watkins SC, et al. Innate gender-based proclivity in response to cytotoxicity and programmed cell death pathway. *J Biol Chem* (2004) 279:38563–70. doi:10.1074/jbc.M405461200
59. Fra A, Yoboue ED, Sitia R. Cysteines as redox molecular switches and targets of disease. *Front Mol Neurosci* (2017) 10:167. doi:10.3389/fnmol.2017.00167
60. Dickinson DA, Forman HJ. Glutathione in defense and signaling: lessons from a small thiol. *Ann N Y Acad Sci* (2002) 973:488–504. doi:10.1111/j.1749-6632.2002.tb04690.x
61. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. *Nutrition* (2002) 18(10):872–9. doi:10.1016/S0899-9007(02)00916-4
62. Pajović SB, Saicić ZS. Modulation of antioxidant enzyme activities by sexual steroid hormones. *Physiol Res* (2008) 57(6):801–11.
63. Massafra C, Gioia D, De Felice C, Picciolini E, De Leo V, Bonifazi M, et al. Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. *J Endocrinol* (2000) 167:447–52. doi:10.1677/joe.0.1670447
64. Massafra C, Gioia D, De Felice C, Muscettola M, Longini M, Buonocore G. Gender-related differences in erythrocyte glutathione peroxidase activity in healthy subjects. *Clin Endocrinol (Oxf)* (2002) 57:663–7. doi:10.1046/j.1365-2265.2002.01657.x
65. Urata Y, Ihara Y, Murata H, Goto S, Koji T, Yodoi J, et al. 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells. *J Biol Chem* (2006) 281(19):13092–102. doi:10.1074/jbc.M601984200
66. Chignalia AZ, Oliveira MA, Debbas V, Dull RO, Laurindo FR, Touyz RM, et al. Testosterone induces leucocyte migration by NADPH oxidase-driven ROS- and COX2 dependent mechanisms. *Clin Sci* (2015) 129:39–48. doi:10.1042/CS20140548
67. Reed DK, Arany I. Sex hormones differentially modulate STAT3-dependent antioxidant responses during oxidative stress in renal proximal tubule cells. *In Vivo* (2014) 28:1097–100.
68. Uchide N, Toyoda H. Antioxidant therapy as a potential approach to severe influenza-associated complications. *Molecules* (2011) 16(3):2032–52. doi:10.3390/molecules16032032
69. Sgarbanti R, Amatore D, Celestino I, Marcocci ME, Fraternale A, Ciriolo MR, et al. Intracellular redox state as target for anti-influenza therapy: are antioxidants always effective? *Curr Top Med Chem* (2014) 14(22):2529–41. doi:10.2174/1568026614666141203125211
70. Botta G, Bizzarri BM, Garozzo A, Timpanaro R, Bisignano B, Amatore D, et al. Carbon nanotubes supported tyrosinase in the synthesis of lipophilic hydroxytyrosol and dihydrocaffeoyl catechols with antiviral activity against DNA and RNA viruses. *Bioorg Med Chem* (2015) 23(17):5345–51. doi:10.1016/j.bmc.2015.07.061
71. Aggarwal BB, Deb L, Prasad S. Curcumin differs from tetrahydrocurcumin for molecular targets, signaling pathways and cellular responses. *Molecules* (2014) 20(1):185–205. doi:10.3390/molecules20010185
72. Di Sotto A, Checconi P, Celestino I, Locatelli M, Carissimi S, De Angelis M, et al. Antiviral and antioxidant activity of a hydroalcoholic extract from *Humulus lupulus* L. *Oxidat Med Cell Long* (2018).
73. Saladino R, Neri V, Checconi P, Celestino I, Nencioni L, Palamara AT, et al. Synthesis of 2'-deoxy-1'-homo-N-nucleosides with anti-influenza activity by catalytic methyltrioxorhenium (MTO)/H2O2 oxyfunctionalization. *Chemistry* (2013) 19:2392–404. doi:10.1002/chem.201201285
74. Bizzarri BM, Botta L, Capecchi E, Celestino I, Checconi P, Palamara AT, et al. Regioselective IBX mediated synthesis of coumarin derivatives with antioxidant and anti-influenza activities. *J Nat Prod* (2017) 80(12):3247–54. doi:10.1021/acs.jnatprod.7b00665
75. Bozzini T, Botta G, Delfino M, Onofri S, Saladino R, Amatore D, et al. Tyrosinase and Layer-by-Layer supported tyrosinases in the synthesis of lipophilic catechols with antiinfluenza activity. *Bioorg Med Chem* (2013) 21(24):7699–708. doi:10.1016/j.bmc.2013.10.026
76. Fioravanti R, Celestino I, Costi R, Cuzzucoli Crucitti G, Pescatori L, Mattiello L, et al. Effects of polyphenol compounds on influenza A virus replication and definition of their mechanism of action. *Bioorg Med Chem* (2012) 20:5046–52. doi:10.1016/j.bmc.2012.05.062
77. Yan Z, Banerjee R. Redox remodeling as an immunoregulatory strategy. *Biochemistry* (2010) 49:1059–66. doi:10.1021/bi902022n
78. Dröge W, Breitkreutz R. Glutathione and immune function. *Proc Nutr Soc* (2000) 59:595–600. doi:10.1017/S0029665100000847
79. Ghezzi P. Role of glutathione in immunity and inflammation in the lung. *Int J Gen Med* (2011) 4:105–13. doi:10.2147/IJGM.S15618
80. Angelini G, Gardella S, Ardy M, Ciriolo MR, Filomeni G, Di Trapani G, et al. Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. *Proc Natl Acad Sci U S A* (2002) 99(3):1491–6. doi:10.1073/pnas.022630299
81. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* (1997) 15:1461–70. doi:10.1038/sj.onc.1201310
82. Voehringer DW, McConkey DJ, McDonnell TJ, Brisbay S, Meyn RE. Bcl-2 expression causes redistribution of glutathione to the nucleus. *Proc Natl Acad Sci U S A* (1998) 95:2956–60. doi:10.1073/pnas.95.6.2956
83. Matarrese P, Nencioni L, Checconi P, Ciarlo L, Gambardella L, Ascione B, et al. Pepstatin A alters host cell autophagic machinery and leads to a decrease in influenza A virus production. *J Cell Physiol* (2011) 226:3368–77. doi:10.1002/jcp.22696
84. Lista P, Straface E, Brunelleschi S, Franconi F, Malorni W. On the role of autophagy in human diseases: a gender perspective. *J Cell Mol Med* (2011) 15:1443–57. doi:10.1111/j.1582-4934.2011.01293.x
85. Regitz-Zagrosek V, Seeland U. Sex and gender differences in clinical medicine. *Handb Exp Pharmacol* (2012) 214:3–22. doi:10.1007/978-3-642-30726-3_1

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