SEX DIFFERENCES IN INFLAMMATORY DISEASES

EDITED BY: Antonietta Rossi, Fiorentina Roviezzo, Luigia Trabace and Joe G. Zein

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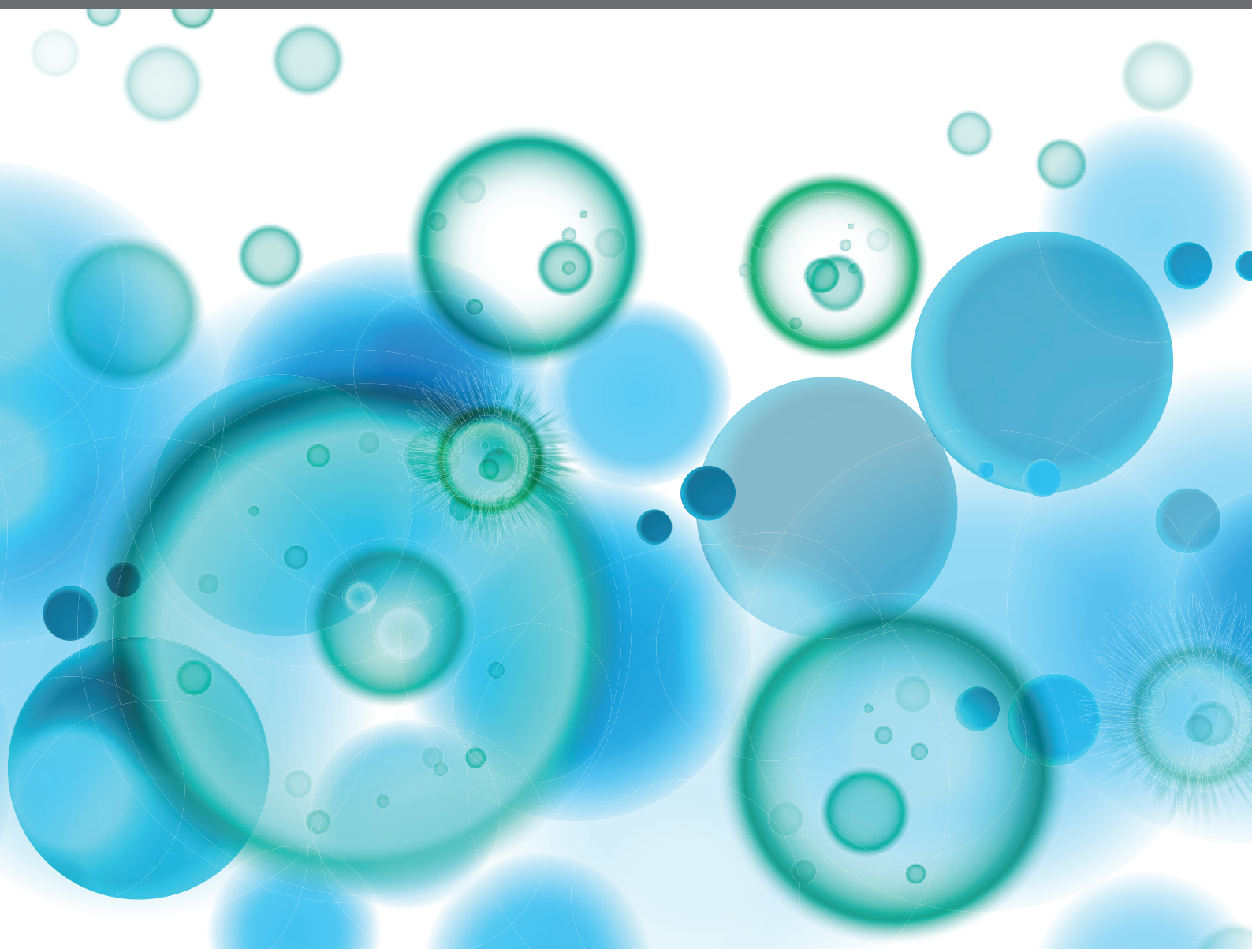

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SEX DIFFERENCES IN INFLAMMATORY DISEASES

Topic Editors:
Antonietta Rossi, University of Naples Federico II, Italy
Fiorentina Roviezzo, University of Naples Federico II, Italy
Luigia Trabace, University of Foggia, Italy
Joe G. Zein, Cleveland Clinic, United States

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EDITORIAL
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Editorial: Sex Differences in Inflammatory Diseases

Luigia Trabace[1]*, *Fiorentina Roviezzo*[2] *and Antonietta Rossi*[2]

[1]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy, [2]Department of Pharmacy, University of Naples Federico II, Naples, Italy

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Editorial on the Research Topic

Sex Differences in Inflammatory Diseases

Inflammatory diseases significantly differ between men and women in regard to their incidence, manifestations, gravity and prognosis, as well as response to pharmacological treatments (Di Florio et al., 2020; Mauvais-Jarvis et al., 2020). Despite the increasing efforts to consider sex as a crucial biological variable impacting on the pathophysiology of inflammatory and autoimmune diseases, cellular and molecular pathways underlying sexual differences in these disorders remain scarcely elucidated. This is also due to the under-representation of the female sex not only in preclinical research, but also in clinical trials.

In this Research Topic, a team of international experts presents the results of their preclinical and clinical studies focused on sex differences in inflammatory and autoimmune disorders affecting different organs. Moreover, findings regarding sex impact on pathogen-induced immune and autoimmune responses are shown and discussed.

An increasing number of evidence points toward a sex-based effects in several disorders of the cardiovascular system, including autoimmune myocarditis and related cardiac fibrosis and dilated cardiomyopathy (Fairweather et al., 2012; Fairweather et al., 2013). In this regard, in a first original article, Barcena et al. assess possible sex-dependent differences in alterations of cardiac function, inflammation and fibrosis development in an animal model of experimental autoimmune myocarditis, showing the presence of a pro-inflammatory phenotype in male animals and of an anti-inflammatory phenotype in females. Macrophage polarization and activation of cardiac fibroblasts have been reported to be crucially involved in myocardial inflammation and remodeling (Kim et al., 2021). In a second original research article, Barcena et al. demonstrate that the polarization of bone marrow-derived macrophages, with a significant overexpression of M1 and M2 markers, as well as increased levels of reactive oxygen species, specifically occurs in male mice. Interestingly, they also show that an inflammatory environment promotes the activation of cardiac fibroblasts, with significant higher levels of the pro-fibrotic markers TGF-β and IL-1β in activated cardiac male fibroblasts compared to female ones.

Recent data have also highlighted a significant impact of sex-dependent differences in the development of cardiac valve alterations, such as aortic stenosis (AS) (Saeed et al., 2020a; Saeed et al., 2020b). In particular, fibrosis has been shown to mainly affects women's aortic valves, whereas higher calcification degrees have been described in men. In this regard, Myasoedova et al. evaluate aortic valve fibrosis in men and women with a severe AS, showing a significant effect of sex on the fibro-calcific process of the aortic valve, both at gene expression and cell type level, with lower content of aortic valve calcium, higher fibrosis and an over-representation of mesenchymal cells in women. Pro-inflammatory pathways, characterized by increased levels of monocytes, macrophages, T and B cells, were, instead, enhanced in men. With respect to vessel autoimmune diseases, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) affects multiple organs, including

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Edited and reviewed by:
Dieter Steinhilber,
Goethe University Frankfurt, Germany

***Correspondence:**
Luigia Trabace
luigia.trabace@unifg.it

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kidney, resulting in ANCA glomerulonephritis (GN). In their retrospective study, Tampe et al. aim to evaluate possible sex differences in patients with AAV and biopsy-proven ANCA GN in both laboratory parameters and histopathological scoring of glomerular and tubulointerstitial lesions, as well as AAV extrarenal manifestations. Although sex was not correlated with short-term clinical AAV course, disease severity and ANCA GN classification, females showed a lower tubulointerstitial inflammation and vasculitis of peritubular capillaries compared to males.

Among autoimmune disorders, psoriasis is the one in which the role of sex has been poorly investigated so far, although it has been widely reported that fluctuations of estrogen levels are implicated in the variety of psoriasis manifestation in women (Ceovic et al., 2013). In this regard, by developing an innate immunity dependent mannan-induced psoriasis model, Wu et al. show an increased severity of the disease in female mice of different strains, as well as a prominent expression of estrogen receptor-β on keratinocytes. Moreover, the expression of genes promoting skin inflammation, including the ones for some specific cytokines, i.e. TNF-α, IL-6, IL-22, IL-23, and IL-17 family, was affected by estrogen levels.

Sex impact on the susceptibility to many infectious diseases, as well as on pathogen-induced immune responses and post-infection viral autoimmunity, has gained increasing attention in last years (Ruggieri et al., 2016), especially following the COVID-19 pandemic (Gebhard et al., 2020; Haitao et al., 2020). In this context, a considerable number of factors has been investigated and proposed to be crucially implicated. In their review, Popescu et al. categorize the highly heterogeneous available literature about the involvement of environmental factors, such as endocrine disrupting chemicals, describing and discussing how they can interfere with immune-related endocrine signaling and contribute to autoimmunity and autoreactivity following infections from Epstein-Barr and Herpes Simplex viruses, as well as SARS-CoV-2. In an interesting HYPOTHESIS AND THEORY article, Spiering and de Vries suggest that females may be significantly protected against severe COVID-19, due to the biallelic Toll-Like Receptor 7, one of the crucial recognition receptors for SARS-CoV-2 ssRNA, resulting in a stronger and more protective interferon-mediated response immediately after infection. In their original research article, Kuipery et al. highlight the impact of sex on the responsiveness of liver myeloid cells following Hepatitis B Virus infection, showing more frequent and severe liver damage, together with increased levels of inflammatory markers of myeloid activation, in men, whereas sex did not impact on the frequency or phenotype of sinusoidal myeloid cells. An evaluation of sex effects on lipid mediators (LMs) of inflammation in a rodent model of inflammatory peritonitis, obtained by zymosan administration in male and female mice after gonadectomy, has been conducted in the original research article by Troisi et al. that shows a predominance of specific pro-inflammatory products in the exudates of males, thus revealing sex differences and a clear role of sex hormones in LM biosynthetic networks during acute self-resolving inflammation. Moreover, genetic factors modifying in a sex-dependent way the response to infectious diseases are discussed in a minireview by Lipoldová and Demant, focusing on 22 autosomal genes/loci affecting, in rodent models, the susceptibility to infections from different pathogens only in females or only in males or in both sexes, but with opposite effects.

The body's response to infections can be severe and result in sepsis that has been described as the leading cause of death in intensive care units, especially when occurring after a trauma (Ma et al., 2016). With respect to this pathological condition, the impact of sex on mortality remains still controversial. In this regard, the retrospective cohort study of Kondo et al., conducted on hospitalized patients with sepsis after trauma, points toward a significant increase of the survival rate in female subjects compared to males, although it is concluded that molecular mechanisms underlying this sex difference need to be further elucidated. On the contrary, the original research article by Scott et al., realized by using a mechanical rat model of traumatic brain injury (TBI), a pathological condition which strongly solicits the immune system (Bao et al., 2021), reports only limited sex-dependent differences in the observed alterations of the blood brain barrier permeability caused by TBI-induced neuroinflammation.

In conclusion, we hope that this Research Topic will be useful for all researchers working in the field of inflammatory and autoimmune diseases and will prompt them to take into adequate consideration possible sex-based differences when designing their studies, in order to obtain reliable results and also to allow a sound translation of preclinical findings into the clinical settings.

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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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REVIEW
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Interplay Between Endocrine Disruptors and Immunity: Implications for Diseases of Autoreactive Etiology

Maria Popescu[1,2,3], *Talia B. Feldman*[2,3] *and Tanuja Chitnis*[1,2,3]*

[1]Harvard Medical School, Boston, MA, United States, [2]Brigham Multiple Sclerosis Center, Department of Neurology, Brigham and Women's Hospital, Boston, MA, United States, [3]Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA, United States

The sex-bias of disease susceptibility has remained a puzzling aspect of several autoimmune conditions, including post-infection viral autoimmunity. In the last half of the twentieth century, the incidence rate of female-biased autoimmunity has steadily increased independent of medical advances. This has suggested a role for environmental factors, such as endocrine disrupting chemicals, which have been described to interfere with endocrine signaling. Endocrine involvement in the proper function of innate and adaptive immunity has also been defined, however, these two areas have rarely been reviewed in correlation. In addition, studies addressing the effects of endocrine disruptors have reported findings resulting from a broad range of exposure doses, schedules and models. This experimental heterogeneity adds confusion and may mislead the translation of findings to human health. Our work will normalize results across experiments and provide a necessary summary relevant to human exposure. Through a novel approach, we describe how different categories of ubiquitously used environmental endocrine disruptors interfere with immune relevant endocrine signaling and contribute to autoimmunity. We hope this review will guide identification of mechanisms and concentration-dependent EDC effects important not only for the sex-bias of autoimmunity, but also for other conditions of immune dysfunction, including post-infection autoreactivity such as may arise following severe acute respiratory syndrome coronavirus 2, Epstein-Barr virus, Herpes Simplex virus.

Keywords: endocrine disruptors, immunity, autoimmunity, environmental factors, sex hormones

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Edited by:
Luigia Trabace,
University of Foggia, Italy

Reviewed by:
Claudio Pirozzi,
University of Naples Federico II, Italy
Hexin Chen,
University of South Carolina, United States
Marina Ziche,
University of Siena, Italy

***Correspondence:**
Tanuja Chitnis
tchitnis@rics.bwh.harvard.edu

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INTRODUCTION

The gradual increase in the female to male disease susceptibility ratio, including for autoimmune and metabolic disease, has been globally reported despite progressive medical advancements. In parallel, production of synthetic materials and their implementation in commercial goods has seen a similar upward trend. To meet consumer demand, a variety of products have now been manufactured using chemical additives. Several of these synthetic compounds have the ability to disrupt normal endocrine function and have appropriately been termed endocrine-disrupting chemicals (EDCs), or xenohormones. Widespread detection of EDCs in the serum and adipose tissue of humans and wildlife alike has drawn attention to the role of environmental factors. Sex-hormones, most notably estrogen, are established modulators of immune cell populations (Orton, Herrera et al., 2006; Bove and Chitnis 2014). EDCs with estrogenic activity may have therefore contributed to the steady

increase in the female to male disease susceptibility ratio, raising concern over their continued ability to modulate immunity in the future. To understand the diversity and sex-bias of immune responses as well as potential triggers for autoimmunity and post-infection autoimmunity, we will provide a comprehensive account of estrogen signaling modalities and the role of estrogen-like EDCs in innate and adaptive immunity.

The choice of focused categories herein presented is relative to the vast diversity of EDCs. The risk for environmental contamination and human exposure depends on the degree and use frequency of EDCs in consumer products. Three such ubiquitous categories are (1) phenols, (2) parabens, and (3) phthalates. Xenoestrogens inclusive of each category exhibit various immunomodulatory functions and are therefore implicit in immune dysfunction, as well as, in the onset and progression of diseases with autoreactive attributes. Together with the inherent dependency of female biology on estrogen, EDCs emerge as driving factors for the increased female to male disease susceptibility ratio. To illustrate, adaptive immune dysfunction induced by phenols results in reduced influenza A viral titers in females, but enhanced production of autoantibodies and overall IgM secretion (Yurino et al., 2004; Peretz et al., 2016). Likewise, parabens are associated with diabetic autoimmunity, such as gestational diabetes and the increased onset risk for future diabetes (Liu et al., 2019). Phthalate compounds and their metabolites similarly enhance insulin resistance and lead to an increased risk of type 1 and 2 diabetes (Castro-Correia et al., 2018; Radke et al., 2019). Furthermore, phenols, parabens and phthalates all have effects upon innate immunity, including on cytokine secretion, which indicates that EDCs inadvertently play an additional role in the dysfunction of adaptive responses.

Despite compelling evidence, studies of EDC effects have reported results across a broad range of exposure doses, schedules and models, which has added heterogeneity and confusion in translating findings to human health. Our review will normalize results across experiments for each EDC category, to provide a summary of effects relevant to innate and adaptive immunity, as well as for autoimmunity. Because EDCs have rarely been reviewed in the context of immunoendocrine interactions, we also describe normal estrogen signaling modalities and the likely influence of the three EDC categories. To the best of our knowledge, this approach will be novel and can expand environmental considerations for different aspects of immunity. We hope this review will guide the identification of mechanisms and exposure effects that are important not only for the sex-bias of autoimmune diseases, but also for related conditions of immune dysfunction, including the post-infection autoreactivity characterized for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Epstein-Barr virus (EBV), Herpes Simplex virus and others (Alexopoulos et al., 2018; Maslinska, 2019; Dalakas, 2020; Lazarian et al., 2020; Mallapaty, 2020).

Categorization of EDCs

EDCs belong to the broad classification of chemical additives, including plasticizers, resins, and antimicrobial agents. The formal definition for EDCs describes them as, "environmental agents that interfere with the normal function of endogenous hormones". Exposure results from either the direct use of diverse household commodities, such as personal care products, toys, electronics and others, or from contaminated environmental sources, such as, soil and water (Di Nisio and Foresta, 2019). Toxicological findings have identified effects of EDCs upon critical periods of development, including prenatal, perinatal, and pubertal development (Gorski, 2002). Such early developmental disruptions cause hormonal dysfunction and establish lifelong consequences, such as increased susceptibility to disease (Collman, 2011).

Xenoestrogens are a subset of EDCs that exhibit structural and functional mimicry of estrogen, including phenols, parabens and phthalates. Phenol EDCs include benzene-derived compounds, for which exposure generally occurs through inhalation, ingestion, or dermal contact. In humans, phenol urine levels less than 1,000 μg/L are considered normal (Ong and Lee, 1994). Past reports have indicated that 55% of Americans had detectable 4-tertiary-octylphenol (OP) in urine (0.2–20.6 μg/L) and high bioaccumulation of nonylphenol (NP) in breast milk (56.3 μg/L) (Calafat et al., 2008; Hwang et al., 2015). Levels of bisphenol A (BPA, 0.7–2.3 ug/L), bisphenol S (BPS, 0.2–0.8 ug/L) and bisphenol F (BPF, LOD-0.7 ug/L) have also been reported (Jacobson et al., 2019).

The second category of EDCs, parabens, include alkyl esters of *p*-hydroxybenzoic acid, such as methyl (MP), ethyl (EP), propyl (PP) and butyl (BP) parabens. Exposure occurs primarily through dermal absorption (2,400 μg/kg/d), but also through ingestion of food (13 μg/kg/d) and pharmaceuticals (417 μg/kg/d). Similar with phenols, parabens are also detected in human breast milk (total: 1.87–49 μg/L) (Park et al., 2019; Dualde et al., 2020).

Phthalates, derived from the esterification of phthalic acids, comprise the third EDC category. Akin to phenols and parabens, exposure includes oral, dermal and inhalation pathways. Although phthalates do not generally bioaccumulate, they have low target binding and can leak from products into the environment (Heudorf et al., 2007). Manufacturing changes have led to decreased detection of certain parabens, such as dibutyl phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP). In contrast, levels of substitute phthalates, including diisobutyl phthalate (DiBP) and diisononyl phthalate (DNP), have increased (Wittassek et al., 2007).

Estrogen Signaling

Estrogen Receptor-α and -β

Estrogens are critical for sexual and reproductive development especially in females and come in several similar chemical forms. Estradiol (E2) is the most abundant in cycling females, while estrone (E1) predominates in the postmenopausal period, and estriol (E3) and estrane (E4) predominate during pregnancy. Activity of endogenous estrogens, predominantly estradiol (E2) is primarily potentiated by the estrogen receptor-α (ERα) and ERβ, although an E2-specific G-protein coupled estrogen receptor-1 (GPER-1) has also been described. Hormone diffusion through the plasma membrane followed by receptor binding in the cytoplasm initiates canonical estrogen-to-ER signaling. Ligand-induced receptor dimerization and nuclear translocation lead to

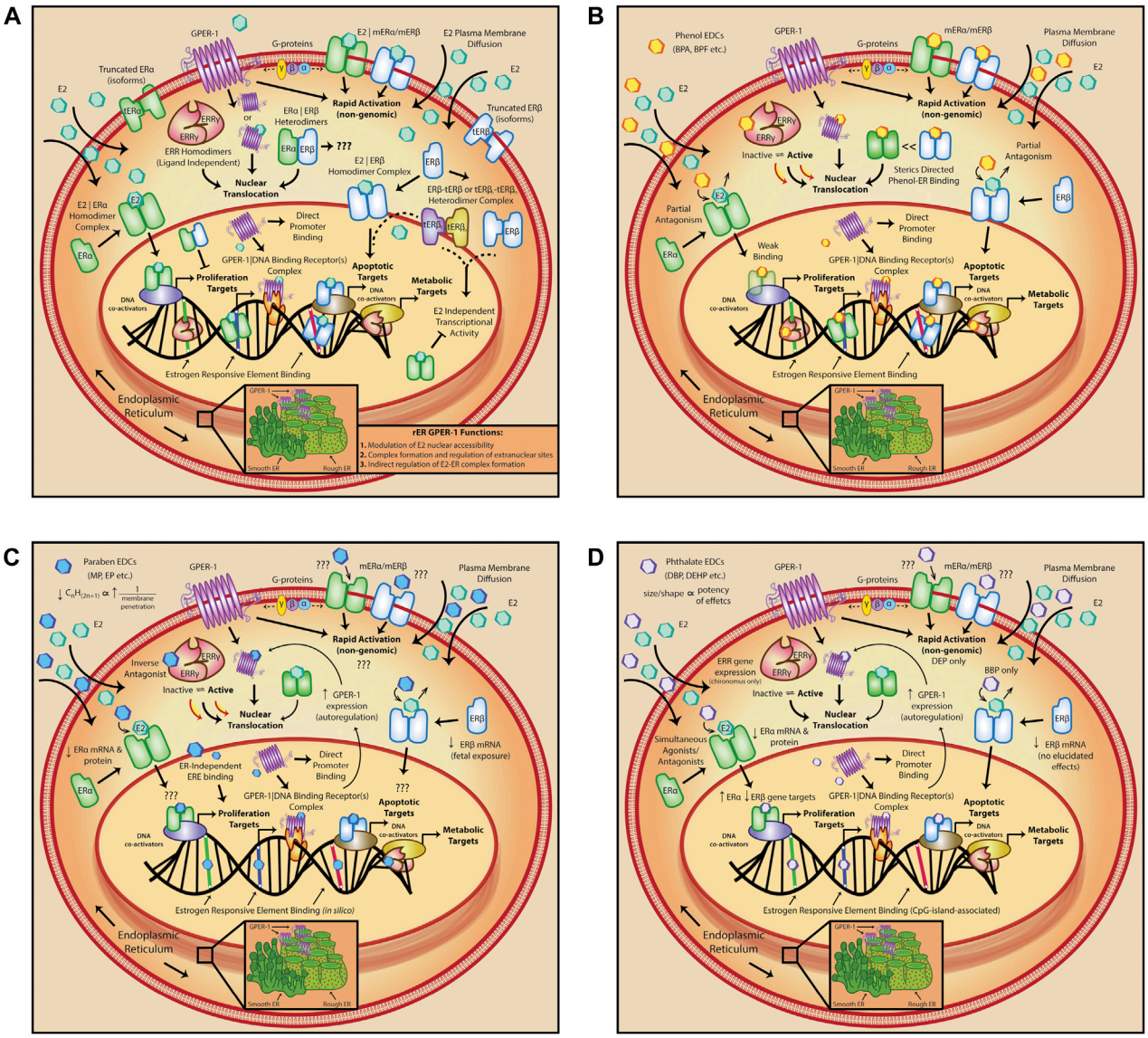

FIGURE 1 | Estrogen signaling and interference by varying categories of EDCs. **(A)** Diversity of estrogen signaling modalities; estradiol (E2) activates several receptor targets to transduce both genomic and non-genomic signaling pathways. **(B)** Phenol-induced deregulation impacts both estrogen receptors, (ER)α and ERβ by genomic/intracellular partial antagonism; binding of phenols is also sterically directed. **(C)** Paraben EDCs exhibit similar deregulation of estrogen signalling pathways, including decreased expression of both estrogen receptors, (ER)α and ERβ, but their activity is largely limited on the basis of alkyl-group size. **(D)** Phthalate compounds display simultaneous agonistic and antagonistic effects on estrogen signaling modalities; similar to parabens, phthalate effects are also size and shape dependent. E2, estradiol; ERR, estrogen related receptor; tER, truncated estrogen receptor; GPER-1, G-protein coupled estrogen receptor 1; rER, rough endoplasmic reticulum; mER, membrane estrogen receptor; G-proteins, G-protein coupled receptor proteins; BPA, bisphenol A; BPF, bisphenol F; MP, methyl paraben; EP, ethyl paraben; BBP, benzyl butyl phthalate; DEP, diethyl phthalate; DEHP, di(2-ethylhexyl) phthalate.

receptor-complexes that either directly bind estrogen responsive DNA elements (EREs) or complex with co-activators and co-repressors to regulate transcription of target genes (Levin and Hammes, 2016; Fuentes and Silveyra, 2019). ERα and ERβ can also heterodimerize, however their function is unclear, especially in contrast with the role of homodimers, such as proliferative ERα-ERα and suppressive/apoptotic ERβ-ERβ (Cowley et al., 1997; Papoutsi et al., 2009; Thomas and Gustafsson, 2011). ER signaling is complex and further diversified by the ability of receptors to localize at the plasma membrane in either truncated or full-length form, where they potentiate rapid non-genomic (non-translational) estrogen signaling (**Figure 1**) (Moss et al., 1997; Flouriot et al., 1998; Chambliss et al., 2000; Govind and Thampan, 2003; Hammes and Levin, 2007; Zhang et al., 2011). Other binding events at the cell membrane may also occur, such as interaction of full length ERα with G-proteins or with truncated isoforms of either ERα or ERβ (Kumar et al., 2007). Isoforms of ERβ, in particular, may further dimerize.

Both homo- and heterodimers of ERβ isoforms have been characterized, each with its own distinct signaling modality. For example, homodimers of ERβ isoforms initiate estrogen-independent transcription whereas only heterodimers can initiate ligand-dependent transcription (Moore et al., 1998; Leung et al., 2006). Heterodimers of ERβ isoforms with full length ERβ can also form, and this increases the estrogen-independent transcriptional activity by four-fold. Activity of some, but not all, heterodimers of ERβ isoforms can be inhibited by ERα (Poola et al., 2005). Evidently, the complexity of estrogen signaling, and the diversity of ER modalities are critical for properly evaluating the effects of EDCs on individual immune populations. Although E2 generally has a higher affinity for its receptors compared to most EDCs, xenoestrogens can activate alternatively available ERs when estrogen is otherwise engaged. These EDC complexes may then act in retrograde upon complexed E2-ERs, ER-genes, or EREs to disrupt their activity.

Non-estrogen Receptor Targets

Alternative targets to the ERs have been extensively described. Estrogen-related receptors (ERRs), notably ERRγ, constitutively (sans-ligand) bind either estrogen-related and EREs or naked DNA and non-E2-related co-activators (Horard and Vanacker, 2003; Audet-Walsh and Giguere, 2015). GPER-1, a G-protein coupled receptor (GPCR) with preferential specificity for 17-β estradiol (E2β), potentiates the rapid, but transient, modulation of several signaling pathways (Filardo et al., 2000; Revankar et al., 2005; Thomas et al., 2005; Ge et al., 2013). Distribution of GPER-1 and its functionality have been comprehensively reviewed elsewhere (Cheng et al., 2011; Xu et al., 2019). In line with its structural classification as a seven transmembrane GPCR (7TMR), GPER-1 undergoes endocytosis, often in the absence of ligand, and has predominantly been localized in the perinuclear space. Constitutive internalization and localization of GPER-1 indicate its potential for nuclear activity, which is corroborated by identification of a putative nuclear localization sequence. This proposes that GPER-1 can initiate gene expression both by interaction with other DNA-binding nuclear receptors, or by direct E2 mediated promoter binding (Madeo and Maggiolini, 2010; Pupo et al., 2013; Rudelius et al., 2015). The way by which EDCs interfere with normal GPER-1 signaling is not well defined. Many EDCs can diffuse through the cell membrane and localize within the same cytoplasmic and/or perinuclear space as occupied by GPER-1. In this vicinity, EDCs can perturb local concentration dynamics or act directly on the GPCR binding site, and thereby interfere with GPER-1 target activation.

Estrogen and Estrogen-Related EDC Interactions

Competitive binding of EDCs to estrogen receptor targets has preceded interference on cell metabolism, nuclear receptor turnover, and hormone sensitization. As a result of binding to ERs, transcriptional enhancement along with modulation of other signaling pathways have been increasingly reported. The diversity of EDC categories also lends diversity to EDC effects. For example, certain xenoestrogens may disrupt activation of nuclear pathways by inherent ability to diffuse and translocate through the lipid membrane, where they promote rapid and direct gene expression. Other EDCs act more distally, on G-protein coupled receptors, to disrupt homeostatic expression of transcription factors, and latently affect gene expression (Yoon et al., 2014). Understanding the interplay of individual exposure, estrogen activity and immunomodulation, along with how this contributes to the collective phenomenon of autoimmunity, will lead to improved healthcare guidelines, reduced exposure burdens and novel therapeutics for the treatment of immune related disease.

Phenols

BPA is the most extensively characterized phenol EDC and has been broadly used in numerous household products (Vandenberg et al., 2009). Past estimates approximated that 93% of Americans had detectable BPA in urine (0.4–149 μg/L) (Calafat et al., 2008). Despite regulatory reassurance in the past decade that BPA exposure is not deleterious within current limits, concern over adverse health effects eventually led to its tapered substitution in many consumer products, by either BPS or BPF. Limited assessment of these bisphenol analogs has permitted their unvalidated substitution in products under the label of safer or "BPA-free" alternatives. Emerging evidence, however, suggests not only that BPS has similar effects to BPA, but that it may, in fact, be more potent than its predecessor (Ferguson et al., 2019; Moon, 2019). Other phenols, such as alkylphenols, metabolites of ethoxylates, including NP monoethoxylates (NP1EOs) and diethoxylates (NP2EOs), all show evidence of bioaccumulation and are toxic (La Guardia et al., 2001). Not surprisingly, adverse phenol effects resulting in endocrine dysfunction have been extensively reported, including abnormal mammary gland development, decreased gonad and epididymis weights, and an increased risk of type 2 diabetes mellitus (Cha et al., 2017; Chamard-Jovenin et al., 2017; Yu et al., 2018). In the context of immunity, phenol interference on endocrine signaling and their activity as xenoestrogens hallmarks the critical influence of environmental factors on immune disease and emphasizes their function as exogenous immunomodulators.

Binding and Mode of Action

The ability of phenols to bind endogenous estrogen receptors, compete with endogenous ligand, and initiate activation of downstream pathways is central to understanding how they may modulate immune cells (**Figure 1**). In contrast with studies on non-genomic ER activation, phenols have been more extensively characterized in the context of genomic signaling. Relative to E2, BPA has reduced affinity for ERα (60–70% transactivation of E2) and exhibits preferential binding for ERβ (80%) over ERα. In consequence, phenols behave as partial antagonists in the presence of E2, in that binding of the EDC is less efficacious. Related factors such as bioaccumulation and tissue distribution of phenols, as well as generation of metabolites, render these EDC compounds with a complexity that exceeds E2 binding affinity alone (Matthews et al., 2001; Takayanagi et al., 2006). Moreover, variations in steric complexity seem to direct binding orientation of different phenols, which controls whether transduced effects will be

agonistic (BPA) or antagonistic (BPC). This is also true of co-activator recruitment and/or binding, whereby BPA is a weak agonist, but bisphenol C (BPC) serves as an antagonist, and binding of both is typically favored in the presence of high co-activator concentrations (Delfosse et al., 2012). Despite this, bisphenols do act as full ERα agonists on cell growth, suggesting that this may be induced by direct ER-bisphenol binding to ERE DNA regions. Similar to ER-binding, phenol-GPER-1 agonist binding has been demonstrated, with relatively high affinity reported for BPA and NP (Thomas and Dong, 2006). In this context, GPER-1 on the rough endoplasmic reticulum can prevent phenol nuclear accessibility and allow E2-ER nuclear complex formation. Alternatively, pre-existing GPER-1 occupancy by E2, such as in the endoplasmic reticulum, can free up cytosolic or nuclear receptor targets for EDC binding and vice-versa. Thus, the physiological state of the organism at the time of exposure is critical for transduction of xenoestrogen effects and may help explain how EDCs function as triggers for disease in some individuals, but not in others. As well, synergistic effects of multiple pathways should similarly be considered. Phenols can further act as inverse antagonists on other receptor targets. BPA, in particular, has been shown to increase ERRγ constitutive activity, and could thereby lead to a deregulated cell metabolism with implications for both cancer and autoreactivity (Liu et al., 2007). Subsequent to their direct binding activity, phenols can augment the rate of receptor turnover. BPA decreases ubiquitination and degradation of ERβ, as well as intracellular ERα protein levels and mRNA transcripts (Masuyama and Hiramatsu, 2004; La Rosa et al., 2014). Similarly, BPF increases ERβ, however, other EDCs, such as NP, have yielded more complex, potentially sex and species specific effects upon receptor turnover (Sakimura et al., 2002; Seo et al., 2006; Okazaki et al., 2017). Collective observations have thereby surmised that many phenols display characteristics of selective ER modulators (SERMs), most notably in their ability to behave in a discriminatory manner (Delfosse et al., 2012). This functional classification is an important consideration for study design and should be deliberated when inferring results to human health.

The turnover rate of ERs and endogenous ligand are precisely balanced to control the downstream signaling necessary for proper function of immune cells. Docking of estrogen to ERs does not produce simple, all-or-nothing target activation. Instead, this binding event appears to induce a state that depends on the equilibrium between receptor and ligand. EDC-induced changes to such a system may impact either the perceived ligand concentration or the receptor availability, leading to complex and sometimes dichotomous results on immune function. Interpretation of immunological findings is further complicated by contrasting pre-clinical and clinical results, likely both influenced by study design and experiment dose.

Innate Immunity Effects

Dichotomy of immune findings is best illustrated by pre-clinical, innate immune findings. For example, phenols decrease downstream signaling and key regulators of inflammation, such as TNF-α, nitric oxide (NO), and NF-κB in both murine macrophages (BPA 228–22,800 μg/L) and immortalized macrophages (BPA 5,700–22,800 μg/L; NP 2,200 μg/L; OP 2,000 μg/L) (Byun et al., 2005; Yoshitake et al., 2008). Alternatively, BPA at similar doses can, instead, increase levels of NO, reactive oxygen species (ROS), NF-κB activation and mRNA transcripts of IL-1β and IL-10 pro-inflammatory cytokines. Interestingly, macrophage bactericidal function was improved following low-dose BPA exposure (0.10–10 μg/L), whereas administration of high dose BPA (100–10,000 μg/L) induced apoptosis, presumably in an oxidative stress-dependent manner (Yang et al., 2015). Dose-independent effects on cytokine secretion have been further observed for both BPA (0.10–1,000 μg/L) and NP (0.10–100 μg/L), such as increased transcripts for IL-1β, IL-10, TNF-α, IFN-γ, and MyD88. Consistently, NO metabolism was affected only by the high dose of BPA or NP (Xu et al., 2013). This dichotomy of results is not only biphasic but may reflect a species specificity for estrogen regulation that results in varying outcomes on inflammatory factors (**Table 1**).

Paradoxical results from clinical studies have likewise been reported. Human macrophages and T lymphocytes responding to BPA (2,850–45,700 μg/L) showed a dose-dependent decrease in neopterin and IFN-γ, respectively (Gostner et al., 2015). In contrast, production of IL-6, TNF-α and IFN-γ from human adipocytes was increased after exposure to BPA (0.02–22.8 μg/L

BOX 1 | Relevance of EDC-immune modulation to COVID-19 disease and post-infection autoimmunity.

Modulation of adaptive immune responses by EDCs presents critical considerations for the current COVID-19 pandemic, caused by the SARS-CoV-2 virus. Recent studies have linked the incidence and severity of SARS-CoV-2 infection with development of post-infection autoimmunity, including diabetes, Guillain-Barre syndrome and autoimmune hemolytic anemia (Dalakas, 2020; Lazarian et al., 2020; Mallapaty, 2020). Herein, we describe findings that identify immune modulatory activity as similarly reported for COVID-19 disease but following EDC exposure. In both contexts, there exists an increased predisposition for autoreactivity. Therefore, we underscore the likelihood of synergism between EDC-induced immune dysfunction and SARS-CoV-2 infection. The sex-bias of immune self-reactivity, conveyed by estrogen mimicry with EDCs, may also explain the increased severity of COVID-19 in males as well as the age-dependency of infection frequency (F: 10–50 years, estrogen^{high}; M: 10< and >50 years, testosterone^{low}) (Scully et al., 2020). To illustrate, adaptive immunity in females may be better equipped to initiate antiviral defenses in the initial stages of infection, in part likely due to the same processes responsible for the female autoimmune bias. Several of the studies we outlined demonstrate the capacity of EDCs to increase the expression of cytokines including IL-6, IL-1β, TNF-α, and IFN-β. Therefore, it is plausible that under noninfectious circumstances, elevated levels of these cytokines may result in an increased baseline state of immune activation. During a pathogen defense response, this heightened state may convey an advantage. However, without a specific target, it could trigger autoreactivity. In contrast, male sex-hormones (testosterone^{high}/estrogen^{low}), which are otherwise preventive against EDC exposure effects and autoimmunity, could dampen the initial response to SARS-CoV-2 infection. This would result in increased viral burden, which may thereby trigger hyperinflammatory syndrome and severe COVID-19 disease in males. Interestingly, early data has suggested an association between male-sex and viral burden duration (Scully et al., 2020). Furthermore, the risk to develop severe disease and mortality rate were both found to be male-biased (Maleki Dana et al., 2020; Scully et al., 2020)

TABLE 1 | Diversity of immune effects by phenol EDCs.

	Dose (μg/L)	Species	Cell type		Immune impact
In vitro					
BPA	100–10,000 (Yang et al., 2015)	Carp	MΦ	↑	NO, ROS, NF-κB, IL-1β mRNA and apoptosis
	0.10–10 (Yang et al., 2015)			↓	Bactericidal function
	228–22,800 (Byun et al., 2005) 5,700–22,800 (Yoshitake et al., 2008)	Mouse	MΦ	↓	TNF-α, NO and NF-κB
	228–22,800 (Sakazaki et al., 2002)		N/A	↓	Lymphoproliferation (B cells > T cells)
	228–22,800[a] (Jang et al., 2020)		Splenocytes	↓	Proliferation
	228 (Yurino et al., 2004)		B1 B cells	↑	IgM
	1,000 (Bodin et al., 2015)		Splenocytes	↓	IL-10, TNF-α, IFN-γ and IL-4
	30, 300 μg/kg (Xu et al., 2019)		Serum (F)	↑ ↑ ↑ ↑ ↑	GM-CSF IFN-γ MIP-1α MIP-1β Development of T1D
	2,850–45,700 (Gostner et al., 2015)	Human	PBMCs (MΦ |T cells)	↓	Neopterin and IFN-γ
	0.02–22.8 (Ben-Jonathan et al., 2009) 0.228 (Valentino et al., 2013)[72]		Adipocytes	↑ ↓ ↑	IL-6, TNF-α and IFN-γ Adiponectin and glucose metabolism JNK, STAT3 and NF-kB
	228–22,800 (Balistrieri et al., 2018)		PMNs	↑ ↓	ROS Chemotaxis and bactericidal function
	22,800[a] (Jang et al., 2020)		WiL2-NS (B cells)	↓ ↑ ↓ ↑	Proliferation Sub G0/G1phase G2/M phase and S phase ROS
	228,000 (Alhomaidan et al., 2019)		Whole blood (DNA-HC) Serum (SLE)	↑ ↑	Hypochromicity/Stability Ab affinity/Recognition for BPA-DNA
BPF	20,000[a] (Jang et al., 2020)	Mouse	Splenocytes	↓	Proliferation
	20,000[a] (Jang et al., 2020)	Human	WiL2-NS (B cells)	↓ ↑ ↑	Proliferation Sub G0/G1 phase, G2/M phase ROS
BPS	7,500–25,000[a] (Jang et al., 2020)	Mouse	Splenocytes	↓	Proliferation
	25,000[a] (Jang et al., 2020)	Human	WiL2-NS (B cells)	↓ ↑ ↑	Proliferation Sub G0/G1, G2/M phase and S phase ROS
NP	2,200 (Yoshitake et al., 2008)	Mouse	MΦ	↓	TNF-α, NO and NF-κB
	220–22,000 (Sakazaki et al., 2002)		N/A	↓	Lymphoproliferation
	220 (Yurino et al., 2004)		B1 B cells	↑	IgM
OP	2,000 (Yoshitake et al., 2008)	Mouse	MΦ	↓	TNF-α, NO and NF-κB
In vivo					
BPA	100–1,000 (Xu et al., 2013)	Zebrafish	Embryo	↑	NO, NOS and ROS
	0.10–1,000[a] (Xu et al., 2013)			↑	mRNA: IL-1β, IL-10, IFN-γ, MyD88 and TNF-α[a]
	300–3,000 μg/kg (*in-utero*) (Yoshino et al., 2004)	Mouse	Serum	↑ ↑	IL-4, IFN-γ and HEL-IgGs Lymphoproliferation (CD8 > CD4)
	2.28–228 (Luo et al., 2016)		Splenic cells/Serum	↑	TH17 differentiation, IL-17, IL-21, IL-6 and IL-23 (F)
	300–350 mg/kg (Yurino et al., 2004)	Mouse (SLE)	B1 cells	↑	Anti-Br-RBC autoantibodies
	10,000 (Krementsov et al., 2013)	Mouse (EAE)	N/A	↑ ↑	First episode severity (F) Relapse incidence (M)
	10 mg/kg (Brinkmeyer-Langford et al., 2014)	Mouse (TVID)	N/A	↑	Temporal onset of MS, disease severity

(Continued on following page)

TABLE 1 | (*Continued*) Diversity of immune effects by phenol EDCs.

	Dose (μg/L)	Species	Cell type		Immune impact
	50 μg/kg (Pirozzi et al., 2020)	Mouse	Serum/Liver	↑	mRNA: IL-6, TNF-α, IFN-γ, MyD88 and NF-κB
				↑	Progression of liver fibrosis
	1,000 (Bodin et al., 2015)		N/A	↑	Diabetic incidence
			Pancreatic islet cells	↑	Severity of insulitis
				+	Regulator of apoptosis
			MΦ	↓	Infiltrates and phagocytic function
	1,000–10,000 (Bodin et al., 2014)		N/A	↑	Diabetic incidence[a] (F)
			Pancreatic islet cells	↑	Severity of insulitis[a](F)
			MΦ	↑	Apoptosis
				↓	Infiltrates
NP	10–100 (Xu et al., 2013)	Zebrafish	Embryo	↑	NO, NOS and ROS
	0.10–100[a] (Xu et al., 2013)			↑	mRNA: IL-1β, IL-10, IFN-γ, MyD88 and TNF-α[a]

Dose-dependent results are underlined. [a]Immune effect at high dose(s) only; (F), female bias; (M), male bias; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; NP, nonylphenol; OP, 4-tertiary-octylphenol; TLR-4, toll-like-receptor-4; NO, nitric oxide; ROS, reactive oxygen species.

Ben-Jonathan et al., 2009; 0.228 μg/L Valentino et al., 2013). This corresponded with a lowered production of the anti-inflammatory hormone, adiponectin, and an impaired glucose metabolism (Ben-Jonathan et al., 2009; Valentino et al., 2013). In the context of clinical obesity and chronic inflammation, low-dose EDC exposure effects suggest that EDCs may exacerbate or even cause autoimmunity, including post-infection autoreactivity of metabolic etiology. Such potential has been identified for BPA, which increased activation of JNK, STAT3 and NF-kB signaling pathways. Conversely, adipogenesis was unaffected despite previous pre-clinical reported effects (BPA 0.228 μg/L; Valentino, D'Esposito et al., 2013; Ariemma, D'Esposito et al., 2016).

In other clinical findings, phenol effects were transduced by GPER-1 activation to induce human polymorphonuclear neutrophils (PMNs) toward a pro-inflammatory phenotype (Rodenas, Tamassia et al., 2017). Despite limited research into EDC effects upon PMNs, administration of BPA (228–22,800 μg/L) did increase ROS production in these cells, in a dose-dependent manner. ROS activity was also associated with diminished bactericidal function and inhibition of PMN chemotaxis, whereas phagocytic function remained unaffected (Balistrieri, Hobohm et al., 2018).

Adaptive Immunity Effects

Phenols have additionally been characterized for their effects on adaptive immunity. Gestational BPA treatment (300–3,000 μg/kg) alters the humoral response to immunization, leading to higher levels of antigen specific immunoglobulin (Ig) Gs and increased lymphoproliferation (Yoshino et al., 2004). Increased IFN-γ and IL-4 were also noted as a result of BPA effects on T cell helper phenotypes, particularly TH1 differentiation. Furthermore, despite an overall increase in T cells, the ratio of CD8/CD4 T cells was skewed in favor of CD8 T cells. Collectively, these results suggest that phenols may prime the humoral response toward sustained inflammation (Yoshino et al., 2004). In the context of pathogen response or autoimmunity, sustained humoral activation as induced by BPA can degenerate into chronic inflammation and the onset or exacerbation of autoreactivity. This is further substantiated by evidence of BPA-induced (2.28–228 μg/L) increased differentiation of TH17 cells, including increased secretion of IL-17 and IL-21, as well as TH17 differentiating cytokines, IL-6 and IL-23. Such changes were altogether dose-dependent and sex-specific (female bias), further suggesting that BPA alters dynamics of adaptive immunity and leads toward a proinflammatory phenotype that may cause adverse health outcomes (Luo, Li et al., 2016).

In contrast, *in vitro* treatment with BPA (2,280–22,800 μg/L) or NP (220–22,000 μg/L) decreased lymphoproliferation, with BPA demonstrating preferential inhibition of B cells over T cells (Sakazaki et al., 2002). Comparable with BPA (22,800 μg/L), analog compounds, BPF (20,000 μg/L) and BPS (7,500 and 25,000 μg/L), decreased splenocyte proliferation and augmented human B cell cycle transitions. The reduced viability of human B cells, likely as a result of enhanced ROS production by all three bisphenols, was comparable with effects previously described for innate cells (Yang et al., 2015; Jang et al., 2020). This ability of EDCs to downregulate B cell responses may be critical in the context of viral or other chronic infections, which have been linked to the onset of several autoimmune phenotypes (**Box 1**). By affecting TH differentiation, altering the distribution of CD8 to CD4 T cells, and diminishing B cell responses, EDCs may directly contribute toward an ineffective adaptive response, one which may allow pathogen escape and influence the development of chronic inflammation and self-reactivity.

Autoimmunity

Exposure to phenols is a potential risk factor for autoimmunity, however studies are similarly limited by complex, often biphasic results. In animal models of autoimmune disease, such as systemic lupus erythematosus (SLE), long-term BPA exposure (300–350 mg/kg) enhanced production of anti-Br-RBC autoantibodies. Elevated levels of IgM secretion from B1 cells have also been reported following exposure to both BPA (228 μg/L) and NP (220 μg/L) (Yurino et al., 2004). Gestational BPA (10,000 μg/L) followed by induction of autoimmune

TABLE 2 | EDC population effects.

EDC compound	Population/sample		Biomarker and/or disease association
Phenols			
BPA	Thyroid autoimmunity/serum (Chailurkit et al., 2016)	↑ ↑ ↑	TgAb TPOAb TRAb
Parabens			
MP	Case report (Henry et al., 1979; Macy et al., 2002)	↑	Hypersensitivity and contact urticaria
	Urine [pregnant (F)] (Watkins et al., 2015)	↓ ↑ ↑ ↑	C-reactive protein IL-6 IL-10 IL-1β
	Urine (M) (Quiros-Alcala et al., 2019)	↑	Asthma morbidity
EP	Urine (Liu et al., 2019)	↑	Gestational diabetes mellitus
PP	Urine [Pregnant (F)] (Watkins et al., 2015)	↑	Oxidative stress
	Urine[a] (Ward et al., 2020)	↓	Diabetic incidence
	Urine (M) (Quiros-Alcala et al., 2019)	↑	Asthma morbidity
BP	Urine/Serum [pregnant (F)] (Watkins et al., 2015)	↑ ↑ ↑ ↑ ↑	Oxidative stress IL-1β IL-6 IL-10 TNF-α
	Urine[a] (Ward, Casagrande et al., 2020)	↓	Diabetic incidence
Phthalates			
MEP	Human cord blood (Herberth et al., 2017)	↓	T Regulatory cells
MiBP	Human cord blood (Herberth et al., 2017)	↓	T Regulatory cells
DEHP	Urine (Yang et al., 2019)	↑ ↑	Anti-HBs IgM responses following post-natal HBV immunization Modulation of gut microbiota composition
DiBP	Urine (children) (Castro-Correia et al., 2018)	↑	New onset type 1 diabetes mellitus

[a]*Immune effect at high detected concentrations; (M), male bias; BPA, bisphenol A; MP, methyl paraben; EP, ethyl paraben; PP, propyl paraben; BP, butyl paraben; MEP, monoethyl paraben; MiBP, monoisobutyl phthalate; DEHP, di (2-ethylhexyl) phthalate; DiBP, diisobutyl phthalate.*

encephalomyelitis (mild EAE; $MOG_{35–55}$/CFA-only) did not affect the clinical course of disease. Conversely, trends in severity of EAE were observed, such as an increased disease score during the first EAE episode in female mice and a higher relapse incidence in male mice (Krementsov et al., 2013). Similarly, an earlier onset of MS symptoms and worsened disease severity were shown in a virus-induced demyelination model of MS (BPA 10 μg/kg) (Brinkmeyer-Langford et al., 2014). Interestingly, colitis was more severe in MS mice exposed to BPA, which corroborates clinical data on the co-occurrence of MS and inflammatory bowel diseases (IBDs) in humans (Kimura et al., 2000; Alkhawajah et al., 2013; Brinkmeyer-Langford et al., 2014).

Consideration of phenols in the context of other autoimmune conditions provides additional evidence for their harmful effects. For example, EDC levels (BPA 1,000 μg/L) relevant to human exposure increased diabetic incidence and corresponded with higher grade insulitis in pancreatic islets, concurrent with decreased macrophage infiltrates of lowered phagocytic potential (Bodin et al., 2015). Similar findings on type-1 diabetes identify BPA as pro-apoptotic for pancreatic islet cells, which suggests a mechanism by which BPA promotes self-antigen auto-activation and diabetic onset (Bodin et al., 2014; Bodin et al., 2015). Furthermore, high-fat diet and BPA exposure (50 ug/kg) were associated with immune-metabolic dysfunction, characterized by increased transcripts for toll-like-receptor-4 and related signaling pathways, such as NF-kB and NLRP3 inflammasome, which amplified proinflammatory cytokine production to promote liver damage (Pirozzi et al., 2020). More specifically, in diabetic animals, decreased levels of secreted IL-10, TNF-α, IFN-γ and IL-4 have also been reported. Animal findings thus suggest that BPA and other phenols can alter the severity of autoimmune disease, likely through a mélange of effects, including increased production of autoantibodies, altered cytokine secretion and decreased macrophage scavenging. Altogether, these changes contribute to the persistence of self-antigen and may exacerbate autoimmunity (Bodin et al., 2015). There is also evidence that

TABLE 3 | Diversity of immune effects by paraben EDCs.

	Dose (μg/L)	Species	Cell type		Immune impact
In vitro					
BP	2 ($\times 10^8$) (Matsuoka et al., 2018) (Indirect/sensitization)	Mouse	Brachial LN cells Dendritic cells	↑ ↑	IL-4 and IFN-γ Skin to dLN trafficking
	1.17 ($\times 10^{-2}$) (Bairati et al., 1994)	Human	Lymphocytes	↓	Lysozyme release
HP-DP	2,360–2,780 (Uramaru et al., 2014)	Rat	Peritoneal mast cells	↑	Histamine release
PP-DDP	20,800–30,600[a] (Uramaru et al., 2014)	Rat	Peritoneal mast cells	↑	Histamine release
IPP	20,800 (Uramaru et al., 2014)	Rat	Peritoneal mast cells	↑	Histamine release
In vivo					
BP	200 mg/kg (Hegazy et al., 2015)	Rat (M)	N/A (brain lysates)	↑ ↓	NO, IL-6 and TNF-α IL-1β mRNA

phenol exposure could contribute to sex-bias in autoimmune disease outcomes. In non-obese diabetic mice, BPA affected both development and pathology of T1D in a sex-specific manner. In females, development of T1D was accelerated and accompanied by a shift in proinflammatory immune factors, whereas disease development in males was delayed, associating with an increase in anti-inflammatory factors (Xu et al., 2019). These results highlight the potential of phenol EDCs to induce sex-specific changes in the development and course of autoimmune diseases.

In humans, the role of phenol EDCs in autoimmunity is less defined. Nevertheless, clinical studies have provided compelling evidence on the detriment of EDCs to human autoimmunity (**Table 2**). When isolated DNA from healthy individuals was exposed to BPA (228,000 μg/L), formation of DNA-BPA complexes and BPA-induced conformational changes (48.2% hypochromicity, 260 nm) were observed. Relative to native DNA, DNA-BPA complexes had increased stability, which suggests a novel process for how environmental factors may influence the development of self-reactive autoantibodies. In conformity, serum IgG antibodies from SLE patients showed affinity and recognition for such DNA-BPA complexes, but were otherwise undetected by IgG antibodies purified from healthy individuals, presumably because the latter have no affinity for self-antigens, such as anti-double stranded DNA (dsDNA) recognition. Interestingly, the SLE IgG antibodies showed preferential recognition of BPA-altered DNA over native DNA, which could help explain how an otherwise weak immunogen (dsDNA) is enhanced to autoimmunity (Rekvig and Mortensen, 2012; Alhomaidan et al., 2019). An impact of phenols has also been reported in autoimmune thyroid disease, where serum BPA showed a positive trend with antibody levels for thyroglobulin (TgAb), thyroid peroxidase (TPOAb) and thyroid receptor (TRAb). BPA was also an independent predictor of TgAb and TPOAb, but not TRAb (Chailurkit et al., 2016). Whether modulation of immune populations by phenols has the capacity to influence the onset or progression of autoimmune disease remains controversial, however, animal findings implicate immune effects that are dependent on several factors including study design (**Table 1**).

Parabens

The paraben category of EDCs has also been described for its effects on immunity (**Tables 2** and **3**). The size of alkyl groups is a defining characteristic for paraben compounds and has been proposed to correlate directly with the degree of ERα and ERβ estrogenic activity (Gomez et al., 2005). However, the size of paraben alkyl groups is correlated with their penetration potential across the cell membrane. This suggests that although higher molecular weight parabens may have increased estrogen-like activity, exposure to such compounds from goods and other commodities is largely limited to their less estrogenic counterparts. In routes of direct exposure, such as intravenous administration, bioaccumulation is also minimal, with paraben levels quickly declining from the serum or maintained at nontoxic levels (Andersen, 2008). Nevertheless, paraben-induced chromosomal aberrations, including gaps, breaks, exchanges and rings have been described, suggesting that lower molecular weight compounds that cross the membrane may directly impact expression of immune genes, despite having lower estrogenic activity (Matsuoka et al., 1979). Alternatively, contributions of the endocrine system in normal immune function are vital, such that even milder disruptions by parabens may be detrimental to human health.

Binding and Mode of Action

Paraben-ER activity and binding mechanism depends on the octanol-water partition coefficient (K_{ow}), an index of cell membrane transfer. Parabens with a relative high K_{ow}, such as BP, can diffuse across the cell membrane and directly bind DNA *in silico* (Manzetti, 2018). Ability to bind DNA directly is not exclusive to BP. Transcriptional and translational products of ERE-genes have also been reported following high dose (1,000 mg/kg/d) exposure to isopropyl and isobutyl parabens. The concurrent decrease in mRNA and protein expression of ERα further suggests that parabens may not always follow an ER-to-ERE DNA activation pattern, and can instead directly bind to the ERE (Vo and Jeung, 2009). Alternatively,

TABLE 4 | Diversity of immune effects by phthalate EDCs.

	Dose (μg/L)	Species	Cell type		Immune impact
In vitro					
MBP	22,200 (Hansen et al., 2015)	Human	T Cells	↑	IL-6
MEHP	20 (Li et al., 2018)	Mouse (F)	M Φ	↓	Phagocytosis
DEP	22,200 (Hansen et al., 2015)	Human	M Φ	↑	IL-6
				↑	CXCL8
				↑	IL-10
				↓	TNF-α
			T Cells	↓	IL-2
				↓	IL-4
				↓	TNF-α
				↓	IFN-γ
DBP	27,800 (Hansen et al., 2015)	Human	MΦ	↑	IL-6
				↑	CXCL8
				↑	IL-10
				↓	TNF-α
			T Cells	↓	IL-2
				↓	IL-4
				↓	TNF-α
				↓	IFN-γ
BBP	31[a] (Kuo et al., 2013)	Human	Plasmacytoid dendritic cells	↓	IFN-α
			T Cells	↑	IFN-β
				↓	IFN-γ
				↑	IL-13
DEHP	≥1,560 (Martins et al., 2015)	Rainbow trout	B Cells	↓	Proliferation
	≥6,250 (Martins et al., 2015)		IgM plasmablasts/Plasma cells	↓	Proliferation
	60 and 600 μg/kg/d (Li et al., 2018)	Mouse	MΦ	↓	TNF-α mRNA (F)
				↓	IL-1 mRNA (F)
				↑	IL-6 mRNA (F)
	3,900 (Ito et al., 2012)		Dendritic cells	↑	Differentiation and maturity
	30, 300, 3,000 μg/kg (Han et al., 2019)		T_{FH}	↑	Bcl-6
				↑	c-MAF
				↑	IL-21
				↑	IL-4
	39[a] (Kuo et al., 2013)	Human	Plasmacytoid dendritic cells	↓	IFN-α
				↑	IFN-β
			T Cells	↓	IFN-γ
				↑	IL-13
In vivo					
BBP	3,000 (Elter et al., 2020)	Mouse	N/A	↑	Severity of RA in progeny
			Serum	↑	IgG1
				↑	IgG2a
			Splenocytes	↑	IFN-γ
				↑	IL-17

(Continued on following page)

TABLE 4 | (*Continued*) Diversity of immune effects by phthalate EDCs.

	Dose (μg/L)	Species	Cell type		Immune impact
DEHP	60 and 600 μg/kg/d (Li et al., 2018)	Mouse	MΦ	↓	TNF-α gene expression
	60 μg/kg/d (Li et al., 2018)			↓	Phagocytosis
	30, 300, 3,000 μg/kg (Han et al., 2014)		T_{FH}	↑	Co-stimulatory activity
				↑	SLAMF1 and SAP
				↑	Germinal center formation
	11.3–13.3 mg/kg/d (Hirai et al., 2015)	Mouse (M with EAO)	MΦ and other cells	↑	Number IFN-γ+
			Lymphocytes	↑	Infiltrates
			Serum	↑	Anti-testicular germ cell autoantibodies

[a]*Immune effect at high dose(s) only; (F), female bias; (M), male bias; MBP, monobutyl phthalate; MEHP, mono-(2-ethylhexyl) phthalate; DEP, diethyl phthalate; DBP, dibutyl phthalate; BBP, butyl benzyl phthalate; DEHP, di (2-ethylhexyl) phthalate; TFH, follicular helper T cells.*

PP induces morphogenic changes in glandular structures via activation of both ERα and ERβ and GPER-1 (Marchese and Silva, 2012). Exposure to MP (3 μg/L), PP (3.6 μg/L) or BP (3.9 μg/L), can also increase GPER-1 gene and protein expression, which indicates that parabens can activate a GPER-1 autoregulatory feedback loop (Wrobel and Gregoraszczuk, 2015). Additional activity of parabens on other estrogen targets involves the inverse antagonism of ERRγ. Parabens (methyl-to-benzyl parabens) are recruited *in silico* to the ERRγ active site, and form bonds with active site residues (Zhang et al., 2013). Binding of parabens to ERα or ERβ conformations that transduce non-genomic signaling has not yet been described, likely due to the size limitation on membrane permeability. However, in the intercellular space, size-excluded parabens could preferentially target surface ERα or ERβ to activate non-genomic signaling. Alternatively, the alkyl bulk of parabens may impede ligation to the active site of surface ERs. Nevertheless, activation of estrogen targets by parabens with a high K_{ow} offers sufficient insight to raise concern regarding the interference of paraben EDCs with normal endocrine signaling in immune populations.

Innate Immunity Effects

Effects of paraben exposure on innate immunity are often derived from cosmetic allergology studies assessing the safety of personal care products. This restricts findings within a narrow niche of innate immune cells, specifically those that relate to allergic sensitivities. Nevertheless, findings can help inform on effective dosages and exposure schedules. Notably, dose ranges often fall within or surpass the doses at which activation of ERs has been described. For example, straight-chain parabens spanning from heptyl-to-decyl (low dose HP-DP, 2,360–2,780 μg/L) or pentyl-to-dodecyl (high dose PP-DDP, 20,800–30,600 μg/L) cause a significant release of histamine from degranulating mast cells (Uramaru et al., 2014). This is also sustained by exposure to the branched isopentyl paraben (IPP, 20,800 μg/L). MP causes immediate hypersensitivity, including contact urticaria, although it is unclear whether paraben-reactivity is a result of innate or adaptive activation, the latter of which would be an anti-paraben allergic recall (Henry et al., 1979; Macy et al., 2002). To date, discerning these effects has been limited by unsuccessful detection of anti-paraben IgE antibodies in the serum. However, this does not rule out a paraben-induced adaptive recall. IgE antibodies have a relative short-half life, which temporally limits their detection, and may lead to the incorrect assumption that paraben hypersensitivity is a localized innate response. Alternatively, other immunoglobulin classes, such as IgG antibodies, may be involved in activation of innate cells, transducing effects otherwise perceived as immediate reactivity to parabens (Kokubu et al., 1989). A more detailed discussion is included in the adaptive immunity subsection on paraben effects.

Observations independent of allergic innate responses have been modestly reported. In the brain, BP (200 mg/kg/d) increases the levels of NO, IL-6 and TNF-α but downregulates IL-1β (Hegazy et al., 2015). Peripherally, it can also enhance dendritic cell (DC) trafficking to draining lymph nodes (dLN) and increases the secretion of IL-4 and IFN-γ from dLNs (Matsuoka et al., 2018). During pregnancy, exposure to BP and PP associate with higher measures of oxidative stress, lower expression of c-reactive protein, and changes in cytokine secretion. BP for example, increases IL-1β, IL-6, IL-10, and TNF-α levels, whereas MP increases only IL-6 and IL-10 and decreases levels of IL-1β (Watkins et al., 2015). The ability of parabens to influence cytokine dynamics implicates not only innate but also adaptive responses, as the latter depend upon the precise balance of inflammatory factors to mount the appropriate humoral defense.

Adaptive Immunity Effects

Effects of parabens on adaptive immunity have primarily remained unexplored, perhaps because of the restrictive interest in parabens outside of cosmetics and personal care products. Alternatively, parabens can be erroneously overlooked since they have minimal bioaccumulation potential and relatively lower toxicity compared to other EDC categories. Nevertheless, clinical case reports identify anomalous serology characterized by both anti-paraben antibodies and paraben-associated auto anti-Jk[a] antibodies (Judd et al., 1982; Judd et al., 2001; Gniadek et al., 2018). Although characterization of paraben-specific IgE antibodies is limited by their half-life, sensitization with BP and HP followed by challenge produces a significant allergic reaction (Uramaru et al., 2014). This suggests that prior paraben priming is sufficient to generate recall upon antigen re-presentation and is a hallmark feature of adaptive immunity. Interestingly, patients presenting with intractable dermatitis resistant to typical corticosteroid creams, achieve rapid and complete recovery when administered paraben-free corticosteroid formulations. This

indirectly confirms the existence of paraben-specific antibodies, although it does not offer an explanation as to how parabens may trigger an adaptive response (Guyton et al., 2005). One possibility involves a combination of effects that involves both innate and adaptive mechanisms. Parabens acting on innate cells result in an unbalanced inflammatory milieu that recruits adaptive immune cells. In turn, recruited cells activate in the presence of cytokines and are then primed by exogenous parabens, generating antibodies with paraben affinity. In the context of dermatitis, this presents an easily remediable effect of exposure. However, such immunomodulation may be detrimental when paraben exposure occurs simultaneous with an ongoing innate or adaptive response, and could trigger hyperinflammatory syndrome and autoreactivity, respectively.

Autoimmunity

The role of parabens in autoimmunity is similarly limited to a few exploratory studies. To the best of our knowledge, no findings have been reported for paraben effects on immune cells that drive autoimmunity, such as B cells or T cells. However, an association of EP urinary levels and gestational diabetes has been reported, which suggests an increased risk of diabetes in later life (Liu et al., 2019). In contrast, high concentrations of methyl-to-butyl parabens in urine were associated with lower odds of diabetes overall (Ward et al., 2020). This suggests that exposure to parabens in the context of diabetic autoimmunity is more complex and requires a molecular approach for validation.

In children, sex-biased associations have been determined between urinary concentrations of methyl and propylparaben, whereby boys have increased asthma morbidity, as modeled by emergency department visits. A similar male-bias has also been reported in paraben-related triclosan exposure and food sensitization among children, further suggesting that EDCs influence human immune disease outcomes in a sex-specific manner (Savage et al., 2012; Quiros-Alcala et al., 2019). Studies on central nervous autoreactivity have shown that paraben exposure, particularly BP (1.17×10^{-2} μg/L), inhibits lysosomal enzyme secretion, which is otherwise elevated extracellularly in MS patients (Bairati et al., 1994; Guyton et al., 2005). Nevertheless, combined findings illustrate the diversity of effects with which paraben EDCs influence immunity.

Phthalates

Phthalates comprise the third category of EDCs. Although a variety of deleterious effects have been identified, phthalate compounds have commonly been described in reference to phthalate syndrome, a condition that causes developmental changes to the reproductive system. Similar to parabens, the strength of phthalate effects is conveyed by the shape (e.g., branching) and length of the ester side chains (Takeuchi et al., 2005). Compounds with relative high potency contain linear ester sidechains of 4–6 carbons, whereas shorter or longer phthalate esters tend to cause less severe effects (Council, 2008). Nevertheless, many of these compounds are known endocrine disruptors and have adverse outcomes, including multigenerational and transgenerational reproductive dysfunction (**Table 2**) (Kay et al., 2013; Zhou et al., 2017). The role of phthalates in reproductive health emphasizes their relevance for all aspects under endocrine control, including innate and adaptive immune responses.

Binding and Mode of Action

Like members of other EDC categories, phthalates both activate and disrupt endocrine signaling, particularly pathways regulated by estrogen and its receptors (**Figure 1**). Although non-genomic activation has been defined for diethyl phthalate (DEP), it has remained unexplored for other compounds (Kumar et al., 2014). Nuclear effects, however, are generally more common. For example, butyl benzyl phthalate (BBP; 3,120 μg/L) caused demethylation of ERα promoter-associated CpG islands and increased ERα gene expression (Kang and Lee, 2005). In GPER-1^{+} cells, BBP (31,200 μg/L), DBP (2,800 μg/L), and DEHP (3,900 μg/L) all led to increased proliferation. In the absence of GPER-1, this effect was undetected for all phthalates, which suggests that they initiate proliferation via GPER-1 (Kim et al., 2004). Additionally, both DBP and diisopentyl phthalate (DiPeP) (500 mg/kg/d) increase GPER-1 gene expression, whereas DiPeP at a lower dose (125–250 mg/kg/d) decreases ERα without affecting levels of ERβ (Curi et al., 2019). Interestingly, phthalates may act simultaneously as agonists and/or antagonists, primarily via ERα; only BBP has been shown to have activity on ERβ. Binding of phthalates to other estrogen targets, including ERRs, has not been extensively characterized. Only one study found that DEHP (50,000 μg/L) increased ERR gene expression (Park and Kwak, 2010). Nevertheless, collective data (**Figure 1**) suggests that phthalates can exert effects via both ERα and GPER-1 and have the potential to disrupt cells that depend on estrogen signaling, which includes cells of the immune system.

Innate Immunity Effects

Phthalate compounds and their metabolites have been identified as adverse modulators of innate immunity (**Table 4**). Perinatal DEHP (60 and 600 μg/kg/d) alters inflammatory functions of peritoneal macrophages and results in suppression of TNF-α and IL-1β gene expression. In contrast, gene expression for IL-6 was significantly upregulated, suggesting that phthalate exposure contributes to the dysregulated continual production of IL-6, which has been associated with the onset of various diseases. Interestingly, both DEHP and its metabolite, mono-(2-ethylhexyl) phthalate (MEHP, 20 μg/L), reduced macrophage phagocytosis (Li et al., 2018). Because phthalates are known to leak from plastic goods and accumulate as contaminants in soil and water sources, there is concern that metabolite exposure may lead to inadvertently persistent exposure. Similar cytokine dysregulation in human innate immune cells has also been reported. DEP (22,200 μg/L) and DBP (27,800 μg/L) upregulate secretion of IL-6, CXCL8 and IL-10 secretion and inhibit TNF-α secretion in macrophages (Hansen et al., 2015). DEHP (3,900 μg/L) further modulates the differentiation and maturation of PBMC-derived DCs (Ito et al., 2012). Even at low doses, both DEHP (39 μg/L) and BBP (31 μg/L) suppress expression of type I interferons, such as IFN-α and IFN-β, from plasmacytoid DCs (Kuo et al., 2013). Collectively, animal and human studies are congruent in implicating phthalates in cytokine dysfunction that can drive aberrant innate immune responses.

Adaptive Immunity Effects

Phthalate-induced dysregulation of adaptive response has unsurprisingly resulted from both parent esters and their metabolites. DEP (22,200 μg/L) and DBP (27,800 μg/L) impaired activation-induced T cell secretion of IL-2, IL-4, TNF-α and IFN-γ, but not levels of IL-6 or IL-10. In contrast, the monoester metabolite of DBP, monobutyl phthalate (MBP, 22,200 μg/L) upregulated expression of IL-6, suggesting that phthalate compounds are twice as immunomodulatory (Hansen et al., 2015). In their intact form, phthalates may disrupt signaling pathways that control normal cytokine expression, such as E2-mediated transduction. However, once inside the cell, they can also be processed into various metabolites. This extends their activity and sometimes produces effects that are independent of the parent compound. Effects induced by monoester metabolites have been reported, including an associated reduction of cord blood T_{reg} cells by both monoethyl (MEP) and monoisobutyl phthalate (MiBP) (Herberth et al., 2017). Nevertheless, not all studies report metabolite activities but they do corroborate adaptive immune dysfunction. For example, DEHP (39 μg/L) and BBP (31 μg/L) suppress IFN-γ and can increase IL-13 secretion from CD4 T cells (Kuo et al., 2013). In B cells, DEHP inhibits proliferation (≥1,560 μg/L), which causes a significant reduction in IgM-secreting plasmablasts and plasma cells (≥6,250 μg/L) (Martins et al., 2015). In contrast, DEHP (30, 300, 3,000 μg/kg) can also increase expression of SLAMF1 and SAP in T_{FH} cells, thereby acting as an immunoadjuvant to promote co-stimulatory activity. DEHP also leads to enhanced formation of germinal centers by elevating expression of transcription factors, Bcl-6 and c-MAF in T_{FH} cells, as well as IL-21 and IL-4 cytokines (Han et al., 2014; Han et al., 2019). Interestingly, early-life DEHP exposure elevates secretion of virus-specific IgM following post-natal hepatitis B immunization. This, however, alters the gut microbiota in newborns and could have lasting repercussions on immune responses in later-life, including onset of autoimmune disease (Yang et al., 2019).

Autoimmunity. Contributions of phthalates to autoimmunity have been thoroughly characterized, especially their potential to exacerbate, or perhaps, induce autoreactivity. In testicular autoimmunity, DEHP (0.01% or 11.3–13.3 mg/kg/d) increases severity of mild experimental autoimmune orchitis (EAO) and is accompanied by an increase in IFN-γ^+ cells and overall macrophages. Elevated lymphocytic infiltrates and anti-testicular germ cell autoantibodies further suggest that phthalates can exacerbate autoimmune disease (Hirai et al., 2015). In rheumatoid arthritis (RA), maternal BBP administration (3,000 μg/L) similarly increases the prevalence and severity of RA in the progeny, illustrating a transgenerational effect of phthalate exposure. This is associated with elevated levels of serum IgG1 and IgG2a, as well as enhanced secretion of IFN-γ and IL-17 from splenocytes (Elter et al., 2020). DEHP (7.5 mg/kg) can also increase levels of autoantibodies and associated proteinuria-induced renal dysfunction (Lim and Ghosh, 2005). Effects of phthalates on other autoimmune conditions have also been identified. In children with new onset of type 1 diabetes, higher levels of DiBP metabolites were detected by comparison with healthy controls (Castro-Correia et al., 2018). Furthermore, although the temporal context of immune deregulation is yet debated, inappropriate inflammatory responses and altered functions of lymphocytes have been proposed to be causative for and/or correlative with type 2 diabetes (de Candia et al., 2019). At levels relevant to human exposure, phthalates induce both transient and life-long metabolic dysfunction, including an increased risk of type 2 diabetes and insulin resistance (Radke et al., 2019). Combined data thus illustrates that phthalates directly and indirectly (transgenerationally) disrupt the normal function of both innate and adaptive immune processes, which may trigger onset of autoimmunity, especially in predisposed individuals. In addition, phthalate immunomodulation may lead to somewhat milder, but chronic, effects, such as persistent inflammation. This disruption in cytokines may, over time, provide a favorable context for self-reactivity to develop, even in individuals otherwise disinclined to autoimmune disease.

DISCUSSION

The sex-bias of disease susceptibility has remained an unresolved phenomenon of autoimmunity, including in MS, RA and SLE. This puzzling bias has also gained momentum with post-infection viral autoimmunity and has particular relevance to the current SARS-CoV-2 pandemic. In considering these two, seemingly unrelated, settings, we draw attention to the incidence rate of female-biased autoimmunity and emphasize its steady increase, which has suggested a role for environmental factors. Similar influence may be exerted by the environment, not only on viral-associated autoreactivity, but also on the immunomodulation of the viral adaptive response. In this review, we have emphasized the importance of the environment, particularly EDCs and estrogen mimicry, and the role played in the disruption of immune dynamics. We show that both innate and adaptive response rely upon hormone transduction for several key functions, including cytokine secretion and proliferation.

EDCs set forth a spectrum of effects and determining how immune populations are affected is constrained by several challenges: (1) dose, (2) dose schedule, (3) biological model, (4) cumulative exposure, and (5) metabolites. For many EDCs, dose, including frequency and duration is a critical factor, especially as it may mask biphasic EDC activities. The choice of biological model should also be considered in parallel with dose, as EDCs can produce dramatically different results across species. This variability is likely characteristic of circulating hormone levels and the degree of endocrine involvement, both of which should be taken into account whenever studies are designed, or findings are inferred to human health. In an effort to address this, we hope our review will provide a summary to direct comparisons across both the dose and biological model.

Furthermore, we caution against experimental oversimplification. Although fewer conditions generally allow for clear and translatable interpretations, this may also result in a context that is too artificial to recapitulate the biological phenomenon. Current EDC studies have not adequately addressed effects of cumulative exposure, despite the regular occurrence for this in our environment. Evaluation of effects for combined EDCs can reveal critical information to help identify mechanisms and risks of disease onset that are

more relevant to natural exposure. EDC metabolites are similarly important in this context of cumulative exposure. Both the inherent ability to metabolize EDCs and the rate at which this occurs warrant consideration and may be useful to resolve the variation of results observed across species and cell types. To aid in this, we have generated mechanism-of-action guides that describe how different categories of environmental endocrine disruptors, including their metabolites, are most likely to interfere in immune-endocrine signaling.

In writing this review, we hope the information herein detailed will remove some of the complexities associated with inferring experimental findings of EDC exposure to human health and may be used as a guide for future research.

AUTHOR CONTRIBUTIONS

MP and TF researched data for the review. TC made a substantial contribution to the discussion of content. All authors have equally contributed in writing and editing of the manuscript prior to submission.

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Sex-Specific Differences of the Inflammatory State in Experimental Autoimmune Myocarditis

Maria Luisa Barcena[1,2], Sarah Jeuthe[2,3†], Maximilian H. Niehues[1†], Sofya Pozdniakova[1,4], Natalie Haritonow[1], Anja A. Kühl[5], Daniel R. Messroghli[2,3,6] and Vera Regitz-Zagrosek[7,8]*

[1] Department of Geriatrics and Medical Gerontology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [2] DZHK (German Centre for Cardiovascular Research), Berlin Partner Site, Berlin, Germany, [3] Department of Internal Medicine – Cardiology, Deutsches Herzzentrum Berlin, Berlin, Germany, [4] Climate and Health Program (CLIMA), Barcelona Institute for Global Health (ISGlobal), Barcelona, Spain, [5] iPATH Berlin-Immunopathology for Experimental Models, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt – Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [6] Department of Internal Medicine and Cardiology, Charité - Universitätsmedizin Berlin, Berlin, Germany, [7] Institute for Gender in Medicine, Center for Cardiovascular Research, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt - Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [8] Department of Cardiology, University Hospital Zürich, University of Zürich, Zürich, Switzerland

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***Correspondence:**
Maria Luisa Barcena
maria-luisa.barcena@charite.de

†These authors have contributed equally to this work

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Increasing evidence suggests male sex as a potential risk factor for a higher incidence of cardiac fibrosis, stronger cardiac inflammation, and dilated cardiomyopathy (DCM) in human myocarditis. Chronic activation of the immune response in myocarditis may trigger autoimmunity. The experimental autoimmune myocarditis (EAM) model has been well established for the study of autoimmune myocarditis, however the role of sex in this pathology has not been fully explored. In this study, we investigated sex differences in the inflammatory response in the EAM model. We analyzed the cardiac function, as well as the inflammatory stage and fibrosis formation in the heart of EAM male and female rats. 21 days after induction of EAM, male EAM rats showed a decreased ejection fraction, stroke volume and cardiac output, while females did not. A significantly elevated number of infiltrates was detected in myocardium in both sexes, indicating the activation of macrophages following EAM induction. The level of anti-inflammatory macrophages (CD68+ ArgI+) was only significantly increased in female hearts. The expression of Col3A1 and fibrosis formation were more prominent in males. Furthermore, prominent pro-inflammatory factors were increased only in male rats. These findings indicate sex-specific alterations in the inflammatory stage of EAM, with a pro-inflammatory phenotype appearing in males and an anti-inflammatory phenotype in females, which both significantly affect cardiac function in autoimmune myocarditis.

Keywords: sex differences, inflammation, experimental autoimmune myocarditis, cytokines, cardiac dysfunction

INTRODUCTION

Myocarditis is a cardiovascular disease that is associated with myocardial inflammation and infiltration of immune cells into the heart muscle (1). Of those immune cells, it is predominantly macrophages and T-cells that infiltrate the cardiac tissue during viral or toxic injury in myocarditis (1–3). Impaired regulation of the autoimmune response against auto-myocardial proteins can lead to chronic inflammation followed by fibrosis, dilated cardiomyopathy (DCM), and heart failure at the end stage of myocarditis (4, 5). Mice infected with coxsackievirus B3 (CVB3) develop a chronic myocarditis, associated with the presence of anti-myosin autoantibodies, myocardial fibrosis, and cardiac remodeling (6–8), leading to alterations in the extracellular matrix (ECM) (9). In addition, mice or rats immunized with cardiac myosin and Complete Freund's Adjuvant (CFA) exhibit experimental autoimmune myocarditis (EAM) (10, 11). Pro-inflammatory cytokines e.g., interleukin (IL)-6, IL-1β and tumor necrosis factor α (TNF-α) together with enhanced reactive oxygen species (ROS) production play a crucial role in the development of autoimmune myocarditis (7, 12). In the EAM model, male animals show an increased fibrotic remodeling of cardiac tissue, which is linked to DCM development (13). Moreover, male animals develop cardiac autoimmunity and chronic inflammation more often than females (14).

Sex differences in cardiovascular diseases leading to heart failure have been well documented (15, 16). Interestingly, men show higher prevalence and severity of cardiovascular diseases than premenopausal women (17–19). However, the risk of negative cardiovascular events increases in women after menopause (20). The male sex is more susceptible to the development of DCM or heart failure due to impaired cardiac remodeling and the cardiac response to stress (21, 22). Furthermore, female mice show less acute inflammation compared to male mice in a viral myocarditis model, although the rate of viral replication is not significantly different between the sexes (23, 24). Several pathological conditions in the heart are associated with increased testosterone levels, promoting increased collagen deposition, fibrosis formation, and remodeling of the ECM (25–28). Fibroblasts are responsible for preserving ECM balance (29–31). In cardiac tissue, the most prominent collagen fibers are collagen type I and collagen type III (32). Sex-related differences, regulated by sex hormones such as estrogen and testosterone, are also observed in the immune system (33, 34). In turn, the immune system also regulates sex hormone production and secretion (33). Sex hormones have an effect on cardiomyocytes, endothelial cells and fibroblasts and dramatically modulate the tissue response to inflammation in a sex-dependent manner (35, 36), e.g., *via* p38 and ERK signaling (37). It is interesting to note that male animals have a higher number of classically activated M1 macrophages, whereas females develop a population of alternatively activated TIM3-positive M2 macrophages (38, 39). Moreover, male mice can present a M2 macrophage subpopulation, which expresses the M1 macrophage marker toll-like receptor (TLR4) and IL-1β. It has been proposed that this M2 macrophage population is strongly involved in fibrotic remodeling of cardiac tissue (6, 40). Furthermore, estrogen decreases TNF-α expression in peripheral blood mononuclear cells (PBMC) (33, 41, 42) and increased TNF-α secretion was detected in premenopausal women who underwent oophorectomy (43). In contrast, testosterone induces a TH1-type immune response in both humans and rodents (23, 24, 44–46). Macrophages activate fibroblasts *via* TGF-β, platelet-derived growth factor (PDGF), and TNF-α (47, 48). Activated fibroblasts produce ECM and favor fibrosis formation after cardiac damage (49, 50). Even though these sex differences in molecular and cellular mechanisms in the immune system are well documented, their interplay in specific diseases is not yet fully understood.

In this study, we investigated sex-related alterations in the inflammatory state in EAM accompanied by fibrosis formation and decreased cardiac function. The functional analyses revealed an impaired cardiac function in male but not female animals. Sex differences were also found in macrophage polarization and fibrosis formation. EAM is associated with an increased expression of inflammatory markers in male hearts.

MATERIAL AND METHODS

Animals

Lewis rats were housed in cages with controlled temperature and humidity on a 12h light/12h dark cycle. They were kept in groups of four or five with free access to food and water. Male and female rats (age: male: 42-56 days and female: 50-80 days; body weight 230-260g, n=16) (Janvier, Le Genest-St-Isle, France) were immunized as previously described with a myosin dose of 0.25 mg to the rear food pads on day 0 (51). 21 days later the animals were euthanized; their hearts, spleens, tibias, lungs, livers, and kidneys were extracted and snap frozen in liquid nitrogen and stored at -80°C. Non-immunized Lewis male and female rats were used as the control (n=10). All procedures and experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the relevant local authorities (Landesamt für Gesundheit und Soziales).

Cardiac Magnetic Resonance Imaging

Cardiac function was evaluated by electrocardiographically triggered cardiac magnetic resonance imaging (CMR) as described in an earlier study (13). Left ventricular ejection fraction, end-diastolic volume, end-systolic volume, and cardiac output were measured before, 14 days, and 21 days after immunization.

Analysis of Heart Weight to Body Weight Ratio

Body weight (BW) was measured before performing CMR. After euthanasia, the hearts without atria were weighed, and the relative heart weight (HW) to body weight (BW) ratio (HW/BW) was calculated as described in (13).

Analysis of Muscle Hypertrophy and Immune Cell Infiltrate in Heart Tissue

Using the H&E staining, heart muscle hypertrophy score and the amount of immune cell infiltrates was counted in myocardium from male and female immunized and non-immunized rats (n=12).

Immunohistochemistry

Paraffin-embedded cardiac tissue sections were incubated with anti-arginase 1 (clone N-20, 1:100, Santa Cruz, USA) primary antibody followed by incubation with secondary antibody biotinylated rabbit anti-goat (1: 400, Dianova, Germany). Biotin was detected with alkaline phosphatase-labelled streptavidin (Agilent, USA) and visualized using RED (Agilent, USA) as a chromogen. Proteins and enzymes were inactivated with heat and alkaline pH prior to incubation with anti-CD68 (1:250, Amsbio #1518), followed by incubation with Alexa488-labelled secondary antibody (1:400, donkey anti-rabbit, Invitrogen, Germany). DAPI (Sigma, Germany) was used to stain nuclei and sections mounted with Fluoromount-G (Southern Biotech, USA). Negative controls were performed by omitting the primary antibodies. Images were acquired with an AxioImager Z1 (Zeiss MicroImaging GmbH, Germany). All evaluations were performed in a blinded manner.

5 µm paraffin-sections of rat LV myocardium were stained with picrosirius red to obtain collagen content (52).

RNA Extraction and Quantitative Real-Time PCR

The total RNA from cardiac rat tissue was isolated with RNA-Bee (Amsbio, UK) and a quantitative real-time PCR was performed with Brilliant SYBR Green qPCR master mix (Applied Biosystems, USA). The relative amount of target mRNA was determined using the comparative threshold (Ct) method as previously described (53). The mRNA contents of target genes were normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT).

Protein Extraction and Immunoblotting

LV myocardium from male and female EAM rats was homogenized in Laemmli buffer (253mM Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200mM DTT, 0.4% bromophenol blue) (54). Proteins were quantified with the BCA Assay (Thermo Scientific Pierce Protein Biology, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: Col3A1 (1:400, Santa Cruz, USA), ERK (1:1000, Santa Cruz, USA), p-ERK (1:2000, Santa Cruz, USA), p38 (1:500, Santa Cruz, USA) and p-p38 (1:500, Santa Cruz, USA). Equal sample loading was confirmed through an analysis of actin (1:1500, Santa Cruz, USA). Immunoreactive proteins were detected with ECL Plus (GE Healthcare, UK) and quantified with ImageLab (Bio-Rad Laboratories, USA).

Statistical Analysis

All data are given as mean ± SEM. The data were evaluated with the non-parametric Mann-Whitney test for two independent groups or with two-way ANOVA analysis. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, USA). Statistical significance was accepted when $p < 0.05$.

RESULTS

Impaired Cardiac Function in Male EAM Rats

Male rats showed a decline in stroke volume 21 days after immunization with cardiac myosin and CFA ($p< 0.05$), while no significant changes in female rats were detected ($p> 0.05$) (**Figure 1A**). Ejection fraction and cardiac output were significantly decreased in EAM male rats at 21 days after immunization in a sex-dependent manner ($p< 0.05$) (**Figures 1B, C**).

While male EAM rats had higher body and heart weights than female EAM rats ($p< 0.05$) (**Supplementary Figures 1A, B**), the relative heart weight to body weight ratio did not vary between sexes in the EAM rats ($p> 0.05$) (**Supplementary Figure 1C**).

The spleen, liver, and kidneys were significantly heavier in male EAM rats when compared to females, while the weight of the lungs was similar in both sexes ($p< 0.05$ and $p> 0.05$, respectively) (**Supplementary Figures 1D–G**).

CD68+ ArgI+ Macrophages Are Increased in Myocardial Tissue in Female EAM Rats

EAM rats did not show a higher immunohistochemical score for heart muscle hypertrophy when compared to healthy rats ($p> 0.05$) (**Figure 2A**). Despite an increased number of infiltrates detected both in male and female myocardial tissue after immunization ($p< 0.01$) (**Figure 2B**), female EAM rats showed significantly fewer immune cell infiltrates than male EAM rats ($p< 0.05$) (**Figure 2B**).

The number of cardiac CD68+ immune-reactive macrophages was similar in male and female EAM rats ($p> 0.05$) (**Figure 2C**). However, female EAM hearts had an increased number of cardiac anti-inflammatory CD68+ ArgI+ macrophages ($p< 0.05$) (**Figure 2D**), indicating an enhanced infiltration of M2 associated macrophages in females.

Male EAM Rats Show More Fibrosis in Myocardial Tissue

To explore sex differences in collagen expression and fibrosis formation in EAM rats, the RNA and protein expression of collagen (Col3A1, Col1A1, Col4 and Col6), matrix metallopeptidase (MMP9), tissue metallopeptidase inhibitor 1 (TIMP1) and the pro-fibrotic factor, TGF-β were examined.

RNA and protein Col3A1 expression was significantly increased in the heart of EAM rats when compared to healthy controls in a sex-dependent manner ($p< 0.05$ and $p> 0.05$, respectively) (**Figures 3A, B**). Female EAM hearts had significantly less Col3A1 than males ($p< 0.01$ and $p< 0.05$) (**Figures 3A, B**). In accordance with these data, immunized male rats showed significantly higher amounts of fibrosis in comparison to female EAM hearts or non-immunized male hearts ($p< 0.05$) (**Figure 3C**).

Col1A1 mRNA expression was also significantly up-regulated in hearts from male but not female immunized rats ($p< 0.05$) (**Supplementary Figure 2A**). No changes in Col4 and Col6 expression were detected in EAM hearts ($p> 0.05$) (**Supplementary Figures 2B, C**). MMP-9 mRNA expression was up-regulated in female EAM hearts in comparison to male immunized rats ($p< 0.05$) (**Supplementary Figure 2D**). In addition, TIMP-1 expression was significantly up-regulated in female EAM hearts ($p< 0.05$ vs male EAM hearts) (**Supplementary Figure 2E**). Moreover, immunized female rats showed a significantly decreased TGF-β mRNA expression when compared with male immunized rats ($p< 0.01$) (**Supplementary Figure 2F**).

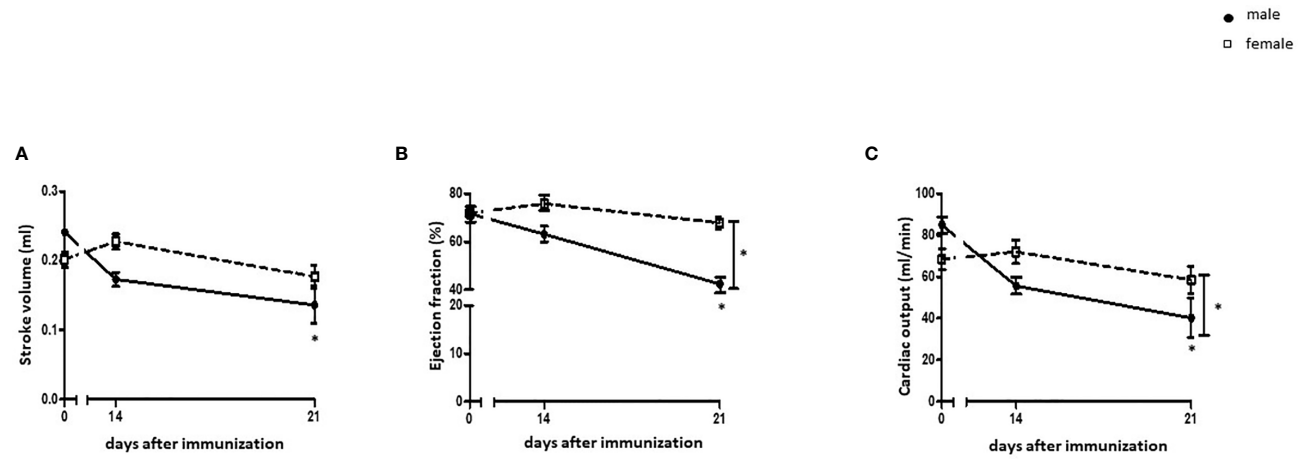

FIGURE 1 | Cardiac function in EAM rats. Cardiac function parameters from the left ventricle were measured by CMR. **(A)** Stroke volume (SV) **(A)**, ejection fraction (EF) **(B)**, and cardiac output **(C)** were assessed before immunization, 14, and 21 days after immunization with cardiac myosin and CFA in male and female rats (n= 9-21). Data are shown as mean ± SEM. *p < 0.05.

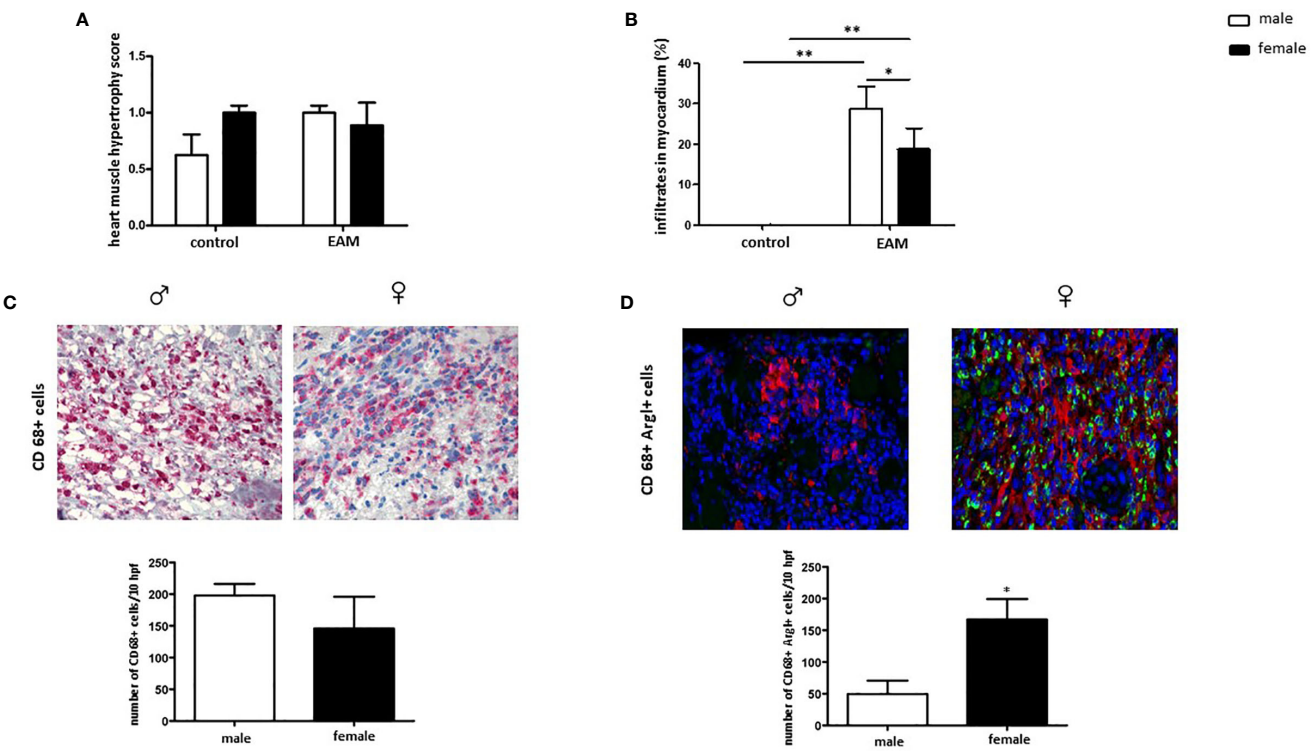

FIGURE 2 | Increased number of cardiac CD68+ ArgI+ macrophages in female EAM rats. Immunohistochemical analysis of heart muscle hypertrophy **(A)**, myocardial immune infiltrates **(B)**, CD68+ immune-reactive cells **(C)**, and CD68+ ArgI+ cells **(D)** in myocardial tissue in male and female EAM animals. Data are shown as mean ± SEM (n= 4-12). *p < 0.05, **p < 0.01. Representative images of cardiac cryosections stained with antibodies against CD68 **(C)** and CD68 and ArgI **(D)** in myocardial tissue in male (♂) and female (♀) EAM animals (n= 4-12). Magnification 200x.

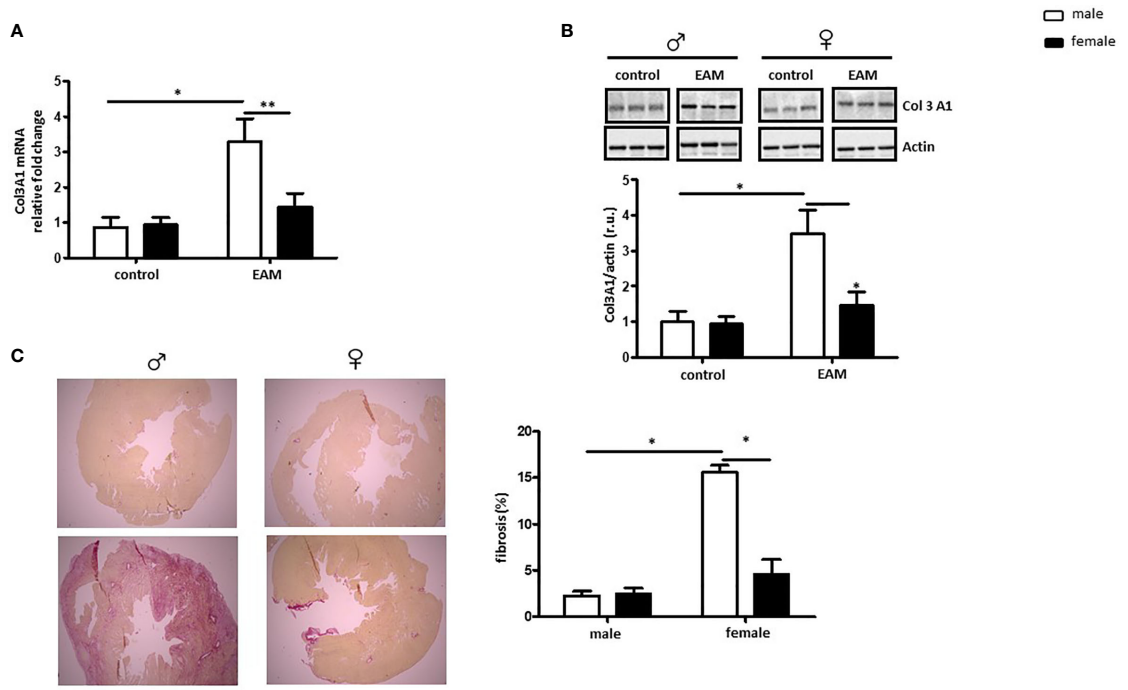

FIGURE 3 | Male EAM rats develop more fibrosis in myocardial tissue. Analysis of Col3A1 mRNA **(A)** and protein expression **(B)** in cardiac tissue from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 4-12). *p < 0.05, **p < 0.01. Representative imaging of western blot analysis; the lanes were run in the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.). **(C)** Representative Sirius red–dyed staining of cardiac tissue of 6 µm from male (♂) and female (♀) animals and corresponding statistics showing enhanced fibrosis in EAM rats (n= 4-12). Magnification 100x.

Sex Differences in the Inflammatory Response in the EAM Model

Sex differences in the inflammatory response in myocarditis have been documented in both human and animal models (3). Both ERK and p38 activation are modulated *via* ER activation (55) and play a crucial role in the polarization of pro-inflammatory macrophages (56). Thus, we examined whether ERK and/or p38 activation (phosphorylation rate) are impaired in EAM hearts in a sex-dependent manner.

ERK phosphorylation was significantly increased in both male and female EAM rats ($p< 0.05$), while the amount of total ERK was unaffected ($p> 0.05$) (**Figure 4A**). In accordance with these findings, the pp38/p38 ratio was significantly increased in EAM rats in both sexes ($p< 0.05$) (**Figure 4B**). No significant changes in the p38 expression in EAM rats were found ($p> 0.05$) (**Figure 4B**).

The mRNA expression of the pro-inflammatory marker TLR4 was significantly increased in both male and female hearts from EAM rats when compared to healthy hearts ($p< 0.05$) (**Figure 5A**), however TLR4 mRNA was significantly up-regulated in male EAM hearts in comparison to female EAM hearts ($p< 0.05$) (**Figure 5A**). Furthermore, the pro-inflammatory markers c-fos, IL-6, iNOS, and IL-1β were only up-regulated in hearts from male but not female EAM rats ($p< 0.05$) (**Figures 5B–E**). In accordance with these data, IL-10 mRNA expression was significantly up-regulated in immunized female rats in comparison to immunized male rats ($p< 0.01$) (**Figure 5F**). The expressions of TNF-α, NFκB, c-jun, and STAT1 were unchanged in both sexes after immunization ($p> 0.05$) (**Supplementary Figures 3A–D**).

DISCUSSION

In the current study, we investigated sex-dependent alterations in inflammation, collagen deposition and fibrosis formation in EAM rats. The main findings are: 1) Cardiac function was preserved in female rats after immunization, while the cardiac function was impaired in male EAM rats; 2) the number of cardiac anti-inflammatory CD68+ ArgI+ macrophages was only increased in female EAM rats; 3) collagen deposition and pathological fibrosis was only enhanced in hearts from male immunized rats; 4) pro-inflammatory mediators were significantly altered only in male EAM hearts. To summarize, an impaired inflammatory response and an exaggerated collagen deposition affecting the cardiac function were revealed in male EAM rats, while females demonstrated a protective response to adjuvant-induced EAM.

To the best of our knowledge, this is the first study to demonstrate sex differences in the inflammatory stage and in fibrosis formation, with a decline in cardiac function in an EAM rat model.

In clinical setting, men are more likely to develop myocarditis and DCM than women (17, 18, 57–59). More pronounced

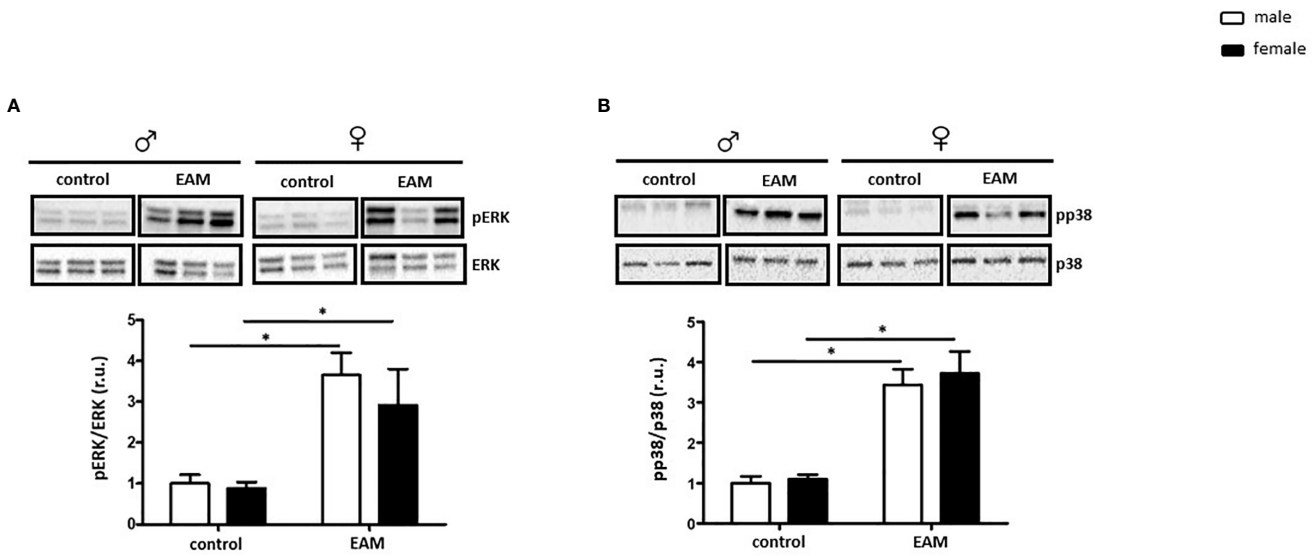

FIGURE 4 | Sex-independent ERK and p38 activation in the EAM model. Western blot analysis of pERK/ERK ratio **(A)** and pp38/p38 ratio **(B)** in cardiac tissue lysates from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12). *$p < 0.05$. Representative imaging of western blot analysis; the lanes were run in the same gel. All data were normalized to the corresponding control and expressed in relative units (r.u.).

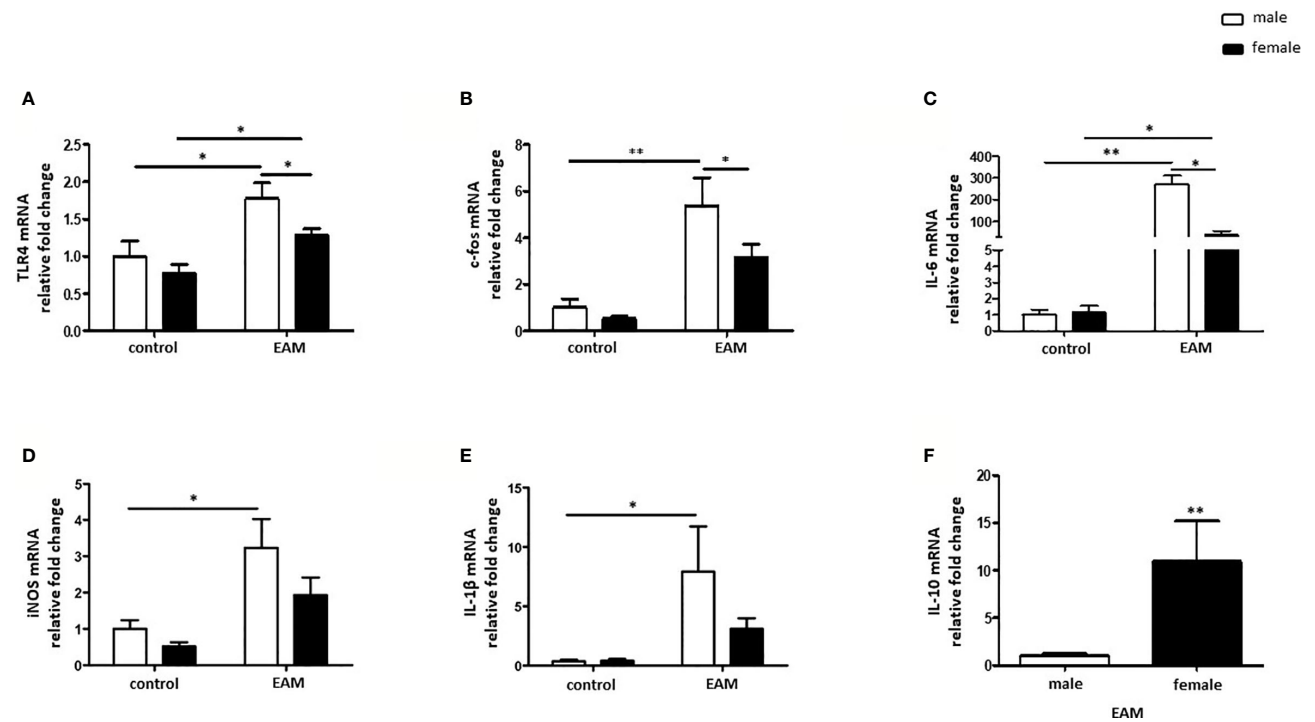

FIGURE 5 | Sex differences in the inflammatory response in the EAM model. Real-time PCR analysis of TLR4 **(A)**, c-fos **(B)**, IL-6 **(C)**, iNOS **(D)**, IL-1β **(E)** and IL-10 **(F)** in rat cardiac tissue lysates from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12). *$p < 0.05$, **$p < 0.01$.

inflammation and fibrosis have been reported in male individuals with myocarditis than in female individuals (21, 22). A potential contribution of sex hormones may be a factor, as the association of DCM and heart failure with high testosterone levels has been previously reported (25, 60–62). These sex differences in humans correspond to sex differences in the mouse model. It is also interesting to note that female mice develop less inflammation after infection with CVB3 by similar viral replication (23, 24).

The EAM immunization protocol is used as a model of the chronic inflammatory phase of post-viral myocarditis (13), characterized by ongoing inflammation, fibrotic remodeling, appearance of anti-myosin antibodies, and development of DCM in the end-stage (63). Schmerler et al. have shown that male EAM rats had decreased ejection fraction and stroke volume (13). In keeping with those results, in our study the ejection fraction and the stroke volume showed a prominent decline in male EAM animals but no significant changes in females were detected, suggesting a preserved cardiac function in females.

Male EAM rats developed autoimmune myocarditis 21 days after immunization with cardiac myosin and CFA in the paw, accompanied by an increased amount of myocardial immune cell infiltrates and CD68+ immune reactive cells (13). In accordance with this study, we detected an increased number of immune cell infiltrates in the heart of male rats after immunization. In female EAM rats, although the infiltrates in the myocardial tissue were increased, it was significantly less than in male rats, indicating a weaker immune response in females.

Macrophages are the central regulator of the immune system in the heart in a normal state as well as during cardiac inflammation (64), and their crucial role in pro-fibrotic processes during chronic inflammation has been reported elsewhere (65). Though we found no sex differences in the number of immune reactive CD68+ macrophages in our EAM model, hearts from female EAM rats were infiltrated with an increased amount of anti-inflammatory CD68+ ArgI+ macrophages, suggesting that a predominant phenotype in females is alternative activated macrophages (M2) that favor an anti-inflammatory environment thus attenuating inflammation in female hearts in autoimmune myocarditis. However, M2 macrophages seem to be involved in the production of collagen and fibrosis formation (66), associated with an increased arginase activity (67). In agreement with this, sex differences in viral myocarditis and post-myocarditis complications, e.g., development of cardiac autoimmunity and DCM, are not caused by the virus itself, but rather by sex-related differences in the immune response (11). Moreover, Fairweather et al. have shown that, in a viral induced myocarditis model, the detrimental immune response in male individuals is driven by a predominant M1 response, while female animals show a stronger M2 response (38, 68). Our results suggest that macrophage polarization plays a crucial role in the development of sex differences in cardiac inflammation. The activation of the M2 response counteracts the detrimental effects of the pro-inflammatory macrophage polarization during acute inflammation, suggesting that a predominant M2 response is cardio protective (42). Our results indicate a pro-inflammatory M1-mediated and M2-mediated anti-inflammatory immune reaction in the heart of male and female rats, respectively.

Chronic activation of the inflammatory response leads to increased collagen deposition and pathological fibrosis is part of many diseases including myocarditis (69). Here it is important to remember that macrophages play a key role in the regulation of fibrosis (70) and activate fibroblasts *via* TGF-β, platelet-derived growth factor (PDGF) and TNF-α (47, 48). In our study, we detected an increased expression of Col3A1 and Col1A1 in the cardiac tissue from male EAM rats, while female EAM rats expressed similar amounts of Col3A1 and Col1A1 as healthy rats. In accordance, the anti-fibrotic factor, TIMP1 was up-regulated in immunized female rats, while the pro-fibrotic factor, TGF-β was decreased in females. Additionally, male EAM rats develop pathological fibrosis in the heart after immunization, while female EAM rats do not, suggesting that they undergo a different, fibrosis-independent, immune response. Indeed, severe fibrosis was previously reported in hearts from males with EAM (13), which may potentially be caused by increased testosterone levels (25–28, 71).

Enhanced ERK and p38 activity was detected in EAM rats in comparison to non-immunized rats of both sexes, arguing that other cascades are involved in activation of pro-inflammatory mediators in male EAM rats. In fact, a stronger M1 response and altered pro-inflammatory mediators were demonstrated solely in male animals. Male EAM rats showed an increased expression of TLR4, IL-6, c-fos, and iNOS when compared to healthy animals, while no significant changes were detected in female EAM rats, indicating that females did not develop a pro-inflammatory response after immunization. In accordance, Roberts et al. demonstrated sex-differences in the cardiac TLR4 expression in CVB3 infected mice, increasing the pathogenicity in male but not female infected mice (72). Of note, c-fos is a key transcription factor for the M1 spectrum, and iNOS is a signature M1 enzyme, reinforcing the observation of sex-dependent macrophages polarization in EAM (42, 73, 74).

Fairweather et al. have proposed a pivotal role for sex hormones in the sex-related differences in cardiac inflammation (75). While estrogen has cardio-protective properties in females, characterized by reducing cardiomyocyte apoptosis, counteracting fibrosis (76, 77), and deactivating cellular pathways that induce hypertrophy (17, 78, 79), testosterone increased cardiac inflammation in a myocarditis mice model (24) and encouraged a M1 response of macrophages in male individuals (25). Moreover, Koenig et al. also reported pro-inflammatory actions of androgens and anti-inflammatory actions of estrogen in CVB3 induced experimental myocarditis (80). Recent studies have also demonstrated that estrogen directly regulates macrophage polarization in different pathological tissue states (81–83), suggesting that E2 is directly involved in the polarization into M2 macrophages in female EAM rats. However, the spectrum of macrophage phenotypes to be researched is larger (42) and the role of sexual hormones should be investigated in the EAM model more in depth.

In conclusion, the present study revealed that autoimmune myocarditis is associated with an increased pro-inflammatory response in males, leading to fibrotic formation, while in females

the model is associated with a muted pro-inflammatory response, balanced immune-regulation, and preserved cardiac function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales, Berlin.

AUTHOR CONTRIBUTIONS

MB conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. MN performed the molecular experiments and analyzed the data. SJ generated the model, performed the functional experiments, obtained the tissue, and analyzed data. SP analyzed the data and wrote the main manuscript text. NH performed molecular experiments and analyzed the data. AK performed the immunohistochemical experiments, analyzed the data, and revised the manuscript. DM designed the functional experiments, provided the EAM tissue, and revised the manuscript. VR-Z generated research funds, initiated, and coordinated the project. SJ and VR-Z revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.686384/full#supplementary-material

Supplementary Figure 1 | Sex differences in heart weight to body weight ratio. Body weight (BW) **(A)** and heart weight (HW) **(B)** and relative heart weight to body weight (HW/BW) **(C)** were measured 21 days after immunization with cardiac myosin and CFA. In addition, the weight from spleen **(D)**, lung **(E)**, liver **(F)**, and both kidneys **(G)** was assessed 21 days after immunization with cardiac myosin and CFA (n= 4-12). Data are shown as mean ± SEM. *p< 0.05, **p< 0.01.

Supplementary Figure 2 | Sex differences in the expression of pro- and anti-fibrotic factors in EAM. Real-time PCR analysis for Col1A1 **(A)**, Col4 **(B)**, Col6 **(C)**, MMP9 **(D)**, TIMP1 **(E)** and TGF-β **(F)** performed with rat cardiac tissue from control or EAM male (♂) and female (♀). Data are shown as the mean ± SEM (n= 4-12). *p< 0.05, **p< 0.01.

Supplementary Figure 3 | TNF-α and NFκB are not increased in EAM. Real-time PCR analysis for TNF-α **(A)**, NFκB **(B)**, c-jun **(C)** and STAT1 **(D)** performed with rat cardiac tissue from control or EAM, male (♂) and female (♀). Data are shown as the mean ± SEM (n= 5-12).

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Impact of Sex Differences on Mortality in Patients With Sepsis After Trauma: A Nationwide Cohort Study

Yutaka Kondo, Atsushi Miyazato, Ken Okamoto and Hiroshi Tanaka*

Department of Emergency and Critical Care Medicine, Juntendo University Urayasu Hospital, Chiba, Japan

Objective: Sepsis is the leading cause of death in intensive care units, and sepsis after trauma is associated with increased mortality rates. However, the characteristics of sepsis after trauma remain unknown, and the influence of sex on mortality remains controversial. This study aimed to assess the role of sex in in-hospital mortality in patients with sepsis after trauma.

Methods: We performed a retrospective cohort study involving several emergency hospitals (n=288) in Japan. The data of patients with trauma who developed sepsis after admission from 2004 to 2019 were obtained from the Japan Trauma Data Bank. We divided the patients into two groups according to sex and compared their in-hospital mortality. We also performed subgroup analysis limited to the elderly population (age ≥ 65 years) and evaluated in-hospital mortality between men and women.

Results: A total of 1935 patients met the inclusion criteria during the study period. Of these, 1204 (62.2%) were allocated to the male group and 731 (37.8%) to the female group. Multivariable Cox proportional-hazards analysis showed a significantly lower risk of in-hospital mortality in the female group than in the male group (hazard ratio (HR): 0.74, 95% confidence interval (CI): 0.62–0.89; p=0.001). In the subgroup analysis, multivariable Cox proportional hazards still showed significantly lower risks of in-hospital mortality in the female group than in the male group (HR: 0.72, 95% CI: 0.58–0.88; p=0.002).

Conclusion: The present study shows a significantly increased survival in the female group when compared to that in the male group of patients with sepsis after trauma. The underlying mechanism remains unclear, and further investigations are required.

Keywords: sex, female, trauma, sepsis, mortality

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Andrea Baragetti, University of Milan, Italy

****Correspondence:***
Yutaka Kondo
kondokondou2000@yahoo.co.jp

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INTRODUCTION

Sepsis is the leading cause of death in intensive care units (ICUs) and accounts for more than five million deaths worldwide each year (1, 2). Moreover, sepsis-associated mortality increases after trauma because of injuries, surgeries, adverse events, and complications, which weaken the immune system. Therefore, a fundamental understanding of sepsis following injury is important to improve clinical outcomes.

Biological sex is known to modulate inflammatory responses during immune processes (3). Several studies using animal models have shown that the sex hormones testosterone and estrogen play a central role in the regulation of posttraumatic immunosuppression (4–6). Testosterone depletion and estrogen supplemental therapy can improve mortality (6). Furthermore, non-hormonal, genetic factor related sexual dimorphism also account for differences in incidence or clinical manifestations of autoimmune diseases, malignancies, and infection (7).

In contrast, the influence of sex on mortality in patients with sepsis remains controversial in clinical settings. Several studies have reported that mortality is lower in women with sepsis than in men with sepsis (8, 9) whereas others have reported increased mortality among women (10, 11). Thus, the influence of sex on mortality in patients with sepsis after trauma remains unknown. We hypothesized that being female sex is associated with lower mortality in cases of sepsis after trauma. This study aimed to elucidate how sex affects in-hospital mortality in patients with sepsis after trauma using a nationwide Japanese cohort dataset.

MATERIALS AND METHODS

The present study was approved by the Ethics Committee of the Juntendo University Urayasu Hospital. The requirement for informed consent was waived because of the anonymous nature of the data set. This study followed the STROBE statement (12).

Study Design and Data Collection

This was a multicenter retrospective cohort study using data from patients treated for sepsis after trauma between 2004 and 2019. The data were available at the Japan Trauma Data Bank (JTDB), which was established in 2003 by the Japanese Association for the Surgery of Trauma (Trauma Registry Committee) and the Japanese Association for Acute Medicine (Committee for Clinical Care Evaluation) as the main parties (13). The database contains nationwide data on patients with trauma and includes patient characteristics, vital signs during the prehospital phase and upon hospital arrival, examinations and treatment records, types and causes of trauma, diagnostic codes using the abbreviated injury scale score (14), several trauma severity scores, hospitalization length, and discharge status (15).

The data derived from 288 emergency hospitals in Japan, which were voluntarily enrolled in the study. The database contains data from approximately 71% of tertiary-level emergency hospitals in Japan (16). Approximately 40% of the participating hospitals are comparable to level I trauma centers in the United States (17, 18).

Study Participants

We included trauma patients who developed sepsis after admission. The definition of sepsis is "sepsis with multiple organ failure", which is equivalent to "severe sepsis" in the conventional systemic inflammatory response syndrome criteria (Sepsis-1 or -2) (19), or current "sepsis" criteria (Sepsis-3) proposed at 2016 (20). Patients lacking sex and systolic blood pressure data or whose systolic blood pressure was zero upon arrival were excluded.

Eligible patients were divided into two groups: male and female.

Variables and Outcomes

In this study, we examined the following patient characteristics: age, sex, vital signs upon arrival, cause of injury, injury severity score (ISS), type of trauma (blunt, penetrating, burn, or others), cause of trauma (accident, suicide attempt, or others), admission year, treatment (resuscitative endovascular balloon occlusion of the aorta, transcatheter arterial embolization, or surgical operation), hospitalization length, and in-hospital mortality.

The primary outcome in this study was in-hospital mortality among men and women with sepsis after trauma.

Statistical Analysis

Continuous variables are presented as median and interquartile ranges. Categorical variables appear as numbers and percentages. Baseline characteristics and crude outcomes were compared using the Mann-Whitney U test for the skewed distribution of continuous variables and the chi-square test or Fisher's exact test for categorical variables.

We performed multivariable Cox proportional hazards regression analysis for the time until death during hospitalization and adjusted for all the variables mentioned above (21). Hazard ratios (HRs) and the associated 95% confidence intervals (CIs) were calculated for in-hospital mortality. For sensitivity analysis, we have added a traumatic brain injury variable which showed head AIS≥1 for adjustment.

We also performed subgroup analysis using the multivariable Cox proportional hazards regression method. The variables used in the main analysis were also used in the subgroup analysis, which involved patients aged ≥65 years only because the effects of sex hormones are attenuated in the elderly.

The two-sided significance level for all tests was set at $p < .05$. All analyses were performed using IBM SPSS, version 26 (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 1935 patients met the inclusion criteria during the study period. Of these, 1204 (62.2%) were allocated to the male group and 731 (37.8%) to the female group (**Figure 1**).

The baseline characteristics and outcomes are shown in **Table 1**. The median age and temperature were higher in women than in men. The median heart rate, respiratory rate, and ISS were lower in women than in men. Glasgow Coma Scale scores and causes of trauma were significantly different between the groups. There were no significant differences in systolic blood pressure, type of trauma, year, or treatment between men and women. Regarding outcomes, the median length of hospitalization was 36.5 days in women and 44 days in men. Crude in-hospital mortality was 36.5% among women and 47.4% among men.

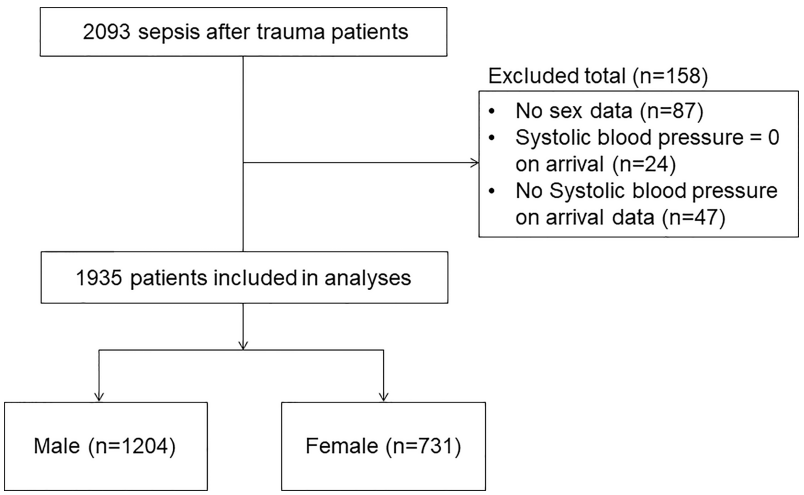

FIGURE 1 | Flow diagram showing the process for selecting patients.

TABLE 1 | Baseline characteristics of eligible patients.

	Male (n = 1204)	Female (n = 731)	*p*-value	Number of patients with missing data, n (%)
Age, years (range)	68 (53–80)	79 (65–85)	<0.001	2 (0.10)
Systolic blood pressure, mmHg	129 (97-156)	132 (105-157)	0.056	0 (0)
Heart rate, beats/min	93 (76-112)	89 (75-104)	0.004	37 (1.9)
Respiratory rate, breaths/min	22 (18-28)	21 (18-26)	0.009	164 (8.5)
Temperature, °C	36.2 (35.6)	36.4 (36.8)	0.001	204 (10.5)
GCS			0.002	111 (5.7)
3-5	119 (10.4)	60 (3.8)		
6-8	129 (11.3)	56 (8.2)		
9-11	116 (10.2)	44 (6.4)		
12-14	325 (28.5)	215 (31.4)		
15	451 (39.6)	309 (45.2)		
ISS	25 (16–29)	16 (9–25)	<0.001	10 (0.52)
Type of trauma			0.78	11 (0.57)
Blunt	906 (75.8)	555 (76.2)		
Penetrating	22 (1.8)	16 (2.2)		
Burn	253 (21.2)	151 (20.7)		
Others	15 (1.3)	6 (0.8)		
Cause of trauma			<0.001	13 (0.67)
Accident	915 (76.6)	616 (84.7)		
Suicide attempt	103 (8.6)	79 (10.9)		
Others	177 (14.8)	32 (4.4)		
Year			0.41	8 (0.41)
2004–2007	91 (7.6)	55 (7.6)		
2008–2011	342 (28.5)	192 (26.4)		
2012–2015	507 (42.3)	335 (46.1)		
2016–2019	260 (21.7)	145 (19.9)		
REBOA	35 (2.9)	17 (2.3)	0.44	0 (0)
TAE	142 (11.8)	73 (10.0)	0.22	0 (0)
Surgical operation	858 (71.3)	546 (74.7)	0.10	0 (0)
Hospitalization length, days	44 (19–82)	36.5 (19–68)	0.012	32 (1.7)
In-hospital mortality	556 (47.4)	260 (36.5)	<0.001	50 (2.6)

Data are presented as number (%) or median (interquartile range).

GCS, Glasgow coma scale; ISS, injury severity score; REBOA, resuscitative endovascular balloon occlusion of the aorta; TAE, transcatheter arterial embolization.

The number of patients with missing data is shown in **Table 1**.

Multivariable Cox proportional hazards analysis showed significantly lower risk of in-hospital mortality among women than among men (HR: 0.74, 95% CI: 0.62–0.89; p=0.001) (**Table 2** and **Figure 2**). Sensitivity analysis also showed significantly lower risk of in-hospital mortality among women than among men (HR: 0.76, 95% CI: 0.63–0.91; p=0.003) (**Supplemental File 1**).

Subgroup analysis included 1237 elderly patients (≥65 years of age). Multivariable Cox proportional-hazards analysis showed a significantly lower risk of in-hospital mortality in women than in men (HR: 0.72, 95% CI: 0.58–0.88; p=0.002) (**Supplemental File 2**).

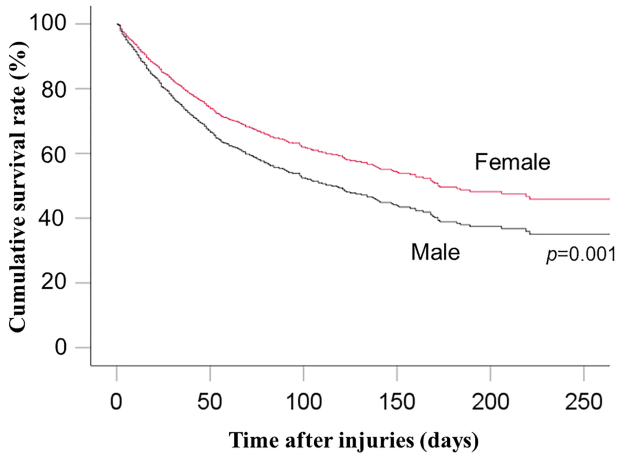

FIGURE 2 | Cumulative survival rate between men and women.

DISCUSSION

The present nationwide retrospective cohort study showed that the in-hospital mortality in men with sepsis after trauma is significantly higher than in women. In our subgroup analysis on elderly patients, in-hospital mortality among women was also lower than that among men. Therefore, the main results are in line with our hypothesis. Similar results were shown in sensitivity analysis.

There is limited evidence on the role of sex in sepsis after trauma. In 2000, an observational study found no difference in the mortality rates among men and women (men 64.9%, women 65.5%) with severe sepsis or septic shock in surgical patients (22). In 2014, another observational study reported that female sex was associated with improved clinical outcomes in cases of sepsis and organ failure after traumatic injury and hemorrhagic shock (23). Our findings are in line with the results of these studies. However, studies assessing several clinical outcomes are required because our study evaluated only in-hospital mortality in men and women.

Several studies have reported that mortality among women with trauma and hemorrhagic shock was significantly lower than in men (24–26) although the underlying reasons have not been fully elucidated. In 2019, an observational study showed that women were more likely to have a hypercoagulable profile when compared to men, and consequently were less likely to develop traumatic coagulopathy after severe trauma (24). Furthermore, the association between hypercoagulability and female hormones has been previously reported (27, 28) and the association between estrogen and the clotting cascade in trauma patients has been confirmed in a previous study (29). Altogether, these data suggest that hypercoagulability in women contributes to reducing mortality due to trauma. Similarly, in our study, hypercoagulability in women might have also contributed to decreasing in-hospital mortality in patients with sepsis after trauma.

Some studies consider that hormonal differences are the primary underlying cause of the mortality differences between men and women (30, 31). Moreover, a study suggested that younger women had lower mortality, unlike elderly women due to the age-dependent hormonal status (30). To evaluate the

TABLE 2 | Cox proportional hazards regression for in-hospital mortality.

	Hazard ratio	95% confidence interval	*p*-value
Sex			
Male	Reference		
Female	0.74	0.62-0.89	0.001
Age, by 1-year increase	1.02	1.02-1.03	<0.001
Systemic blood pressure, by 1-mmHg increase	0.99	0.99-1.00	0.024
Heart rate, by 1-beat/min increase	0.99	0.99-1.00	0.37
Respiratory rate, by 1-time/min increase	1.02	1.01-1.03	0.002
Temperature, by 1-centigrade increase	0.91	0.86-0.96	0.001
GCS			
3-5	Reference		
6-8	0.68	0.49-0.95	0.025
9-11	0.54	0.37-0.77	0.001
12-14	0.55	0.42-0.73	<0.001
15	0.48	0.36-0.64	<0.001
ISS, by 1-point increase	1.01	1.01-1.02	<0.001
Type of trauma			
Blunt	Reference		
Penetrating	1.10	0.55-2.19	0.78
Burn	1.96	1.61-2.40	<0.001
Others	0.91	0.36-2.31	0.84
Cause of trauma			
Accident	Reference		
Suicide attempt	1.25	0.93-1.70	0.14
Others	1.07	0.80-1.42	0.65
Year			
2004–2007	Reference		
2008–2011	0.56	0.41-0.75	<0.001
2012–2015	0.54	0.41-0.73	<0.001
2016–2019	0.50	0.37-0.69	<0.001
REBOA	0.79	0.45-1.38	0.40
TAE	1.13	0.85-1.50	0.40
Surgical operation	0.43	0.36-0.52	<0.001

GCS, Glasgow coma scale; ISS, injury severity score; REBOA, resuscitative endovascular balloon occlusion of the aorta; TAE, transcatheter arterial embolization.

effects of sex hormones on in-hospital mortality, we performed a subgroup analysis including exclusively elderly patients. If the difference in in-hospital mortality between men and women were strongly dependent on female hormones, the mortality among postmenopausal women might be higher than that in young women, and might not significantly differ from that of men. However, subgroup analysis shows that the in-hospital mortality in elderly women was lower than that of elderly men. This result implies that multiple factors are involved in in-hospital mortality in addition to hormonal differences. In line with this observation, several studies have reported that the treatment and care provided at hospitals differ between male and female patients (32, 33). Alternatively, high estrogen levels throughout a woman's life may be associated with health benefits even after menopause. Another possible explanation is non-hormonal, genetic factor related sexual dimorphism; several genes encoding for innate immune molecules are located on the X chromosome (7). In fact, an animal study reported that X-linked genetic variations in genes involved in the immune response were associated with differences in defense against infections between males and females (34).

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) play an important role in sepsis as potent activators of initiating innate immune system (35, 36). Inflammation, organ injury, and death that follows infection were due to the body's response to PAMPs and DAMPs. This mechanism of immune system may differ in sex and may lead to different mortality rate.

Our study showed that the female group was older than the male group. The male group had higher ISS scores than the female group. These findings might come from different patterns of trauma between the groups (37). We therefore used the variables, age, ISS scores, and cause of trauma, for main analysis and still got lower mortality rate in the female group even after adjustment.

We excluded following patients with no sex data because sex data is essential for the design of present study. We also excluded systolic blood pressure was zero on arrival and no systolic blood pressure data because the number of was small, and clinical characteristics differ between cardiac arrest before arrival and the others.

There are several limitations in this study. First, the definition of sepsis changes over time and may affect sepsis-associated mortality data assessments. Second, some data on the evaluated variables were missing and excluded from our analysis, which may have affected the main results. Third, we could not perform sample size calculation because differences of anticipated mortality rate between the groups are unknown. However, we have used a large database and therefore statistically underpower is unlikely. Furthermore, sample size calculation is mostly required prospective design because it needs to determine study period and number of included patients. Our study is retrospective design and includes long study period and many patients. Finally, we have selected independent variables by clinical importance. We retrospectively analyzed the data, and some unadjusted confounders might have been overlooked. Selection of variables could affect results although our sensitivity analysis supported main results.

CONCLUSION

The present retrospective cohort study contained data from hospitals throughout Japan and showed that mortality due to sepsis after trauma was significantly lower among females than among males. However, further studies are required to unveil the underlying causes of the sex-associated mortality differences observed in this study.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data are available upon reasonable request although data are not in public. Requests to access these datasets should be directed to YK, kondokondou2000@yahoo.co.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Juntendo University Urayasu Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YK conceived this study, analyzed the data, and drafted the manuscript. AM partially revised the manuscript. KO and HT advised on the design and revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.678156/full#supplementary-material

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Comprehensive Analysis of Sex Differences at Disease Manifestation in ANCA-Associated Glomerulonephritis

Désirée Tampe[1], Peter Korsten[1], Philipp Ströbel[2], Samy Hakroush[2†] and Björn Tampe[1*†]*

[1] Department of Nephrology and Rheumatology, University Medical Center Göttingen, Göttingen, Germany, [2] Institute of Pathology, University Medical Center Göttingen, Göttingen, Germany

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***Correspondence:**
Samy Hakroush
samy.hakroush@med.uni-goettingen.de
Björn Tampe
bjoern.tampe@med.uni-goettingen.de

†These authors have contributed equally to this work and share senior authorship

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Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis affecting multiple organ systems, including the kidney. Besides investigations focusing on renal outcomes, sex differences associated with distinct clinical and histopathological findings in ANCA glomerulonephritis (GN) have not been systematically investigated. Therefore, we here aimed to systematically analyze sex differences in patients with AAV and biopsy-proven ANCA GN. We provide a comprehensive analysis of 53 kidney biopsies with ANCA GN retrospectively included between 2015 and 2020 and identified specific sex differences in ANCA GN concerning laboratory parameters and systematic scoring of renal histopathology glomerular and tubulointerstitial lesions, and extrarenal manifestations of AAV. We did not observe any correlation between sex and short-term clinical AAV course or disease severity by comparing general AAV parameters. AAV manifestations in females occurred at an older age with more joint involvement. Regarding histopathological findings, we, again, observed no sex difference among ANCA GN classification, but a significant correlation between females and distinct histopathological findings with less tubulointerstitial inflammation and vasculitis of peritubular capillaries. Finally, we here identified fewer associations between clusters of clinical, laboratory parameters, and histopathological findings in females as compared to males. These findings are of great relevance and further improve our understanding of sex differences in the pathogenesis of ANCA GN. While future studies about specific sex differences and conclusions in these clusters are crucial, our observations further support that sex differences are relevant, affect distinct parameters, and influence clinical, laboratory parameters, and histopathological findings in AAV, particularly ANCA GN.

Keywords: sex differences, autoimmune disease, systemic vasculitis, ANCA-associated vasculitis, ANCA glomerulonephritis

INTRODUCTION

According to the 2012 revised Chapel Hill Consensus Conference Nomenclature of Vasculitides, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis, most frequently presenting as microscopic polyangiitis (MPA) or granulomatosis with polyangiitis (GPA) (1, 2). Acute kidney injury (AKI) due to necrotizing and crescentic ANCA glomerulonephritis (GN) is a common and severe complication of AAV as it can cause progressive chronic kidney disease (CKD), end-stage kidney disease (ESKD), or death (3, 4). Several studies have investigated determinants of renal outcomes in ANCA GN, including baseline kidney function and histopathological lesions (5, 6). Proteinase 3 (PR3) and myeloperoxidase (MPO) are two major autoantigens in patients with AAV. The genes encoding these autoantigens are abnormally expressed in peripheral neutrophils of patients with active AAV (7). Mechanistically, neutrophils are activated by pathogenic ANCAs causing the release of inflammatory cytokines, reactive oxygen species and lytic enzymes, resulting in excessive formation of neutrophil extracellular traps (NETs) (8–10). Pathogenic ANCAs, in particular proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA), trigger a deleterious immune response resulting in a pauci-immune necrotizing and crescentic GN, a common manifestation of glomerular injury in AAV (11). Unlike many other autoimmune diseases, AAV has a slight predominance and higher prevalence of PR3-ANCA compared to MPO-ANCA in males (12–16). With regard to outcomes, males show a higher risk of progression to ESKD, especially in crescentic class ANCA GN (16). However, recent evidence suggests that PR3-ANCA is more prevalent than MPO-ANCA in males without any outcome differences regarding sex, potentially attributed to the known latitudinal gradient of ANCA specificity (17, 18). Besides investigations focusing on renal outcomes, sex differences in association with distinct clinical and histopathological findings in ANCA GN have not been systematically investigated (18). Therefore, we systematically analyzed sex differences in patients with biopsy-proven ANCA GN, emphasizing laboratory parameters, systematic scoring of renal histopathology including glomerular and tubulointerstitial lesions, and extrarenal manifestations of AAV.

METHODS

Study Population

A total of 53 kidney biopsies with ANCA GN at the University Medical Center Göttingen were retrospectively included between 2015 and 2020, the patient cohort was previously described (19–25). While no formal approval was required to use routine clinical data, a favorable ethical opinion was granted by the local Ethics committee (protocol no. 22/2/14 and 28/09/17). The Birmingham Vasculitis Activity Score (BVAS) version 3 was assessed (26). Medical records were used to obtain data on age, sex, duration of disease onset before admission, diagnosis (MPA or GPA), and laboratory results including the predominant serological ANCA autoantigens (all patients were positive for MPO-ANCA or PR3-ANCA). The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (27). The simplified acute physiology score (SAPS) II was calculated

TABLE 1 | Clinical and laboratory parameters of the total ANCA GN cohort.

Clinical data	
Females – no. (%)	23 (43.4)
Median age (IQR) – years	65 (54.5-74.5)
Disease onset – median days before admission (IQR)	18 (7–46)
Kidney biopsy – median days after admission (IQR)	6 (3-9.5)
MPA/GPA subtype – no./no. (%/%)	26/27 (49.1/50.9)
History of vasculitis – no. (%)	8 (15.1)
Median SAPS II (IQR) – points	24 (19–32)
ICU supportive care – no. (%)	24 (45.3)
RRT within 30 days after admission – no. (%)	16 (30.2)
AAV manifestations	
Median BVAS (IQR) – points	18 (15-20.5)
Extrarenal manifestation – no. (%)	44 (83)
Lung involvement – no. (%)	31 (58.5)
Sinus involvement – no. (%)	9 (17)
Joint involvement – no. (%)	12 (22.6)
Ear involvement – no. (%)	4 (7.5)
Eye involvement – no. (%)	3 (5.7)
Nerve involvement – no. (%)	6 (11.3)
Skin involvement – no. (%)	9 (17)
Pulmonary hemorrhage – no. (%)	7 (13.2)
Laboratory data	
MPO-ANCA/PR3-ANCA – no./no. (%/%)	26/27 (49.1/50.9)
Median serum creatinine (IQR) – mg/dL	3.04 (1.315-4.94)
Median eGFR (IQR) – mL/min/1.73 m^2	19 (9.7-50.2)
Median CRP (IQR) – mg/L	57.4 (19.1-106.7)
Median C3c (IQR) – g/L	1.295 (0.9925-1.413)
Median C4 (IQR) – g/L	0.26 (0.195-0.3025)
Urinary data	
Median uPCR (IQR) – mg/g	904.3 (505.2-1653)
Median uACR (IQR) – mg/g	445.2 (164.2-854.6)
Median α_1-microglobulin (IQR) – mg/g	69.63 (34.8-172.5)
Median α_2-macroglobulin (IQR) – mg/g	5.055 (2.924-11.13)
Median IgG (IQR) – mg/g	44.05 (20.5-190.8)
Hemoglobinuria – no. (%)	52 (98.1)
Acanthocytes – no. (%)	8 (15.1)
Comorbidities	
Arterial hypertension – no. (%)	24 (45.3)
Diabetes mellitus – no. (%)	8 (15.1)
Malignancies – no. (%)	1 (1.9)
Histopathological subgrouping	
Crescentic class – no. (%)	17 (43.3)
Focal class – no. (%)	26 (49.1)
Sclerotic class – no. (%)	3 (5.7)
Mixed class – no. (%)	7 (13.2)
ARRS	
High risk – no. (%)	8 (15.1)
Medium risk – no. (%)	23 (43.4)
Low risk – no. (%)	22 (41.5)

ANCA, anti-neutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; C3c, complement factor 3 conversion product; C4, complement factor 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate (CKD-EPI); GN, glomerulonephritis; GPA, granulomatosis with polyangiitis; ICU, intensive care unit; IQR, interquartile range; MPA, microscopic polyangiitis; MPO, myeloperoxidase; no., number; PR3, proteinase 3; RRT, renal replacement therapy; SAPS II, simplified acute physiology score II; uPCR, urinary protein-to-creatinine ratio; uACR, urinary albumin-to-creatinine ratio.

according to published guidelines (28). The requirement of intensive care unit (ICU) supportive care was defined at the time of admission; all patients required critical care treatment for more than 24 hours. Renal replacement therapy (RRT) was performed intermittently in all cases. Indications for RRT included severe electrolyte and acid-base abnormalities, volume overload, or encephalopathy. Comorbidities were evaluated according to the medical records, none of the patients had type 1 diabetes mellitus or documented information about a family history of diabetes mellitus.

Renal Histopathology

Two renal pathologists (SH and PS) independently evaluated kidney biopsies and were blinded to data analysis. Each kidney biospsy was routinely stained for periodic acid Schiff, Masson's trichrome, silver stain, IgA, IgG and IgM to confirm pauci-immune ANCA GN, and the extent of interstitial fibrosis/tubular atrophy (IFTA) was also assessed. Furthermore, each glomerulus was scored for the presence of necrosis, crescents, and global sclerosis. Based on these scores, histopathological subgrouping according to Berden et al. into focal, crescentic, mixed, or sclerotic classes was performed (5). The ANCA renal risk score (ARRS), according to Brix et al. into low, medium, or high risk, was calculated (6). Kidney biopsies were also evaluated analogously to the Banff scoring system for allograft pathology as described previously (29). In brief, Banff score lesions included interstitial inflammation (*i*), tubulitis (*t*), arteritis (*v*), glomerulitis (*g*), interstitial fibrosis (*ci*), tubular atrophy (*ct*), arteriolar hyalinosis (*ah*), peritubular capillaritis (*ptc*), total inflammation (*ti*), inflammation in areas of IFTA (*i-IFTA*) and tubulitis in areas of IFTA (*t-IFTA*) (29). Systematic histological scoring of acute tubular injury (ATI) lesions was evaluated as previously described (30, 31). In brief, epithelial simplification and tubular dilatation, nonisometric cell vacuolization, cellular, red blood cell (RBC), and hyaline casts were given a score between 0 and 4 as a percentage of the total affected cortical area of the biopsy (score 0: <1%, 1: ≥1-10%, 2: ≥10-25%, 3: ≥25-50%, 4: >50%). In addition, infiltrates of neutrophils, eosinophils, plasma cells, and mononucleated cells (macrophages and T lymphocytes) were quantified as a fraction of the total area.

Plasma Exchange and Remission Induction Therapy

Glucocorticoids (GCs) were administered either as intravenous pulse therapy or orally with a tapering schedule. At the time of kidney biopsy, all patients received GCs and further remission induction therapy was initiated thereafter based on histopathological confirmation of ANCA GN. Plasma exchange (PEX) was administered during the induction period at the discretion of treating physicians. Rituximab (RTX) was administered in four intravenous doses at 375 mg/m^2 every week; RTX was not administered within 48 hours before PEX treatment.

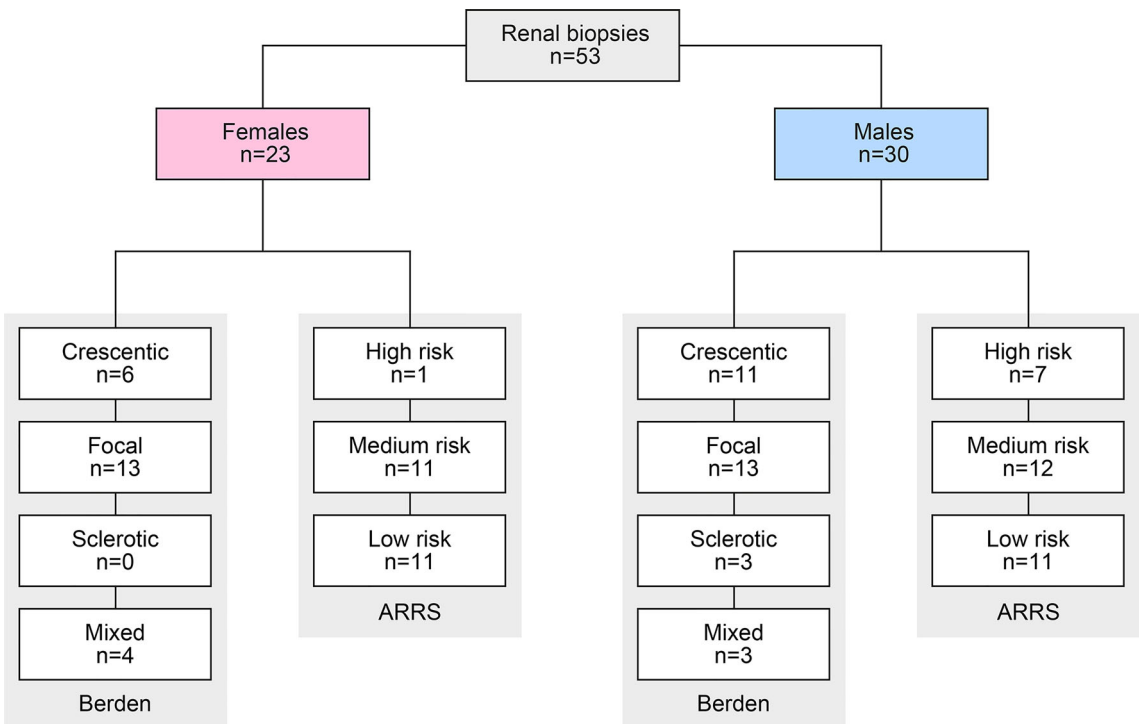

FIGURE 1 | Total patient cohort of ANCA GN. STROBE flow chart of the patient disposition. ANCA, anti-neutrophil cytoplasmic antibodies; ARRS, ANCA renal risk score; GN, glomerulonephritis; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology.

Cyclophosphamide (CYC) was administered in three intravenous doses up to 15 mg/kg every two weeks and every three weeks after that, adjusted for age and renal function. Combination therapy was administered in four intravenous doses at 375 mg/m^2 RTX every week and two intravenous doses at 15 mg/kg CYC every two weeks. At the discretion of treating physicians, remission induction therapy depended on previous regimens and individual patient factors. RTX was preferred in younger patients, with toxicity being the main reason for this choice (32). Prophylaxis to prevent *Pneumocystis jiroveci* infection was given according to local practice.

Statistical Methods

Variables were tested for normal distribution using the Shapiro-Wilk test. Statistical comparisons were not formally powered or prespecified. Non-normally distributed continuous variables are shown as the median and interquartile range (IQR), categorical variables are presented as frequency and percentage. For group comparisons, the Mann-Whitney U-test was used to determine differences in medians. Non-parametric between-group comparisons were performed with Pearson's Chi-square test. Spearman's correlation was performed to assess the correlation between clinical, laboratory, and histopathological parameters, and heatmaps reflecting the mean values of Spearman's ρ are shown, the asterisks indicating significant correlations. Data analyses were performed with GraphPad Prism (version 8.4.3 for macOS, GraphPad Software, San Diego, California, USA). Multiple regression analyses were performed using IBM SPSS Statistics (version 27 for MacOS, IBM Corporation, Armonk,

TABLE 2 | Sex differences among clinical and laboratory parameters at disease onset in ANCA GN.

	Females	Males	P-value
Clinical data			
Median age (IQR) – years	69 (63–76)	57 (49.75-74)	***0.0291***
Disease onset – days before admission (IQR)	13 (7-24.5)	29 (13–60)	*0.0726*
Kidney biopsy – days after admission (IQR)	7 (5–9)	5 (3-10.25)	*0.3258*
MPA/GPA subtype – no./no. (%/%)	8/15 (34.8/65.2)	18/12 (60/40)	*0.0687*
History of vasculitis – no. (%)	5 (21.7)	3 (10)	*0.2367*
Median SAPS II (IQR) – points	27 (21–32)	24 (19-31.25)	*0.2693*
ICU supportive care – no. (%)	9 (39.1)	15 (50)	*0.4308*
RRT within 30 days after admission – no. (%)	5 (21.7)	11 (36.7)	*0.2407*
AAV manifestations			
Median BVAS (IQR) – points	18 (15–21)	18 (15-20.25)	*0.9679*
Extrarenal manifestation – no. (%)	21 (91.3)	23 (76.7)	*0.1595*
Lung involvement – no. (%)	14 (60.9)	17 (56.7)	*0.7583*
Sinus involvement – no. (%)	5 (21.7)	4 (13.3)	*0.4192*
Joint involvement – no. (%)	9 (39.1)	3 (10)	***0.0120***
Ear involvement – no. (%)	3 (13)	1 (3.3)	*0.1847*
Eye involvement – no. (%)	1 (4.4)	2 (6.7)	*0.7173*
Nerve involvement – no. (%)	5 (21.7)	1 (3.3)	***0.0361***
Skin involvement – no. (%)	3 (13)	6 (20)	*0.5038*
Pulmonary hemorrhage – no. (%)	3 (13)	4 (13.3)	*0.9754*
Laboratory data			
MPO-ANCA/PR3-ANCA – no./no. (%/%)	9/14 (39.1/60.9)	17/13 (56.7/43.3)	*0.2056*
Median serum creatinine (IQR) – mg/dL	1.97 (1.29-3.94)	3.64 (1.318-5.44)	*0.2261*
Median eGFR (IQR) – mL/min/1.73 m^2	25.3 (12.5-44.2)	16.35 (9.35-54.3)	*0.9539*
Median CRP (IQR) – mg/L	47.1 (20.5-109.3)	63.7 (16-108.1)	*0.9752*
Median C3c (IQR) – g/L	1.3 (1.093-1.418)	1.27 (0.895-1.413)	*0.7313*
Median C4 (IQR) – g/L	0.245 (0.163-0.293)	0.29 (0.195-0.35)	*0.2801*
Urinary data			
Median uPCR (IQR) – mg/g	819.6 (556.9-1492)	1339 (495.3-3020)	*0.2143*
Median uACR (IQR) – mg/g	445.2 (99.-697.2)	486.3 (211.2-1763)	*0.1888*
Median α_1-microglobulin (IQR) – mg/g	69.63 (28.4-150.3)	78.45 (34.95-185.8)	*0.8137*
Median α_2-macroglobulin (IQR) – mg/g	4.935 (2.95-10.12)	5.139 (2.84-13.01)	*0.8719*
Median IgG (IQR) – mg/g	32.98 (19.2-81.2)	102.2 (20.1-248.4)	*0.0976*
Hemoglobinuria – no. (%)	22 (95.7)	30 (100)	*0.2489*
Acanthocytes – no. (%)	3 (13)	5 (16.7)	*0.7150*
Comorbidities			
Arterial hypertension – no. (%)	11 (47.8)	13 (43.3)	*0.7447*
Diabetes mellitus – no. (%)	3 (13)	5 (16.7)	*0.7150*
Malignancies – no. (%)	1 (4.3)	0 (0)	*0.2489*

For group comparisons, the Mann-Whitney U-test was used to determine differences in medians. In addition, non-parametric between-group comparisons were performed with Pearson's Chi-square test. Bold indicates statistically significant values at group level.

ANCA, anti-neutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; C3c, complement factor 3 conversion product; C4, complement factor 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate (CKD-EPI); GN, glomerulonephritis; GPA, granulomatosis with polyangiitis; ICU, intensive care unit; IQR, interquartile range; MPA, microscopic polyangiitis; MPO, myeloperoxidase; no., number; PR3, proteinase 3; RRT, renal replacement therapy; SAPS II, simplified acute physiology score II; uPCR, urinary protein-to-creatinine ratio; uACR, urinary albumin-to-creatinine ratio.

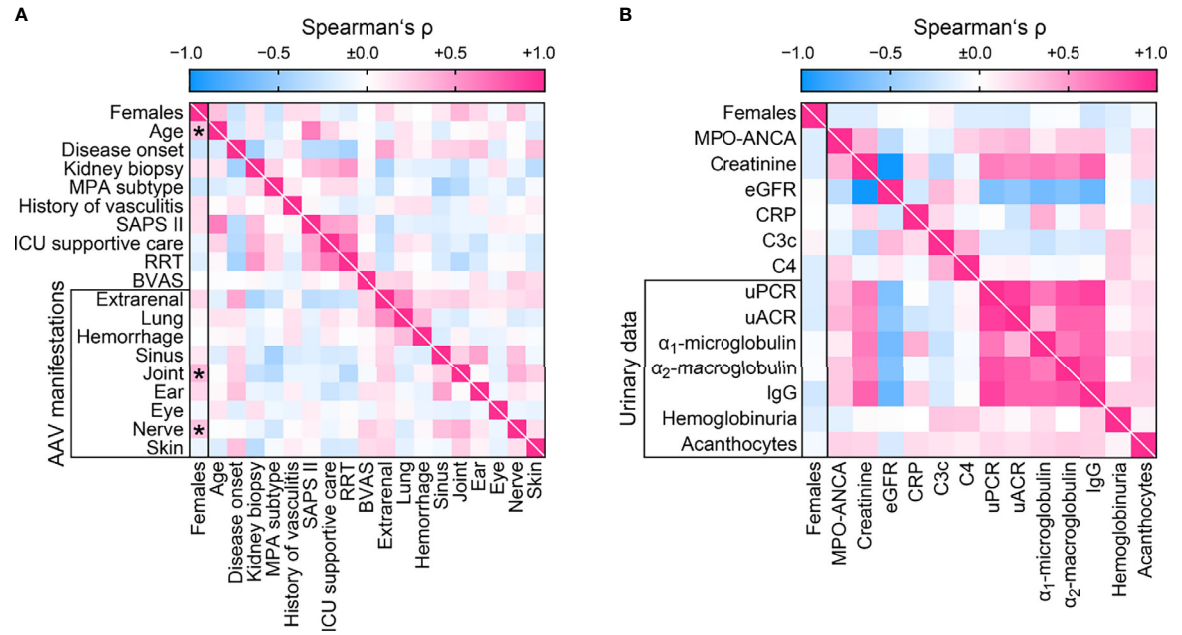

FIGURE 2 | Sex differences among clinical characteristics and laboratory parameters at disease manifestation in AAV. **(A)** Sex in association with clinical findings are shown by heatmap reflecting mean values of Spearman's ρ, asterisks indicate $p < 0.05$. **(B)** Sex in association with laboratory parameters are shown by heatmap reflecting mean values of Spearman's ρ. AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; C3c, complement factor 3 conversion product; C4, complement factor 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; IgG, immunoglobulin G; MPA, microscopic polyangiitis; MPO, myeloperoxidase; RRT, renal replacement therapy; uACR, urinary albumin-to-creatinine ratio; uPCR, urinary protein-to-creatinine ratio.

New York, USA). We retained covariates significantly associated with complement component measurements in a multivariable regression model, limiting the model covariates to avoid model over-fit. A probability (p) value of <0.05 was considered statistically significant.

RESULTS

Description of Demographic and Clinical Characteristics

A total of 53 renal biopsies with ANCA GN was included. The baseline characteristics of the cohort are shown in **Table 1**. In this cohort, 23/53 (43.4%) were females, the median (IQR) age at diagnosis was 65 (54.5-74.5) years, and all patients were Caucasian. The median (IQR) disease onset before admission was 18 (7–46) days, and kidney biopsy was performed within 6 (3-9.5) days after admission to confirm renal involvement of AAV. Based on clinical characteristics, 26/53 (49.1%) patients were diagnosed as MPA, and the remainder as GPA. A total number of 8/53 (15.1%) patients had a history of vasculitis. The median (IQR) BVAS was 18 (15-20.5). The median (IQR) SAPS II at admission was 24 (19–32), and 24/53 (45.3%) of patients required ICU supportive care. There were 44/53 patients (83%) with some extrarenal manifestation of AAV (31 with lung, 9 with sinus, 12 with joint, 4 with ear, 3 with eye, 6 with peripheral nerve, and 9 with skin involvement), and 7/53 (13.2%) had alveolar hemorrhage. Based on laboratory findings, 26/53 (49.1%) positive for MPO-ANCA and 27/53 (50.1%) positive for PR3-ANCA. The worst median (IQR) eGFR at disease onset was 19 (9.7-50.2) mL/min/1.73 m^2, and 16/53 (30.2%) required RRT within 30 days after admission. Histopathological subgrouping revealed 17/53 (43.3%) crescentic, 25/53 (49.1%) focal, 3/53 (5.7%) sclerotic, and 7/53 (13.2%) mixed class ANCA GN (5). ARRS was high in 8/53 (15.1%), intermediate in 23/53 (43.4%), and low-risk class ANCA GN in 22/53 (41.5%) of cases (**Figure 1**) (6).

Sex Differences Among Clinical Characteristics and Laboratory Parameters at Disease Manifestation in AAV

We first analyzed sex differences among clinical characteristics and laboratory parameters in AAV. We did not observe any correlation between sex and ANCA subtype, short-term clinical

TABLE 3 | Multiple regression analyses of parameters attributed to females.

	ß	SE	P-value
Parameter			
Age – years	0.2849	0.0043	***0.0276***
Joint involvement	0.2751	0.1603	***0.0475***
Nerve involvement	0.1778	0.2117	*0.1951*

Bold indicates statistically significant values.
SE, standard error.

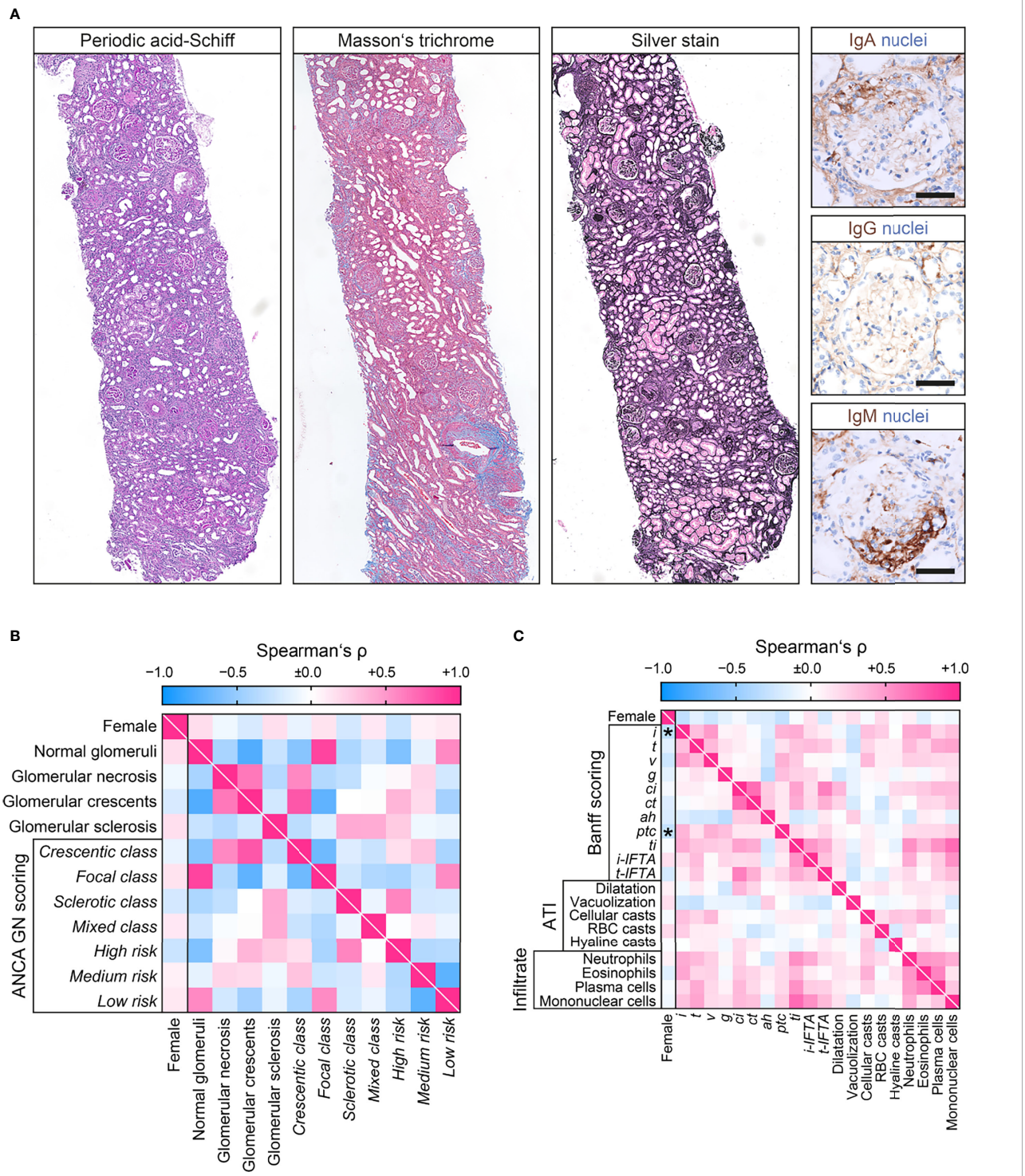

FIGURE 3 | Sex differences among histopathological findings at disease manifestation in ANCA GN. **(A)** Representative photomicrographs of pauci-immune ANCA GN showing crescent formations with IgM entrapment within cellular proliferations and injured glomerular capilaries. Note, lack of granular or linear staining of IGA and IgA along the GBM typical for pauci-immune GN (scale bars: 50 µm). **(B)** Sex in association with glomerular lesions and ANCA GN scoring are shown by heatmap reflecting mean values of Spearman's ρ. **(C)** Sex in association with tubulointerstitial lesions analogous to the Banff scoring system, ATI lesions, and distinct inflammatory infiltrates are shown by heatmap reflecting mean values of Spearman's ρ, asterisks indicate p<0.05. ah, arteriolar hyalinosis; ANCA, anti-neutrophil cytoplasmic antibodies; ATI, acute tubular injury; ci, interstitial fibrosis; ct, tubular atrophy; g, glomerulitis; GN, glomerulonephritis; i, interstitial inflammation; i-IFTA, inflammation in IFTA; RBC, red blood cell; t, tubulitis; ptc, peritubular capillaritis; ti, total inflammation; t-IFTA, tubulitis in IFTA; v, intimal arteritis.

AAV course (disease onset, admission or time of kidney biopsy), or severity (SAPS II, need for ICU supportive care or RRT within 30 days after admission). Interestingly, females were significantly older at time of biopsy despite disease onset before admission was equally distributed (**Table 2** and **Figure 2A**), implicating that AAV manifestation in females occured at an older age. While systemic disease activity assessed by BVAS did not differ, females had significantly more joint and peripheral nerve involvement among extrarenal AAV manifestations (**Table 2** and **Figure 2A**). Multiple regression analyses confirmed that identified parameters age and joint involvement were independently attributed to females (**Table 3**). In contrast, we did not observe any sex-specific associations among systemic and urinary laboratory parameters including predominant ANCA autoantibodies (**Table 2** and **Figure 2B**). In summary, we observed no sex difference among general AAV parameters, but AAV manifestations in females occurred at an older age with more involvement of joints.

Sex Differences Among Histopathological Findings at Disease Onset and Choice of Remission Induction Therapy in ANCA GN

We next analyzed sex differences among histopathological findings in pauci-immune ANCA GN (**Figure 3A**). The number of normal glomeruli, glomerular necrosis, crescents, or sclerosis did not differ with regard to sex, also reflected by ANCA GN scoring (**Table 4** and **Figure 3B**) (5, 6). Interestingly, females had significantly less interstitial inflammation (*i*) and peritubular capillaritis (*ptc*) among tubulointerstitial lesions according to the Banff scoring system (**Figure 3C**) (29). In contrast, we did not observe an association between sex and ATI lesions or inflammatory infiltrates (**Table 4** and **Figure 3C**) (30, 31). Furthermore, choice of PEX and remission induction therapy did not differ with regard to sex (**Table 5**). In summary, we observed no sex differences among general ANCA GN scores or choice of remission induction therapy. Interestingly, there was a significant correlation with distinct histopathological findings including fewer interstitial inflammation and vasculitis manifestation in peritubular capillaries in females.

TABLE 5 | Sex differences in choice of PEX and remission induction therapy in ANCA GN.

	Females	Males	P-value
PEX therapy			
PEX – no. (%)	6 (26.1)	14 (46.7)	*0.1255*
Median sessions of PEX (IQR) – no.	5 (4.75-7.25)	5 (5–5)	*0.5236*
Remission induction therapy			
RTX – no. (%)	7 (30.4)	11 (36.7)	*0.3668*
CYC – no. (%)	13 (56.5)	12 (40)	
RTX/CYC – no. (%)	2 (8.7)	6 (20)	

For group comparisons, the Mann-Whitney U-test was used to determine differences in medians. In addition, non-parametric between-group comparisons were performed with Pearson's Chi-square test.

ANCA, anti-neutrophil cytoplasmic antibodies; CYC, cyclophosphamide; GCs, glucocorticoids; IQR, interquartile range; no., number; PEX, plasma exchange; RTX, rituximab.

Sex-Specific Cluster Analysis for the Association Between Clinical, Laboratory Parameters and Histopathological Findings at Disease Manifestation in ANCA GN

Finally, we aimed to identify sex-specific associations between clinical, laboratory, and histopathological parameters in ANCA GN by a separate analysis of females and males. Overall, we identified a significant association between 208/3844 (5.4%) parameters included in females (**Figure 4**) compared to 302/3844 (7.9%) parameters in males (**Figure 5**). The decreased association in females was attributed to less correlation between clusters of clinical, laboratory parameters, glomerular lesions, and ANCA GN scoring *versus* all other included parameters (**Table 6**). Notably, there was a less robust association of the cluster between serological and clinical parameters and scoring of glomerular lesions in ANCA GN (**Figures 4**, **5**). In addition, there was a low correlation between

TABLE 4 | Sex differences among histopathological findings at disease onset in ANCA GN.

	Females	Males	P-value
Glomerular lesions			
Median total glomeruli (IQR) – no.	19 (11–33)	14.5 (10.75-27)	*0.1721*
Median normal glomeruli (IQR) – %	54.55 (35.29-75.76)	43.43 (11.6-72.88)	*0.2365*
Median glomerular necrosis (IQR) – %	12.5 (0-42.86)	16.67 (0-51.39)	*0.5919*
Median glomerular crescents (IQR) – %	27.27 (8.33-43.75)	41.8 (11.88-63.4)	*0.1581*
Median glomerular sclerosis (IQR) – %	12.5 (0–30)	1.85 (0-19.41)	*0.2704*
Histopathological subgrouping			
Crescentic class – no. (%)	6 (26.1)	11 (36.7)	*0.2892*
Focal class – no. (%)	13 (56.5)	13 (43.3)	
Sclerotic class – no. (%)	0 (0)	3 (10)	
Mixed class – no. (%)	4 (17.4)	3 (10)	
ARRS			
High risk – no. (%)	1 (4.3)	7 (23.3)	*0.1586*
Medium risk – no. (%)	11 (47.8)	12 (40)	
Low risk – no. (%)	11 (47.8)	11 (36.7)	

For group comparisons, the Mann-Whitney U-test was used to determine differences in medians. In addition, non-parametric between-group comparisons were performed with Pearson's Chi-square test.

ANCA, anti-neutrophil cytoplasmic antibodies; ARRS, ANCA renal risk score; GN, glomerulonephritis; IQR, interquartile range; no., number.

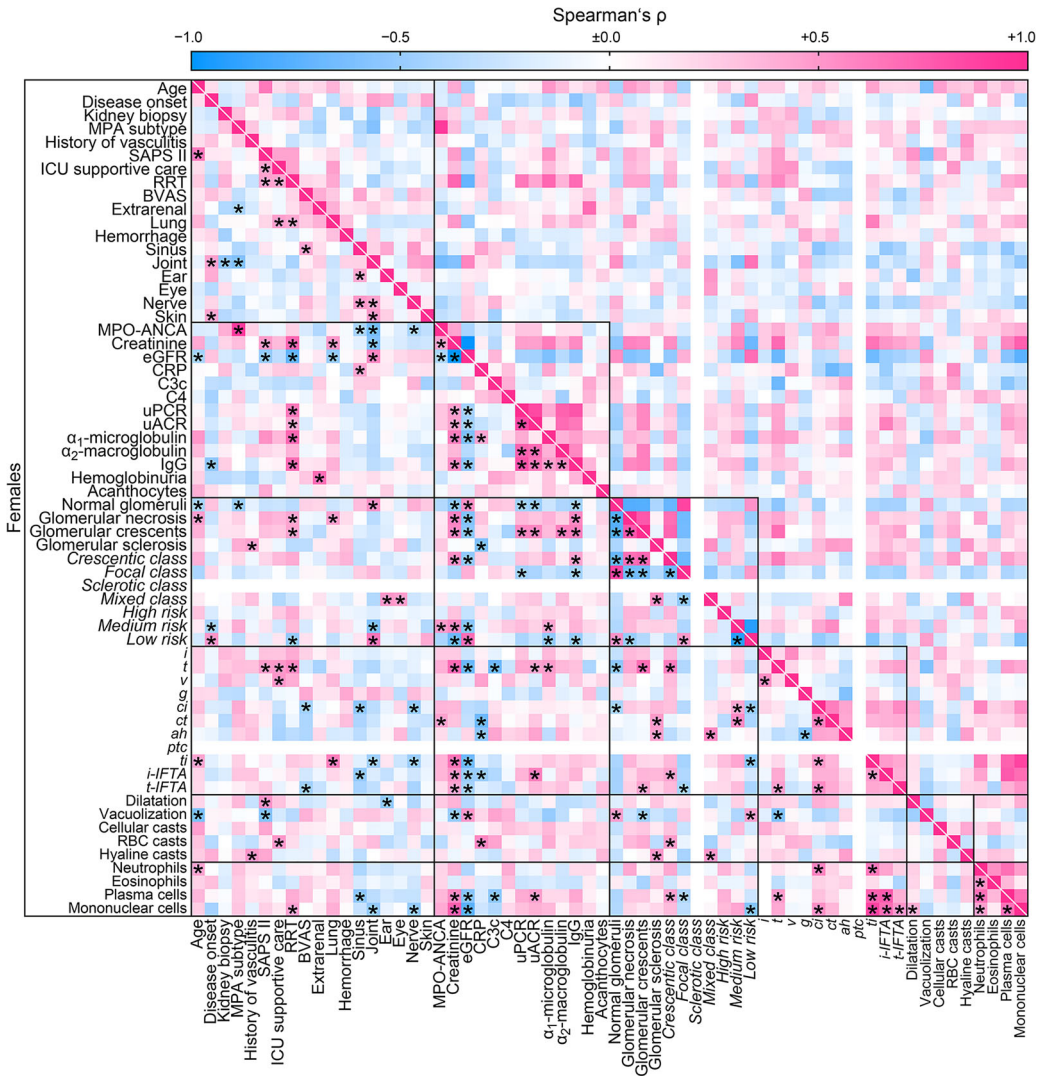

FIGURE 4 | Female-specific cluster analysis for the association between clinical, laboratory parameters, and histopathological findings in ANCA GN. Associations of clinical, laboratory parameters, and histopathological clusters in ANCA GN specifically in females are shown by heatmap reflecting mean values of Spearman's ρ, asterisks indicate $p < 0.05$. Clusters are depicted by black boxes, the empty lines for sclerotic class ANCA GN and *ptc* within the heatmap reflect no data analysis because the respective parameters were absent in all cases. ah, arteriolar hyalinosis; ANCA, anti-neutrophil cytoplasmic antibodies; ATI, acute tubular injury; BVAS, Birmingham Vasculitis Activity Score; C3c, complement factor 3 conversion product; C4, complement factor 4; ci, interstitial fibrosis; CRP, C-reactive protein; ct, tubular atrophy; eGFR, estimated glomerular filtration rate; g, glomerulitis; GN, glomerulonephritis; i, interstitial inflammation; IgG, immunoglobulin G; i-IFTA, inflammation in IFTA; MPA, microscopic polyangiitis; MPO, myeloperoxidase; RBC, red blood cell; RRT, renal replacement therapy; t, tubulitis; ptc, peritubular capillaritis; ti, total inflammation; t-IFTA, tubulitis in IFTA; uACR, urinary albumin-to-creatinine ratio; uPCR, urinary protein-to-creatinine ratio; v, intimal arteritis.

the cluster of glomerular scoring and tubulointerstitial lesions in ANCA GN (**Figures 4**, **5**). Thus, we identified a lesser, female-specific association between clusters of clinical and serological parameters and histopathological findings in ANCA GN.

DISCUSSION

We here provide a comprehensive analysis and identified specific sex differences in ANCA GN concerning serologic parameters, systematic scoring of renal histopathology including glomerular and tubulointerstitial lesions, and extrarenal manifestations of AAV. Comparing general AAV parameters, we did not observe any correlation between sex and short-term clinical AAV course or severity. In our cohort, AAV manifestations in females occurred at an older age, as reported previously (33, 34). In addition, we observed more involvement of joints and peripheral nerves in females. Regarding histopathological findings, we, again, observed no sex differences among general ANCA GN scoring but a significant correlation with distinct histopathological findings including less tubulointerstitial inflammation in females.

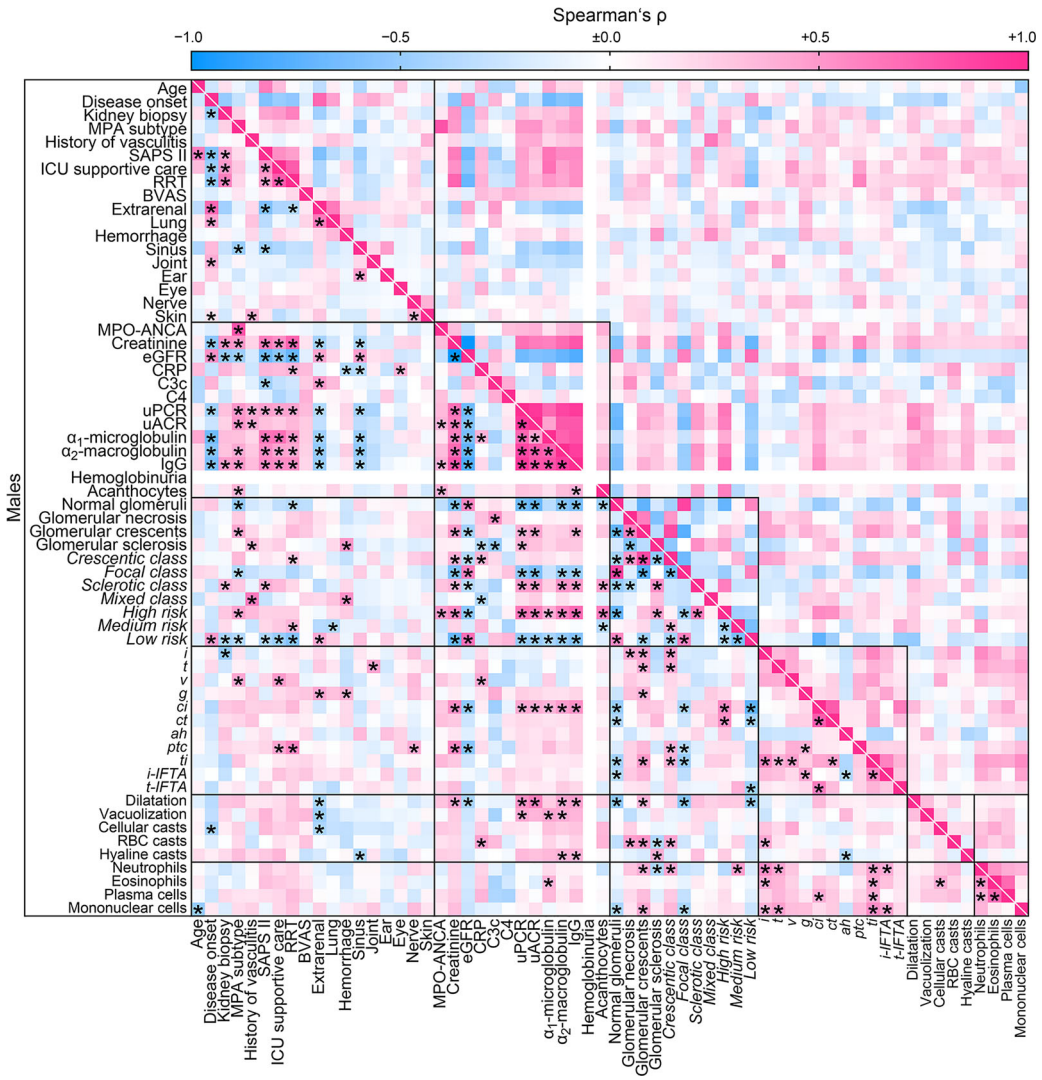

FIGURE 5 | Male-specific cluster analysis for the association between clinical, laboratory parameters, and histopathological findings in ANCA GN. Associations of clinical, laboratory parameters, and histopathological clusters in ANCA GN specifically in males are shown by heatmap reflecting mean values of Spearman's ρ, asterisks indicate $p < 0.05$. Clusters are depicted by black boxes, the empty line for hemoglobinuria within the heatmap reflects no data analysis because the respective parameter was present in all cases. *ah*, arteriolar hyalinosis; ANCA, anti-neutrophil cytoplasmic antibodies; ATI, acute tubular injury; BVAS, Birmingham Vasculitis Activity Score; C3c, complement factor 3 conversion product; C4, complement factor 4; *ci*, interstitial fibrosis; CRP, C-reactive protein; *ct*, tubular atrophy; eGFR, estimated glomerular filtration rate; *g*, glomerulitis; GN, glomerulonephritis; *i*, interstitial inflammation; IgG, immunoglobulin G; *i-IFTA*, inflammation in IFTA; MPA, microscopic polyangiitis; MPO, myeloperoxidase; RBC, red blood cell; *t*, tubulitis; *ptc*, peritubular capillaritis; RRT, renal replacement therapy; *ti*, total inflammation; *t-IFTA*, tubulitis in IFTA; uACR, urinary albumin-to-creatinine ratio; uPCR, urinary protein-to-creatinine ratio; *v*, intimal arteritis.

TABLE 6 | Sex differences among clusters of clinical, laboratory parameters, and histopathological findings in ANCA GN.

	Females	Males	P-value
Clinical parameters *versus* other – no. (%)	59 (7.4)	90 (11.4)	***0.0076***
Laboratory parameters *versus* other – no. (%)	73 (11.3)	127 (19.6)	***<0.0001***
Glomerular lesions and ANCA GN scoring *versus* other – no. (%)	66 (11.8)	108 (19.3)	***0.0005***
Tubulointerstitial lesions *versus* other – no. (%)	53 (7)	54 (7.1)	*0.9201*
ATI lesions *versus* other – no. (%)	17 (5.8)	29 (9.8)	*0.0654*
Inflammatory infiltrates *versus* other – no. (%)	24 (10.3)	22 (9.5)	*0.7560*

Non-parametric between-group comparisons were performed with Pearson's Chi-square test. Bold indicates statistically significant values at group level.
ANCA, anti-neutrophil cytoplasmic antibodies; ATI, acute tubular injury; GN, glomerulonephritis; no., number.

Based on previous observations, male patients with ANCA GN had a significantly higher risk of progression to ESKD than females in a Norwegian cohort of patients with ANCA GN (16). The most crucial sex difference has been reported in crescentic class ANCA GN, representing active glomerular lesions and supporting the concept that observed outcome differences are caused by sex-specific inflammatory differences and responses to immunosuppressive therapy. In contrast, no significant sex-specific difference in ANCA GN outcomes has been observed when combining ESKD and death as a composite outcome in Irish and British patients with ANCA GN (18). These observations might potentially be attributed to the known latitudinal gradient of ANCA specificity (17, 18). Our observation of sex-specific differences in tubulointerstitial inflammation is relevant since tubulointerstitial inflammation has previously been associated with active glomerular lesions (35). In addition, interstitial inflammation is more pronounced in MPO-ANCA than in PR3-ANCA GN, further supporting the hypothesis that interstitial lesions differ between ANCA GN subtypes (20, 35). In the current study, we did not observe sex-specific differences with regard to the ANCA subtype. Still, less interstitial inflammation in females was observed, further supporting that sex may affect AAV manifestations and outcomes. While only limited data are available, distinct inflammatory lesions have previously been shown to affect the long-term renal outcomes in ANCA GN (36).

In addition, we observed fewer vasculitis manifestations in peritubular capillaries in females with ANCA GN. The prevalence of interstitial vasculitis manifestations has been described in a considerable subset of patients with ANCA GN ranging from 10 to 35% (35, 37–41). Generally, histopathological subgrouping of ANCA GN into four classes (focal, crescentic, mixed, and sclerotic) as defined by Berden et al. in 2010 was proposed to predict long-term renal survival rates (5). However, unlike Berden's classification, Brix et al. in 2018 suggested the ANCA renal risk score (ARRS) by incorporation of baseline glomerular filtration rate (GFR) to the histopathological findings (percentage of normal glomeruli, tubular atrophy/interstitial fibrosis) to predict ESKD in patients with AAV (6). Recently, interstitial vasculitis has been shown to improve long-term outcome prediction in ANCA GN in both scoring systems (42). These observations underscore the pathogenic role of interstitial vasculitis in ANCA GN, and our findings of more minor vasculitis manifestations in peritubular capillaries in females further improve our understanding of sex differences in AAV.

Finally, we identified a less pronounced association between clusters of clinical and laboratory parameters and histopathological findings in ANCA GN in females. There was a less robust association of the cluster of serological with clinical parameters and scoring of glomerular lesions in ANCA GN in our cohort. In addition, there was a low correlation between the cluster of glomerular scoring and tubulointerstitial lesions in ANCA GN. While future studies regarding specific sex differences in these clusters are crucial, these observations further support that sex differences affect distinct parameters. Furthermore, they suggest an interplay between clinical, laboratory parameters, and histopathological findings in AAV, particularly in ANCA GN.

The main limitations of our study are its retrospective design, the small patient number, and no long-term follow-up data on renal outcomes. Furthermore, we here aimed to sex-specific data by clustering associative data with regard of clinical, laboratory parameters, and histopathological findings in ANCA GN, requiring further investigation with regard to specific parameters. Nevertheless, we here provide a comprehensive analysis and identified specific sex differences in ANCA GN regarding laboratory parameters, systematic scoring of renal histopathology including glomerular and tubulointerstitial lesions, and extrarenal manifestations of AAV.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the University Medical Center Göttingen, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BT conceived the study, collected and analyzed data, and wrote the first draft. SH and DT collected and analyzed data. SH and PS evaluated histopathological findings. PK analyzed data and edited the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

MINI REVIEW
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Gene-Specific Sex Effects on Susceptibility to Infectious Diseases

Marie Lipoldová[1]* *and Peter Demant*[2]

[1] Laboratory of Molecular and Cellular Immunology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czechia, [2] Department of Molecular and Cellular Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States

Inflammation is an integral part of defense against most infectious diseases. These pathogen-induced immune responses are in very many instances strongly influenced by host's sex. As a consequence, sexual dimorphisms were observed in susceptibility to many infectious diseases. They are pathogen dose-dependent, and their outcomes depend on pathogen and even on its species or subspecies. Sex may differentially affect pathology of various organs and its influence is modified by interaction of host's hormonal status and genotype: sex chromosomes X and Y, as well as autosomal genes. In this Mini Review we summarize the major influences of sex in human infections and subsequently focus on 22 autosomal genes/loci that modify in a sex-dependent way the response to infectious diseases in mouse models. These genes have been observed to influence susceptibility to viruses, bacteria, parasites, fungi and worms. Some sex-dependent genes/loci affect susceptibility only in females or only in males, affect both sexes, but have stronger effect in one sex; still other genes were shown to affect the disease in both sexes, but with opposite direction of effect in females and males. The understanding of mechanisms of sex-dependent differences in the course of infectious diseases may be relevant for their personalized management.

Keywords: sex-bias, sex-dependent gene, mouse model, susceptibility to infection, sex influence, viruses, bacteria, parasites

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Antonietta Rossi,
University of Naples Federico II, Italy

Reviewed by:
Joe G. Zein,
Cleveland Clinic, United States
Victor Ortega,
Wake Forest School of Medicine, United States

***Correspondence:**
Marie Lipoldová
lipoldova@img.cas.cz

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INTRODUCTION

Sex plays an important role in immune response, including susceptibility to infectious diseases (1), outcome of vaccination (2–6) and response to treatment (7). Sex differences in susceptibility to infectious and inflammatory diseases are widespread – both in terms of number of pathogens and diseases they influence and in terms of the number of vertebrate and invertebrate species and genera where they were observed. In humans they were demonstrated in a number of diseases discussed in detail below and hence they form a significant but hitherto unexplained component of clinical inter-patient heterogeneity. Their individual prediction and functional explanation may therefore significantly improve individual management of disease. A part of this phenomenon may be under genetic control, but there is presently little evidence for this in humans. However, there are extensive data from studies in mice that described 22 autosomal gene-loci controlling the sex differences in response to 12 infectious or inflammatory agents. We are presenting a comprehensive summary of this information, as it may help to proceed to clarification of the manifestations and mechanisms of sex differences in these pathologies.

Sexual dimorphism takes place already in healthy individuals. In most cases, basal immune responses are higher in females than in males. It has been described that women have higher several immunology-related parameters than males: blood levels of mature B cell subsets, IgM-only B cells, proliferating and memory (CD45RA-) Treg cells, NK bright (CD56++CD16-) subsets (8), immunoglobulin M (9), neutrophil and platelets (10), and higher CD4+/CD8+ ratio (11). TLR7 ligands induce higher IFNα production in woman peripheral blood lymphocytes (12). Neutrophils of men exhibit lower responses to cytokine stimulation and decreased ability to form neutrophil traps (13), whereas in women neutrophils were characterized by enhanced type I IFN pathway activity and enhanced proinflammatory responses (14). Male and female neutrophils differ also in bioenergetics. Metabolic assays of oxygen consumption rate (OCR), which is a key metric of mitochondrial function, and the extracellular acidification rate (ECAR), which approximates glycolytic activity in male and female neutrophils shown that OCR was higher in male than female neutrophils, whereas there were no differences in ECAR (14). As the immune cells differentiation and function crucially depend on mitochondrial bioenergetics (15, 16), sex differences in mitochondrial functions have a potential to modulate immune responses. Differences in immune responses have been observed also between males and females of other mammals (17–19), birds (20), reptiles (21), echinoderms (22) and insects (23). Such baseline differences can contribute to sex biases in response to pathogens (24,25 and see the next section) and to vaccination (2–6). Females usually have more efficient response to vaccination than males (2–5), but also develop more often adverse reactions to vaccination (4, 5). On the other hand, vaccination of healthy volunteers by Bacille Calmette-Guérin (BCG) led to enhanced cytokine responses to restimulation and reduced systemic inflammation. The effect was much stronger in men than in women (6).

SEX BIASES IN HUMAN INFECTIONS

Viruses

Male sex was associated with higher death rate in hepatitis A virus-hospitalized cases (24). Male sex was also a risk factor for hepatitis B virus (HBV) and hepatitis C Virus (HCV) prevalence and for development of hepatocellular carcinoma subsequent to HBV and/or HCV infection (25). Presence of virus stimulates inflammatory responses and appearance of reactive oxygen (ROS) and nitrogen species, which are described as leading cause of series of alterations that led to DNA damage (25). Estrogen may serve an inhibitory role in these processes by inhibiting inflammation, tumor progression and invasion and stimulating DNA repair (26), whereas androgen induced miR-216a stimulated tumorigenesis (27). The lower survival rate was observed among male patients infected with Ebola virus (28). The higher COVID-19 case mortality rate and increased severity of disease was described in males (29–31). Interestingly, gene encoding ACE2 (angiotensin-converting enzyme 2), which plays an essential role in cell entry of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is localized on X chromosome (Xp22.2), thus females have double gene dose and can be potentially heterozygous compared to males who are definitely hemizygous. It has been speculated that together with X mosaicism it might favor women in counteracting the progression of the SARS-CoV-2 infection (32, 33). Prevalence of herpes simplex virus type 1 (HSV1) and type 2 (HSV2) in persons aged 14-49 in United States in the years 2015-2016 was higher in women than in men (34). Similarly, HSV1 in Europe was more often detected in women than in men (35). Sex differences in measles mortality were compared among 78 countries in years between 1950 and 1989. Regional variations showed excess female mortality of 3% in Europe, 6.2% in North America, 5.9% in Far East Asia, 4.3% in Latin America, and 20.9% in the Middle East. The cumulative excess female mortality in comparison with males was small at age 0-4 (+4.2%), larger at age 5-14 (+10.9%), and peaks at ages 15-44 (+42.6%) (36). The most probable explanation of these variations is the influence of estrogens (36). Several studies have shown that women are more susceptible to human immunodeficiency virus 1 (HIV-1) acquisition than men, as the male-to-female transmission is more efficient than female-to-male transmission (37, 38). Indeed, in Sub-Saharan Africa higher HIV prevalence is observed in women (39). However, in Europe, there are more newly detected HIV infections in males than in woman, because sex between men remains the predominant mode of HIV transmission reported in the EU (European Union)/EEA (European Economic Area) (40). Thus, socioeconomic factors most likely contribute to the sex biases in HIV/AIDS (Acquired Immune Deficiency Syndrome).

Bacteria

Sex differences have also been reported in bacterial infections. Analysis of 4742 randomly selected subjects, aged 12-64, from Northern Ireland shown that *Helicobacter pylori* infection was more common in males than in females (41). A retrospective seroepidemiologic survey of *Chlamydia pneumoniae* infection in patients in Beijing, China between 2008 and 2017 revealed that adult men had both a higher prevalence and higher levels of antibodies than women (42). *Klebsiella* spp. induced bacteremia was higher in males than in females in England, Wales, and Northern Ireland (43). 60% of patients hospitalized in the years 2005-2014 in USA with Lyme disease (infectious agent *Borrelia burgdorferi*) were men (44). On the other hand, reinfection with *B. burgdorferi* in individuals from Sweden that were initially diagnosed with erythema migrans and treated with antibiotics was much higher in women than in men (45). Incidence of tuberculosis that is caused by infection with *Mycobacterium tuberculosis* was described to be higher in men than in women (46), however a consistent female excess for tuberculosis at age 5-29 was observed (47). *Listeria monocytogenes* is a foodborne pathogen that is highly prevalent in pregnant woman, older adults and immunocompromised individuals. Incidence of listeriosis in the years 2008-2016 in USA was higher in males than in non-pregnant females (48). Socioeconomic factors highly influence the spread of syphilis: a sexually transmitted infection caused by *Treponema pallidum*. Sex differences in its incidence, prevalence and geographical variations have been well described. For example, the incidence of maternal syphilis is higher in low- to middle-income countries as compared to high-income countries where syphilis is more common among

men who have sex with men. In Africa the spread of syphilis is also high in female sex workers (49, 50).

Parasites

Male sex is a risk factor for visceral leishmaniasis (51). Men were more susceptible to visceral infection caused by *Leishmania donovani* (52–54) and *L. infantum* (55–58). More variability was observed in studies of sex influence on cutaneous leishmaniasis. Some epidemiological studies revealed in male patients a higher incidence of cutaneous leishmaniasis caused by *L. major* and *L. tropica* (59, 60), *L. major* only (61), and also by *L. guyanensis* (62). However, the study in Afghanistan found that females developed more lesions and scars after *L. tropica* infection (63) and other analyses reported no significant sex differences in registered cases of cutaneous leishmaniasis caused by *L. tropica* (64) and *L. major* (65). No sex bias was observed in intestinal schistosomiasis caused by *Schistosoma mansoni* in adults (66). Infection rates did not differ significantly among various age and sex groups infected with *Schistosoma haematobium* (67).

Fungi

Prior to the AIDS epidemics, cryptococcal disease, caused by *Cryptococcus neoformans* and *Cryptococcus gattii* was rather rare. It was reported in case series 2-3 times more frequently in men as in women (68). In the AIDS era, in the years 2000-2007 were in USA hospitalized 10077 patients with cryptococcal disease, 26% were females. Males had a higher risk of a disease in both HIV-infected and uninfected cohorts. Age- and sex-adjusted death rates were almost threefold higher in males compared to females (69).

Worms

Females were found to be more predisposed to *Ascaris lumbricoides* infection than were males (70). Human neurocysticercosis results from the infection of the central nervous system with the larval stage of the intestinal tapeworm, *Taenia solium*. In Ecuador, the number of transitional cysts in brain was found to be higher in the female than in the male patients (71).

VARIOUS INFLUENCES ON SEX EFFECTS

Thus, differences in susceptibility and prevalence between males and females have been observed in many human infections. The extensive studies showed that some infectious diseases exhibit male (24, 25, 28–31, 41–43, 52–58, 68, 69), the other female bias (34–36, 70, 71), but there are also epidemiological studies with contradictory results; some studies showing male and the other female bias or no sex bias in the same disease (37–40, 46, 47, 59–65). These disparities may be explained by the fact that the occurrence and susceptibility to infectious diseases is influenced by many factors such as presence of pathogen reservoir, presence and properties of pathogen transmission vector in case of vector borne diseases (72), as well as immune status, sex and hormonal status, age, nutrition, microbiome and genotype of the host (72–75) and multiple environmental factors, including climate changes (76). Susceptibility to many human diseases is modified by socio-cultural determinants, behavioral/lifestyle risk factors (50), prevalence of co-morbidities (48) and co-infection with several pathogens (69, 77).

SEX-DEPENDENT RESPONSES REVEALED IN ANIMAL EXPERIMENTS

Sex-dependent differences in response to pathogens could be more effectively analyzed in animal studies. Mouse experiments revealed important features of sex-dependent responses to infectious diseases: dose-dependence, pathogen and pathogen species-dependence, organ specificity and genetic modification.

Dose-Dependent Sex Bias

Dose-dependent sex bias was described in responses to viruses and bacteria. The response of the strain C57BL/6 infected intranasally with the mouse adapted influenza A/PR8;H1N1 was sex-dependent when median infection dose [10^2 or 10^3 $TCID_{50}$ (tissue culture infectious dose)] were used and females exhibited higher mortality than males. The effect of infections with low (10^1 $TCID_{50}$) or high (10^4 or 10^5 $TCID_{50}$) viral inoculi was sex independent (78). Dose-dependent sex bias was observed also in the animal model of gram-negative sepsis. Wistar rats were injected intraperitoneally with bacteria *Escherichia coli* LPS in one of two doses: 1.5 or 15 mg/kg. Day after the LPS injection, serum levels of endotoxin, corticosterone, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activity in the serum and morphological changes in the lung, liver, thymus, and spleen exhibited dose-dependent sex bias. Low-dose LPS led to the serum endotoxin level increase only in males and it was combined with a more pronounced inflammatory response in the lungs (characterized by infiltration of eosinophils and neutrophils) and thymus (characterized by presence of macrophages and dead lymphocytes) and an increase and decrease in ALT and AST activity, in males and females, respectively, without any changes in corticosterone level. High-dose LPS induced systemic inflammatory response syndrome (SIRS) comprises higher blood endotoxin levels in males than in females, lower the volume fraction index of the white pulp of the spleen of males, increase of apoptotic cells in thymus and decrease of corticosteroids in males only. Sex differences of pathological changes in the lungs and liver were not revealed (79). The observed dose-dependent sex differences might be largely caused by different dynamics of induction of different signaling pathways in males and females.

Sex Differences Depend on Species and Sub-Species of Pathogen and on Genotype of the Host

Sex bias in disease susceptibility and prevalence that is dependent on pathogen species is described in *Sex Biases in Human Infections*. Here we describe, that sex bias can depend also on pathogen sub-species. DBA/2 female mice are highly resistant and males susceptible to lesion development after infection with the parasite *L. mexicana*. On the contrary, although both female and male mice developed ulcerating lesions after infection with *L. major*, lesions healed in males, but not females (80). Sex

differentially influenced also infection with *L. tropica* and *L. major* and the response was modified by genotype. Females of strains BALB/c, CcS-11, CcS-16 and CcS-20 are more susceptible than males to development of skin lesions induced by *L. tropica*, whereas no sex bias was observed in strains STS, CcS-3, CcS-5, CcS-12 and CcS-18. On the other hand, infection by *L. major* induced larger skin lesions in males of strains CcS-3, CcS-5 and CcS-18, whereas no difference between males and females was observed in strains BALB/c, STS, CcS-11, CcS-12, CcS-16 and CcS-20 (81).

Sex Affects Pathology of Various Organs Differently and Its Influence Is Modified by the Host Genotype

Strains BALB/c and CcS-11 did not exhibit any sex influence on lesion size induced by *L. major*, but males of strain CcS-11 contained more parasites in spleens than females, and males of both strains had much higher parasite load in lymph nodes (82). Organ-dependent sex response was observed also in animal model of gram-negative sepsis (79). These phenomena might be explained by presence of different defense mechanisms in different tissues (83, 84), as well as by highly tissue-dependent sex-biases in expression of genes observed in intercross between strains C57BL/6 and C3H/HeJ (85).

MECHANISMS OF SEX-DEPENDENT RESPONSES

The observed sex-differential responses to disease susceptibility may be explained by direct and indirect influence of sex hormones and non-hormonal sex-biasing influence of X and Y chromosomes. Sex steroid hormones (estrogen, testosterone and progesterone) influence response to infections by 1) direct effect on pathogen metabolism, growth, and expression of virulence factors. It was shown that physiological concentration of progesterone inhibited replication of *Coxiella burnetii* in JEG-3 cells (86), both testosterone and progesterone inhibited growth of *Staphylococcus aureus* (87). 2) by modification of immune response and physiology of the host. Effects on sex hormones on the host are exerted *via* sex hormone-receptor interactions. These receptors are present in cell nucleus and membrane (88) of non-immune and immune cells and tissues (88–91). Complexes of sex hormone-nuclear steroid receptor bind target DNA through hormone response elements to act as transcription factors (88). They can also bind to DNA-protein complexes and epigenetically modify cell functions (90, 91). Sex hormone-receptor complexes can exert their effects also through DNA-independent mechanisms, such as the activation of cytoplasmic signal transduction pathways (90). These interactions influence pro- and anti-inflammatory signaling pathways (92, 93). Indirect influences might include for example sex-dependent organ development (94) or influence of sex hormones on gut microbiota (95).

Non-hormonal sex-bias effects are mediated by genes localized on X and Y chromosomes (1, 96, 97). The X chromosome carries a number of immune-related genes (96), such as toll-like receptor 7 (TLR7) and interleukin-1 receptor-associated kinase 1 (IRAK1), as well as a number of immune-associated microRNAs (96). X inactivation, or silencing of one X chromosome, in women would be expected to provide dosage compensation of X-linked genes, however certain regions of the X chromosome escape inactivation (96, 98). This can lead to higher transcription levels of specific genes that are involved in sex-specific responses (96, 99). The Y chromosome also influences immune gene expression, regulation, and susceptibility to infections (97). For example, the Y chromosome mediates susceptibility to cocksackie virus independently of serum testosterone level (100). Genetic variation in chromosome Y regulates susceptibility to influenza A virus infection (101).

MOUSE AUTOSOMAL GENES THAT CONTROL SEX-BIASED RESPONSES TO INFECTIONS

Besides X- and Y-linked genes, there are also autosomal genes operating in sex-dependent manner. Sex-dependent autosomal genes modify response to viruses (102–106), bacteria (106, 107), parasites (108–111), fungi (112) and worms (113) (**Table 1**, **Figure 1**). Three models were introduced to explain gene-sex-interactions (114). 1. "Environment specific effect": Sex dependent gene/loci affect susceptibility only in females (102, 104, 107, 111, 112), or males (102–105, 108–110, 112, 113). 2. "A main effect" model for gene by environment (= sex) interaction. A disease can affect both sexes, but is more severe in one sex compared to the other (106, 107, 111). 3. "A flip-flop" model of gene by environment interaction. Gene affects susceptibility in both sexes, but in different directions (102, 108).

Viruses

Theiler's murine encephalomyelitis virus-induced demyelination (TMEVD) is an animal model for virally triggered multiple sclerosis. QTLs (quantitative trait loci) *Tmevd7* and *Tmevd8* modify susceptibility to virus-induced demyelination in males only, *Tmevd9* influences susceptibility to this disease in females and *Tmevd6* affects susceptibility in both sexes, but has an opposite effect on males and females (102). Locus *Rmp-4* (Resistance mousepox 4) modifies virus titer in spleen and liver as well as survival after infection with ectromelia virus (mousepox) (103). Two loci not named by authors *NNI1* (not named influenza 1) and *NNI2* control survival after infection with the mouse-adapted influenza H3N2/Hk/1/98. *NNI1* and *NNI2* operate in females and males, respectively (104). Susceptibility to HSV1 is in males controlled by *Hlr* (herpes resistance locus) (105). Gene *LRRK2* (leucine-rich repeat kinase-2) is a 280 KDa, multi-domain protein that has dual catalytic and kinase activity as well as number of protein-protein interaction domains. Two major inflammatory pathways have been biochemically linked to LRRK2 action: TLR pathway and NFAT pathway (115). It is associated with Parkinson's disease, leprosy and Crohn's disease that are disorders with an important

TABLE 1 | Autosomal genes and loci controlling sex-biased responses to infection in mouse.

Pathogen	Locus/Gene	Chromosome	Cross/Strain	Trait (phenotype) controlled	Sex effect	Reference
Viruses						
Theiler's murine	*Tmevd6*	1	BALB/c x DBA/2J	virus-induced demyelination	opposite effects on females and males	(102)
encephalomyelitis	*Tmevd7*	5	BALB/c x DBA/2J	virus-induced demyelination	males	(102)
virus	*Tmevd8*	15	BALB/c x DBA/2J	virus-induced demyelination	males	(102)
	Tmevd9	1	BALB/c x DBA/2J	virus-induced demyelination	females	(102)
mousepox/ ectromelia	*Rmp-4*	1	C57BL/6 x D2	virus titer in spleen and liver, survival	males	(103)
mouse-adapted	*NNI1*	2	C57BL/6J x A/J	survival	females	(104)
influenzaH3N2/ HK/1/68	*NNI2*	17	C57BL/6J x A/J	survival	males	(104)
herpes simplex virus 1	*Hrl*	6	BALB/c x 129S6	survival	males	(105)
reovirus - T3D	*Lrrk2*- knockout	15	C57BL/6	mortality from encephalitis	females - increased mortality in knockouts	(106)
Bacteria						
Chlamydia	*NNCH1*	5	C57BL/6J x A/J	chlamydial burden in lungs	females	(107)
pneumoniae	*NNCH2*	17	C57BL/6J x A/J	chlamydial burden in lungs	stronger effects on males	(107)
Salmonella typhimurium	*Lrrk2*- knockout	15	C57BL/6	bacterial replication in spleen	female knockouts higher bacteria replication than WT; knockin of pG2019S mutation lower bacteria replication than WT, stronger effect on females	(106)
Parasites						
Leishmania major	*Lmr4*	6	BALB/c x CcS-9	parasite load in lymph nodes	males	(108)
	Lmr14	2	CcS-9 x BALB/c	eosinophil infiltration into lymph nodes	males	(109)
	Lmr14	2	BALB/c x CcS-9	parasite load in lymph nodes	males	(108)
	Lmr15	11	BALB/c x CcS-9	parasite load in lymph nodes	opposite effects on females and males	(108)
	Lmr27	17	BALB/c x CcS-9	parasite load in lymph nodes	males	(108)
Leishmania mexicana	*Il4ra* CD4+ T cell specific expression	7	BALB/c	skin lesions	non-healing phenotype in males	(110)
Trypanosoma	*Tbbr1*	3	BALB/c x CcS-11	survival	females	(111)
brucei brucei	*Tbbr2*	12	BALB/c x CcS-11	survival	stronger effect on females	(111)
Fungi						
Cryptococcus	*Cnes1*	3	C57BL/6J x CBA/J	lung fungal burden	females	(112)
neoformans	*Cnes2*	17	C57BL/6J x CBA/J	lung fungal burden	females	(112)
	Cnes3	17	C57BL/6J x CBA/J	lung fungal burden	males	(112)
Worms						
Trichuris muris	*TM5*	5	C57BL/6J x DBA/2	Serum IFNγ	males	(113)

The Table summarizes position on chromosome, cross used to map certain locus or mouse genetic background, disease phenotype controlled and sex effect.
Cnes, Cryptococcus neoformans susceptibility; Hlr, herpes resistance locus; Il4ra, interleukin 4 receptor alpha; Lmr, Leishmania major response; Lrrk2, leucine-rich repeat kinase-2; NNCH, not named Chlamydia; NNI, not named influenza; Rmp-4, Resistance mousepox 4; Tbbr, Trypanosoma brucei brucei response; TM, Trichuris muris; Tmevd, Theiler's murine encephalomyelitis virus-induced demyelination.

inflammatory component. Shutinoski and co-workers tested hypothesis that *Lrrk2* plays role also in infections with paramount inflammatory responses such as reovirus and *Salmonella typhimurium* (will be discussed in the next subsection). The increase of mortality caused by reovirus-induced encephalitis in *Lrrk2*-knockout mice in comparison with wild type animals was observed in female, but not in male mice (106).

Bacteria

Chlamydia pneumoniae causes a variety of respiratory diseases. Susceptibility to this pathogen was controlled by two sex-dependent QTLs: *NNCH1* (not named *Chlamydia 1*) and *NNCH2*. Effect of *NNCH1* was observed in females, whereas *NNCH2* exerted stronger effect on males (107). Comparison of replication of *S. typhimurium in* spleens of wild type and *Lrrk2*-knockout mice shown increased replication of bacteria in spleen of female knockouts. Knockin of Parkinson's Disease (PD)-linked p.G2019S *Lrrk2* mutation led to lower pathogen burden in spleens. The effect was stronger in females (106).

Parasites

Sex-dependent QTLs operating in *L. major* infected mice are involved in control of pathogen load in lymph nodes (108) and infiltration of eosinophils into lymph nodes (109). *Lmr4* (*Leishmania major* response) and *Lmr27* control parasite load in lymph nodes in males, *Lmr14* influences both parasite load in and eosinophil infiltration into lymph nodes in males and *Lmr15* determines parasite load in lymph nodes in both sexes, but with opposite direction of effect (108, 109). Wild type BALB/c mice infected with *L. mexicana* develop non-healing, progressively growing skin lesions. Monitoring the course of infection with *L. mexicana* in BALB/c mice lacking expression of IL-4Rα (interleukin 4 receptor, alpha) in $CD4^+$T cells revealed that these mice developed small lesions, which subsequently healed in females,

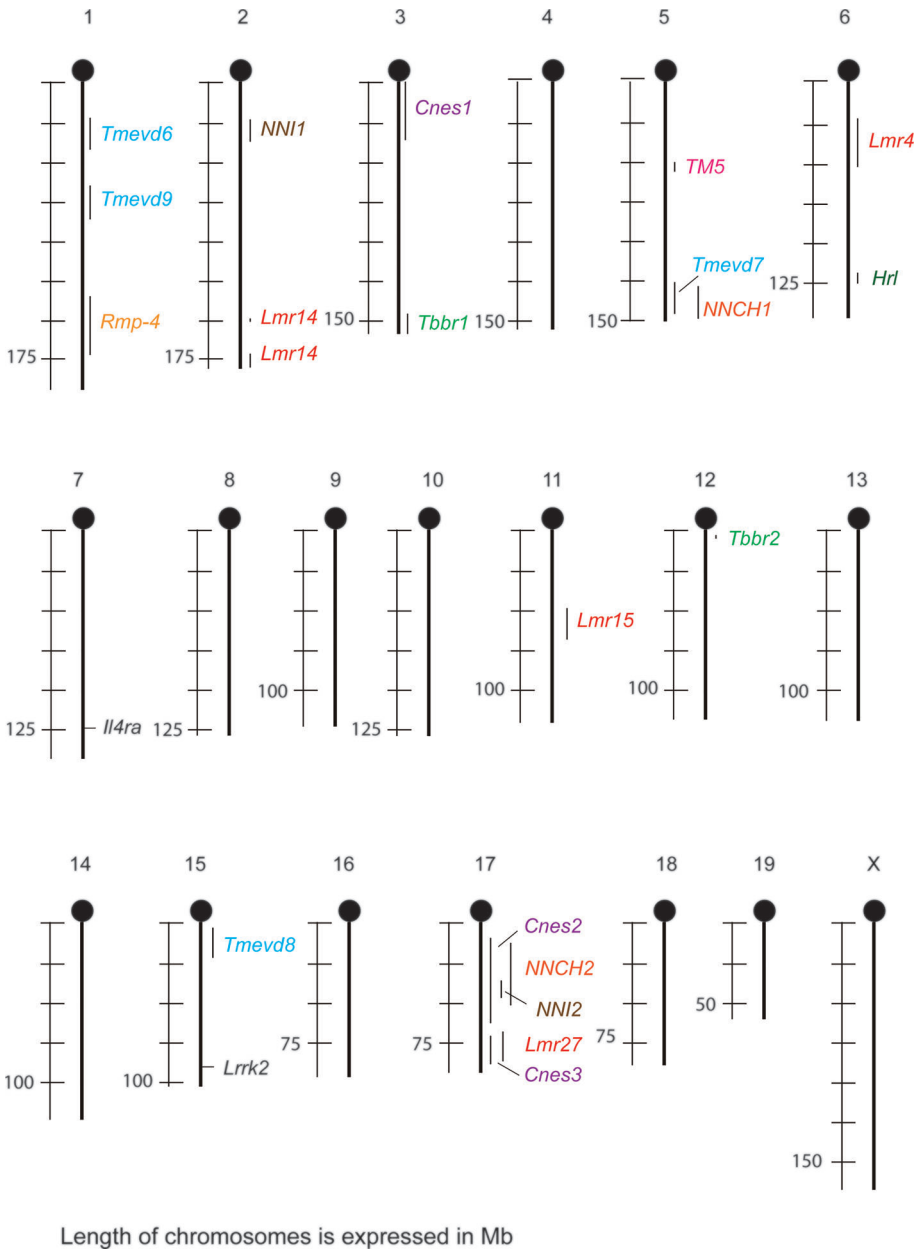

FIGURE 1 | Sex-dependent loci and genes that control susceptibility to infections in mouse and their overlaps. *Cnes*, *Cryptococcus neoformans* susceptibility; *Hlr*, herpes resistance locus; *Il4ra*, interleukin 4 receptor alpha; *Lmr*, *Leishmania major* response; *Lrrk2*, leucine-rich repeat kinase-2; *NNCH*, not named *Chlamydia*; *NNI*, not named influenza; *Rmp-4*, Resistance mousepox 4; *Tbbr*, *Trypanosoma brucei brucei* response; *TM*, *Trichuris muris*; *Tmevd*, Theiler's murine encephalomyelitis virus-induced demyelination.

but persisted in males (110). *Tbbr1* (*Trypanosoma brucei brucei* response 1) and *Tbbr2* control survival after infection with *T. b. brucei*. Effect of *Tbbr1* is visible only in females, *Tbbr2* has stronger effect on females than on males (111).

Fungi

Cryptococcus neoformans is a fungal pathogen that causes pneumonia, meningitis and disseminated disease in immunocompromised host (68, 112). Fungal burden in lungs after infection with this pathogen was controlled by three sex-dependent QTLs. *Cnes1* (*Cryptococcus neoformans* susceptibility 1) and *Cnes2* operate in females, whereas effect of *Cnes3* is observed in males (112).

Worms

TM5 (*Trichuris muris* 5) is associated with IFNγ production in serum of males infected with parasitic nematode *T. muris* (113).

Overlaps and Features of Sex-Dependent Loci

Some sex-dependent loci co-localize (**Figure 1**). Locus *Tmevd7* on chromosome 5 (102) overlaps with locus controlling susceptibility to *Chlamydia* (107). *Cnes2* on proximal and central part of chromosome 17 (112) co-localizes with loci modifying susceptibility to *Chlamydia* (107) and influenza (104). *Cnes3* on distal part on chromosome 17 (112) overlaps with *Lmr27* (108). This suggests the presence either of clusters of functionally related genes, or of genes that are involved in controlling the response to several infections, similarly as *Lrrk2* that controls response to reovirus and bacteria *S. typhimurium* (106).

Loci *Lmr15* (108) and *Tmevd6* (102) exhibit different effect on males and females (flip-flop model) (114) (**Table 1**). Both of them are localized on rather long chromosomal segments, thus we cannot exclude existence of two closely linked genes – one controlling susceptibility of males, the other females. However, it cannot be excluded that the opposite sex-dependent effects are controlled by one gene. Similar situation was described in humans. Polymorphism in rs2069885 (c.350 C>T) in *IL9* (5q31.1) has an opposite effect on the risk of severe respiratory syncytial virus infection in boys and girls (116), as well as on *Aspergillus fumigatus*-induced allergic lung inflammation estimated as IgE level and *IL9/IL9R* mRNA ratio in lung expectorates in males and females suffering by cystic fibrosis (117). The inflammation is stimulated by IL-9 – IL-9R on mast cells – innate lymphoid cells – Th9 pathway (117). Sex influence might be exerted by interaction of IL-9 with IL-9R. *IL9R* is located in the pseudoautosomal regions 2 (PAR2) on Xq28 and Yq12 that behave as autosomes, recombine during meiosis and PAR regions on X escape silencing (118, 119).

CONCLUSIONS AND PERSPECTIVE

Sex differences in response to infections are frequent in human and form a considerable part of interpretation heterogeneity. The genetic studies in mice revealed 22 genes/QTLs influencing these differences, suggesting a genetic heterogeneity of this phenomenon. The mechanisms of effects of these sex-specific mouse genes/QTLs are unknown, but may appear as a result of sex hormone regulation of the polymorphic genes underlying these QTLs or interaction between X- or Y-chromosome-linked genes (96–99). Some of the differences between females and males might be due to sex-specific genetic architecture, characterized by profound gene-sex interactions (85, 117, 120, 121). This would mean that some genes controlling response to infections might operate differently in the two sexes. The understanding of these sex- dependent responses could facilitate personalized medicine that would take into account sexual dimorphism in susceptibility to infectious diseases, outcome of vaccination and response to treatment.

AUTHOR CONTRIBUTIONS

ML and PD wrote the paper. Both authors contributed to the article and approved the submitted version.

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Why Females Do Better: The X Chromosomal TLR7 Gene-Dose Effect in COVID-19

Anna E. Spiering[1,2] *and Teun J. de Vries*[1,2]*

[1] Amsterdam University College, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [2] Department of Periodontology, Academic Centre for Dentistry Amsterdam, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, Netherlands

A male sex bias has emerged in the COVID-19 pandemic, fitting to the sex-biased pattern in other viral infections. Males are 2.84 times more often admitted to the ICU and mortality is 1.39 times higher as a result of COVID-19. Various factors play a role in this, and novel studies suggest that the gene-dose of Toll-Like Receptor (TLR) 7 could contribute to the sex-skewed severity. TLR7 is one of the crucial pattern recognition receptors for SARS-CoV-2 ssRNA and the gene-dose effect is caused by X chromosome inactivation (XCI) escape. Female immune cells with TLR7 XCI escape have biallelic TLR7 expression and produce more type 1 interferon (IFN) upon TLR7 stimulation. In COVID-19, TLR7 in plasmacytoid dendritic cells is one of the pattern recognition receptors responsible for IFN production and a delayed IFN response has been associated with immunopathogenesis and mortality. Here, we provide a hypothesis that females may be protected to some extend against severe COVID-19, due to the biallelic TLR7 expression, allowing them to mount a stronger and more protective IFN response early after infection. Studies exploring COVID-19 treatment via the TLR7-mediated IFN pathway should consider this sex difference. Various factors such as age, sex hormones and escape modulation remain to be investigated concerning the TLR7 gene-dose effect.

Keywords: TLR7, interferon, COVID-19, X chromosome inactivation escape, gene-dose effect, sex differences

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Antonietta Rossi,
University of Naples Federico II, Italy

Reviewed by:
Ezra Aksoy,
Queen Mary University of London, United Kingdom
Michael Paul Gantier,
Hudson Institute of Medical Research, Australia

****Correspondence:***
Teun J. de Vries
teun.devries@acta.nl

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INTRODUCTION

Early in the pandemic, male sex was identified as a risk factor for hospitalization and mortality after SARS-CoV-2 infection (1). While behavioral and socio-economic factors are implied, immunological differences between the sexes may be more important in this disparity. Females typically show a stronger immune response than males and possible factors that could explain this phenomenon are X chromosomal genes, immunomodulatory functions of sex hormones and sex-dependent expression of susceptibility genes (2, 3). Females have better clinical outcomes than males following sepsis and infection (4–7). For instance, males suffer from more severe and intense disease after hepatitis B virus (HBV) or Epstein Barr virus (EBV) infection (7). The immune response differs between males and females in various ways, ranging from innate recognition to downstream adaptive immunity, resulting in distinctly different cytokine responses to infection. Induction of pattern recognition receptors (PRRs), subsequent interferon (IFN) and antibody

production is higher in females than males. Sex hormones display immunomodulatory activity at various levels, and immune response to viruses can vary with natural hormonal fluctuations in females. Novel research suggests also an important role for sex chromosomal gene that can modulate differences in the immune response or cause differential expression of disease susceptibility genes such as class I and II MHC glycoproteins (3).

On the other hand, autoimmune diseases are predominantly present in females (4–6, 8). For instance, the incidence of systemic lupus erythematosus (SLE) is almost nine-fold higher in females and male Klinefelter patients (47, XXY), but rarely develops in female Turner patients (45, X0) (9), indicating the importance of the X chromosome in SLE and immunity. The X chromosome is home to many genes directly or indirectly related to immunity and variability in expression of X chromosomal genes is often associated with sex-related immune disparities

The sex-bias observed in the COVID-19 pandemic fits this pattern. An X chromosomal gene of interest is TLR7, identified as a pattern recognition receptor (PRR), recognizing ssRNA viruses such as SARS-CoV-2. It is one of the PRRs involved in type 1 interferon (IFN) production in COVID-19 (10, 11). In the present article, we hypothesize that contribution of beneficial gene expression of the second female X chromosome could partially explain the sex-skewed ICU admission. In particular, this hypothesis and theory article focuses on TLR7 expression and activation and the consequences for males and females in COVID-19.

SUBSECTIONS

COVID-19 and TLR7

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the single-stranded RNA (ssRNA) virus that causes COVID-19. The spike protein on the viral envelope binds to the angiotensin-converting enzyme receptor 2 (ACE2) on the host cell. ACE2 has a systemic function in lowering blood pressure and is expressed in most organs, but abundantly in lung respiratory epithelium. The spike protein is activated by the serine protease 2 transmembrane protein (TMPRSS2) on the host cell, allowing for viral particles' internalization. The viral RNA is subsequently released into the host cytoplasm, from where it can move to the host ribosome for production of new virions to infect other cells. Alternatively, it can enter the endosome (2, 12). Various cellular sensors can recognize SARS-CoV-2 ssRNA or other intermediates of viral replication and induce downstream signaling to activate the innate immune response. At the level of the lung epithelium, these sensors are cytoplasmic RIG-I and MDA5 (13, 14). The inflammatory mediators produced by the epithelium are picked up and further amplified by PRRs in innate immune cells (15), such as TLR7 and TLR8.

Toll-Like Receptor 7 (TLR7), and also TLR8, is a PRR encoded on the X chromosome. TLR7 is expressed at the endosomal membrane of plasmacytoid dendritic cells (pDCs) and B cells and to a lesser extent in monocytes and macrophages (16). TLR7 can recognize ssRNA derived from viruses and bacteria in humans, and can also respond to RNA-associated autoantigens (17). Following detection of ssRNA, TLR7 activates the MyD88-dependent pathway, activating both the nuclear factor kappa Beta (NFκB) and the type 1 interferon (IFN-α and IFN-β) pathway in pDCs (18). This process is dependent on the gene product of CXorf21, an X chromosomal gene encoding for the TLR adaptor interacting with endolysosomal SLC15A4 (TASL) protein which stimulates nuclear migration of interferon regulatory factor 5 (IRF5) (19). IFN-α transcription in pDCs is further dependent on the nuclear migration of IRF7 (18), as shown in **Figure 1**. pDCs are the most potent IFN producers and while other cells can also produce IFNs, pDCs function as the primary type 1 IFN producers during viral infections (20). In macrophages, TLR7 stimulation can lead to production of pro-inflammatory cytokines (21).

The most important type 1 IFNs are IFN-α and IFN-β. The production of type 1 IFN is essential for the antiviral response, especially in the early stages of infection. Type 1 IFNs have essential functions: activating an antiviral state in infected cells to limit the viral spread, restraining innate immunity, controlling pro-inflammatory pathways, and activating adaptive immunity. They exhibit both direct and indirect antiviral activity. IFNs bind the interferon-alpha/beta receptor (IFNAR) at infected cells, which initiates the JAK-STAT signaling pathway and the following cascade destroys both host and viral RNA. Viral replication and production are directly restricted by the induction of IFN-stimulated genes (ISGs) (22–24).

TLR7 has been implicated as PRR in SARS-CoV-1 and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infections (25). These related viruses possess a high number of binding motifs for TLR7 and bioinformatic analysis showed that the SARS-CoV-2 genome has even more ssRNA motifs that can potentially interact with TLR7 than its family member SARS-CoV-1 (23). Early in the COVID-19 pandemic, the importance of TLR7 after SARS-CoV-2 infection was first demonstrated in a clinical setting by van der Made et al. (10). After four young males (two brother pairs from unrelated families) without medical history were admitted to the intensive care unit (ICU), a genetic cause was suspected. In all four patients, different TLR7 deleterious variants and concurrent low levels of type 1 IFN were identified. Loss of function was confirmed when experiments with primary PBMCs showed no upregulation of TLR7 and a failure to induce IFN-inducible genes (ISGs) after stimulation with a TLR7 agonist (10). Later, more such cases of deleterious TLR7 variants in severe male patients have been described (11, 26, 27), suggesting that a loss-of-function of TLR7 could explain some cases of severe COVID-19 which result in functional defects of type 1 IFN. Likewise, single nucleotide polymorphisms (SNPs) could alter TLR7's effectivity.

As pulmonary epithelial cells do not express TLR7, they alone are not sufficient for the defense against SARS-CoV-2; TLR7 deficiency was pathogenic in patients by impairing the production of large amounts of type 1 IFNs by pDCs (11). After infecting lung epithelial cells, innate phagocytic cells such

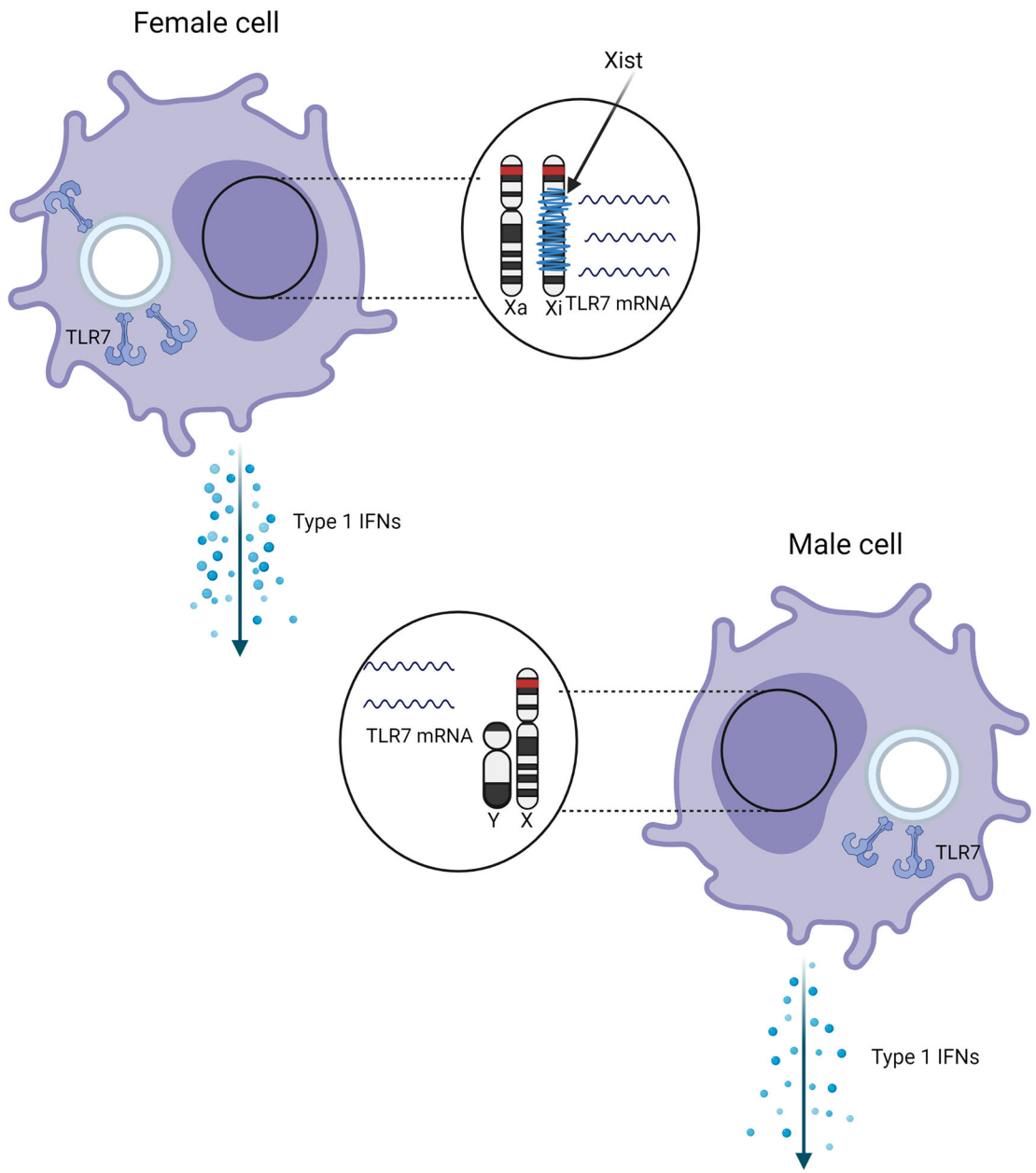

FIGURE 1 | Schematic representation of the effect of sex and X chromosome inactivation escape on the TLR7 expression. TLR7 gene (red) on the inactivated X chromosome (Xi) wrapped with Xist (blue) escapes inactivation. Escape from distal X chromosome inactivation in female pDCs may result in more TLR7 mRNA and protein compared male cells, resulting in higher production of type 1 IFNs.

as pDCs are recruited and can phagocytose the infected cells and produce type 1 IFNs after PRR stimulation. The clinical observations of deleterious TLR7 mutations and a poor type 1 IFN response identify TLR7 as an important PRR in the immune response against COVID-19.

Since TLR7 is located on the X chromosome, mutations in TLR7 will affect males more than females, who bear two X chromosomes per cell. TLR7 deficiency has been reported as a genetic mediator for severe COVID-19 especially in younger males, with percentages of 1-2% found across cohorts (10, 11, 26, 27). Therefore, genetic screening for TLR7 primary immunodeficiency was recommended in young males with severe COVID-19 in the absence of other relevant risk factors (11, 26, 27). Despite the observed prevalence in young males, the penetrance of deleterious TLR7 mutations may be worse in older patients, as both the pDC number and functional IFN secretion

decreases with age, which was associated with a reduced number of pDCs expressing TLR7 (28).

X Chromosome Inactivation

The TLR7 gene is encoded at the distal end of the X chromosome. To avoid double dosage of X chromosomal genes, one female X chromosome is epigenetically silenced in early fetal development. It is random which of the two parental (either maternal or paternal) chromosomes is inactivated, and females are therefore functional mosaics for the active X chromosome (Xa) and the inactive X chromosome (Xi) (29–31). This process is called X chromosome inactivation (XCI). An early study suggests that exactly one chromosome is entirely inactivated in diploid, and half of the X chromosomes in tetraploid cells (32). This view has been modified: certain genes escape from X chromosome inactivation (see later on).

The X inactivation center (Xic) at the centromere controls XCI and codes for several proteins and RNAs that form a nuclear complex during XCI, of which the long non-coding RNA (lncRNA) X inactivation specific transcript (Xist) is a critical member. Xist is expressed only from the inactive X chromosome (Xi) and wraps around the X chromosome, turning it into the inactive Barr body during interphase (31, 33). Recent studies show that XCI is not complete. This phenomenon, XCI escape, could cause extra gene expression of some genes located on the female X chromosome (34, 35) and is a source of (tissue-specific) sex differences (35). The exact mechanism of XCI escape is still unclear. Most genes that escape XCI are at the distal end of the X chromosomes' short arm, suggesting that the chromosomal location is relevant for escape susceptibility (36). Since the Xist gene is at the centromere, it has been suggested that the direction of Xist RNA wrapping around the X chromosome is from the center outwards (35, 37, 38). The degree of escape is variable between genes, tissues, and individuals, but this variability is poorly characterized (39, 40). Moreover, it is unclear to what extent the X chromosome inactivation state is variable or stable throughout the course of life (41).

Approximately 15 to 20% of human X chromosomal genes escape inactivation and are transcribed from both X chromosomes. Escape is characterized when the Xi alleles' mRNA or protein expression is at least 10% of that of the Xa allele (42). XCI escape can be studied in various ways, such as bulk RNA-sequencing (RNA-seq) to assess differential allelic expression or RNA fluorescence *in situ* hybridization (RNA FISH) to detect nascent RNA transcripts. XCI status can also be examined indirectly *via* epigenetic markers such as methylation patterns (43).

Evidence for XCI Escape of TLR7

TLR7 escapes XCI in different female immune cells: pDCs (20, 44), monocytes and B cells (44). A study by Souyris et al. in 2018 was the first to demonstrate the XCI escape of TLR7 in primary human cells. Souyris et al. confirmed biallelic mRNA expression of TLR7 in 30% of pDCs, monocytes, and B cells from healthy females and male Klinefelter Syndrome (KS) patients (47, XXY). TLR7 transcription from both X chromosomes was observed using RNA FISH in B cells, confirming XCI escape. The relative abundance of TLR7 mRNA transcripts in female biallelic B cells was up to 50% higher than male monoallelic B cells. Western blot testing confirmed this at the protein level: a 1.38- and 1.31-fold increase in TLR7 protein was found in biallelic peripheral blood mononuclear cells (PBMCs) compared to female monoallelic PBMCs and male cells, respectively. Additionally, female B cells differentiated more efficiently into plasmablasts than male B cells upon TLR7 stimulation. None of these effects were observed upon TLR9 stimulation.

Souyris et al. (44) hypothesized that the increased TLR7 dosage would result in increased TLR7-induced type 1 IFN levels, which was confirmed by Hagen et al. (20). This study confirmed 30% female pDCs with biallelic expression and a similar 50% higher abundance of TLR7 mRNA transcripts. Moreover, Hagen et al. observed that biallelic pDCs showed significantly higher type 1 IFN mRNA and protein in the first 2 hours after TLR7 stimulation with a TLR7 agonist (2020). As visualized in **Figure 2**, these results confirmed that human female pDCs produce more type 1 IFN after TLR7 stimulation, showing that TLR7 XCI escape is correlated with the antiviral type 1 IFN response.

Before these two publications, it was already established that female pDCs produced more type 1 IFN after TLR7 stimulation and expressed more interferon alpha/beta receptors (IFNAR) than male pDCs (45–48), especially in females after puberty (49). Similarly, Berghöfer et al. found higher induction of IFN-α in female PBMCs stimulated with TLR7 agonists and not by TLR9 agonists, confirming a skewed and beneficial TLR7 response which could be explained by XCI escape. However, this could not be confirmed in immortalized B-cell lines (46). Others did not investigate XCI escape as a cause of their observations and sought other explanations. Estrogens were suggested as a possible cause of increased IFN signaling in females, as *in vitro* blocking of estrogen receptor (ER) signaling inhibited TLR7-mediated type 1 IFN production by pDCs. However, when female pDCs were transplanted into male mice, an enhanced TLR7-mediated type 1 IFN production was still seen, suggesting that female sex hormones and the number of X chromosomes independently contributed to the enhanced type 1 IFN production (48). Webb et al., who compared genetic deviations with more than one X chromosome, also showed that increased IFN production was dependent on the number of X chromosomes present in the cell (49). A study by Sarmiento et al. found slightly contradictory results: they observed that stimulated female and Klinefelter syndrome (KS) PBMCs had higher TLR7 and IFN-α mRNA levels than male and Turner's syndrome female PBMCs (45, X0), supporting TLR7 XCI escape, but the KS cells did not show higher IFN-α levels after TLR7 stimulation. In comparison, the female cells showed higher levels compared to the male and TS cells: in the case of the KS cells with two X chromosomes, their higher TLR7 expression by XCI escape did not result in higher IFN, while it did in the female cells with two X chromosomes. Accordingly, the increased IFN production was not dependent on the number of X chromosomes (50), opposite to what others observed (49). One of the explanations provided by Sarmiento et al. is that male KS cells were used, which cannot rule out epigenetic modification of

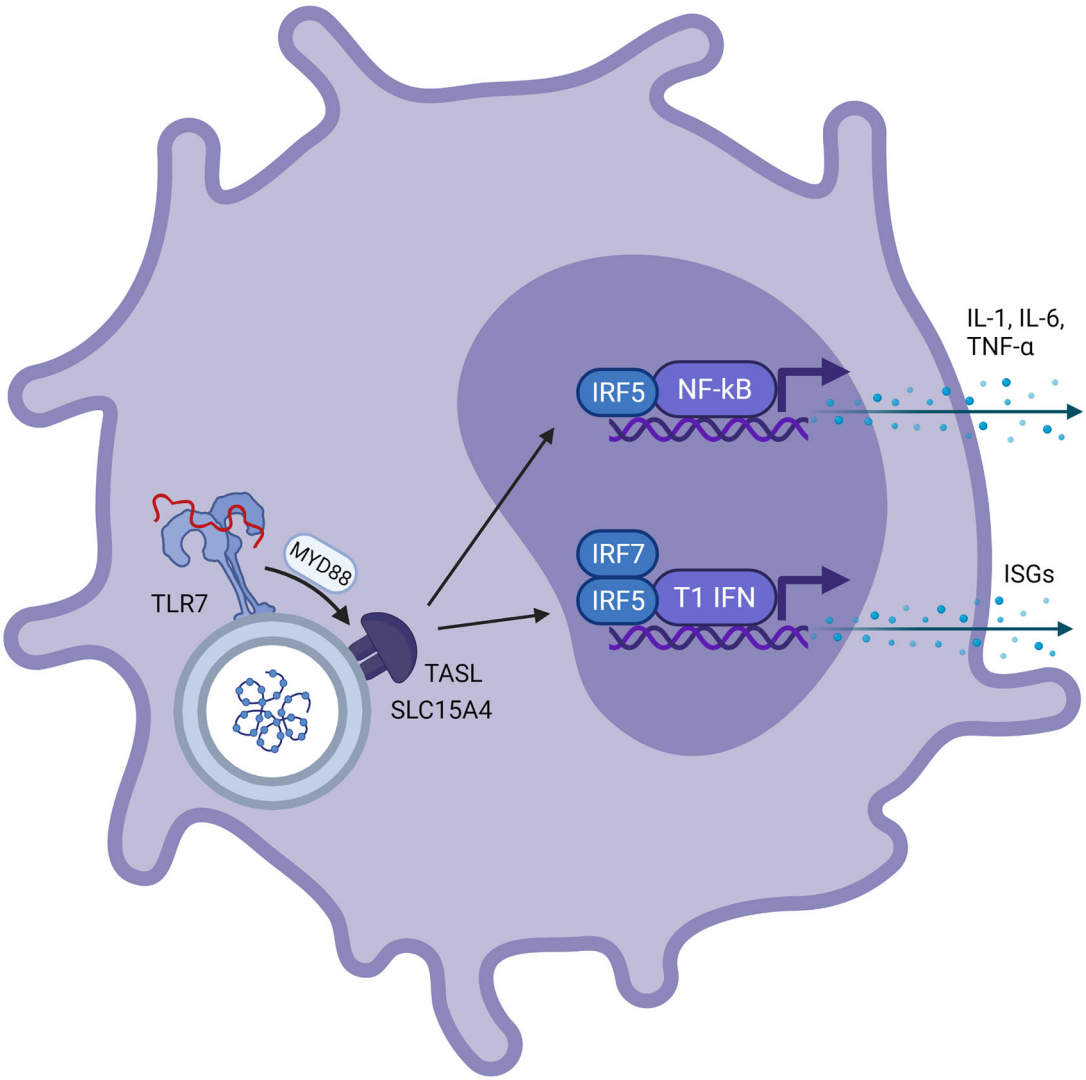

FIGURE 2 | Schematic representation of a plasmacytoid dendritic cell with viral ssRNA in the endosome. This is recognized by TLR7 on the endosomal membrane. *Via* the MYD88 pathway, 'TLR adaptor interacting with SLC15A4 on the lysosome' (TASL) is activated which is needed for recruitment of Interferon Regulatory Factor 5 (IRF5), a transcription factor involved in transcription of NF-kβ and type 1 interferons. This results in further transcription of IL-1, IL-6 and TNF-α for NF-kβ and interferon stimulated genes for type 1 interferons, respectively.

TLR7 by testosterone (50). Altogether, these results strongly suggest that increased type 1 IFN secretion after TLR7 stimulation in female pDCs is linked to biallelic TLR7 expression by XCI escape.

Possible Implications of TLR7 XCI Escape

This gene-dose effect may play a role in the COVID-19 pandemic, where a significant sex bias has been observed. A meta-analysis of over 3 million global cases between the 1st of January to the 1st of June 2020 revealed that males are 2.84 times more often admitted to the ICU with severe COVID-19 and die 1.39 more often than females, while there is no difference in the proportion of males and females infected with SARS-CoV-2. Socio-economic, lifestyle, and behavioral factors may be partly responsible for this sex bias, but it is equally likely that differences in males' and females' immune responses are the driving factor for various reasons. Firstly, the difference was consistently and ubiquitously observed around the world in the selected time period (1). Additionally, another meta-analysis found that while age is another critical risk factor for severe COVID-19, males have a significantly higher risk of death than females at all ages above 30 years (51) and a similar sex bias had been previously observed in the SARS-CoV-1 and MERS-CoV outbreak (52), two ssRNA viruses closely related to SARS-CoV-2. This is consistent

with the male sex bias observed in other ssRNA viruses such as hepatitis C and ebola (1, 53). TLR7 gene dosage is a known risk factor and area of interest in SLE, as overexpressing TLR7 in mice leads to SLE-like disease and TLR7 XCI escape has been connected to the high incidence of SLE in females (9).

Altogether, this suggests an actual biological and immunological difference in response to SARS-CoV-2 infection. XCI escape of TLR7 and subsequent increased expression of type 1 IFN could be part of the immunological explanation for the observed sex differences concerning severe COVID-19 susceptibility.

In COVID-19, the immune response is a double-edged sword. Its role after viral infection is to rapidly recognize and eliminate the virus but a late and uncontrolled immune response can lead to immunopathogenesis, such as observed in severe COVID-19 patients (22, 54, 55). The double-edged sword is particularly evident in the distinct clinical phases of severe COVID-19. Patients who later develop severe COVID-19 often show only mild symptoms early on, with low levels of cytokines, leukocytes, and type 1 IFNs. This early phase of severe COVID-19 is characterized by rapid viral replication without detectable pattern recognition receptor (PRR) and IFN triggering, opposite to what is typically seen in pathogenic ssRNA influenza viruses (56). Later, around 7-10 days after symptom onset, these patients rapidly deteriorate. Levels of inflammatory cytokines and chemokines (IL-1, IL-6 and TNF-α) increase to a delayed peak at this stage while lymphocyte levels are low and inflammatory dysregulation develops into acute respiratory distress syndrome (ARDS) (22, 57–59). These high levels of inflammatory cytokines is also called cytokine storm (CS) and is not uncommon in infectious diseases. It can result in systemic inflammation, organ failure, acute lung pathology, and ARDS, as seen in COVID-19 (22, 24, 55, 57).

Mild patients may induce a type 1 IFN-mediated innate immune response shortly after infection, obstructing viral replication at an early stage (60), opposite to what is seen in patients infected with highly pathogenic influenza viruses (56). Moreover, this is in contrast to severe patients who either show a delayed IFN peak or no peak at all. Continuously low as well as delayed peak levels of type 1 IFNs concurrent with a decreased viral load have been observed, suggesting that cause of the symptoms lies, at this stage of the disease, rather at the immune system than at the level of the initial viral infection (22, 57–59). A reduced type 1 IFN response concurrent with a consistent chemokine production has been observed both *in vitro* and in COVID-19 patients (61). A strong and timely, not delayed IFN response is considered protective. But if this response is uncontrolled and late, the increased cytokine and chemokines, many of which are interferon stimulated genes (ISGs), can contribute to development of ARDS. Increases in IL-6 and TNF-α are independent predictors of COVID-19 severity (62). As chemokines are often expressed by an induction of the NF-kB pathway, these results suggest that the NF-kB pathway is induced more than the type 1 IFN pathway (61). Demonstrating the importance of type 1 IFNs are observations of deleterious IRF7 mutations (63) and auto-antibodies against IFNs (64) associating with mortality in COVID-19 patients. Various studies reported an early IFN peak in mild to moderate patients and low IFN levels associated with severe disease and mortality (56–58, 60, 65–68). This profound inflammation is considered one of the leading causes of severity in the later phases of COVID-19 (22, 55, 57–59, 69). A study investigating immune differences in COVID-19 found that increased severity in male patients correlated to high cytokine levels, which was not the case for the females in this cohort (70). With biallelic expression of TLR7 by XCI escape in at least part of their immune cells, females could be expected to mount a more distinct type 1 IFN response early on after SARS-CoV-2 infection, associated with mild disease and possibly preventing them from progressing to severe disease and immunopathology. This is supported by the observed sex bias early in the pandemic of increased hospitalization and mortality in males compared to females and data about the differential role of the immune system in COVID-19. An experimental or clinical study that examines sex differences in the TLR7 and type 1 IFN response in COVID-19 is necessary to further explore this hypothesis.

Additionally, more recent SARS-CoV-2 strains and long-COVID are topics of interest. Analysis of 4000 COVID-19 cases in an app in which individuals can self-report their symptoms showed that female sex is associated with long COVID prevalence (71). This could be explained by the observation in a recent preprint of elevated IFN levels in long COVID patients at 8 months after infection (72), as the IFN response is usually more distinct in females (45–48).

The observation of the sex bias in COVID-19 severity was done in cases up until June 2020 (1), and with a rapidly mutating virus, this may be different in newer strains. While studies into more recent SARS-CoV-2 strains are slowly appearing, it is already emerging that the delta (B.1.617.2) variant come with an increased hospitalization risk (73). Studies about a possible sex bias in this variant have, to the best of our knowledge, not yet been published.

X linked differences in TLR7 expression may not only contribute to COVID-19 susceptibility, but also to hepatitis C virus (HCV) or human immunodeficiency virus (HIV) susceptibility (74). Females are more likely to clear HCV in acute infection than males and develop cirrhosis less often in chronic infection. These differences could in part be explained by increased TLR7 signaling, which is beneficial in HCV (74). SARS-CoV-1 and MERS-CoV showed a male sex bias and similarly to SARS-CoV-2, high IFN levels 'pre-crisis' and low IFN levels in the 'crisis' phase have been observed in these two viruses (56, 75–78). In contrast to COVID-19, females show faster HIV disease progression to acquired immune deficiency syndrome (AIDS) than males, which could be explained by more persistent inflammation in females contributing to immune impairment. It has been demonstrated that females have a higher TLR7-mediated pDC response to HIV-derived ligands, leading to increased type 1 IFN expression, resulting in increased activation of CD8+ T cells (45).

Finally, pediatric cases are of particular interest. Sex differences based on behavioral or hormonal factors may take place later in life, while genetic mechanisms, amongst which XCI

escape, may be evident from birth onwards. XCI takes place in the fetus, but it remains unclear when XCI escape takes place. Patterns of XCI escape have been observed already in trophoblastic cells, but in the embryo proper, where XCI escape is probably still absent (43). Initial XCI research already suggested inactivation state may be dynamic over the course of life (79), and later studies suggest that XCI gene silencing may indeed be variable and gene- and tissue specific (41). An experimental mouse study showed age-associated loss of XCI for the *Atp7a* gene: the inactivated X chromosome (Xi) allele of *Atp7a* was silenced in young adult mice but Xi expression levels could rise up to 5% of the active X chromosome (Xa) in older mice (80). Such studies have not been conducted with human cells *in vitro* nor *in vivo*. It would certainly be interesting to examine possible differences in XCI status in the course of female life, for TLR7 and other genes alike (43, 81).

Generally, pediatric autoimmune diseases are more severe and more prevalent in females (82) and male newborns are more vulnerable to infections and death than their female counterparts (83), similar to the pattern seen in adults. Male sex is a risk factor for severe disease after respiratory syncytial virus (RSV) infection, a common childhood infection (82). In pediatric COVID-19, a wide spectrum of severity is seen. Generally, the COVID-19 prevalence is much lower than in adults and it seems that children are less at risk of a severe clinical outcome, although it is yet unclear why (84–90). One possibility is the higher basal expression of MDA5 and RIG-I in respiratory epithelium than adults, which results in a stronger innate response upon SARS-CoV-2 infection (91). Pediatric studies are underrepresented in COVID-19 research (89). Large scale age-stratified COVID-19 studies often do not further specify below the age of 30 or even 50, much less sex-specific studies (1, 92). Interestingly, a review analyzing 12,306 pediatric COVID-19 cases in the USA found no sex bias with equal male and female propensity (84). Another study analyzed age- and sex-stratified excess deaths in 29 high-income countries that possibly associated with the COVID-19 pandemic in 2020 and found no sex difference in excess deaths in children. The deaths in children were at lower or expected levels in this time period (93). In light of XCI escape, a possible explanation for this discrepancy is that escape of XCI increases over age (41, 80). Large COVID-19 cohort studies with clear age- and sex-stratified groups that include XCI parameters could shed light on this issue in the future.

Treatment Options

Despite the current world-wide increasing vaccination rate (94), effective treatment options are still necessary for those who are not (yet) vaccinated or for breakthrough cases in the already vaccinated population (95). There is a wide array of possible treatment strategies, some of which include the TLR7-IFN axis. TLR7 agonists have been proposed as treatment (22, 25, 96–99), which may prevent the onset of severe COVID-19 in symptomatic patients and even synergize with active antiviral therapy such as remdesivir. TLR7 agonists could be used to stimulate the innate immune response and induce type 1 IFNs and ISGs early in the disease to prevent progression to more severe phases. TLR7 agonists with several modes of administration are already available for other inflammatory diseases with various degrees of efficacy (100) and would need further examination for use in COVID-19, but, no clinical trial was started. Importantly, administration in later phases of COVID-19 is likely to lead to further immune dysregulation and pathology. As this stage is already characterized by immunopathology, further immune stimulation is not likely to relieve symptoms (25, 98). Successful early interventions which prevent late-stage immunopathology could even help to overcome sex-biased COVID-19 severity.

A possible danger that comes with TLR7 agonists is stimulation of both the pro-inflammatory NFkβ and IFN downstream pathways. Other upstream stimulators of IFNs independent of NFkβ could be considered, e.g. STING agonists which have been explored before in oncology immunotherapy and are recently studied in COVID-19 (101).

Since SARS-CoV-2 showed sensitivity *in vitro* to type 1 IFNs, administration of IFNs may be possible and especially beneficial in patients with a very dysregulated IFN response (60, 102) or patients with deleterious TLR7 or IFN mutations (27, 63, 64). No randomized clinical trials have been performed for type 1 IFNs in SARS-CoV-1, but clinical benefits have been suggested by comparing patients' clinical outcomes. A retrospective cohort of 446 patients showed that early administration of IFN was associated with reduced hospital mortality while late IFN administrated increased mortality and delayed recovery (103), emphasizing that timing of the administration is crucial, similarly to TLR7 stimulation. Experiments showed that the addition of type 1 IFN to SARS-CoV-2 infected cells resulted in a striking decrease in viral replication, significantly more than in SARS-CoV-1 infected cells (60, 61). The potential window of opportunity for therapeutic IFNs is early after infection, since late administration may exacerbate the inflammatory state of advance disease (61, 104). There are currently several single and combination treatment trials with different administration routes of type 1 IFNs going on (24, 78, 104).

SARS-CoV-2 can obstruct IFN production: a transcriptome profiling study, which was conducted using *in vitro* tissue culture, *ex vivo* infection of primary cells and *in vivo* samples from COVID-19 patients and animals, found that SARS-CoV-2 yields lower levels of type 1 IFN, a lower response of ISGs, and high levels of chemokines, in comparison to other respiratory viruses. These results suggest viral IFN suppression (61). Additionally, host risk factors have been found: IFN-neutralizing autoantibodies or genes suppressing IFN production (63, 64, 67, 104). It has to be explored to what extent SARS-CoV-2 can really interfere with IFNs, and what this means for possible treatment strategies.

Interestingly, treatments with TLR7 antagonists have also been proposed (105). These may ameliorate the cytokine storm (CS) in patients in a later stage of COVID-19. Currently, a phase II clinical trial of a TLR7 antagonist (NCT04448756) is being completed, which investigated the antagonist's possibilities in modulating the CS and immunopathology in severe COVID-19 patients. Suppression of the inflammatory response in later stage

COVID-19 is a strategy which can be pursued in various ways. For instance, dexamethasone showed positive results in an early open-label trial (106).

TLR7 and type 1 IFN agonists as well as antagonists have been proposed, depending on the stage and timing of disease, emphasizing the immune system as a double-edged sword. When considering either of these strategies or other immunomodulatory strategies, the difference in TLR7 dosage between males and females needs to be taken into account.

DISCUSSION

COVID-19 is a disease where the immune system is a double-edged sword, as visible in the different clinical stages. In severe patients, IFN levels are low early on and symptoms are moderate but later, IFN levels rise, and a cytokine storm subsequently occurs. Males are significantly more likely than females to experience severe disease and TLR7 XCI escape in females may be partly responsible for this. TLR7 has been observed to escape XCI in 30% of female immune cells, resulting in biallelic TLR7 expression and increased IFN production compared to males. Severe COVID-19 disease has been associated with a delayed IFN response and increased TLR7-mediated IFN production in females may prevent females from progressing to severe disease.

This sex-determined gene-dose effect may have important implications for managing the COVID-19 pandemic. While sex-based immunological differences are not new (2), sex as a biological variable is often neglected in clinical and especially pre-clinical research, especially in inflammatory diseases (107, 108). Unfortunately, COVID-19 research is no exception. A recent preprint established that the vast majority of many registered clinical trials for COVID-19 do not mention sex or gender as a recruitment criterium nor bring up sex or gender in the description of the analysis phase (109). Fortunately, two major vaccine trials (Pfizer, Moderna) did include sex-disaggregated primary outcome data. However, further analysis of sex-disaggregated adverse events and secondary outcomes was lacking, which could have set a reporting benchmark for future medical intervention reporting (108). Sex-disaggregated data are not provided by all countries and clinical reports about infection or mortality rate are often not sex-disaggregated, further complicating sex-specific analyses (110, 111). The Global Health 50/50 research initiative and the International Center for Research on Women have started the Sex, Gender and COVID-19 Project which tracks sex-disaggregated data and aims to further attract attention for this issue (111).

In various articles calling for investigation of TLR7 agonist or IFN administration early in COVID-19, sex is also not named as a factor to be taken into account (22, 24, 60, 78, 98, 99). This trend is especially surprising considering the gendered hospitalization and mortality rates of COVID-19 and many have called to reverse this (1, 108, 110, 112, 113). While several articles noted the possible sex differences in immune response to COVID-19, only few clinical and experimental studies have been conducted to investigate this further (70). While it is no exception, it is unfortunate to see this, especially in the light of a possible role for higher expression of TLR7 and IFN in females, probably allowing for sex-specific treatments. In treatment based on modulating TLR7 or administrating IFNs, the sex-determined TLR7 gene-dose effect by XCI escape may be especially important considering the importance of proper timing in the different disease phases. While many questions remain about sex differences in the TLR7-mediated IFN response, it is well-established that these differences exist. Not taking these into account in clinical studies is counter beneficial for exploring these differences. For example, it could be studied whether males need a higher dose of TLR7 agonist or antagonist, or IFN administration than females. Another question is the possible difference in TLR7 XCI status between ICU admitted and non-ICU admitted females.

Thus far, two recent studies have shown proof of XCI escape of TLR7 (20, 44) or examined its effect on IFN expression (20). Replications of these results would further support these findings, in conjunction with previously reported increased IFN response of female immune cells to TLR7 stimulation (45, 47, 48). In these studies, it seems likely that some degree of TLR7 XCI escape may play a role. However, little is known about the stability and duration of XCI and XCI escape: TLR7 is no exception. Insights in the mechanisms of Xist spreading and Xist maintenance may provide more answers into stability, modulation and timeline of XCI escape (50). A recent study demonstrated that Xist is continually required in adult human B cells to regulate several X-linked immune genes including TLR7 (81). Single-cell transcriptome data of female patients with either systemic lupus erythematosus (SLE) or COVID-19 revealed Xist dysregulation and overexpression of TLR7 in PBMCs and atypical B cells. These results suggest that Xist RNA maintenance is crucial for TLR7 silencing, and that Xist regulation may be subject to change in disease states such as COVID-19 or SLE and that Xist maintenance may alter TLR7 expression from the X chromosomes (81). Recently, methods for manipulating Xist have been developed for mouse pre-implantation embryos (114). However, there is still much to be learned about Xist maintenance and TLR7 transcription regulation alike, which could open up possibilities for further investigation of XCI escape and possible manipulation.

Other mechanisms than XCI escape may increase the protein dosage. Both TLR7 and TLR8 expression are under the influence of single nucleotide polymorphisms (SNPs) which can impact expression. For instance, the TLR7 SNP rs179008 is carried by approximately 30% of females of European descent and lowers TLR7 protein dosage in pDCs *via* controlling TLR7 mRNA translation. Affected pDCs from both pre and postmenopausal females showed an impaired TLR7-mediated IFN production, but affected male pDCs did not (115, 116). The TLR8 SNP rs3764880 influences expression of TLR8 isoforms in human monocytes and increases the amount of TLR8 protein (21). Additionally, genomic copy number variation (CNV) can also control TLR7 protein dosage, and this CNV has been associated to SLE incidence (117, 118). Thus, the amount of protein dosage is not only under control of epigenetic mechanisms such as XCI escape, but also by CNV and SNPs.

Other X-chromosomal genes may also be implicated in COVID-19, specifically Toll-Like Receptor 8 (TLR8) and TLR

adaptor interacting with SLC15A4 on the lysosome (TASL). TLR8 is in many ways related to TLR7: both are encoded on the X chromosome in close proximity to each other. Both are activated by ssRNA and are complementary to each other in the detection of specific ssRNA motifs. TLR8 is absent in pDCs and B cells, but is expressed in myeloid cells such as monocytes, macrophages and neutrophils, where it can drive IFN-β production dependent on IRF5 (21, 119–121). Compared to TLR7, TLR8 is studied to a lesser extent (119, 122). TLR8 has been suggested to escape XCI in mice but this has not been studied yet in humans (123). In SLE, TLR7 dosage as well as TLR8 dosage are important determinants for the observed sex differences (19, 116, 120).

While SARS-CoV-2 ssRNA can trigger a TLR8-dependent inflammatory response from macrophages *in vitro* (124), it was suggested by others that TLR8 is of lower clinical importance in COVID-19, due to its lack of expression on pDCs and that no deleterious TLR8 variants were found in severe patients (11). TASL interacts with lysosomal SLC15A4 to activate IRF5 after TLR7 activation and is an interferon stimulated gene (ISG) (116). Similar to TLR7, both TLR8 and TASL are involved in the etiology of systemic lupus erythematosus (SLE), a female-biased autoimmune disease (19, 116, 120). TASL has not been associated with COVID-19 directly, but indirectly *via* its adaptor function in the TLR7-mediated IFN pathway. Despite its location on the X-chromosome, XCI escape of TASL has not been studied, so it is unclear if there are sex-based dosage differences of TASL.

This article proposed a hypothesis that XCI escape of TLR7 and the resulting gene-dose effect in females is beneficial in COVID-19. Unfortunately, it will prove difficult to establish a causal link between this TLR7 gene-dose effect and increased hospitalization and mortality in males. It seems important to first establish interindividual variation in biallelic expression between mild and severe COVID-19 female patients. Recently, software has been developed to include X chromosomal associations, such as XCI escape, into GWAS analyses (30), but this cannot associate gene dosage, only genetic variation such as SNPs. However, it could be a way forward, for instance in identifying the genetic make-up of female patients who experience severe COVID-19. Various methods are available to study XCI escape (43, 125). RNA sequencing (RNAseq) and expression data to identify XCI escape relies on good methods to differentiate alleles, usually SNP frequencies to distinguish alleles at multiple loci. Single-cell hybridization methods such as RNA fluorescence *in situ* hybridization (RNA-FISH) used by Souyris et al. (44) do not rely on SNPs and can be used to identify cell types within a tissue. Bulk RNAseq methods could be used for larger-scale investigations into quantitative differences in TLR7 expression between males and females, between males and females of various ages, interindividual variability between females and tissue variability.

Age is another risk factor for severe COVID-19. Ageing differentially affects the male and female immune system, with a male trend towards accelerated immune ageing (1, 126). Ageing might also have an effect on XCI escape, which could be established in bulk RNAseq studies. Especially the role of estrogens may be important here, considering that females after menopause experience a significant drop in estrogen levels. Estrogens are known to modulate the female immune response and protect against infection severity *via* upregulating both the innate and adaptive immune response (2). Estrogens and to a lesser extent progesterone can enhance the TLR7-mediated IFN production by pDCs (45, 127) and blocking estrogen receptor (ER) signaling inhibits this IFN production (48, 127). Treating SARS-CoV-1 infected female mice with ovariectomy or an ER antagonist significantly increased mortality, indicating a protective effect of ER signaling in mice (52). Possibly, this protective effect was mediated by TLR7-mediated IFN signaling, but this was not studied. If ER signaling indeed enhances TLR7 signaling, the menopausal estrogen drop could mean that the protective effect of increased TLR7 signaling in females would be partly lost and the female sex bias in COVID-19 would be diminished.

TLR7 is also expressed in stimulated B cells, where it is involved in B cell receptor activation, antibody production and recognizing foreign nucleic acids (128). TLR7 XCI escape was also demonstrated in B cells (44)and increased B cell stimulation and its effects may also be relevant in COVID-19.

Besides the TLR7 gene-dose effect caused by XCI escape, other biological differences between the sexes may influence the sex bias observed in COVID-19. Other factors are differential ACE2 expression, generally increased T cell activity and immunoglobulin production by B cells in females, other X-encoded immune genes, and activity of sex hormones (1, 51).

Interestingly, ACE2, the entry receptor of SARS-CoV-2, is encoded on the short arm of the X chromosome in sites that are known to escape XCI. Despite this localization, XCI escape of ACE2 has not been experimentally demonstrated in the way that TLR7 has been with RNA fluorescence *in situ* hybridization (RNA FISH). Male-female expression differences could be used as an indirect proxy measurement for XCI status. Data from a genotype-tissue expression study shows a tissue-specific heterogenous expression pattern which is *male*-biased in most tissues (40) while XCI escape would show a female-biased expression pattern. However, there are indications that ACE2 expression does not necessarily correlate to ACE2 enzyme activity due to regulation by sex hormones. Therefore, it is necessary to study at the transcriptional, translational and post-translational level (40, 62).

Unexpectedly, ACE2 was recently identified as an ISG (interferon-inducible gene) in respiratory epithelial cells (129). The antiviral type 1 IFN response may allow for viral entry in neighboring cells by upregulating ACE2 expression as SARS-CoV-2 exploits the ACE2-mediated tissue-protective IFN response to gain cellular entry. This raises questions about IFN treatment, because of the possibility of increased cellular entry by increased ACE2 levels. However, patients receiving angiotensin II receptor blockers and ACE inhibitors, widely used antihypertensives that increase ACE2 expression, showed a lower COVID-19 mortality rate compared to patients not receiving these antihypertensives (130). These results imply that ACE2 is important but not sufficient for viral entry, and that other co-receptors such as serine protease 2 transmembrane

protein (TMPRSS2) may also be needed. Of interest, IFN-β increases ACE2 expression, while IFNα does not, suggesting that the type 1 IFN subtypes may have distinct functions (130).

Various other immune genes are encoded on the X chromosome and mutations in X-linked immune genes are known to affect males more than females. For instance, a mutation in interleukin-2 receptor subunit gamma (IL2RG) causes severe combined immunodeficiency (SCID). Males with SCID have impaired cellular and humoral immunity, resulting in increased susceptibility for infections, with a symptom onset before the age of one (131). Likewise, other X-linked immune genes may add to the observed sex bias in COVID-19, possibly by escape of XCI. IL-1 receptor-associated kinase-1 (IRAK-1) is the most studied gene regarding the sex bias in inflammatory responses. It escapes XCI, favoring an increased NFκB-dependent gene transcription response in females (132). CD40L and CXCR3, which have an important role in the T cell response, are both encoded at the X chromosome and escape XCI. It is unknown if the sex-differential role of T cells has a role in COVID-19 (51).

Additionally, The X chromosome encodes for 10% of human microRNAs (miRNAs), many of which are associated with modulation of the immune response. Dysregulated expression of miRNAs has been associated with various inflammatory diseases and may also contribute to the sex differences in COVID-19 (54, 132) but there is yet too little evidence to attribute specific sex-typical immune responses to X-linked miRNAs (2).

To conclude, there are clear indications for an X chromosomal TLR7 gene-dose effect in COVID-19. As sex-determined variables are currently understudied, and the effects of other X-related immune genes and TLR7 modulators are unclear, future research should consider to shed more light on the sex bias in COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Research topic was mutually agreed on by both authors, research was primarily conducted by AS, writing was initiated by AS. AS was supervised by TV during the research period, TV supervised and corrected writing. All authors contributed to the article and approved the submitted version.

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Male Macrophages and Fibroblasts from C57/BL6J Mice Are More Susceptible to Inflammatory Stimuli

Maria Luisa Barcena[1,2*], *Maximilian H. Niehues*[1], *Céline Christiansen*[1], *Misael Estepa*[1,3], *Natalie Haritonow*[1], *Amir H. Sadighi*[1], *Ursula Müller-Werdan*[1], *Yury Ladilov*[4] *and Vera Regitz-Zagrosek*[4,5]

[1] Department of Geriatrics and Medical Gerontology, Charité –Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany, [2] DZHK (German Centre for Cardiovascular Research), Berlin Partner Site, Berlin, Germany, [3] Department of Internal Medicine and Cardiology, Deutsches Herzzentrum Berlin, Berlin, Germany, [4] Institute for Gender in Medicine, Center for Cardiovascular Research, Charité University Hospital, Berlin, Germany, [5] Department of Cardiology, University Hospital Zürich, University of Zürich, Zürich, Switzerland

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Joe G. Zein,
Cleveland Clinic, United States

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VU University Amsterdam, Netherlands
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VU University Amsterdam, Netherlands

***Correspondence:**
Maria Luisa Barcena
maria-luisa.barcena@charite.de

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Mounting evidence argues for the significant impact of sex in numerous cardiac pathologies, including myocarditis. Macrophage polarization and activation of cardiac fibroblasts play a key role in myocardial inflammation and remodeling. However, the role of sex in these processes is still poorly understood. In this study, we investigated sex-specific alterations in the polarization of murine bone marrow-derived macrophages (BMMs) and the polarization-related changes in fibroblast activation. Cultured male and female murine BMMs from C57/BL6J mice were polarized into M1 (LPS) and M2 (IL-4/IL-13) macrophages. Furthermore, male and female cardiac fibroblasts from C57/BL6J mice were activated with TNF-α, TGF-β, or conditioned medium from M1 BMMs. We found a significant overexpression of M1 markers (c-fos, NFκB, TNF-α, and IL-1β) and M2 markers (MCP-1 and YM1) in male but not female activated macrophages. In addition, the ROS levels were higher in M1 male BMMs, indicating a stronger polarization. Similarly, the pro-fibrotic markers TGF-β and IL-1β were expressed in activated cardiac male fibroblasts at a significantly higher level than in female fibroblasts. In conclusion, the present study provides strong evidence for the male-specific polarization of BMMs and activation of cardiac fibroblasts in an inflammatory environment. The data show an increased inflammatory response and tissue remodeling in male mice.

Keywords: sex differences, inflammation, bone marrow macrophages, macrophage phenotype, activated fibroblasts

INTRODUCTION

In several cardiovascular diseases, inflammatory and pro-fibrotic responses play a detrimental role (1, 2). Inflammatory processes are tightly regulated by signals that initiate and maintain inflammation and promote resolution of the inflammation (3, 4). An imbalance in these mechanisms may promote cellular and tissue damage (3).

Macrophages are a crucial part of the cardiac immune response since they are the most abundant immune cells in the heart (5). It is worth noting that cardiac macrophages interact with other cells in the heart and directly or indirectly regulate different phases of cardiac diseases: acute inflammation, immune-regulation, and resolution, as well as cardiac remodeling (6). In addition, macrophages modulate the response to various stressful conditions in the heart (7). During cardiac stress, e.g., myocardial infarction or myocarditis, the population of tissue-resident macrophages expands by recruiting from the bloodstream or local proliferation (8–10). Macrophages can be polarized into M1 macrophages, which have a pro-inflammatory signature, or into M2 macrophages, which are involved in anti-inflammatory actions, wound healing, tissue remodeling, and immune regulatory actions (11, 12). An aberrant expression of pro-inflammatory cytokines during inflammatory processes leads to the macrophage phenotype switching into a pro-inflammatory phenotype, promoting the perpetuation of the inflammation (13). Key pro-inflammatory Th1-related cytokines, e. g., interferon gamma (IFN-γ) or toll-like receptor (TLR4) signaling, induce a M1 phenotype (14), which releases pro-inflammatory mediators like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and reactive oxygen species (ROS) (15). Macrophage differentiation into the anti-inflammatory M2 phenotype is induced by exposure to IL-4 and IL-13 (16, 17). M2-macrophages express and release anti-inflammatory molecules including IL-10, transforming growth factor beta (TGF-β), and interleukin-1 receptor antagonist (IL-1ra) (18).

Macrophages play a significant role in the cardiac remodeling of the extracellular matrix (19) by activating cardiac fibroblasts *via* TGF-β, IL-1β, and TNF-α (20, 21). The depletion of monocytes and macrophages in the myocardium following cardiac stress decreases both fibroblast activation and collagen deposition (22).

Numerous factors may modulate macrophage polarization by cytokines. In particular, macrophage polarization may be affected by sex hormones, e.g., by the main female sex hormone, estradiol (E2). Although both pro- and anti-inflammatory actions of E2 have been described (23, 24), most studies argue for the anti-inflammatory effects of estrogen receptor (ER) activation in the heart (25, 26). Several reports propose the anti-inflammatory effects of E2 are caused by the inhibition of production and the release of pro-inflammatory cytokines with a M1 signature (27). Furthermore, the ERα seems to be involved in the promotion of M2 macrophage polarization, leading to an anti-inflammatory phenotype (25, 26, 28). In keeping with that profile, E2 loss leads to the expression of pro-inflammatory cytokines e.g., IL-1β, TNF-α, and IFN-γ in humans (29). The anti-inflammatory actions of E2 in male and female peripheral blood mononuclear cells are also observed after activation with lipopolysaccharide (LPS) (25, 30). Altogether, E2 seems to suppress pro-inflammatory and promote anti-inflammatory responses. The difference in E2 blood concentration in males and females may, therefore, be responsible for the sex difference in the inflammatory response.

In this study, we investigated sex-related alterations in the polarization of murine bone marrow macrophages (BMMs) and polarization-related changes in murine fibroblast activation. The analyses revealed that male BMMs are more susceptible to LPS treatment and promote a prominent M2 phenotype. Sex differences were also found in oxidative stress, i.e., less total ROS formation in female BMMs in a pro-inflammatory environment. Moreover, we demonstrated an activation of cardiac fibroblasts with the pro-inflammatory supernatant of cultures of M1 macrophages. Finally, 17β estradiol treatment improved the pro-inflammatory phenotype in male BMMs.

MATERIAL AND METHODS

Animals

Young age-matched male and female C57/BL6J mice (n= 18) (Forschungseinrichtungen für Experimentelle Medizin (FEM), Charité -Universitätsmedizin Berlin) were euthanized and heart, femur, and tibia were collected in ice-cold DPBS (Gibco, Germany) for further processing. A 12 h/12 h light and dark cycle was applied. Water and food were provided *ad libitum*. All experimental procedures were performed according to the established guidelines for the care and handling of laboratory animals and were approved by the Animal Care Committee of the Senate of Berlin, Germany (Approval number: T0333/08).

Cell Culture

Isolation and Cultivation of Murine Bone Marrow-Derived Macrophages

Bone marrow cells were collected by flushing the femur and tibia in DMEM (phenol red) (Gibco, Germany), 10% fetal bovine serum (FBS) (Biochrom, Germany), 1 mmol/l penicillin/streptomycin (Biochrom, Germany), and 1 mmol/l sodium pyruvate (Sigma, Germany) using a 20-gauge needle and were passed through a 70 μm cell strainer. Cells were cultivated for ten days (10% CO_2 and 37°C) in DMEM (phenol red), 0.05 mmol/l β-mercaptoethanol (Sigma-Aldrich, Germany), 1% non-essential amino acids (Thermo Scientific, Germany), 1 mM penicillin/streptomycin, 1 mM sodium pyruvate, 20% donor horse serum (Sigma-Aldrich, Germany), 10% FBS (Biochrom, Germany), and 20% L929-conditioned medium.

Isolation and Cultivation of Murine Cardiac Fibroblasts

Hearts were cut into small pieces and digested 5 times by incubation in a collagenase/dispase buffer for two minutes at 37°C (31). The supernatant was carefully removed and diluted with ice cold growth medium (DMEM with phenol red, 10% fetal bovine serum (FBS), 1 mmol/l glutamine, 1 mmol/l penicillin/streptomycin, 1 mmol/l sodium pyruvate) and centrifuged at 1200 rpm for 5 min at 4°C. Cells were cultivated in fibroblast growth medium (DMEM, 10% FBS, 1 mmol/l penicillin/streptomycin, 1 mmol/l sodium pyruvate, and 1 mmol/l glutamine) until 80% confluence.

Macrophage Polarization

BMMs were polarized into M1 macrophages with 10 ng/ml LPS (Sigma-Aldrich, Germany) and into M2 macrophages with 10 ng/ml recombinant mouse IL-4 (PeproTech, Germany) and 10 ng/ml recombinant mouse IL-13 (PeproTech, Germany) for 24 h.

Fibroblast Activation

Fibroblasts were activated using 20 ng/ml TNF-α (32) (PeproTech, Germany), 10 ng/ml TGF-β (PeproTech, Germany) (33) or 10 ng/ml LPS (Sigma, Germany) for 24 h in fibroblast starvation medium (with 2.5% charcoal-stripped FCS, Biochrom, Germany).

Activation of ERs

Cells were starved with a phenol free medium and 2.5% charcoal-stripped FCS (Biochrom, Germany) for 24 h prior to E2 treatment. After starvation, cells were treated with 10 nmol/l water soluble E2 (Sigma-Aldrich, Germany) or with 10 nmol/l dextrin (Sigma-Aldrich, Germany) as vehicle for 24 h.

Treatment With Conditioned Medium From M1-BMMs

Mice cardiac fibroblasts were cultivated with mixture (1:1) of the fibroblast-starvation medium and conditioned medium from M1-BMMs for 24 h. To produce the conditioned medium from M1-BMMs, BMMs were treated with 10 ng/ml LPS (Sigma-Aldrich, Germany) for 24h. The cell culture medium was collected and centrifuged at 1200 rpm for 5 min at 4°C. The supernatant was stored at -80°C.

Flow Cytometry

The purity of the BMM population was determined *via* flow cytometry analysis. $1x10^6$ cells were taken from the freshly harvested BMMs, processed and stained with the required antibodies according to the manufacturer's protocol. The fluorescently labeled monoclonal antibodies (mAbs) that specifically recognize proteins expressed by macrophages were used for phenotypical characterization. The used two-color panel included two surface antigens, F4/80 (1:100, Miltenyi Biotec, Germany) and CD11b (1:100, Miltenyi Biotec, Germany). In this two-color immunofluorescence protocol, the samples were single stained with each antibody, and then stained using both antibodies. Data were acquired with a MACS-Quant device (Miltenyi Biotec, Germany) using the MACSQUANTIFY™ software (**Figure 1A**).

Immunofluorescence

BMMs and murine cardiac fibroblasts were cultivated in 8-chamber slides (Sigma-Aldrich, Germany). Cells were fixed with 4% Histofix (Roth, Germany) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich). BMMs were stained against F4/80 (1:100, Abcam, UK) or CD11b (1:100, Abcam, UK) (**Figures 1B–E**). Fibroblasts were stained with antibodies against vimentin (1:100), CD31 (negative control for endothelial cells) (1:100), and desmin (negative control for smooth muscle cells) (1:100) (**Figures 1F–K**). The secondary antibodies anti-mouse FITC (1:100) (Dianova, Germany) or anti-mouse Cy3 (Dianova, Germany) were applied according to the manufacturer's protocol. Nuclei were stained using DAPI (1:50000) (Sigma, Germany) and cells were mounted with Fluoromount G (Southern Biotech). Negative controls were performed by omitting the primary antibodies. Images were acquired using a BZ-9000E fluorescence microscope (Keyence, Germany). All evaluations were performed in a blinded manner.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from BMMs or murine cardiac fibroblasts was homogenized in RNA-Bee (Amsbio, UK). Quantitative real-time PCR was performed using the Brilliant SYBR Green qPCR master mix (Applied Biosystems, USA). The relative amount of target mRNA was determined using the comparative threshold (Ct) method as previously described (34). The mRNA content of target genes was normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT).

Protein Extraction and Immunoblotting

BMMs were homogenized in a Laemmli buffer (253 mmol/l Tris/HCL pH 6.8, 8% SDS, 40% glycerin, 200 mmol/l Dithiothreitol, 0.4% bromophenol blue) (35). Proteins were quantified using the BCA Assay (Thermo Scientific Pierce Protein Biology, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: NFκB (1:1,000, Santa Cruz, USA), ERα (1:100, Santa Cruz, USA), ERβ (1:200, Santa Cruz, USA), GPR30 (1:500, Santa Cruz, USA), ERK (1:1,000, Santa Cruz, USA) and p-ERK (1:2000, Santa Cruz, USA). Equal sample loading was confirmed by an analysis of actin (1:1,500, Santa Cruz, USA). Immunoreactive proteins were detected using ECL Plus (GE Healthcare, Buckinghamshire, UK) and quantified with ImageLab [version 5.2.1 build 11, Bio-Rad Laboratories (USA)].

Total ROS Measurements

BMMs were loaded with 0.01 mmol/l DCF (2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester) for total ROS measurement for 30 min. Subsequently, the cells were washed twice with PBS containing calcium-chloride (1 mmol/l) and lysed with a 0.5% TritonX-100 buffer. The fluorescence intensity was analyzed by excitation at 485 ± 10 nm and emission at 530 ± 10 nm using a ViktorX Multilable Plate reader and subsequently normalized to protein level.

Statistical Analysis

The data are given as the mean ± SEM. The data were evaluated using the non-parametric test (Mann-Whitney test for two independent groups) or two-way ANOVA analysis. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, USA). Statistical significance was accepted when $p < 0.05$.

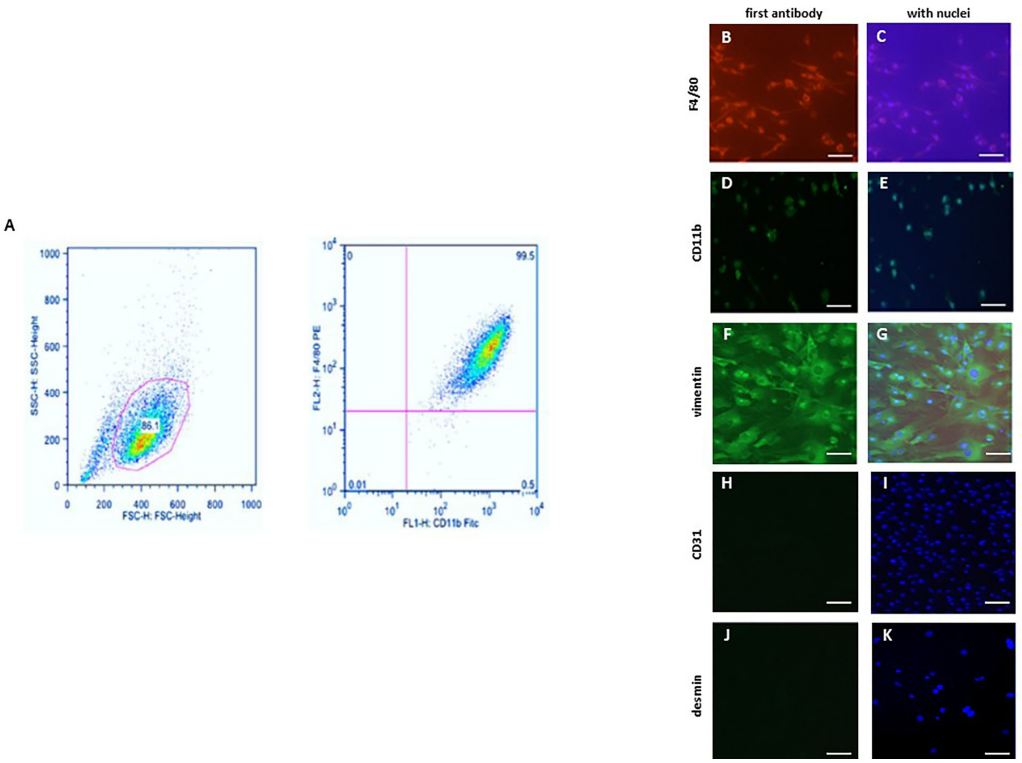

FIGURE 1 | Characterization of bone marrow derived macrophages (BMMs) and cardiac fibroblasts. **(A)** Flow cytometric analysis of the F4/80 and CD11b expression in BMMs. Representative images of BMMs stained against **(B, C)** F4/80 and **(D, E)** CD11b. Representative images of cardiac fibroblasts stained against **(F, G)** vimentin, **(H, I)** CD31 (endothelial marker; negative control) and **(J, K)** desmin (smooth muscle marker; negative control). Nuclei were stained with DAPI **(C, E, G, I, K)** Magnification: 40x; scale bar: 50 µm. Data are representative of 3 independent experiments with similar results.

RESULTS

Male and Female BMMs Express Estrogen Receptors

To investigate the effect of E2 on the polarization of macrophages, we first analyzed the expression of the estrogen receptors in male and female BMMs. All three estrogen receptors (ERα, ERβ and GPR30) are expressed in male and female BMMs (**Figures 2A–E**). Since the stimulation of ER activates ERK1/2 (36), the effects of E2 on ERK1/2 phosphorylation in BMMs were shown. 24h E2 treatment increased ERK phosphorylation in both sexes (**Figure 2F**).

LPS Elicits Stronger Pro-Inflammatory Response in Male Than in Female BMMs

To investigate sex differences in the M1 polarization of macrophages, male and female murine BMMs were treated with LPS. Mitogen-activated protein kinase p38 (p38) is a known downstream target of LPS and plays a crucial role in M1 macrophage polarization (12, 37). p38 was activated (indirectly highlighted by the phosphorylation rate) in male but not female BMMs after 24 h LPS treatment (**Figure 3A**). LPS treatment significantly increased c-fos and TLR4 expression at the RNA level in both sexes (**Figures 3B, C**). LPS treatment also significantly increased the NFκB mRNA in male and female macrophages, whereas the NFκB mRNA elevation in males was about four-fold higher than that in females (**Figure 3D**). Correspondingly, a significant increase of NFκB expression at the protein level was observed only in male macrophages after LPS treatment (**Figure 3E**). It is important to note that western blot assay also revealed a two-fold higher NFkB protein expression in males than females under basal conditions (**Figure 3E**), suggesting a pro-inflammatory phenotype in male BMMs under basal conditions. In accordance with these findings, TNF-α and IL-1β expression was about two times higher in male macrophages after LPS treatment (**Figures 3F, G**).

In contrast, LPS treatment downregulated the expression of the M2 marker, TIM-3, in male macrophages (**Figure 3H**). Both IL-10 as well as MCP-1, prominent M2 markers, were similarly upregulated in male and female macrophages after pro-inflammatory stimulus (**Figures 3I, J**).

Male BMMs Show a More Prominent M2 Phenotype Than Females After IL4/IL13 Treatment

To evaluate the sex differences in the polarization of M2 macrophages, the expression of specific markers was investigated. IL-4/IL-13 co-treatment significantly increased the

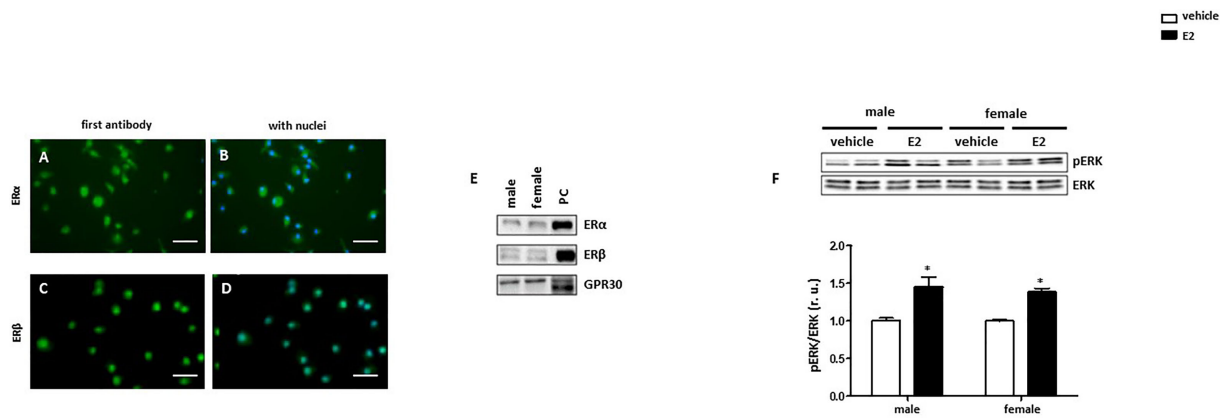

FIGURE 2 | Male and female BMMs express active estrogen receptors. Representative images of BMMs cells stained against (green) **(A, B)** ERα and **(C, D)** ERβ. Nuclei were stained with DAPI (blue) **(B, D)**. Magnification: 40x, scale bar: 50 μm. Western blot analysis of **(E)** ERα, Erβ, and GPR30 and **(F)** pERK/ERK ratio in male and female BMMs with or without E2 treatment for 24 hrs. Data are representative of 3 independent experiments with similar results. Data are normalized to the male untreated group and expressed in relative units (r. u.). *p < 0.05, untreated *vs.* treated. PC = positive control (MCF7 lysate).

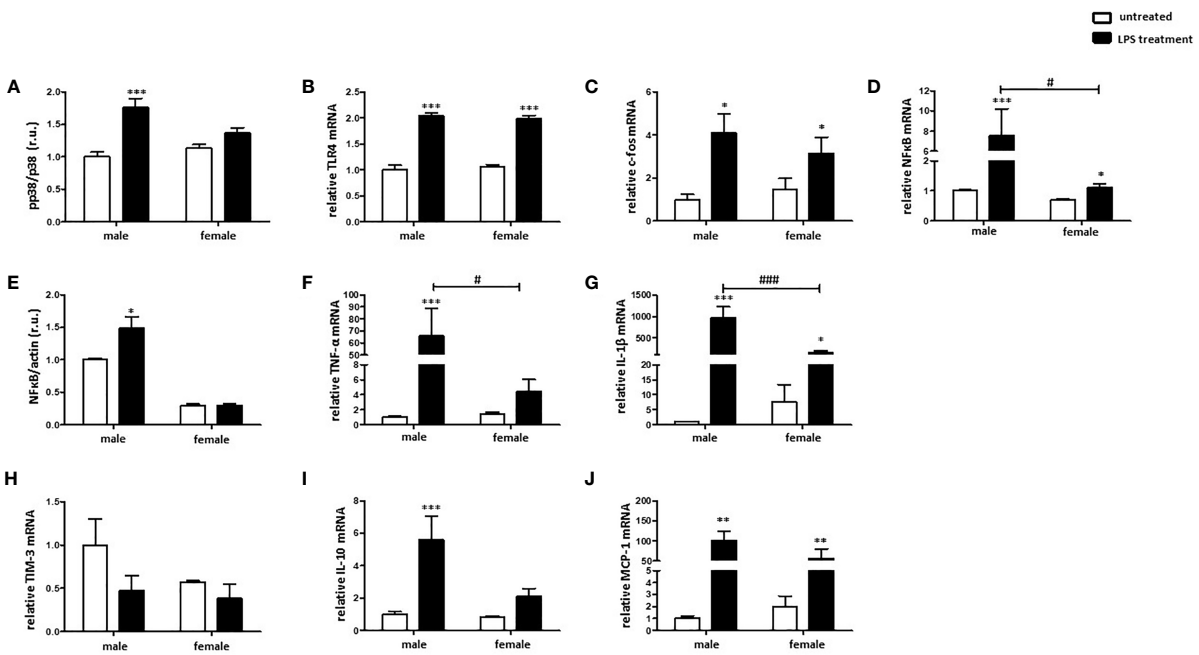

FIGURE 3 | LPS elicits stronger pro-inflammatory response in male than in female BMMs. Expression analyses of **(A)** pp38/p38 ratio, **(B)** TLR4 mRNA, **(C)** c-fos mRNA, **(D)** NFκB mRNA, **(E)** NFκB protein, **(F)** TNF-α mRNA, **(G)** IL-1β mRNA, **(H)** TIM-3 mRNA, **(I)** IL-10 mRNA, and **(J)** MCP-1 mRNA performed with bone marrow macrophages lysates from male and female mice treated with 10 ng/ml LPS for 24 h. Data are shown as means ± SEM (n = 9; independent experiments with technical duplicates). Data are normalized to the male untreated group and expressed in relative units (r.u.). *p < 0.05, **p < 0.01, ***p < 0.001, untreated *vs.* treated; $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001, male *vs.* female.

MCP-1 mRNA expression in male and female BMMs (**Figure 4A**). In addition, YM1 mRNA expression was also upregulated in male and female M2 macrophages, whereas male macrophages showed about 2-fold stronger response than female cells (**Figure 4B**). The M2 marker RELM-α was also markedly upregulated in both male and female macrophages after IL-4/IL-13 co-treatment (**Figure 4C**). As might be expected, prominent pro-inflammatory markers, e. g., TNF-α was downregulated in male M2 macrophages (**Figures 4D**). Surprisingly, IL4/IL13 treatment had any effect on the expression of IL-1β neither in male nor in female macrophages (**Figure 4E**).

TNF-α, but Not TGF-β, Activates a Pro-Fibrotic Phenotype in Mouse Cardiac Fibroblasts

Sex differences in collagen expression and fibrosis formation are well established (31). To investigate the role of sex in fibroblast activation, cultured male and female mouse cardiac fibroblasts were treated with TNF-α or TGF-β. 24 h treatment with TNF-α significantly increased the mRNA expression of the pro-fibrotic markers MCP-1 and IL-1β, in male and female cardiac fibroblasts and the mRNA expression of TGF-β in male cardiac fibroblasts (**Figures 5A–C**), while the TGF-β expression was not increased in female cardiac fibroblasts after TNF-α treatment (**Figure 5B**). Furthermore, male fibroblasts showed a higher elevation of TGF-β and IL-1β (about 2.0-fold) under TNF-α treatment than female fibroblasts (**Figures 5B, C**). In contrast, 24 h treatment with TGF-β did not affect the expression of the pro-fibrotic marker MCP-1 in female fibroblasts, while it increased it in male cells (**Figure 5E**). Neither TNF-α, nor TGF-β affected the expression of Col1A1, a key marker involved in fibrosis formation, in cardiac fibroblasts (**Figures 5D, F**). In addition, MCP-1, TGF-β and IL-1β were increased in male cardiac fibroblasts after LPS treatment, while in female fibroblasts only MCP-1 and IL-1β were increased (data not shown).

Pro-Inflammatory Macrophage Environment Promotes a Pro-Inflammatory and Pro-Fibrotic Fibroblast Phenotype

To investigate sex differences in the macrophage-fibroblast interaction, male and female cardiac fibroblasts were cultivated with the corresponding male or female conditioned medium from pro-inflammatory M1 BMMs.

Cultivation of male and female cardiac fibroblasts with a pro-inflammatory male or female M1 conditioned medium, respectively, increased the expression of MCP-1, TNF-α, NFκB, and IL-1β at the mRNA level in both sexes (**Figures 6A–D**). It is worth noting that the responses were more prominent in male than in female cells. Male fibroblasts showed in the TNF-α and IL-1β expression an about two-fold stronger response than female cells.

Female BMMs Are More Protected Against Oxidative Stress After Pro-Inflammatory Stimulus

To investigate the role of sex on ROS formation, total ROS level was measured in non-differentiated, as well as in M1 polarized male and female murine BMMs. M1 macrophages showed a two-fold higher ROS formation compared with untreated cells (**Figure 7**). Elevation of ROS was also more pronounced in male than in female M1 macrophages (**Figure 7**).

E2 Treatment Promoted The Pro-Inflammatory Phenotype in Male BMMs

Both pro- and anti-inflammatory actions of E2 have been described (23, 24). To test the effects of E2, male and female M1 and M2 macrophages were post-treated with E2 for additional 24 h. The treatment significantly increased the expression of pro-inflammatory markers, e.g., c-fos, NFκB, and TNF-α, in male M1 macrophages, while it had no effects on female cells (**Figures 8A–C**). In M2 BMMs, E2 significantly decreased the expression of MCP-1 in male but not in female M2 macrophages (**Figure 8D**), while E2 had no effect on the expression of TNF-α (**Figure 8E**).

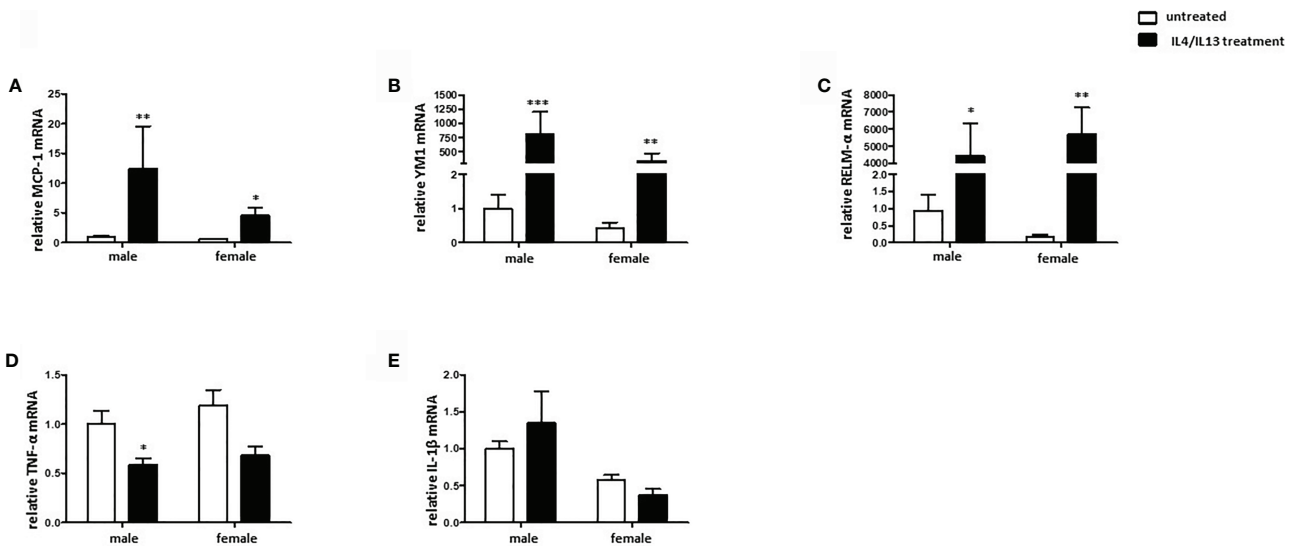

FIGURE 4 | Male BMMs show a more prominent M2 phenotype compared with females. Real-time PCR analyses of **(A)** MCP-1, **(B)** YM1, **(C)** RELM-α, **(D)** TNF-α, and **(E)** IL-1β performed with bone macrophage lysates from male and female mice treated with 10 ng/ml IL-4 and 10 ng/ml IL-13 for 24h. Data are shown as means ± SEM (n = 9; independent experiments with technical duplicates). Data are normalized to the male untreated group. *p < 0.05, **p < 0.01, ***p < 0.001, untreated *vs.* treated.

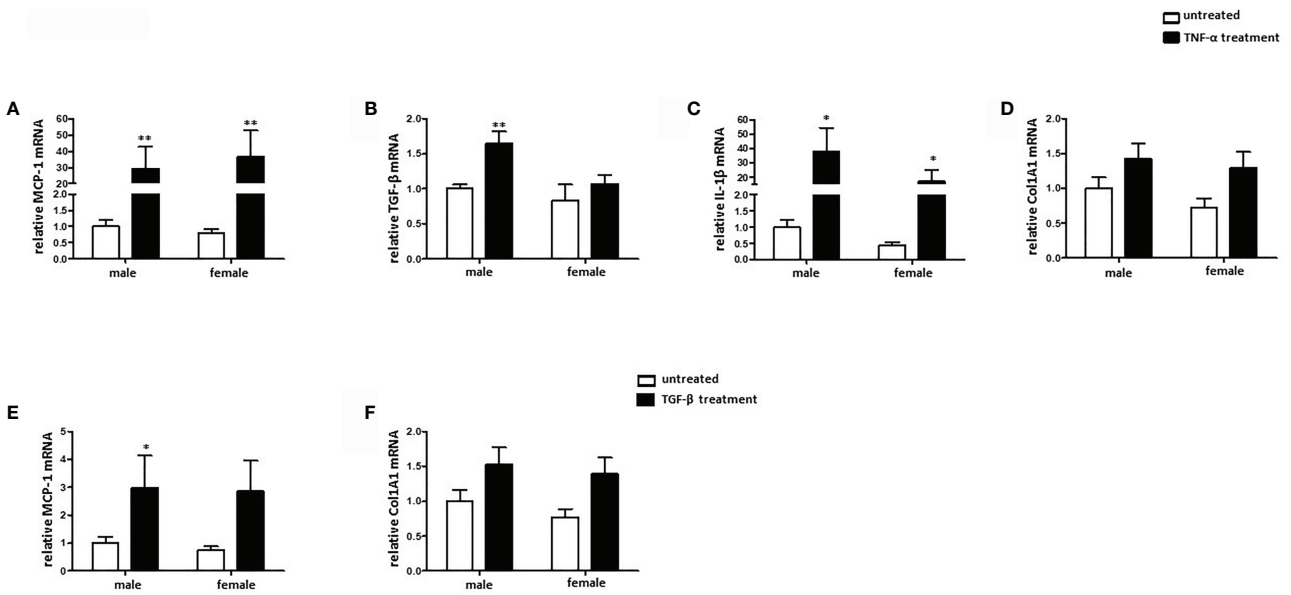

FIGURE 5 | TNF-α, but not TGF-β, induces pro-fibrotic phenotype in mouse cardiac fibroblasts. Real-time PCR analyses of **(A)** MCP-1, **(B)** TGF-β, **(C)** IL-1β, and **(D)** Col1A1 performed with lysates from male and female mice cardiac fibroblasts treated with 20 ng/ml TNF-α for 24 h. Real-time PCR analyses of **(E)** MCP-1 and **(F)** Col1A1 performed with lysates from male and female mice cardiac fibroblasts treated with 10 ng/ml TGF-β for 24 h. Data are shown as means ± SEM (n = 6; independent experiments with technical duplicates). Data are normalized to the male untreated group. *p < 0.05, **p < 0.01, untreated *vs.* treated.

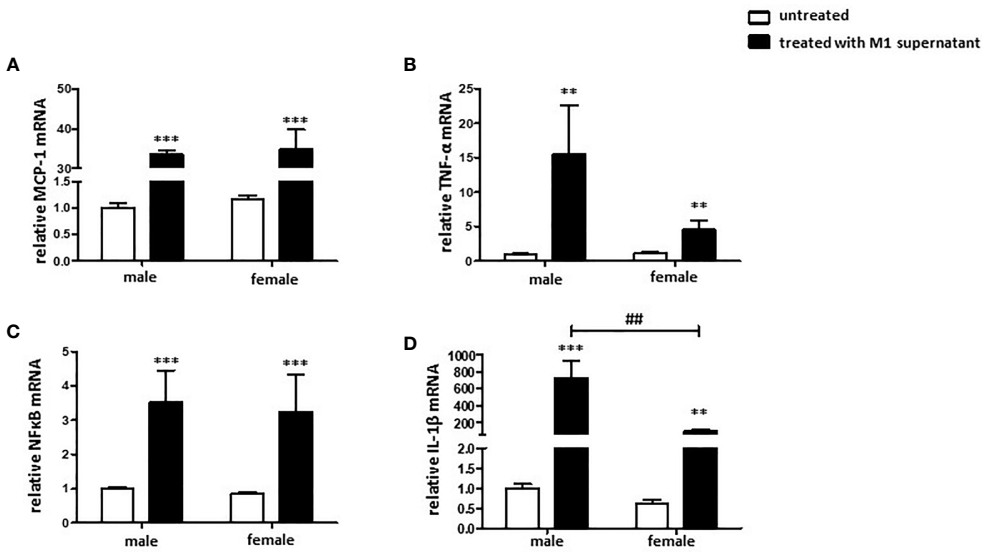

FIGURE 6 | Pro-inflammatory environment promotes a pro-inflammatory and pro-fibrotic fibroblast phenotype. Real-time PCR analyses of **(A)** MCP-1, **(B)** TNF-α, **(C)** NFκB, and **(D)** IL-1β performed with lysates from male and female mice cardiac fibroblasts cultivated with conditioned medium from M1 polarized BMMs for 24 h. Data are shown as means ± SEM (n = 6; independent experiments with technical duplicates). Data are normalized to the male untreated group. **p < 0.01, ***p < 0.001, untreated *vs.* treated; ##p < 0.01, male *vs.* female.

DISCUSSION

In the current study, we investigated sex-related alterations in the polarization of murine BMMs. The main findings are as follows: 1) Male BMMs show a stronger pro-inflammatory response to the LPS treatment than female BMMs; 2) Male BMMs show a more prominent M2 phenotype than females under IL4/IL13 treatment; 3) Treatment of cardiac fibroblasts with TNF-α or

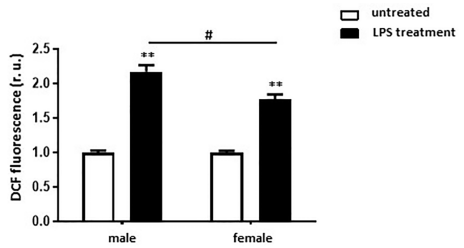

FIGURE 7 | LPS treatment leads to stronger ROS formation in male than in female BMMs. Analysis of ROS formation (DCF fluorescence, r.u.) in untreated and M1 (10 ng/ml LPS for 24 h) male and female macrophages. Data are shown as means ± SEM (n = 6-7; independent experiments with technical duplicates). Data are normalized to the male untreated group. $^{**}p < 0.01$, untreated *vs.* treated; $^{\#}p < 0.05$, male *vs.* female.

conditioned medium from M1 macrophages promotes a stronger pro-inflammatory and pro-fibrotic response in male cells; 4) E2 treatment promotes the pro-inflammatory and suppresses the anti-inflammatory phenotype in male BMMs.

Sex-Dependent M1 and M2 Polarization

Activation of p38 is known as a downstream signaling of LPS and is important in M1 macrophage polarization signaling (12, 37). Our study revealed that p38 was activated *via* LPS in male but not female murine BMMs, suggesting that sex influences the activation of p38. In accordance with our results, p38 activation was higher in male than female myocardium after ischemia-reperfusion injury in rats, leading to a lower myocardial inflammatory response in females (38). Importantly, p38 activation promotes the expression of transcription factors such as NFκB or other pro-inflammatory mediators, e.g., TNF-α and IL-1β (39), suggesting that the male-specific p38 phosphorylation observed in this study may be translated into the stronger pro-inflammatory response.

Indeed, the majority of the pro-inflammatory markers show stronger responses to LPS treatment in male than in female macrophages. Particularly, the key pro-inflammatory transcription factor NFκB (12) was strongly upregulated in M1 male BMMs after 24 h LPS treatment. In addition, typical M1 signature cytokines, i.e., TNF-α and IL-1β, were also strongly upregulated in male cells. These data suggest that LPS treatment elicits a stronger pro-inflammatory response in male than in female BMMs. In line with our findings, the pro-inflammatory response increased in male hearts in a model of experimental autoimmune myocarditis, while female hearts showed less inflammation and an increased number of M2 macrophages, leading to a stronger induction of cardiac inflammation and cardiac dysfunction in male rats (40). It is interesting to note that MCP-1 and YM1 were significantly more upregulated in male than in female M2 macrophages polarized with IL4/IL13, however the expression of RELM-α was similar in male and female BMMs. This sex-dependent difference in the expression of anti-inflammatory markers in M2 BMMs might be explained by the difference in polarization of different M2 macrophage subspecies, e.g., M2a, M2b, M2c, and M2d, which have distinct functions and are activated by different stimuli (15, 30, 41). In contrast, pro-inflammatory M1 markers such as TNF-α were strongly downregulated in male M2 BMMs, suggesting that male macrophages are more susceptible to M2-macrophage polarization.

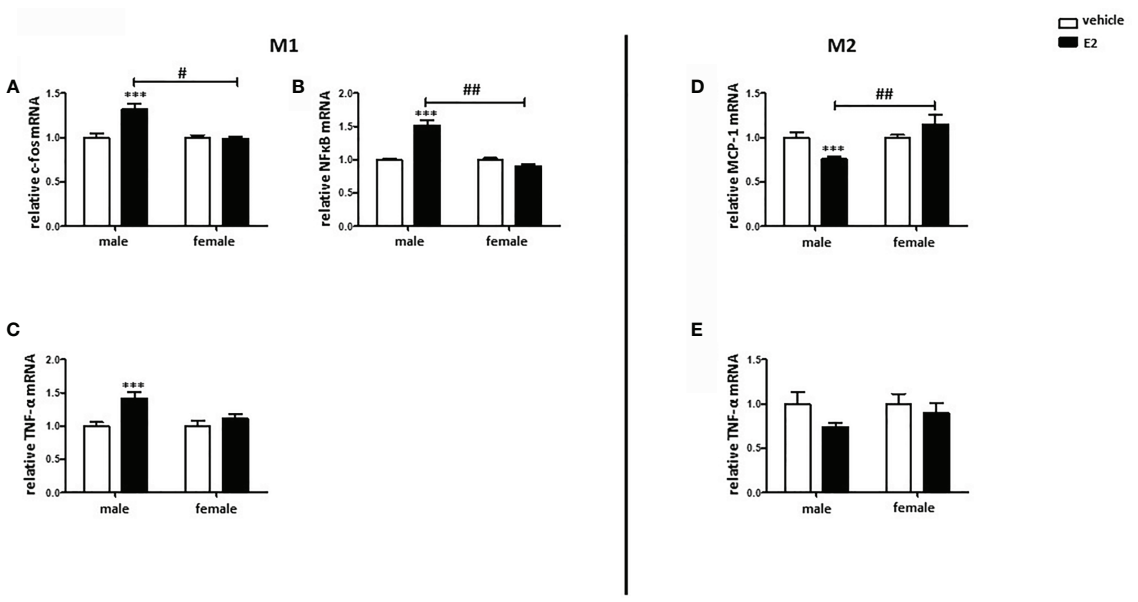

FIGURE 8 | E2 treatment promoted the pro-inflammatory phenotype in male BMMs. Real-time PCR analyses of **(A)** c-fos, **(B)** NFκB, and **(C)** TNF-α in M1 macrophages, as well as **(D)** MCP-1 and **(E)** TNF-α in M2 macrophages with or without E2 treatment (10 nmol/l) for 24 h. Data are shown as means ± SEM (n=6; independent experiments with technical duplicates). Data are normalized to vehicle. $^{***}p < 0.001$, vehicle *vs.* E2 treated; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, male *vs.* female.

Effects of M1 Polarization on ROS Formation

ROS formation plays a central role in many inflammatory diseases, as it is an important mediator of inflammation and cell injury (42). ROS formation was significantly increased after M1 polarization in both male and female BMMs, however the ROS level was significantly higher in male M1 macrophages than in female cells. In accordance with our results, Lagranha et al. reported that several mitochondrial-related sex differences are involved in the modulation of ROS homeostasis (43). Of note, sex hormones, especially E2, modulate mitochondrial ROS production (44, 45). Furthermore, it has been shown, in an atherosclerosis model using ovariectomized female mice, that E2 treatment decreased the expression of NADPH oxidase and the superoxide anion formation, while it increased the expression of two ROS-scavenging enzymes (Cu/ZnSOD and MnSOD), which suppose the E2 anti-oxidative effect (46).

E2 Effects on BMM Polarization

E2 seems to play a crucial role in inflammatory processes (47, 48), and both pro-inflammatory as well as anti-inflammatory effects of E2 have been described (49, 50). In the present study, E2 treatment upregulated the expression of the pro-inflammatory markers, e.g., TNF-α, c-fos, and NFκB, in male M1 macrophages in a pro-inflammatory environment (LPS treatment), whereas it reduced the expression of the anti-inflammatory MCP-1 in male M2 macrophages exposed to an anti-inflammatory environment (IL4/IL13 treatment), suggesting that E2 promotes pro-inflammatory responses in male macrophages. In contrast, E2 had no effects on the expression of any markers investigated in female macrophages. In this regard, it was shown that E2 promotes sex-specific differences in the polarization of macrophages in an asthma animal model, as both male and female macrophages showed an increased expression of M2 genes induced by IL-4 after treatment with a specific ERα agonist, with stronger effects in females (51), thus profoundly impacting the immune system (52). In addition, Villa et al. proposed that E2 treatment decreases the M1 pro-inflammatory phenotype of macrophages, promoting the switch into M2c macrophages (53).

Sex-Dependent Effects of Pro-Inflammatory Stimuli in Cardiac Fibroblasts

Activation of a pro-fibrotic program in cardiac fibroblasts may lead to pathological cardiac remodeling and heart failure (54). Activated fibroblasts express markers like MCP-1 and pro-fibrotic cytokines such as IL-1β (55–57). Moreover, Van Linthout describes TNF-α as crucial for fibroblast activation (21). In accordance with this, we demonstrated that TNF-α treatment significantly increased the expression of pro-fibrotic factors in a sex-independent manner. However, the TNF-α induced fibroblast activation was more prominent in male than in female fibroblasts and female fibroblasts expressed lower levels of pro-fibrotic factors such as TGF-β and IL-1β. Nevertheless, TNF-α did not change the expression of Col1A1 in neither male nor female fibroblasts. Col1A1, produced by fibroblasts *via* the TFG- β pathway, is fundamental for extracellular matrix synthesis and has been shown to play a key role in the development of diseases characterized by pathological fibrosis as well as the metastasis of various tumors (58, 59). Furthermore, TNF-α has been shown to decrease the Col1A1-expression in cultured fibroblasts (60), however our results did not support that finding.

In addition, TNF-α plays a crucial role in crosstalk between macrophages and fibroblasts (21). Depending on the macrophage subtype, macrophages can promote fibroblast activation or inhibition (41). Our study demonstrates an activation of cardiac fibroblasts with the pro-inflammatory supernatant of cultures of M1 macrophages, suggesting that a pro-inflammatory environment promotes a pro-inflammatory and pro-fibrotic phenotype in fibroblasts. Importantly, male fibroblasts showed more prominent effects from this treatment than female fibroblasts.

In addition, exposure to M1 supernatant strongly increased the ROS levels in male fibroblasts (unpublished data).

In conclusion, the present study revealed (i) a sex-dependent pro-inflammatory response to the M1 polarization stimuli in murine BMMs and (ii) a sex-dependent pro-inflammatory and pro-fibrotic response to the M1 macrophage environment in murine cardiac fibroblasts. The data suppose sex hormones and biological sex differences may play a pivotal role in the human immune system, which may dramatically affect cardiac inflammatory diseases, such as myocarditis.

Limitations

We only investigated sex differences in macrophage polarization in C57/Bl6J mice. Since strain differences in the immune cell population have been reported (61, 62), we might consider strain differences in the macrophage polarization.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of the Senate of Berlin, Germany, approval number: T0333/08.

AUTHOR CONTRIBUTIONS

MB conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. MN performed the molecular biological experiments and analyzed the data. CC performed the FACS analysis, characterization of ERs and ROS measurements,

and analyzed the data. ME prepared figures and wrote the main manuscript text. NH performed molecular biological experiments and analyzed the data. AS performed the ROS measurements and analyzed the data. UM-W revised the manuscript. YL analyzed the data and wrote the main manuscript text. VR-Z generated research funds and coordinated the project. All authors commented on the manuscript.

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Sex Hormone–Dependent Lipid Mediator Formation in Male and Female Mice During Peritonitis

*Fabiana Troisi[1], Simona Pace[1], Paul M. Jordan[1], Katharina P. L. Meyer[1], Rossella Bilancia[2], Armando Ialenti[2], Francesca Borrelli[2], Antonietta Rossi[2], Lidia Sautebin[2], Charles N. Serhan[3] and Oliver Werz[1]**

[1]Department of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Jena, Germany, [2]Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Naples, Italy, [3]Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States of America

Introduction: Sex differences in inflammation are obvious and contribute to divergences in the incidence and severity of inflammation-related diseases that frequently preponderate in women. Lipid mediators (LMs), mainly produced by lipoxygenase (LOX) and cyclooxygenase (COX) pathways from polyunsaturated fatty acids (PUFAs), regulate all stages of inflammation. Experimental and clinical studies revealed sex divergences for selected LM pathways without covering the entire LM spectrum, and only few studies have addressed the respective role of sex hormones. Here, we performed the comprehensive LM profile analysis with inflammatory peritoneal exudates and plasma from male and female mice in zymosan-induced peritonitis to identify the potential sex differences in LM biosynthesis during the inflammatory response. We also addressed the impact of sex hormones by employing gonadectomy.

Methods: Adult male and female CD1 mice received intraperitoneal injection of zymosan to induce peritonitis, a well-established experimental model of acute, self-resolving inflammation. Mice were gonadectomized 5 weeks prior to peritonitis induction. Peritoneal exudates and plasma were taken at 4 (peak of inflammation) and 24 h (onset of resolution) post zymosan and subjected to UPLC–MS-MS–based LM signature profiling; exudates were analyzed for LM biosynthetic proteins by Western blot; and plasma was analyzed for cytokines by ELISA.

Results: Pro-inflammatory COX and 5-LOX products predominated in the peritoneum of males at 4 and 24 h post-zymosan, respectively, with slightly higher 12/15-LOX products in males after 24 h. Amounts of COX-2, 5-LOX/FLAP, and 15-LOX-1 were similar in exudates of males and females. In plasma of males, only moderate elevation of these LMs was apparent. At 4 h post-zymosan, gonadectomy strongly elevated 12/15-LOX products in the exudates of males, while in females, free PUFA and LOX products were rather

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***Correspondence:**
Oliver Werz
oliver.werz@uni-jena.de

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPLA2, cytosolic phospholipase A2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FLAP, 5-lipoxygenase-activating protein; LPS, lipopolysaccharide; HDHA, hydroxy-DHA; LM, lipid mediator; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; NSAID, nonsteroidal anti-inflammatory drugs; PUFAs, polyunsaturated fatty acids; PG, prostaglandin; Rv, resolvin; SPM, specialized pro-resolving mediators; TX, thromboxane.

impaired. In plasma, gonadectomy impaired most LMs in both sexes at 4 h with rather up-regulatory effects at 24 h. Finally, elevated 15-LOX-1 protein was evident in exudates of males at 24 h which was impaired by orchiectomy without the striking impact of gonadectomy on other enzymes in both sexes.

Conclusions: Our results reveal obvious sex differences and roles of sex hormones in LM biosynthetic networks in acute self-resolving inflammation in mice, with several preponderances in males that appear under the control of androgens.

Keywords: sex differences, lipoxygenase, lipid mediator, specialized pro-resolving mediators, peritonitis, inflammation

INTRODUCTION

Acute inflammation upon tissue damage or invasion of pathogens is an orchestrated response of the immune defense system, regulated by a variety of signaling molecules including lipid mediators (LMs) (Medzhitov 2010; Serhan 2014; Calder P. C. 2020). LMs are polyunsaturated fatty acid (PUFA)-derived metabolites that encompass pro-inflammatory eicosanoids such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) as well as inflammation-resolving specialized pro-resolving mediators (SPMs, i.e., lipoxins, resolvins, maresins, and protectins) that critically regulate the inflammatory response in a temporal manner (Funk 2001; Serhan 2014; Calder P. C. 2020). While PGs/TXs and LTs are rapidly produced at the onset of inflammation where they initialize and maintain the inflammatory process, the formation of SPMs is delayed and is crucial for the termination of inflammation and for the return to homeostasis (Serhan and Savill 2005; Serhan 2014; Serhan and Levy 2018). Imbalances of these pro-inflammatory and inflammation-resolving LMs may result in excessive and persistent inflammation where PG/TX and LT dominate and SPM levels are low (Tabas and Glass 2013; Chiang and Serhan 2020). In fact, chronic inflammatory diseases like asthma and arthritis, cardiovascular diseases, Alzheimer's disease, type 2 diabetes, and cancer are afflicted with increased ratios of pro-inflammatory eicosanoids vs SPMs (Serhan and Levy 2018; Dalli and Serhan 2019; Chiang and Serhan 2020).

The biosynthesis of LMs is organized in a complex network where multiple enzymes are arranged in connected cascades that act in single cells or *via* transcellular metabolism (Capra et al., 2015; Christie and Harwood 2020). The release of free PUFA from phospholipids by phospholipase A_2 is a critical step in

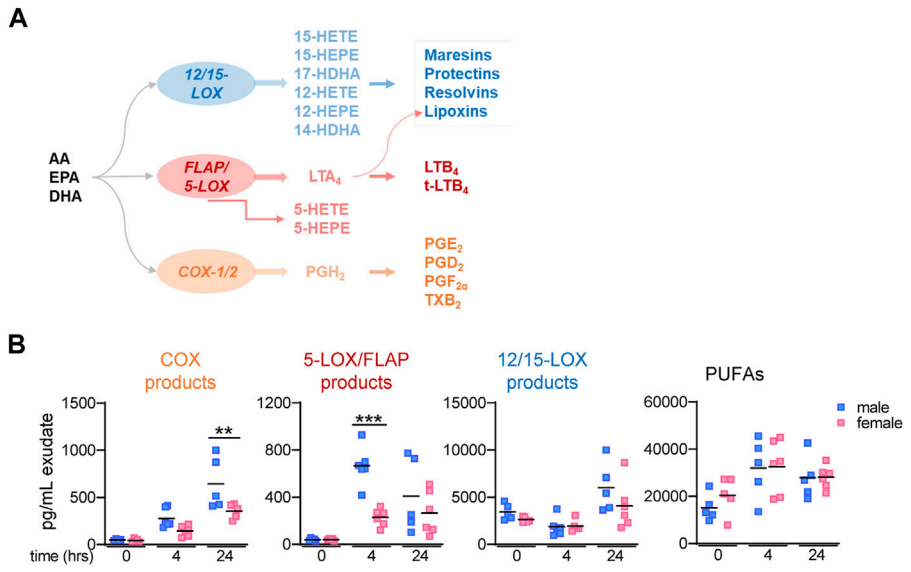

FIGURE 1 | Lipid mediator biosynthetic pathway and lipid mediator levels in mouse peritoneal exudates. **(A)** Scheme of LM biosynthetic pathways yielding 12/15-LOX–, 5-LOX/FLAP–, and COX-1/2–derived products and respective enzymes involved. **(B)** Peritoneal lavages (exudates) from male and female mice were taken at different time points post-zymosan/vehicle i. p. injection. After solid phase extraction, LMs were analyzed by UPLC–MS-MS; time = 0 h: peritoneal lavages immediately taken from mice after treatment with vehicle (no zymosan); t = 4 h and t = 24 h: peritoneal lavages (exudates) post-zymosan. Amounts of relevant LM groups related to respective biosynthetic enzymes: COX products (sum of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2), 5-LOX products (sum of LTB_4, t-LTB_4, 5-HETE, and 5-HEPE), 12/15-LOX products (sum of 12-HETE, 12-HEPE, 14-HDHA, 15-HETE, 15-HEPE, and 17-HDHA), and PUFA (sum of AA, EPA, and DHA). Results are shown in pg/mL exudate in scatter dot plots of individual data points and the mean in black lines; n = 5–6; ***$p < 0.001$ male vs. female; two-way ANOVA with Šídák's multiple comparison test.

providing sufficient amounts of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as LM substrates (Astudillo et al., 2019; Murakami et al., 2017). Conversion of AA *via* the cyclooxygenase (COX) pathway yields mainly pro-inflammatory prostanoids such as PGE_2, PGD_2, $PGF_2\alpha$, and TXA_2 (Smith et al., 2011), while the AA metabolism *via* the 5-LOX pathway, aided by 5-LOX-activating protein (FLAP), leads to pro-inflammatory LTB_4 and cysteinyl-containing LT (Radmark et al., 2015) (**Figure 1A**). In SPM formation, PUFAs are first oxygenated by 12- or 15-LOX to generate 12- or 15-hydroxyeicosatetraenoic acid (12- or 15-HETE) from AA, 12- or 15-hydroxyeicosapentaenoic acid (12- or 15-HEPE) from EPA, and 14- and 17-hydroxydocosahexaenoic acid (14- or 17-HDHA) from DHA, respectively (Chiang and Serhan 2020) (**Figure 1A**). In a second step, these monohydroxylated products are converted by 12/15-LOXs to generate the di-hydroxylated protectins and maresins, while 5-LOX or 15-LOX accomplish the formation of tri-hydroxylated lipoxins and resolvins *via* hydroperoxidation, epoxide formation, and epoxide hydrolysis; reduction of these hydroperoxides yields di-hydroxylated resolvins (Chiang and Serhan 2020) (**Figure 1A**).

Sex differences in immune responses are obvious and contribute to divergences in the incidence and severity of inflammation-related disorders, especially autoimmune diseases (Klein and Flanagan 2016; Rosen et al., 2017). Notably, a variety of inflammatory diseases related to elevated PG and LT levels are sex-biased and preponderate in women, for example, rheumatoid arthritis and asthma (Pace et al., 2017c; Pace and Werz 2020). The side-by-side analysis of LT formation in neutrophils, monocytes, and macrophages from male and female human beings and/or rodent subjects revealed superior LT levels in samples from females (Pergola et al., 2008; Pergola et al., 2011; Rossi et al., 2014; Pace et al., 2017a). Androgens are causative for these sex divergences, as they suppressed LT formation by preventing the intracellular assembly of the LT-biosynthetic 5-LOX/FLAP complex (Pergola et al., 2008; Pace et al., 2017a), involving extracellular signal–regulated protein kinase (ERK)-1/2 (Pergola et al., 2008) and/or phospholipase (PL)D (Pergola et al., 2011) signaling routes. In an asthma model, LT levels increased only in lungs of female but not in those of male mice during allergen sensitization along with superior airway hyperreactivity and pulmonary inflammation (Rossi et al., 2019). Androgen administration to sensitized female mice abrogated this sex bias in LT-mediated asthma (Cerqua et al., 2020). Also sex differences in the PG biosynthesis were reported (for review, see Pace et al. (2017c)), where PG levels often dominate in males, even though opposite findings were apparent, depending on the type of organ or tissue and the experimental settings, for example, the time point of analysis and age of the subjects with consequences for the hormonal status (Pace et al., 2017a; Pace et al., 2017b). Finally, sex differences in the SPM biosynthesis were evident upon the analysis of plasma from healthy or diseased humans and from experimental studies with humans or rodents. Obviously, EPA and DHA are more efficiently biosynthesized in females, and females have higher plasma levels of DHA than males (Calder PC. 2020). However, there is currently no consistent sex bias in the levels of SPM and their precursors, which in some studies dominate in males (English et al., 2017; Barden et al., 2018; Pullen et al., 2020), while in other studies, they preponderate in female subjects (Barden et al., 2015; Rathod et al., 2017; Halade et al., 2020; Trotta et al., 2021; Yaeger et al., 2021). Moreover, the influence of sex hormones on the SPM biosynthesis is rather elusive.

There is obviously no consisting pattern of sex differences that unequivocally proves preponderances of all different LMs in males or females. With some few exceptions (English et al., 2017; Rathod et al., 2017; Yaeger et al., 2021), most studies addressing sex differences and/or the impact of sex hormones related to the LM biosynthesis focused on single selected pathways, namely, either 5-LOX/LTs or COX/prostanoids, or SPM, and neglected the others. Here, we aimed at obtaining comprehensive LM signature profiles including pro-inflammatory LT and PG/TX and inflammation-resolving SPM in inflammatory exudates and plasma from male and female mice at the peak (4 h) and the resolution (24 h) of acute inflammation employing zymosan-induced peritonitis as an appropriate experimental model (Cash et al., 2009). Moreover, using this model, we aimed at revealing the impact of sex hormones on the LM biosynthesis in acute inflammation by employing gonadectomy of male and female mice in order to abrogate endogenous sex hormone production (Scotland et al., 2011; Rossi et al., 2014).

MATERIALS AND METHODS

Animals

Adult (5–6 weeks) male and female CD1 mice (Charles River, Calco, Italy) were housed at the animal care facility of the Department of Pharmacy of the University of Naples "Federico II" and kept under controlled environment (i.e., temperature 21 ± 2°C and humidity 60 ± 10%) with free access to normal chow and water. Experiments were conducted after 4 days of acclimation of the mice during the light phase of a 12-h light/dark schedule. The experimental procedures were approved by the Italian Ministry and carried out in accordance with the EU Directive 2010/63/EU and the Italian DL 26/2014 for animal experiments, and in compliance with the ARRIVE guidelines and Basel declaration including the 3R concept.

Surgical Removal of Gonads

In order to investigate the impact of sex hormones, male and female mice underwent gonadectomy. Orchidectomy was performed as previously described (Rossi et al., 2014). In brief, male mice were anesthetized using xylazine (10 mg/kg) and ketamine (100 mg/kg), and subsequently immobilized, shaved, and disinfected. Testes were removed through a perineal raphe access. Then, the scrotum was closed by a single stitch. For the ovariectomies, female mice were immobilized with a ventral decubitus after anesthesia, and ovaries were removed by applying two cuts on the lateral side of the rachis (left and right); then, a suture with a single stitch was applied. For both

groups of mice with orchidectomy and ovariectomy, respective control groups (male and female) that were "sham-operated" without the removal of the gonads were used. Animals were allowed to recover for 5 weeks after the surgery which is the time necessary for the turnover of the sexual hormone levels in the body.

Zymosan-Induced Peritonitis in Mice

Peritonitis was induced in male and female "sham-operated" and gonadectomized mice (five to six per group), respectively, 5 weeks after surgery. The protocol utilized for the induction of peritonitis followed the procedures and conditions that were described by us before (Rossi et al., 2014; Pace et al., 2017a; Pace et al., 2017b). Briefly, after intraperitoneal (i.p.) injection of 1 mg zymosan as suspension in 0.5 ml of saline per mouse, the animals were euthanized in a saturated atmosphere with CO_2 at selected time points (4 and 24 h post-zymosan), and blood and peritoneal exudates were collected. A control group "time zero" (no zymosan) for sham-operated and gonadectomized mice was included. Peritoneal exudates were obtained by washing the cavity with 3 ml of sterile filtered cold PBS. These peritoneal exudates were then centrifuged at 20,000 × g for 20 min in order to remove cells. Samples were immediately frozen at −80°C. Blood (0.7–0.9 ml) was collected by intracardiac puncture (22-gauge needle) using citrate as an anticoagulant (3.8% (w/v)), immediately after killing of mice with CO_2, and centrifuged at 800 × g at 4 °C for 10 min in order to obtain plasma. Cell-free exudates and plasma were used for the measurements of LMs, Western blot analysis, and cytokine analysis.

Cytokine Analysis

ELISA kits for the detection of murine IL-1β, IL-6, and TNF-α in peritoneal exudates (R&D systems, Wiesbaden-Nordenstadt, Germany) were used according to the instructions of the manufacturers.

Sample Preparation and Solid Phase Extraction of Lipid Mediators

Peritoneal exudates (1 ml) and plasma (0.15 or 0.2 ml) were diluted in ice-cold methanol (ratio 1:3, v/v). Deuterium-labeled internal standards were added to each sample, as follows: d8-5S-HETE, d4-PGE_2, d4-LTB_4, d5-LXA_4, d5-RvD2, and d8-AA (500 pg, each). Samples were kept in methanol at −20°C for 60 min to allow protein precipitation and then centrifuged (1,200 × g, 4 °C, 10 min). Samples were diluted with 8 ml of acidified water (pH 3.5) immediately before solid phase extraction. The solid phase C18 cartridges (SepPak Vac C18 6cc, Waters, Milford, MA, United States) were equilibrated with 6 ml of methanol before the addition of 2 ml of water. Acidified samples were loaded onto the conditioned C18 cartridges and washed with 6 ml of water and 6 ml of *n*-hexane. LMs were eluted with 6 ml of methyl formate. Subsequently, eluted samples (6 ml) were evaporated until dryness using an evaporation system (TurboVap LV, Biotage, Uppsala, Sweden) with nitrogen (5–10 psi). Samples were then resuspended in 100 μL methanol/water (1:1, v/v) and centrifuged twice (15,000 × g, 5 min, 4 °C) for UPLC–MS-MS automated injections.

Analysis of Lipid Mediators by UPLC–MS-MS

LMs were analyzed with an Acquity™ UPLC system (Waters, Milford, MA, United States) and a QTRAP 5500 mass spectrometer (ABSciex, Darmstadt, Germany) equipped with a Turbo V™ Source and electrospray ionization. LMs were separated using an ACQUITY UPLC® BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Eschborn, Germany) at 50°C with a flow rate of 0.3 ml/min and a mobile phase consisting of methanol–water–acetic acid of 42:58:0.01 (v/v/v) that was ramped to 86:14:0.01 (v/v/v) over 12.5 min and then to 98:2:0.01 (v/v/v) for 3 min (Werner et al., 2019). QTRAP 5500 was operated in the negative ionization mode using scheduled multiple reaction monitoring (MRM) coupled with information-dependent acquisition. The scheduled MRM window was 60 s, optimized LM parameters were adopted (Werner et al., 2019), and the curtain gas pressure was set to 35 psi. The retention time and at least six diagnostic ions for each LM were confirmed by means of an external standard (Cayman Chemical/Biomol GmbH, Hamburg, Germany). Quantification was achieved by calibration curves for each LM. Linear calibration curves were obtained for each LM and gave r^2 values of 0.998 or higher. Additionally, the limit of detection for each targeted LM was determined (Werner et al., 2019).

Western Blot Analysis

Exudates corresponding to equal amounts of proteins were separated by electrophoresis on SDS–polyacrylamide gels (10% for 5-LOX, p12-LOX, 15-LOX-1, COX-2, and β-actin; 16% for FLAP) and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Freiburg, Germany). Ponceau in 5% (v/v) acetic acid was used as a stain to confirm the correct loading and protein transfer to the membrane. After blocking with 0.1% TBS/Tween containing 5% BSA (1 h, room temperature), the membranes were washed and incubated overnight at 4 °C with primary antibodies as follows: monoclonal mouse anti–5-LOX-1:1,000 (Clone 33/5-Lipoxygenase, BD Biosciences, San Jose, CA, United States); polyclonal rabbit anti–15-LOX-1, 1:200 (Abcam, ab80221); COX-2 rabbit, polyclonal 1:1,000 (Cat#4842S, Cell Signaling, Danvers, MA, United States); FLAP rabbit, polyclonal 1:1,000 (Cat#ab85227, Abcam, Cambridge, UK); platelet-type 12-LOX mouse monoclonal, 1:300 (Cat#sc-365194, Santa Cruz Biotechnology, Heidelberg, Germany); and β-actin rabbit, monoclonal 1:1,000 (Cat#3700, Cell Signaling). After incubation, membranes were washed with 0.1% TBS-Tween (3 × 10 s) and were incubated for 1.5 h at room temperature with fluorescence-labeled secondary antibodies IRDye 800 CW (dilution 1:10.000) and IRDye 680 LT (dilution 1:80.000) for 1 h, at RT. After washing and overnight drying, immune-reactive bands were visualized by an Odyssey infrared imager (LI-COR Biosciences).

TABLE 1 | Lipid mediator profiling in peritoneal exudates of male and female mice. Amounts of LM in peritoneal exudates from male and female mice taken at different time points post-zymosan/vehicle i.p. injection (see legends **Figure 1**). Data are given in pg/mL exudate expressed as mean ± SEM of n = 5 to 6 animals per group and as heatmap. Fold-change of female mean values compared to male mean values. *$p < 0.05$, **$p < 0.01$ male 0 h vs. Four or 24 h; #$p < 0.05$, ##$p < 0.01$ female 0 h vs. Four or 24 h; unpaired Student's *t* test.

No	0 h			4 h			24 h		
	Male	Female	Fold	Male	Female	Fold	Male	Female	Fold
COX products									
PGE_2	13 ± 2.3	12 ± 1.5	0.9	41 ** ± 6.9	34## ± 4.2	0.8	80 * ± 18	54## ± 3.7	0.7
PGD_2	5.6 ± 1.1	3.2 ± 0.5	0.6	18 ** ± 2.6	10## ± 1.4	0.6	23 * ± 5.1	16## ± 1.2	0.7
PGF_2	7.7 ± 2.9	4.3 ± 0.6	0.6	106 ** ±18	41# ± 9.4	0.4	230 ** ± 23	115## ± 10	0.5
TXB_2	38 ± 9.1	24 ± 6.6	0.6	113 ** ±18	60# ± 8.9	0.5	314 * ± 81	189##± 20	0.6
5-LOX products									
LTB_4	n.d	n.d	-	65 ** ± 8.5	17##± 2.4	0.3	32 ± 14	21# ± 7.5	0.7
t-LTB_4	21 ± 4.1	15 ± 1.4	0.7	15 ± 1.7	6.7## ± 1.0	0.4	17 ± 4.0	15 ± 3.9	0.9
5-HEPE	3.5 ± 0.6	5.7 ± 1.7	1.6	48 ** ± 6.5	26## ± 4.9	0.5	26 ± 11	15# ± 2.7	0.6
5-HETE	16 ± 2.1	20 ± 2.9	1.3	538** ±56	179## ± 23	0.3	334 * ± 114	216# ± 62	0.6
12/15-LOX products									
14-HDHA	339 ± 49	286 ± 23	0.8	266 ± 41	534 ± 164	2.0	767 ± 180	669 ± 165	0.9
12-HEPE	1,393 ± 144	1,000 ± 67	0.7	534** ± 110	567## ± 78	1.1	2,186 ± 408	1,536 ± 408	0.7
12-HETE	1,327 ± 137	952 ± 64	0.7	509** ± 105	540## ± 74	1.1	2,082 ± 389	1,001 ± 162	0.5
17-HDHA	84 ± 22	91 ± 12	1.1	101 ± 13	186 ± 46	1.8	317 ± 97	278# ± 66	0.9
15-HEPE	43 ± 3.8	53 ± 6.6	1.2	35 ± 3.1	68 ± 8.3	2.0	90 * ± 13	65 ± 10	0.7
15-HETE	239 ± 57	235 ± 20	1.0	249 ± 24	268 ± 42	1.1	561 * ± 105	283 ± 50	0.5
PDX	<3	<3	-	n.d	n.d	-	n.d	n.d	
PD1	<3	<3	-	n.d	n.d	-	3.8 ± 0.8	5.9 ± 1.7	1.6
RvD5	4.5 ± 0.5	n.d	-	12 ± 2.1	4.0## ± 0.7	0.3	4.5 ± 2.0	3.5# ± 1.3	0.8
LXA_4	<3	<3	-	n.d	n.d	-	<3	<3	-
PUFAs									
AA	10,790 ± 1,496	14,536 ± 2,422	1.3	18,086 * ± 2,614	17,695 ± 2,126	1.0	19,187 * ± 2,092	18,697 ± 1,716	1.0
EPA	834 ± 106	847 ± 162	1.0	1,950* ± 417	2,135# ± 474	1.1	1,682 ± 411	1,471# ± 183	0.9
DHA	3,499 ± 968	5,111 ± 1,222	1.5	11,935 * ± 2,679	12,755#± 2,120	1.1	7,022 ± 1,488	7,941 ± 442	1.1

Statistical Analysis

Results are expressed as mean ± S.E.M. of the mean of *n* observations, where *n* represents the number of animals or number of samples analyzed. Results were analyzed by 2-tailed Student's *t* test, one-way ANOVA followed by Tukey *post hoc* tests, or two-way ANOVA followed by Šídák's multiple comparison test. A *p*-value less than 0.05 was considered significant. Outliers were identified by Grubbs'outlier test.

RESULTS

Sex Differences in the Lipid Mediator Formation in Zymosan-Challenged Peritoneum of Mice

In order to investigate if LM signature profiles produced during acute inflammation differ in mammals between sexes, we employed the well-established and suitable model of zymosan-induced peritonitis in male and female mice (Cash et al., 2009; Rossi et al., 2014). Targeted LM metabololipidomics using UPLC–MS-MS of peritoneal exudates were obtained from mice 4 h after zymosan injection, which is the temporal peak of inflammatory reactions (Rossi et al., 2014), and 24 h post-zymosan, which corresponds to the onset of inflammation resolution (Serhan, 2014). In addition, exudates from mice where vehicle was injected i.p. instead of zymosan and sacrificed right thereafter (designated as t = 0) were used as samples of unstimulated "healthy" controls. For the sake of simplicity in presentation, we grouped LMs into COX products (sum of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2), 5-LOX products (sum of LTB_4, t-LTB_4, 5-HETE, and 5-HEPE), 12/15-LOX products (sum of 12-HETE, 12-HEPE, 14-HDHA, 15-HETE, 15-HEPE, and 17-HDHA), and PUFAs (sum of AA, EPA, and DHA). In vehicle-treated "healthy" mice (t = 0), COX and 5-LOX products in the exudates were low, while substantial amounts of 12/15-LOX products and PUFAs were detectable, without obvious sex differences for any LM group (**Figure 1B**). At 4 and 24 h post-zymosan, COX and 5-LOX products were strongly elevated with a tendency of higher levels in males vs females, being significant for 5-LOX products at 4 h and for COX products at 24 h (**Figure 1B**). In contrast, the amounts of 12/15-LOX products at 4 h were not changed vs vehicle-treated mice but elevated at 24 h after zymosan with a higher tendency in males.

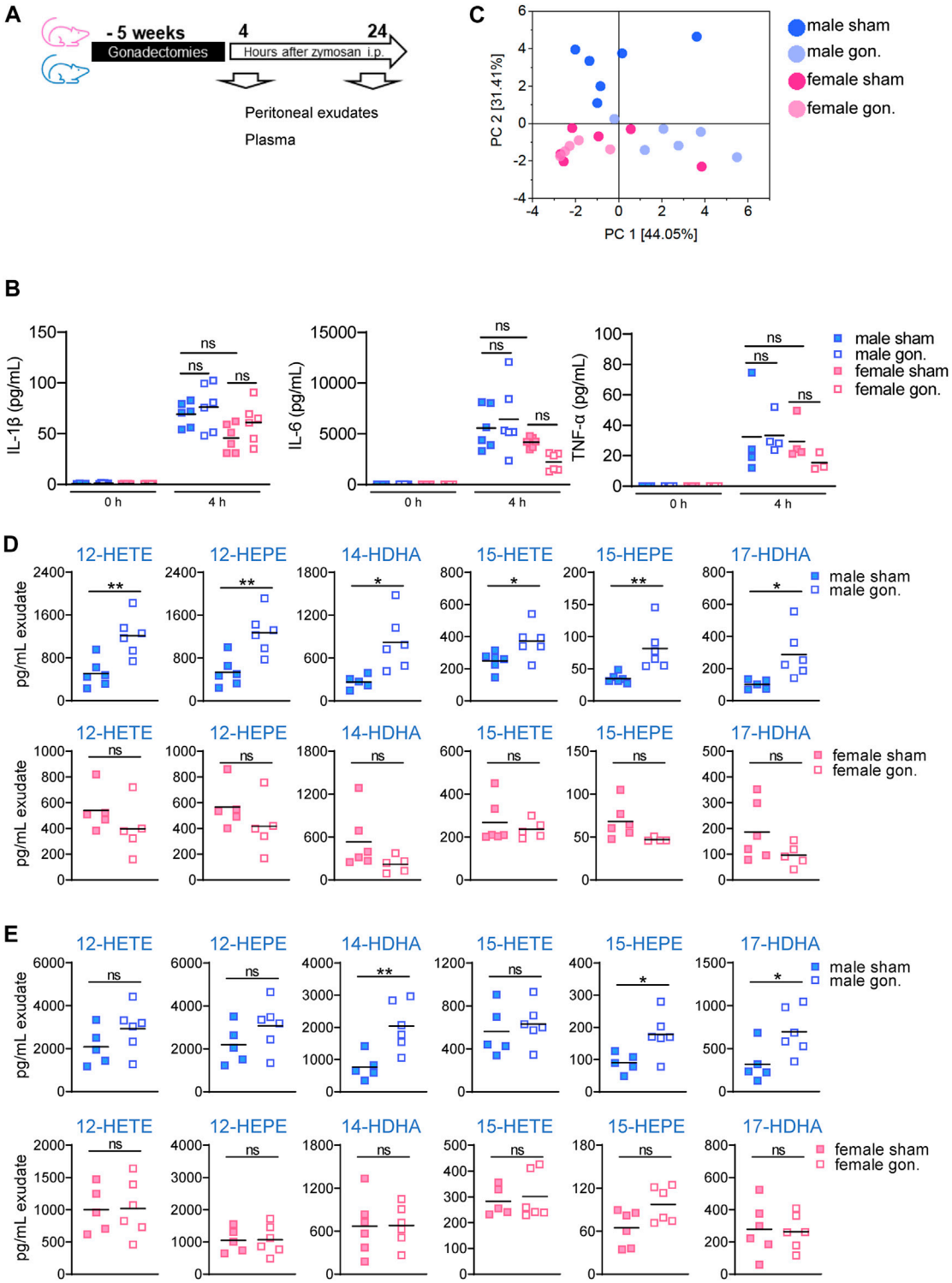

FIGURE 2 | Effect of hormone depletion on the LM content in peritoneal exudates from male and female mice upon peritonitis induction. **(A)** Experimental setup and time line. **(B)** Amounts of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in peritoneal exudates of gonadectomized and non-gonadectomized (sham-operated) male and female mice after treatment with vehicle (no zymosan = 0 h) and 4 h after zymosan injection. Results are shown in pg/mL exudate in scatter dot plots as individual data points and means as black line; n = 4–6; ordinary one-way ANOVA with Tukey's multiple comparison test. **(C)** Principal component analysis of LM profiles in the exudates from gonadectomized and non-gonadectomized (sham-operated) male and female mice, 4 h after zymosan injection (corresponding raw data are shown in **Table 2**). **(D,E)** Amounts of 12/15-LOX products in peritoneal exudates of gonadectomized and non-gonadectomized (sham-operated) male and female mice 4 h **(D)** and 24 h **(E)** after zymosan injection. Results are shown in pg/mL exudate in scatter dot plots as individual data points and the means as black lines; n = 5–6; $^{*}p < 0.05$, $^{**}p < 0.01$ non-gonadectomized vs. gonadectomized; unpaired Student's *t* test; ns = not significant.

TABLE 2 | Effect of hormone depletion on lipid mediator profiles in peritoneal exudates from male and female mice at 4 h post-zymosan injection. Amounts of LM in peritoneal exudates from male and female mice 4 h post zymosan/vehicle i.p. injection that underwent gonadectomy or sham-operation (see legend **Figure 2**). Data are given in pg/mL exudate expressed as mean ± SEM of n = 5 to 6 animals per group and as heatmap. Fold-change of gonadectomy mean values compared to sham mean values of male and female mice.

No	4 h					
	Male sham	Male gon	Fold	Female sham	Female gon	Fold
COX products						
PGE_2	41 ± 6.9	71 ± 8.9	1.7	34 ± 4.2	43 ± 3.2	1.3
PGD_2	18 ± 2.6	22 ± 3.1	1.2	10 ± 1.4	12 ± 1.2	1.2
PGF_2	106 ± 18	61 ± 12	0.6	41 ± 9.4	53 ± 5.8	1.3
TXB_2	113 ± 18	139 ± 19	1.2	60 ± 8.9	81 ± 8.2	1.4
5-LOX products						
LTB_4	65 ± 8.5	25 ± 3.2	0.4	17 ± 2.4	11 ± 2.4	0.7
t-LTB_4	15 ± 1.7	9.0 ± 0.9	0.6	6.7 ± 1.0	5.1 ± 0.9	0.8
5-HEPE	48 ± 6.5	20 ± 4.4	0.4	26 ± 4.9	6.6 ± 1.1	0.3
5-HETE	538 ± 56	244 ± 51	0.5	179 ± 23	69 ± 11	0.4
12/15-LOX products						
14-HDHA	266 ± 41	821 ± 159	3.1	534 ± 164	220 ± 51	0.4
12-HEPE	534 ± 110	1,275 ± 161	2.4	567 ± 78	417 ± 96	0.7
12-HETE	509 ± 105	1,214 ± 153	2.4	540 ± 74	397 ± 91	0.7
17-HDHA	101 ± 13	287 ± 62	2.8	186 ± 46	96 ± 19	0.5
15-HEPE	35 ± 3.1	82 ± 14	2.3	68 ± 8.3	47 ± 1.2	0.7
15-HETE	249 ± 24	372 ± 43	1.5	268 ± 42	236 ± 19	0.9
RvD5	12 ± 2.2	4.6 ± 1.3	0.4	4.0 ± 0.7	<3	
PUFAs						
AA	18,086 ± 2,614	16,158 ± 2,011	0.9	17,695 ± 2,126	8,763 ± 620	0.5
EPA	1,950 ± 417	1,341 ± 206	0.7	2,135 ± 474	943 ± 186	0.4
DHA	11,935 ± 2,679	9,638 ± 1,630	0.8	12,755 ± 2,120	3,874 ± 524	0.3

Moderate elevations without differences between sexes were found for PUFAs (**Figure 1B**).

More detailed analysis of single LM members within the COX/LOX groups at t = 0, 4, and 24 h is shown in **Table 1**. Among COX products, PGE_2 levels were somewhat elevated in males vs females at all three time points, but $PGF_2\alpha$ and TXB_2 clearly dominated in exudates of males, especially at 4 h. Similarly, all 5-LOX products were higher in exudates of males after 4 and 24 h, especially the major products, namely, LTB_4 and 5-HETE. In contrast, 12/15-LOX-derived 14-HDHA, 17-HDHA, and 15-HEPE were higher in female mice but only at 4 h, even though RvD5 was lower. At t = 0 and at 24 h, these 12/15-LOX products were hardly different between the sexes or somewhat lower in females (**Table 1**). Taken together, sex differences in LM levels in exudates during acute peritonitis are obvious not only for pro-inflammatory COX and 5-LOX products that were elevated in males at 4 and 24 h but also for 12/15-LOX products elevated in males at 24 h, without accompanying the differences in free PUFA levels.

Gonadectomy-Mediated Sex Hormone Depletion Differentially Affects Lipid Mediator Abundance in Male and Female Mice.

Since sex hormones are known to affect 5-LOX product levels *in vitro* and *in vivo*, where androgens such as 5α-DHT act as endogenous suppressors in neutrophils, monocytes, and macrophages (Pace et al., 2017a; Pergola et al., 2008; Pergola, et al., 2011), and progesterone down-regulates LTs in monocytes (Pergola et al., 2015), we studied how ablation of sex hormone production in mice would affect LM levels during subsequently induced peritonitis. Male and female mice were gonadectomized or sham-operated and allowed to recover for 5 weeks after surgery, which is the time necessary for the turnover of the sex hormone levels (Scotland et al., 2011; Rossi et al., 2014). Then, zymosan was injected, and animals were sacrificed after 4 and/or 24 h for the analysis of cytokines and LMs in peritoneal exudates and systemically in plasma (**Figure 2A**).

The levels of the pro-inflammatory cytokines IL-1β, TNFα, and IL-6 in the exudates 4 h post-zymosan were not significantly different between the sexes of sham-operated mice, although a tendency for elevated levels of IL-1β and IL-6 in exudates of males vs females was observed (**Figure 2B**). Also, gonadectomy did not significantly alter peritoneal cytokine levels in either sex, but a trend toward lower levels for TNFα and IL-6 was seen in female but not in male mice (**Figure 2B**). At 4 h post-zymosan, pro-inflammatory 5-LOX products were lower in the exudates after gonadectomy of females but also of males, while the amounts of COX products in parallel were somewhat increased, except $PGF_2\alpha$ in males (**Table 2**). In contrast, significant and striking elevation of the 12/15-LOX products 17-HDHA, 14-HDHA, 15-HETE, 15-

TABLE 3 | Effect of hormone depletion on lipid mediator profiles in peritoneal exudates from male and female mice at 24 h post-zymosan injection. Amounts of LM in peritoneal exudates from male and female mice 24 h post-zymosan/vehicle i.p. injection that underwent gonadectomy or sham-operation (see legend **Figure 2**). Data are given in pg/mL exudate expressed as mean ± SEM of n = 5 to 6 animals per group and as heatmap. Fold-change of gonadectomy mean values compared to sham mean values of male and female mice.

No	4 h					
	Male sham	Male gon	Fold	Female sham	Female gon	Fold
COX products						
PGE_2	80 ± 18	75 ± 5.6	0.9	54 ± 3.7	42 ± 6.8	0.8
PGD_2	23 ± 5.1	29 ± 5.6	1.3	16 ± 1.2	12 ± 2.3	0.7
PGF_2	230 ± 23	159 ± 11	0.7	115 ± 10	100 ± 16	0.9
TXB_2	314 ± 81	282 ± 29	0.9	189 ± 20	147 ± 36	0.8
5-LOX products						
LTB_4	32 ± 14	45 ± 9.2	1.4	21 ± 7.5	5.2 ± 0.7	0.2
t-LTB_4	17 ± 4.0	23 ± 3.2	1.3	15 ± 3.9	6.5 ± 0.7	0.4
5-HEPE	26 ± 11	50 ± 13	1.9	15 ± 2.7	17 ± 3.1	1.2
5-HETE	334 ± 114	459 ± 76	1.4	216 ± 62	120 ± 31	0.6
12/15-LOX products						
14-HDHA	767 ± 180	2,041 ± 304	2.7	669 ± 165	680 ± 115	1.0
12-HEPE	2,186 ± 408	3,072 ± 453	1.4	1,052 ± 170	1,072 ± 188	1.0
12-HETE	2,082 ± 389	2,926 ± 431	1.4	1,001 ± 162	1,021 ± 179	1.0
17-HDHA	317 ± 97	696 ± 111	2.2	278 ± 66	264 ± 45	1.0
15-HEPE	90 ± 13	178 ± 27	2.0	65 ± 10	97 ± 10	1.5
15-HETE	561 ± 105	632 ± 78	1.1	283 ± 50	301 ± 38	1.1
PD1	3.8 ± 0.8	12 ± 1.0	3.1	5.9 ± 1.7	6.7 ± 1.2	1.1
RvD5	4.5 ± 2.0	7.3 ± 1.1	1.6	3.5 ± 1.3	<3	
LXA_4	<3	<3	-	<3	<3	-
PUFAs						
AA	19,187 ± 2,092	26,529 ± 929	1.4	18,697 ± 1,716	19,272 ± 2,501	1.0
EPA	1,682 ± 411	3,271 ± 562	1.9	1,471 ± 183	2,377 ± 275	1.6
DHA	7,022 ± 1,488	13,950 ± 1,382	2.0	7,941 ± 442	9,044 ± 703	1.1

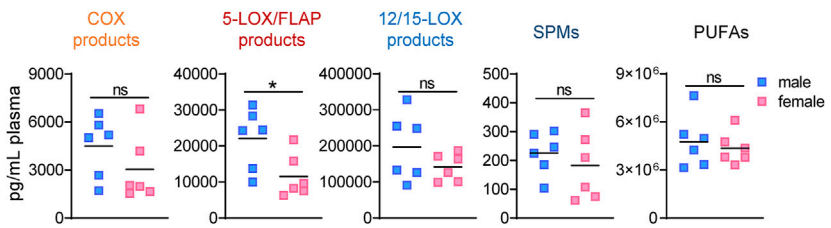

FIGURE 3 | Lipid mediator levels in plasma of male and female mice during peritonitis. Amounts of COX products (sum of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2), 5-LOX products (sum of LTB_4, t-LTB_4, 5-HETE, and 5-HEPE), 12/15-LOX products (sum of 12-HETE, 12-HEPE, 14-HDHA, 15-HETE, 15-HEPE, and 17-HDHA), and PUFA (sum of AA, EPA, and DHA) in plasma of male and female mice 4 h after zymosan injection. Results are shown in pg/mL plasma in scatter dot plots as individual data points and the means as black lines; n = 5–6; *$p < 0.05$, male vs. female; unpaired Student's *t* test; ns = not significant.

HEPE, 12-HETE, and 12-HEPE was evident after orchiectomy in exudates of male mice but rather impaired in those of female animals after ovariectomy (**Table 2**). Gonadectomy caused lower free PUFA levels in exudates of female mice with only very small effects in males (**Table 2**). We found marked differences in the LM profiles between the various groups of mice by employing the unbiased principal component analysis (PCA). In detail, LM metabolomes from male sham-operated mice clustered in a different manner from those derived from other mice, implying an obvious impact of sex hormones, seemingly androgens (**Figure 2C**).

The increased levels of 12/15-LOX products in exudates of male mice after orchiectomy were essentially maintained also at 24 h post-zymosan, where again in female animals such elevations due to ovariectomy were absent, except a tendency for 15-HEPE (**Table 3**; **Figure 2D**). Again, ovariectomy caused lower 5-LOX products in exudates of females at 24 h after zymosan, while in contrast to 4 h, 5-LOX products were

TABLE 4 | Effect of hormone depletion on lipid mediator profiles in plasma from male and female mice at 4 h post-zymosan injection. Amounts of LM in plasma from male and female mice 4 h post zymosan/vehicle i.p. injection that underwent gonadectomy or sham-operation. Data of n = 5 to 6 animals per group are shown as mean ± SEM in pg/mL plasma and as heatmap. Fold-change of gonadectomy mean values compared to sham mean values of male and female mice.

No	4 h					
	Male sham	Male gon	Fold	Female sham	Female gon	Fold
COX products						
PGE_2	2,512 ± 487	790 ± 237	0.3	1,581 ± 494	234 ± 139	0.1
PGD_2	1,365 ± 262	504 ± 163	0.4	896 ± 292	142 ± 87	0.2
PGF_2	177 ± 22	58 ± 12	0.3	117 ± 20	14 ± 10	0.1
TXB_2	434 ± 17	272 ± 31	0.6	439 ± 54	93 ± 46	0.2
5-LOX products						
LTB_4	140 ± 20	39 ± 7.2	0.3	100 ± 34	66 ± 16	0.7
t-LTB_4	311 ± 51	85 ± 26	0.3	175 ± 55	83 ± 13	0.5
5-HEPE	1,075 ± 87	672 ± 99	0.6	726 ± 84	892 ± 106	1.2
5-HETE	20,508 ± 3,305	7,770 ± 2,085	0.4	10,531 ± 2,388	4,516 ± 927	0.4
12/15-LOX products						
14-HDHA	66,682 ± 15,812	37,727 ± 4,506	0.6	53,620 ± 5,559	32,120 ± 4,562	0.6
12-HEPE	18,230 ± 5,189	7,142 ± 1,154	0.4	12,196 ± 2,055	10,382 ± 1890	0.9
12-HETE	82,404 ± 16,431	45,061 ± 3,486	0.5	59,519 ± 6,788	34,559 ± 6,476	0.6
17-HDHA	5,060 ± 535	2,478 ± 339	0.5	3,131 ± 441	1,660 ± 93	0.5
15-HEPE	409 ± 22	276 ± 41	0.7	314 ± 50	387 ± 23	1.2
15-HETE	24,205 ± 2,339	7,698 ± 1,630	0.3	12,563 ± 2,739	5,257 ± 994	0.4
PD1	29 ± 2.6	n.d		22 ± 3.5	n.d	
PDX	58 ± 5.3	27 ± 3.6	0.5	51 ± 13	22 ± 4.1	0.4
MaR1	90 ± 19	n.d	-	80 ± 29	n.d	-
RvD1	289 ± 38	85 ± 22	0.3	248 ± 80	29 ± 16	0.1
RvD4	2,412 ± 399	920 ± 280	0.4	1,278 ± 425	514 ± 154	0.4
RvD5	49 ± 5.9	n.d	-	30 ± 12	n.d	-
PUFAs						
AA	2,686,452 ± 344,713	3,494,389 ± 268,824	1.3	2,654,887 ± 265,662	2,630,517 ± 443,140	1.0
EPA	692,395 ± 117,398	966,984 ± 86,084	1.4	736,517 ± 66,330	992,826 ± 158,138	1.3
DHA	1,376,142 ± 250,885	1,214,286 ± 141,497	0.9	959,017 ± 163,038	1,174,683 ± 317,718	1.2

slightly elevated in male exudates due to orchiectomy (**Table 3**). After 24 h, COX products in exudates were rather decreased due to gonadectomy, particularly in female mice. Note that PUFA levels were up-regulated in male exudates after orchiectomy and to some extent, at least for EPA, also in female animals due to ovariectomy (**Table 3**). To sum up, gonadectomy strongly up-regulates 12/15-LOX products in peritoneal exudates of male mice 4 and 24 h post-zymosan, along with elevated PUFAs and 5-LOX products after 24 h, but not so in female mice, where free PUFA (after 4 h) and 5-LOX products (4 and 24 h) are lowered as a consequence of ovariectomy. Therefore, in this experimental model, androgens may repress PUFA release and 12/15-LOX product formation in males at 4 and 24 h, while progesterone and/or estrogens are rather stimulatory in this respect.

The analysis of LM profiles in plasma of the respective animals showed divergent effects due to gonadectomy, especially in males at 4 h post-zymosan. As shown in **Figure 3** and **Table 4**, the levels of PUFAs, COX, and 12/15-LOX products, and SPMs at 4 h post-zymosan were not significantly different between the sexes (no gonadectomy), while 5-LOX products were higher in plasma from males vs females. At 24 h post-zymosan, COX and 5-LOX product levels were minute, while 12/15-LOX products were still abundant and clearly higher in plasma of male mice; PUFA levels hardly differed between the sexes (**Table 5**). Orchiectomy caused an overall decrease of COX, 5-LOX, and 12/15-LOX products after 4 h of zymosan in plasma (**Table 4**). Note that PUFA levels were not impaired, but AA and EPA were rather elevated after gonadectomy. For female animals 4 h post-zymosan, ovariectomy lowered plasma levels of 5-LOX and 12/15-LOX products like in exudates, and strongly suppressed the amounts of COX products (**Table 4**), which were instead elevated in the corresponding exudates. Like in male mice, plasma PUFA levels in female animals were not altered due to gonadectomy, which is in contrast to the corresponding exudates (**Table 4**). Interestingly, these main detrimental effects of gonadectomy in male and female mice on LM plasma levels after 4 h vanished after 24 h of zymosan, and a small trend toward higher levels of LOX products and PUFAs became apparent (**Table 5**). It should be noted that COX products were sparsely present in plasma, regardless of gonadectomy (**Table 5**). Together, despite elevated

TABLE 5 | Effect of hormone depletion on lipid mediator profiles in plasma from male and female mice at 24 h post-zymosan injection. Amounts of LM in plasma from male and female mice 24 h post zymosan/vehicle i.p. injection that underwent gonadectomy or sham-operation. Data of n = 5 to 6 animals per group are shown as mean ± SEM in pg/mL plasma and as heatmap. Fold-change of gonadectomy mean values compared to sham mean values of male and female mice.

No	24 h					
	Male sham	Male gon	Fold	Female sham	Female gon	Fold
COX products						
PGE_2	28 ± 3.0	30 ± 4.5	1.0	n.d	n.d	
PGD_2	n.d	26 ± 5.4		n.d	n.d	
PGF_2	n.d	n.d		n.d	n.d	
TXB_2	100 ± 32	127 ± 32	1.3	n.d	n.d	
5-LOX products						
LTB_4	n.d	n.d	-	n.d	n.d	-
t-LTB_4	n.d	n.d	-	n.d	n.d	-
5-HEPE	951 ± 95	540 ± 57	0.6	469 ± 70	623 ± 29	1.3
5-HETE	906 ± 91	1,158 ± 114	1.3	827 ± 82	1,076 ± 100	1.3
12/15-LOX products						
14-HDHA	16,370 ± 3,713	21,385 ± 5,322	1.3	7,245 ± 1,534	10,127 ± 3,614	1.4
12-HEPE	8,112 ± 2,893	7,813 ± 2,508	1.0	2,041 ± 533	3,273 ± 1,092	1.6
12-HETE	22,278 ± 2,184	35,561 ± 8,643	1.6	10,836 ± 2,208	14,327 ± 4,034	1.3
17-HDHA	768 ± 119	954 ± 233	1.2	388 ± 49	487 ± 119	1.3
15-HEPE	208 ± 23	209 ± 46	1.0	108 ± 20	123 ± 20	1.1
15-HETE	709 ± 116	898 ± 163	1.3	551 ± 70	570 ± 96	1.0
PD1	n.d	n.d	-	n.d	n.d	-
PDX	n.d	n.d	-	n.d	n.d	-
MaR1	n.d	n.d	-	n.d	n.d	-
RvD1	n.d	n.d	-	n.d	n.d	-
RvD4	21 ± 1.9	24 ± 6.6	1.1	n.d	n.d	-
RvD5	n.d	n.d	-	n.d	n.d	-
PUFAs						
AA	2,239,879 ± 231,728	3,618,336 ± 637,031	1.6	2,101,980 ± 151,143	2,985,021 ± 673,892	1.4
EPA	893,062 ± 58,082	1,406,104 ± 232,881	1.6	797,472 ± 86,664	1,192,464 ± 132,302	1.5
DHA	1,103,504 ± 284,606	1,462,360 ± 363,695	1.3	775,753 ± 79,763	985,133 ± 214,450	1.3

PUFA levels in plasma, gonadectomy causes impaired levels of COX, 5-LOX, and 12/15-LOX products in both sexes at 4 h, but not at 24 h.

Effects of Sex and Sex Hormone Depletion on the Expression Levels of Lipid Mediator Biosynthetic Enzymes

The cellular capacity to generate LMs depends primarily on the abundance of the respective biosynthetic enzymes and the availability of free PUFAs as substrates, at least for COX and 12/15-LOX pathways. For 5-LOX, the interaction with FLAP and CLP, the subcellular localization, and phosphorylation at serine residues are additional events that govern product formation (Radmark et al., 2015). Since we already observed some elevated PUFA levels due to gonadectomy, we next studied the amounts of 5-LOX, FLAP, platelet-type 12-LOX, 15-LOX-1, and COX-2 in the peritoneal exudates by Western blot on the protein level. After 4 h of zymosan, no sex differences were evident in the abundance of these proteins in sham-operated animals (**Figure 4A**). Gonadectomy of male and female mice caused no significant changes in the expression levels of these proteins 4 h post-zymosan, except 5-LOX that was elevated due to ovariectomy in females and some trends toward higher levels of FLAP (**Figure 4A**). Orchiectomy slightly augmented 5-LOX, FLAP, 12-LOX, and COX-2 proteins in exudates of male mice, which was not significant (**Figure 4A**). At 24 h post-zymosan, sham-operated male mice displayed significant higher amounts of 15-LOX-1 protein in exudates, which were significantly reduced upon orchiectomy (**Figure 4B**). For exudate levels of 5-LOX, FLAP, and COX-2 after 24 h, neither significant sex differences nor effects of gonadectomy were apparent (**Figure 4B**). Together, sex differences in the protein levels of the key LM biosynthetic enzymes/protein are apparent only for 15-LOX-1 in exudates, being superior in male mice after 24 h, while gonadectomy hardly alters the levels of this protein, except the suppression of 15-LOX-1 in males and elevation of 5-LOX in females.

DISCUSSION

Sex differences in inflammation and in the biosynthesis of LMs have been reported before, and are appreciated as important

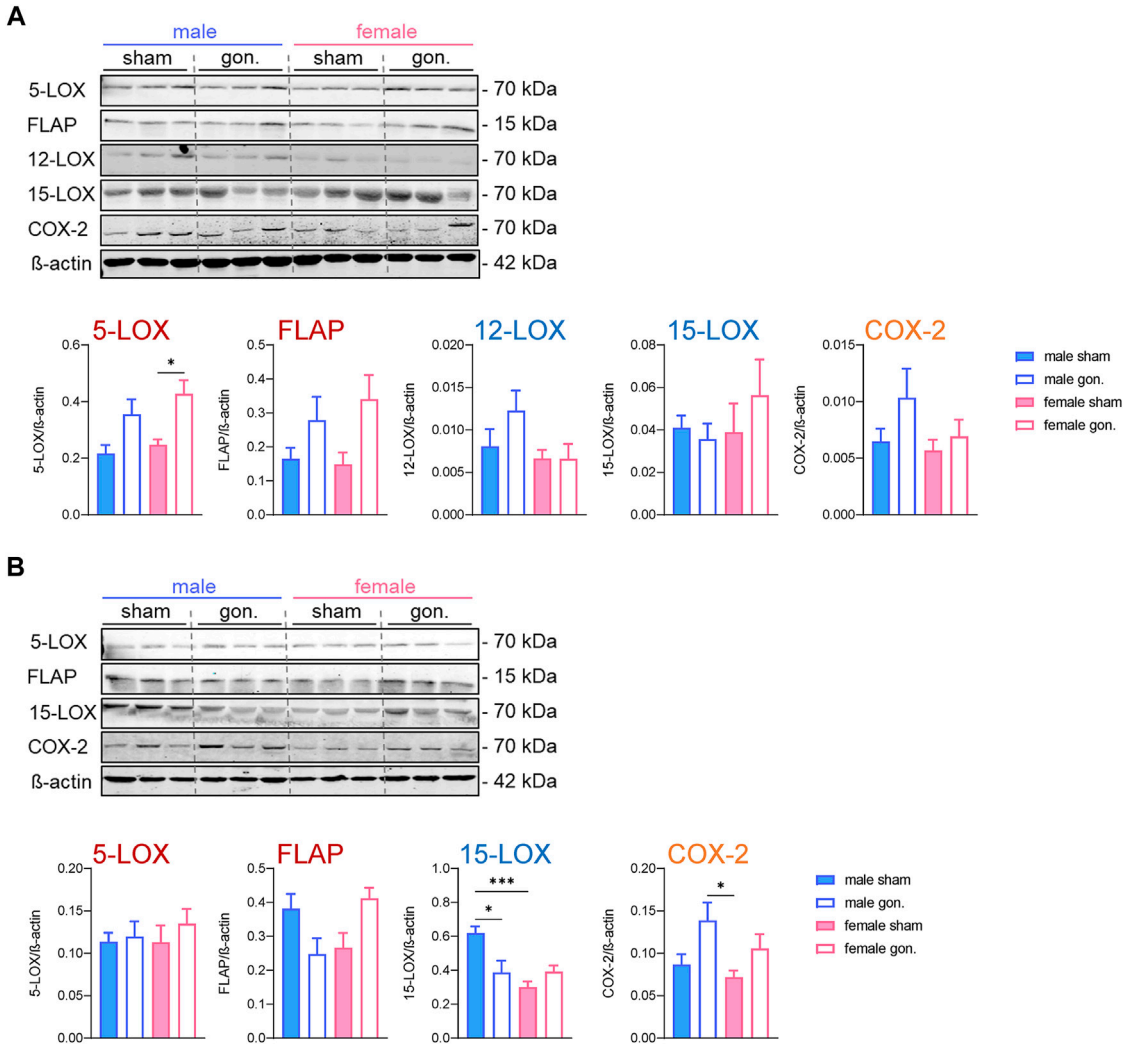

FIGURE 4 | Effect of sex hormone depletion on the expression of LM biosynthetic proteins in peritoneal exudates upon peritonitis induction. Protein levels and the densitometric analysis of 5-LOX, FLAP, platelet-type 12-LOX, 15-LOX, and COX-2 in lysed peritoneal exudates of gonadectomized and non-gonadectomized (sham-operated) male and female mice 4 h **(A)** and 24 h **(B)** after zymosan injection, normalized to β-actin. Western blots are shown as representatives from three different mice and given as mean values of n = 4–6; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA with Tukey's multiple comparison test for statistical analysis.

variables that determine the frequency and severity of inflammation-associated diseases, including many autoimmune diseases (Klein and Flanagan 2016; Pace et al., 2017c; Di Florio et al., 2020; Shabbir et al., 2021). Nevertheless, most studies addressing sex divergences related to LMs focused simply on single selected pathways without covering the entire LM spectrum relevant for progression and resolution of inflammation. Here, we performed the comprehensive LM profile analysis with inflammatory peritoneal exudates and plasma from male and female mice in zymosan-induced peritonitis, a well-established experimental model of acute, self-resolving inflammation (Cash et al., 2009; Rossi et al., 2014), in order to identify potential sex differences in the LM biosynthesis during the inflammatory response. By employing gonadectomy to abrogate endogenous sex hormone production (Cash et al., 2009; Scotland et al., 2011; Rossi et al., 2014), we revealed significant impact of sex hormones on LM formation, particularly in male animals.

Zymosan-induced peritonitis represents an acute local inflammation of the peritoneum with a peak in leukocyte recruitment after 4–12 h (Bannenberg et al., 2005; Rossi et al., 2014) and a subsequent resolution phase after 12–24 h (Schwab et al., 2007). Thus, we have chosen the time point at 4 h as peak and at 24 h as resolution of inflammation in this model. First of all, in healthy mice without zymosan, LM levels were low without apparent sex differences. But clear sex differences in the biosynthesis of pro-inflammatory COX and 5-LOX products were revealed during peritonitis, predominating in males at the peak of inflammation (4 h) and at the onset of resolution (24 h), and a tendency also for 12/15-LOX products that were slightly higher in males after 24 h. This preponderance in

males applied to both local LM production at sites of inflammation (exudates) and at least for 5-LOX and 12/15-LOX products, also systemically in the plasma. Intriguingly, at the peak of inflammation 4 h post-zymosan, sex hormone ablation by gonadectomy elevated 12/15-LOX products in the exudates from males, while in females, by contrast, gonadectomy rather decreased free PUFA, 5-LOX, and 12/15-LOX products. In plasma, gonadectomy impaired COX-, 5-LOX–, and 12/15-LOX–derived products when inflammation peaks but not at the resolution phase. Finally, sex differences for the LM biosynthetic enzyme expression were found for 15-LOX-1 dominating in exudates of males at the resolution phase being suppressed by orchiectomy without the striking impact of gonadectomy on other enzymes.

A sex bias in LT formation in zymosan-induced peritonitis was observed before with, however, higher LTB_4 and cys-LT levels in peritoneal exudates of female mice at least at the very early onset 15–60 min after zymosan injection (Rossi et al., 2014; Pace et al., 2017a), while at later time points (>2 h), a shift to higher LTB_4 levels in males was obvious (Rossi et al., 2014). The latter observation fits well to the higher 5-LOX product levels in peritoneal exudates and plasma of male mice at 4 h post-zymosan, monitored in the present study. Also, exudates of skin blisters 24 h after cantharidin exposure of male human subjects contained more LTB_4 than those of female counterparts (Rathod et al., 2017). It thus appears that the sex differences in 5-LOX product formation is temporally regulated during inflammation dominating in females at the early phase but may switch at later stages prevailing in males. Notably, in various *in vitro* studies using short-term (10–30 min) stimulated human neutrophils and monocytes or murine macrophages, cells derived from males produced consistently lower amounts of 5-LOX products vs cells from females (Pergola et al., 2008; Pergola et al., 2011; Pergola et al., 2015; Pace et al., 2017a). It appears that prolonged (>2 h) stimulation of 5-LOX–positive innate immune cells induces regulatory mechanisms in a sex-specific manner (elevation in males and impairment in females) that remain to be explored. Our present data in line with previous results (Pergola et al., 2008; Rossi et al., 2014; Pace and Werz 2020) exclude sex divergences in 5-LOX and the FLAP protein expression at 4 and 24 h in this respect. Rather, distinct temporal subcellular localization of 5-LOX as a highly mobile enzyme (Radmark et al., 2015) is conceivable, which indeed differed in neutrophils, monocytes, and macrophages derived from males vs those from females (Pergola et al., 2008; Rossi et al., 2014; Pace and Werz 2020).

Our current data on sex-specific formation of COX products with higher levels in exudates of male mice at 4 and especially at 24 h post-zymosan fit well to previous studies where PG formation was elevated in male animals during inflammation at 4 h in zymosan-induced mouse peritonitis or at 2 h in carrageenan-induced rat pleurisy (Pace et al., 2017b). In fact, the COX pathway often dominates in males, mainly due to the higher expression of COX-2 (Sullivan et al., 2005), although this may depend on the type of cell, tissue, and organ; on the inflammatory status; and on the sex hormone level related to the age of the subjects (Pace et al., 2017c). Sex differences in PG biosynthesis and modulation by sex hormones were also observed on the cellular level where, for example, neutrophils or platelets from male donors generated more COX products as than those from female counterparts (Mallery et al., 1986; Pinto et al., 1990; Pace et al., 2017b), again supporting our present data with elevated PGs in males. In our study, the protein expression of COX-2, the inducible COX isoform with varying expression during inflammation being causative for massive PG formation (Smith et al., 2011), was not different between sexes, neither at four nor at 24 h post-zymosan. This agrees with the accompanied elevation of pro-inflammatory cytokines that were not significantly different between the sexes.

Formation of 12/15-LOX products including SPM was hardly sex-biased and only elevated in the exudates of male mice 24 h post-zymosan, where accordingly the protein levels of 15-LOX-1 as a key enzyme of SPM formation (Werz et al., 2018; Jordan et al., 2020) dominated significantly in male samples. Along these lines, SPMs were elevated in the male infarcted heart (Pullen et al., 2020). In studies with humans, however, SPM levels tended to be lower in males. Thus, Rathod et al. reported on lower SPM levels in cantharidin-induced skin blisters at the resolution phase of male human subjects (Rathod et al., 2017). Similarly, 24–48 h after myocardial infarction, SPM plasma levels tended to be lower in male patients, which was also affected by the race (Halade et al., 2020). Similarly, plasma levels of 18-HEPE, 14-HDHA, and 17-HDHA were lower in male metabolic syndrome patients, although SPM levels were not different (Barden et al., 2015). Of interest, not only SPMs (i.e., RvD5) but also PGE_2 were lower in the lungs of male mice after exposure to ozone along with reduced AA and DHA levels compared with females, but ovariectomy had no impact on eicosanoid and SPM production despite depleted pulmonary DHA concentrations (Yaeger et al., 2021). Conversely, in healthy volunteers treated with dexamethasone, the plasma levels of 17-HDHA, RvD1, AT-RvD1, and RvE2 were higher in males (Barden et al., 2018). Also, higher SPM levels were found in emotional tears from men than in those derived from females (English et al., 2017), while retinal RvD1 levels in aged mice were again lower in males vs females (Trotta et al., 2021). Therefore, sex differences in SPM formation are obvious but inconsistent without clear preponderance in one gender, depending on experimental settings, species, race, age, and additional factors that may impact SPM formation.

To gain further insights into how sex impacts LM biosynthetic pathways during acute inflammation, we studied how ablation of sex hormones would modulate LM formation using gonadectomy. Several studies revealed striking suppression of LT formation by androgens in activated human neutrophils (Pergola et al., 2008; Pace et al., 2017a) and monocytes (Pergola et al., 2011), and in murine peritoneal macrophages (Rossi et al., 2014; Pace et al., 2017a). In a mouse asthma model, testosterone treatment during the sensitization phase lowered LT levels in the lung of female animals (Cerqua et al., 2020). Conversely, the ablation of testosterone in male mice by orchiectomy increased LTB_4 and cys-LT formation in the peritoneum 15–30 min post-zymosan i.p. injection (Rossi et al., 2014). Besides androgens, also progesterone was found to rapidly suppress the LT biosynthesis, especially in human

monocytes (Pergola et al., 2015), while estradiol increased the 5-LOX activity in isolated kidneys of metabolic syndrome female rats (Zuniga-Munoz et al., 2015). In our present study, decreased 5-LOX products in peritoneal exudates and plasma of orchiectomized male mice were evident at 4 h post-zymosan; however, at 24 h, 5-LOX products were rather elevated in orchiectomized animals. Despite decreased 5-LOX product formation at 4 h, gonadectomy elevated the protein levels of 5-LOX and FLAP in both sexes. These data suggest that modulation of the 5-LOX pathway by sex hormones proceeds by other processes than the regulation of 5-LOX and the FLAP protein expression, such as the 5-LOX/FLAP interaction, and seemingly depends also on the time point or phase of the inflammatory process (Pergola et al., 2008; Pace et al., 2017a).

We found that sex hormone depletion had only moderate and rather inconsistent impact on PG formation in the zymosan-challenged peritoneum along with slight elevations of COX-2 protein. However, in the plasma of both sexes, the elevated PG levels at the peak of inflammation (4 h) were strongly repressed by gonadectomy, implying that sex hormones are required for systemic induction of COX pathways. Others found that in the brain, estrogen, and/or progesterone suppress the COX-2 pathway, as ovariectomy augmented the COX-2 expression and PGE_2 in female rat hypothalamus (Brito et al., 2016), while estrogen and progesterone treatment of ovariectomized rats suppressed the LPS-induced COX-2 expression (Mouihate and Pittman 2003). In the kidney, however, the mPGES-1 expression was elevated after orchiectomy without changes of COX and cPGES (Sullivan et al., 2005). Together, sex hormones influence COX product formation with not only up-regulatory effects but also down-regulatory effects, which may depend on the organ/tissue and the phase of the inflammatory process.

Little is known about the impact of sex hormones on the 12/15-LOX pathway and related SPM formation. In the present study, the most pronounced effects of gonadectomy were indeed obvious for the 12/15-LOX pathway, where orchiectomy strongly increased 12/15-LOX product formation without the elevation of 15-LOX-1 protein in the peritoneum, but not in the plasma after 4 h; in contrast, ovariectomy was rather suppressive. At 24 h, gonadectomy elevated 12/15-LOX product levels in the peritoneum and plasma of both sexes, with more pronounced effects in males. Thus, androgens may have suppressive and estrogen/progesterone stimulatory impact on 12/15-LOX at the site of inflammation. This fits well to results by others showing that application of estradiol increases the 12-LOX and 15-LOX-2 expression and activity in human umbilical vascular smooth muscle cells (Somjen et al., 2016), and enhances the 12-LOX activity in rat platelets *in vivo* (Chang et al., 1982). Moreover, chronic hyperandrogenism decreased the protein expression of 12-LOX in prepuberal mice (Elia et al., 2011), supporting our hypothesis of a detrimental impact of androgens on the 12/15-LOX pathway and thus on SPM formation.

CONCLUSION

Using UPLC–MS-MS–based profiling of a broad spectrum of LMs in the peritoneum and plasma of male and female mice during the course of acute inflammation revealed significant sex differences for defined LM biosynthetic pathways at the inflamed site. Thus, in peritoneal exudates, pro-inflammatory COX and 5-LOX products predominate in males vs females at the peak of inflammation (4 h) and at the onset of resolution (24 h), while in plasma, only 5-LOX products dominated in males with minor magnitude. Neither the availability of PUFAs as LM substrates nor the expression of the, respective, key enzymes COX-2 and 5-LOX/FLAP in the exudates differed between males and females, and therefore do not readily account for these sex divergences. However, the slightly higher 12/15-LOX products in male exudates after 24 h correlate to elevated 15-LOX-1 protein levels. Our results with gonadectomized mice, and thus impaired sex hormone levels, indicate a pronounced suppression of 12/15-LOX product formation in males by androgens without such impact of estrogen and progesterone, again without clear correlation to PUFA supply and the 15-LOX-1 expression. Moreover, sex hormones influence COX and 5-LOX product formation with not only up-regulatory effects but also down-regulatory effects, which may depend on the organ/tissue and the phase of the inflammatory process. In summary, our data add to the obvious sex differences in LM biosynthetic networks that are, however, inconsistent in view of the respective pathway and the phase of the inflammatory process, and are seemingly further governed by the species, the race, the age of studied subjects, and the experimental settings. Consequent consideration of the sex in experimental and clinical studies of LM biosynthesis and biology may help to better understand how this complex sex bias is regulated, especially by sex hormones.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Italian Ministry.

AUTHOR CONTRIBUTIONS

SP, AI, FB, LS, AR, CS, and OW conceived and designed the experiments. FB, AR, LS, and OW supervised the experiments. FT, SP, and RB performed the experiments. FT, SP, PJ, KM, and RB analyzed the data. FT, SP, PJ, AI, FB, LS, AR, CS, and OW interpreted the data. OW wrote the manuscript. FT, SP, PJ, and OW critically revised the manuscript. All authors have read and approved the final version of the manuscript.

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Determining Sex-Based Differences in Inflammatory Response in an Experimental Traumatic Brain Injury Model

Michael C. Scott, Karthik S. Prabhakara, Andrew J. Walters, Scott D. Olson* and Charles S. Cox Jr.**

Department of Pediatric Surgery, University of Texas Health Science Center at Houston, Houston, TX, United States

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Edited by:
Luigia Trabace,
University of Foggia, Italy

Reviewed by:
Martin Götte,
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Regina Hummel,
Johannes Gutenberg University Mainz, Germany

****Correspondence:***
Michael C. Scott
Michael.c.scott@uth.tmc.edu
Scott D. Olson
Scott.D.Olson@uth.tmc.edu
Charles S. Cox Jr.
Charles.S.Cox@uth.tmc.edu

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Introduction: Traumatic brain injury is a leading cause of injury-related death and morbidity. Multiple clinical and pre-clinical studies have reported various results regarding sex-based differences in TBI. Our accepted rodent model of traumatic brain injury was used to identify sex-based differences in the pathological features of TBI.

Methods: Male and female Sprague-Dawley rats were subjected to either controlled-cortical impact (CCI) or sham injury; brain tissue was harvested at different time intervals depending on the specific study. Blood-brain barrier (BBB) analysis was performed using infrared imaging to measure fluorescence dye extravasation. Microglia and splenocytes were characterized with traditional flow cytometry; microglia markers such as CD45, P2Y12, CD32, and CD163 were analyzed with t-distributed stochastic neighbor embedding (t-SNE). Flow cytometry was used to study tissue cytokine levels, and supplemented with ELISAs of TNF-α, IL-17, and IL-1β of the ipsilateral hemisphere tissue.

Results: CCI groups of both sexes recorded a higher BBB permeability at 72 hours post-injury than their respective sham groups. There was significant difference in the integrated density value of BBB permeability between the male CCI group and the female CCI group (female CCI mean = 3.08 x 108 ± 2.83 x 107, male CCI mean = 2.20 x 108 ± 4.05 x 106, p = 0.0210), but otherwise no differences were observed. Traditional flow cytometry did not distinguish any sex-based difference in regards to splenocyte cell population after CCI. t-SNE did not reveal any significant difference between the male and female injury groups in the activation of microglia. Cytokine analysis after injury by flow cytometry and ELISA was limited in differences at the time point of 6 hours post-injury.

Conclusion: In our rodent model of traumatic brain injury, sex-based differences in pathology and neuroinflammation at specified time points are limited, and only noted in one specific analysis of BBB permeability.

Keywords: traumatic brain injury, sex-based differences, neuroinflammation, blood-brain barrier, neurologic injury

INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of death and disability related to trauma in the United States. In 2014, the CDC estimated that TBI accounted for 2.87 million Emergency Department visits, 288,000 hospitalizations, and 56,800 deaths (1). The Global Burden of Disease Study found that the age-adjusted incidence rate of TBI in 2016 was 369 per 100,000. The global prevalence of individuals living with TBI-related disability was 759 per 100,000; TBI alone accounted for 8.1 million years lived with disability in 2016 (2). TBI is characterized by two phases of injury: primary injury and secondary injury. Primary injury is the initial insult *via* physical impact and damage to brain tissue. Secondary injury from TBI is characterized by an increase in neuronal excitotoxicity and progression of neuroinflammation (3).

Current treatment modalities for severe TBI focus on control of intracranial hypertension, maintenance of adequate cerebral perfusion pressure, and supportive care. Treatment options include surgical decompression, hyperosmolar therapy, temporizing ventilator strategies, optimization of nutrition, infection prophylaxis, and anti-epileptic medication for seizure prophylaxis (4). Despite the benefits of these options, few truly address slowing or halting the progression of TBI. Thus, research in TBI has emphasized understanding its pathophysiology in order to develop targeted therapies that limit injury progression and promote resolution (3).

Sexual dimorphism may influence the progression of TBI and may need to be considered in pre-clinical and clinical studies. Some clinical studies have suggested that there is a difference in outcomes with the female sex deriving some benefit in mortality and complication risk (5); other clinical studies have indicated that there is no significant difference (6). TBI severity may influence these observed differences (7). Pediatric studies have suggested that females may have some survival advantage compared to males after sustaining a TBI. Improved survival in pre- and post-pubescent stage females has been observed in retrospective studies of severe TBI (8, 9). Some retrospective studies allude to a variance in post-injury functional and psychological outcomes. For example, males and females who have sustained a TBI report different clinical symptoms, such as sleep disturbances and noise sensitivity (10, 11). Scott et al. found that psychological issues after TBI may differ in males and females as well; males were more likely to report substance abuse, and females were more likely to report experiencing anxiety or depression (11). In contrast to studies reporting differences, a recently published retrospective study of data from the Chinese Head Trauma Data Bank indicated no significant difference between male and female adult patients in mortality or long-term unfavorable outcomes after acute TBI (12).

Pre-clinical studies have demonstrated potential variability in the secondary injury of TBI. Doran et al. found that the increase in both peripheral myeloid influx and resident microglia proliferation in the injured cerebral hemisphere after controlled-cortical impact (CCI) injury was higher in male rats compared to female rats. Bromberg et al. found that Iba-1 staining of microglia, was higher in male rats in the paraventricular nucleus 7 days after sustaining fluid-percussion injury (FPI), indicating a higher number of microglia (13, 14). Armstead et al. have indicated that cerebral autoregulation may have sex-based differences (15–17). Different mechanisms to induce TBI are used in animal studies, which can influence the results. In a review of animal studies, 55% of studies using CCI as an injury mechanism reported females having better outcomes in post-injury evaluation such as behavioral and motor function studies. When using a closed-head injury (CHI) mechanism, 31% of studies showed females with improved outcomes in post-injury evaluations like cytoskeletal degradation, reactive astrogliosis, and cortical blood flow. Studies using fluid-percussion injury (FPI) reported females having worse survival and regulation of cell apoptosis in 60% of studies. However, these apparent differences may be in part depend on the outcomes measured (7).

After review of the information above and other studies (**Tables 1**, **2**), we used our CCI model with our specific methodological outcome measures to uncover any sex-based differences in neuroinflammation. Experimental groups of male and female Sprague-Dawley rats sustaining either CCI or sham injury were compared. The progression of neuroinflammation at various time intervals after injury was evaluated based on the following features: microglial and splenocyte cell analysis with flow cytometry, blood-brain barrier (BBB) permeability, and tissue cytokine analysis (**Figure 1**).

MATERIALS AND METHODS

Animals

All animal experiments were approved by the institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston, TX, USA, in compliance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments complied with the standards of the American Association for the Accreditation of Laboratory Animal Care. The approved protocol number was AWC-18-0121. Sprague Dawley rats (Envigo Labs, Indianapolis, Indiana) were used in this series of experiments. A total of 62 rats (5-7 weeks) were used in the study; 31 were males (225-250g) and 31 were females (200-225g). Rats were housed in same-sex pairs in rat microisolators under 12-hour light/dark cycles. Housing conditions were temperature controlled; water and standard rodent laboratory chow were available ad libitum. Rats were randomized to CCI versus sham injury prior to use. Rats were weighed immediately prior to injury. The rats were controlled for age. There was a significant difference in weight; female rats were smaller in weight on average compared to male rats. This will be discussed further in the results. We controlled rats for age to ensure they were at a similar stage of brain development; controlling rats by weight would likely require the use of older female rats.

Controlled Cortical Impact Injury Model

We used a previously established protocol of controlled cortical impact injury (CCI) to model TBI in rats (33–36). Briefly, animals were anesthetized with 4% isoflurane and oxygen at a flow rate 5 Liters/minute in a vented chamber and then maintained at 1% to 2% isoflurane and oxygen 3 Liters/minute for the duration of the procedure. Next, the animal was secured on a stereotactic frame. The surgical site on the scalp was prepared with alcohol and iodine solution. Subcutaneous 0.25% bupivacaine was administered for

TABLE 1 | Human studies of sex-based differences in traumatic brain injury.

Study title	Author and year	Study type	Results and findings
The effect of gender on patients with moderate to severe head injuries.	Berry et al., (5).	Retrospective study Age separated	Females had lower risk of mortality and complications. Premenopausal and postmenopausal women had a lower risk of mortality than age matched men. No difference in mortality between premenopausal women and age matched men
Does sexual dimorphism influence outcome of traumatic brain injury patients? The answer is no!	Coimbra et al., (6)	Retrospective study Case controlled	No significant difference in overall outcome or subset analysis. Excluding patients older than 50 years did not change result.
Sex differences in traumatic brain injury: what we know and what we should know.	Gupte et al., (7)	Analysis of literature review	Overall, human studies report worse outcomes in women than men, animal studies report better outcomes in females than males. A greater percentage of human studies show women have better outcomes in moderate-severe TBI.
Gender impacts mortality after traumatic brain injury in teenagers.	Ley et al., (8)	Retrospective study Pediatric, age separated	Lower mortality rate in 0–12-year-old patients than 12-18-year-olds. No difference between males and females in 0–12-year-olds. Reduced mortality in females in 12-18-year-olds.
Use of a pediatric cohort to examine gender and sex hormone influences on outcome after trauma	Phelan et al., (9)	Retrospective study Pediatric, age separated	0-8-year old and 8.1-14.5-year-old patients had equivalent survival rates between genders across all severities. 14.6-20-year-olds had a significantly improved survival rate for women across all subgroups, more pronounced with increasing injury severity score.
A comparison of adult outcomes for males compared to females following pediatric traumatic brain injury	Scott et al., (10)	Observational study of adults with childhood history of TBI	Patients with childhood TBI had high rates of problem behaviors compared to controls. Females more likely to report a history of internalizing problems. Males more likely to report externalizing problems.
Gender differences in self-reported long-term outcomes following moderate to severe traumatic brain injury	Colantonio et al., (11)	Retrospective study	Difference between self-reported symptoms men and women in long term outcomes and symptoms related to daily functioning.
Chinese Head Trauma Data Bank: Effect of gender on the outcome of patients with acute traumatic brain injury	Chinese Head Trauma Study Collaborators (12)	Observational study	No significant difference in the outcome of patients with acute TBI between men and women
Acute serum hormone levels: characterization and prognosis after severe traumatic brain injury	Wagner et al., (18)	Observational study	Acute serum hormone levels were significantly altered after severe TBI. Increased hormone levels were associated with increased mortality and worse global outcomes.
Pituitary function within the first year after traumatic brain injury or subarachnoid hemorrhage	Tölli et al., (19)	Observational study	Perturbations in pituitary function were frequent early after the event but declined after the first year. No relationship seen between hormonal levels and injury variables.
Chronic hypopituitarism after traumatic brain injury: risk assessment and relationship to outcome	Bavisetty et al., (20)	Observational study	TBI patients with hormonal deficiencies had worse disability rating scale, greater rates of depression, worse quality of life, emotional well-being, and general health.

local anesthesia. A midline scalp incision was then made; the right-sided musculature and soft tissue were bluntly dissected away for exposure of the calvarium. Using a dental drill, a 7-mm diameter craniectomy was performed between the right coronal and lambdoid sutures. A CCI device (Impact One Stereotaxic Impactor, Leica Microsystems, Buffalo Grove, Illinois) was utilized to administer a standardized and unilateral severe brain injury. Injury parameters included a depth of 2.7 mm, impact velocity of 5.6 m/s, and a dwell time of 150 microseconds using a 4-mm diameter impactor tip to the parietal association cortex. Immediately after the injury, the incision was closed using sterile wound clips and animals were allowed to recover in newly cleaned microisolator cages provided by the University Center for Laboratory Medicine and Care (CLAMC). Sham injuries were performed by anesthetizing the animals, making the midline incision, and separating the skin, connective tissue and aponeurosis from the cranium. The incision was closed using sterile wound clips. In our lab, this has been our traditional method of creating a sham group. We are of the view that the addition of a sham craniectomy may induce underlying neuroinflammation, which is supported by recent studies (37, 38). Also, the primary intention of this study is to focus on differences between sexes in a true TBI, and not necessarily the sham injury.

Blood Brain Barrier Permeability Measurement

Blood brain barrier (BBB) permeability was measured using a far-red dye (Alexa Fluor 680, ThermoFisher Scientific, MA, USA) bioconjugated to a 10 kDa dextran molecule, as previously reported by our group and used in several additional studies (39–42). CCI and sham injuries were induced as previously described. In this set of injuries, rats were sacrificed 72 hours post-injury. This time point was chosen to keep this study consistent with our prior studies of BBB permeability after injury (41, 43–45). At 72 hours after injury and 30 minutes prior to euthanasia, 0.5 mL of 1 mg/mL Alexa Fluor 680 dye was intravenously injected in rats *via* tail vein. Following exsanguination and 4% paraformaldehyde cardiac perfusion, the brains were harvested, sliced in 2 mm coronal sections and scanned for Alexa Fluor 680 signal using an Odyssey laser scanner (LI-COR Biosciences, NE, USA) in the 700 nm emission channel; auto-fluorescence was visualized in the nonspecific 800 nm emission

TABLE 2 | Pre-clinical animal studies of sex-based differences.

Study	Author and year	Study details	Results and Findings
Sex-dependent pathology in the HPA axis at a sub-acute period after experimental traumatic brain injury	Bromberg et al., (13)	Rat, mild fluid percussion injury	Males had injury induced neuroinflammation and astrocytosis compared with sex match shams, females did not. Glucocorticoid receptor protein levels elevated in females compared with sex match shams, males did not.
Both estrogen and progesterone attenuate edema formation following diffuse traumatic brain injury in rats.	O'Connor et al., (21)	Rat, weight drop impact	Male rats had an increase in BBB permeability compared to female rats after neurologic injury. Administration of estrogen/progesterone reduced BBB permeability.
Sex Differences in Thermal, Stress, and Inflammatory Responses to Minocycline Administration in Rats with Traumatic Brain Injury.	Taylor et al., (22)	Rat, CCI	At 35 days post-injury, ovariectomized female rats had a greater expression of IL-1β and IL-6 in the ipsilateral hippocampus, but males had a greater expression of TNF-α.
Gender influences outcome of brain injury: progesterone plays a protective role	Roof et al., (23)	Rat, bilateral cortical contusions Male vs. pseudo-pregnant vs. normally cycling females vs ovariectomized ± estrogen and/or progesterone replacement	Males and normally cycling females had a significant increase in edema. Pseudopregnant females had no significant increase in edema. All three groups were significantly different from one another. Replacement of estrogen in ovariectomized rats did not alter response, while replacement of progesterone did.
Estrogen-related gender difference in survival rate and cortical blood flow after impact-acceleration head injury in rats	Roof et al., (24)	Rat, Marmarou impact-acceleration head injury. Ovariectomized females and males ± estradiol replacement	Significantly more females survived injury. Females showed less reduction and better recovery of cortical blood flow. Postinjury cortical blood flow was higher in female and male rats with estradiol injections.
Neuropathological protection after traumatic brain injury in intact female rats versus males or ovariectomized females	Bramlett et al., (25)	Rat, fluid percussion injury. Intact females, ovariectomized females, and males compared.	Intact females had smaller cortical contusion compared to males. Non-proestrous group was significantly different from ovariectomized females. Overiectomzed females had larger areas of damage compared to intact females, similar to males.
Evaluation of estrous cycle stage and gender on behavioral outcome after experimental traumatic brain injury	Wagner et al., (26)	Rat, CCI Females proestrous or non-proestrous.	No significant difference between females regardless of estrous cycle. Females performed significantly better than males on motor function tasks.
Sex differences in acute neuroinflammation after experimental traumatic brain injury are mediated by infiltrating myeloid cells	Doran et al., (14)	Mice, CCI	Males had an influx of peripheral myeloid cells, followed by proliferation of microglia. Females had improved motor function at 1 day.
Male and female mice exhibit divergent responses of the cortical vasculature to traumatic brain injury	Jullienne et al., (27)	Mice, CCI	No difference between males and females in lesion volume, neurodegeneration, blood brain barrier alteration, and microglial activation. Females had more astrocytic hypertrophy and heme-oxygenase-1 induction at one day post injury. Males exhibited increased endothelial activation and expression of β-catenin. 7 days: males had an increase in number of vessels and vessel complexity.
Sex-Dependent Macromolecule and Nanoparticle Delivery in Experimental Brain Injury.	Bharadwaj et al., (28)	Mice, CCI	Female mice at random stages of estrous cycle had an increase in macromolecular tracer accumulation, indicating an increase in BBB permeability compared to males at 3 hours and 24 hours. There was no sex-based difference in neuroglial response.
Blood-brain barrier breakdown and edema formation following frontal cortical contusion: does hormonal status play a role?	Duvdevani et al. (29)	Mice, CCI	Using Evans blue dye, no sex-based difference in BBB permeability was observed. Hormonal status did not have an effect on BBB permeability in male or female rats.
Cytoskeletal protein degradation and neurodegeneration evolves differently in males and females following experimental head injury	Kupina et al., 2003 (30)	Mice, weight drop impact	Male peak protein degradation and neurodegeneration at 3 days, females at 14 days after injury.
Lack of a gender difference in post-traumatic neurodegeneration in the mouse controlled cortical impact injury model	Hall et al., (31)	Mice, CCI	A focal CCI showed no gender difference.
Sexual dimorphism in the inflammatory response to traumatic brain injury.	Villapol et al., (32)	Mice, CCI	Male mice had a more rapid influx and activation of microglia to CCI lesion site compared to females. Differences were indistinguishable at 1-week post injury.
Impaired cerebral blood flow autoregulation during posttraumatic arterial hypotension after fluid percussion brain injury is prevented by phenylephrine in female but exacerbated in male piglets by	Armstead et al., (15)	Pig, fluid percussion brain injury Intervention: phenylephrine	Pial artery dilation was impaired more in males than females. Phenylephrine decreased impairment of pial artery dilation in females but caused vasoconstriction in males. Cerebral blood flow, cerebral perfusion pressure, and autoregulatory index decreased in males, less in females.

(Continued)

TABLE 2 | Continued

Study	Author and year	Study details	Results and Findings
extracellular signal-related kinase mitogen-activated protein kinase upregulation			Phenylephrine reduced ERK MAPK upregulation in females, increased upregulation in males.
TBI sex dependently upregulates ET-1 to impair autoregulation, which is aggravated by phenylephrine in males but is abrogated in females	Armstead et al., (16)	Pig, fluid percussion injury Intervention: phenylephrine and antagonists/ scavengers to elucidate mechanism.	Endothelin-1, activated oxygen, and ERK MAPK released in males than females, contributing to impaired autoregulation during hypotension after TBI.
Adrenomedullin reduces gender-dependent loss of hypotensive cerebrovasodilation after newborn brain injury through activation of ATP-dependent K channels	Armstead et al., (17)	Pig, fluid percussion injury Intervention: adrenomedullin	Impaired potassium channel. Adrenomedullin induced pial artery dilation was y greater in female than male piglets. Hypotensive pial artery dilation was blunted to a greater degree in males than females. Topical pretreatment with adrenomedullin reduced the loss of hypotensive pial artery dilation in both genders, but protection was significantly greater in males.

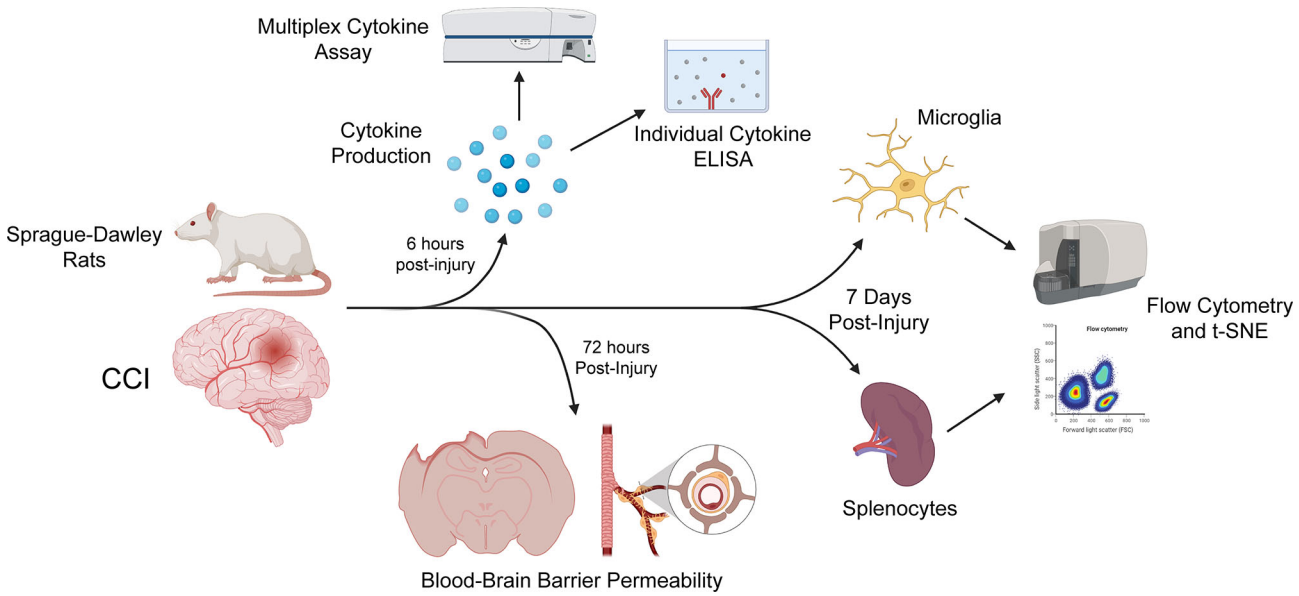

FIGURE 1 | Time points post-injury for each study. All rats underwent either sham or CCI injury. For cytokine studies of brain tissue, including individual ELISAs and a multiplex cytokine assay (performed using the LSR-II flow cytometer [BD Bioscience]), rats were sacrificed 6 hours after injury, to capture the peak of cytokine production. Rats were sacrificed 72 hours post-injury for our assessment of blood-brain barrier permeability. Flow cytometry and t-SNE of microglia and splenocyte immunophenotype using the Beckman-Coulter Gallios flow cytometer was performed 7 days after injury. Created with BioRender.com.

channel. Quantitative measurements of dye extravasation were determined for each brain by using a uniform region of interest and measuring mean intensity and the mean integrated density of the slices using a threshold to exclude background and low intensity autofluorescence (ImageJ).

Microglia Flow Cytometry Immunophenotyping

Upon sacrifice, 7 days after CCI injury, brains were harvested and processed as described previously (41, 46, 47). The 7-day time point was chosen to evaluate microglia to allow for comparison to our previous studies (41). This time point has been viewed as a point of peak microglial activation after CNS injury (48). Briefly, the ipsilateral and contralateral side of the brain was processed separately using the Adult Neural Tissue Dissociation Kit GentleMACS Dissociator (Miltenyi Biotech), using manufacturer protocol. Following enzyme digestion, the myelin was removed by Percoll centrifugation. Approximately 1 x 10^6 cells from the cerebral tissue digestion were used for flow cytometry. A multicolor flow cytometry microglia/myeloid cell panel recently developed in our lab was used to stain and identify microglia and their immunophenotype (46). Our staining method identified the following microglial markers: P2Y12, CD11bc, CD45, CD32, CD163, RT1b. Aliquots of the cerebral

tissue digestion were placed BD Trucount tubes (BD Biosciences) to determine absolute cell counts. Traditional flow cytometry analysis was also performed with FlowJo vr10.6.1 (FlowJo, LLC, Ashland, Oregon). In order to identify P2Y12 positive microglia, live cells were initially gated by P2Y12 expression and then gated on CD11bc and CD45. CD11-positive cells were then gated on phenotypic markers CD32, RT1B, and CD163. To ensure identification of all microglia and myeloid cells, live cells were gated on CD11bc and CD45 without P2Y12. CD11bc-positive cells were then gated on phenotypic markers CD32, RT1B, and CD163. Data for microglial cells was acquired by a Gallios Flow Cytometer (Beckman Coulter).

Splenocyte Flow Cytometry Immunophenotyping

Spleens were harvested from animals at the time of euthanasia, 7 days following injury. Splenocytes were isolated as previously described (49). Briefly, the spleen was washed in 10 mL of PBS, homogenized using a gentleMACS Dissociator (Miltenyi Biotech). The cells were then filtered through a 70-µm filter and centrifuged at 400g for 5 minutes. Next, flow cytometry was performed to characterize lymphoid and myeloid cell populations. The antibody panel consisted of the following cell surface markers: anti-CD3-FITC, anti-CD25-PE, anti-CD8a-PerCP, anti-CD11bc-PECy7, anti-RT1B-APC, anti-CD4-APCCy7, anti-CD45RA-V450. All antibodies were purchased from Beckman Coulter. Myeloid cells were identified as CD11bc-positive, CD3-negative, CD45RA-negative cells. B cells were identified as CD45RA-positive, CD3-negative, CD11bc-negative cells. T cells were identified as CD3-positive, CD11bc-negative, CD45RA-negative cells. Further T-cell subsets were identified using the CD4, CD8, and CD25 markers. Data for the splenocyte samples was acquired by a Gallios Flow Cytometer (Beckman Coulter) using Kaluza acquisition software. Subsequent data analyses were completed utilizing FlowJo (vr10.6.1).

T-Distributed Stochastic Neighbor Embedding Analysis

t-Distributed stochastic neighbor embedding (t-SNE) is a tool that analyzes flow cytometry data, by projecting all data points onto a multi-dimensional graph plot. t-SNE forms a "heat-map" of all cells that were analyzed by flow cytometry. We perform t-SNE to better characterize specific populations of cells and other features such as their morphometry and expression of activation markers. The t-SNE analysis was performed with FlowJo (vr10.6.1). Briefly, live cells were gated on all samples. Within each sample, live cell events were randomly downsampled to 3000 events; analysis was run on an equal number of events per sample. The individual sample files were concatenated to link them together into a single-standard file. t-SNE was run using the FlowJo plugin, which included all fluorophores previously listed. Unique t-SNE was created for both splenocyte and microglia panels with all samples and groups derived from the same t-SNE run, for consistency.

Inflammatory Cytokine Cytometric Profile

Finally, we studied the expression of inflammatory cytokines in brain tissue. The brains were harvested at 6 hours post injury from rats following euthanasia. This time point typically represents peak pro-inflammatory cytokine expression post-injury (48, 50). For each brain, the ipsilateral hemisphere was homogenized in RIPA buffer (Alfa Aesar) containing protein inhibitor cocktail (Thermo Scientific) using PT-3100 Polytron homogenizer (Kinamatia AG). The protein was quantified by BCA kit (Thermo Scientific). Tissue lysate at a concentration of 1mg/mL was run on LEGENDplex™ Rat Inflammation Panel (13-plex) kit, using manufacturer protocol and an LSR-II flow cytometer (BD Biosciences). For further verification of our multiple cytokine panel, more accurate and sensitive ELISAs (Biolegend) of individual cytokines were performed.

Statistics

In analysis, there was no need for blinding, since all assays were performed using unbiased methodology, including quantitative digital image analysis. All the statistical analysis was performed with GraphPad Prism software version 9 (GraphPad Software, Inc., San Diego, CA). All the data were analyzed using ordinary one-way ANOVA with Dunnet's correction for multiple comparisons and presented as mean ± SE, unless otherwise specified. A p-value less than 0.05 was deemed statistically significant. Prism software tests for normality, and no outliers were detected throughout our data.

For *in vivo* flow cytometry data for microglia and splenocytes, cell count means of each inflammatory marker were studied. Blood brain barrier permeability data was analyzed by comparing the mean intensity and the mean integrated density of dye extravasation between all groups. The means were determined after the threshold intensity values for detection were established, similar to prior studies in our lab (39–42). For each group, comparisons of the mean concentrations were performed for our individual ELISA and multiple-cytokine assay data. Cytokine ELISA studies were performed with triplicate samples from each cerebral tissue culture.

RESULTS

Blood Brain Barrier Permeability

In our study of changes in BBB permeability, we did note differences at the 72-hour time point post-injury. When comparing all CCI rats to all sham rats, the mean intensity of detected dye was significantly greater in the CCI group compared to the sham group (**Figure 2A**; CCI mean intensity = 1365 ± 43.6, sham mean intensity = 813.2 ± 16.6). The mean integrated density value of extravasated dye in the CCI groups for both sexes was greater than that of their respective sham groups. This difference was statistically significant (**Figure 2B**; female sham mean = $1.20 \times 10^7 \pm 1.08 \times 10^6$, female CCI mean = $3.08 \times 10^8 \pm 2.83 \times 10^7$, $p<0.0001$; male sham mean = $2.25 \times 10^7 \pm 4.05 \times 10^6$, male CCI mean = $2.20 \times 10^8 \pm 2.08 \times 10^7$, $p<0.0001$).

When comparing the injured groups for each sex, the mean integrated density value of dye extravasation was slightly larger in the female group compared to the male group. This difference was statistically significant (**Figure 2B**; female CCI mean = $3.08 \times 10^8 \pm 2.83 \times 10^7$, male CCI mean = $2.20 \times 10^8 \pm 4.05 \times 10^6$,

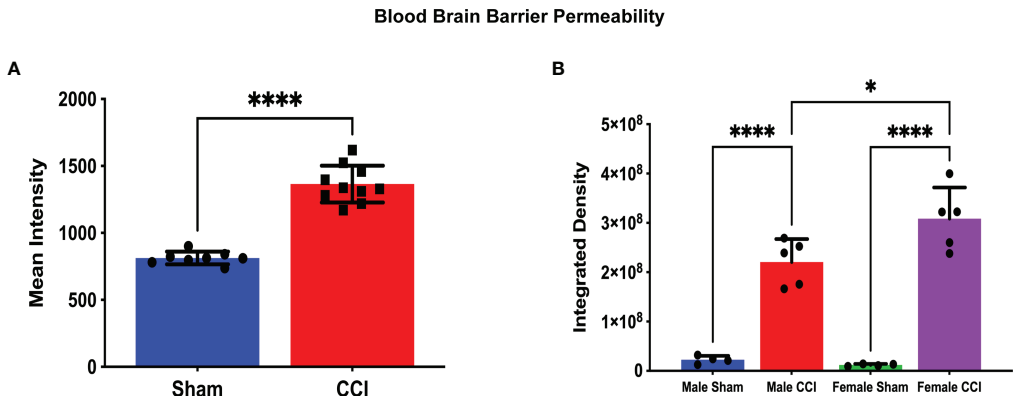

FIGURE 2 | Blood-Brain Barrier Permeability as measured by Alexa Fluor Dye extravasation. In this analysis, a minimum threshold of 1.5k was set for detection of BBB permeability; this threshold was set to eliminate any artifact fluorescence that may be detected by the scanner. The measurements displayed are the mean intensity of dye for all CCI rats compared to all sham rats **(A)** and the mean integrated density for each group **(B)**. There was a significant difference in the mean intensity of dye extravasation when comparing all sham rats to all CCI rats (**A**; CCI mean intensity = 1365 ± 43.61, sham mean intensity = 813.2 ± 16.66, $p < 0.0001$). The integrated density is the sum of detected fluorescence of dye in a specific area of cerebral tissue. A higher integrated density indicates a more permeable BBB in a specific area of cerebral tissue. Both the female CCI (mean integrated density = $3.08 \times 10^8 \pm 2.83 \times 10^7$) and the male CCI ($2.20 \times 10^8 \pm 4.05 \times 10^6$) groups recorded higher mean intensities compared to their respective sham group (female sham mean = $1.20 \times 10^7 \pm 1.08 \times 10^6$, male sham mean = $2.25 \pm 4.05 \times 10^6$). The female CCI group did record a higher integrated density compared to the male group, and this difference did reach statistical significance (mean difference = 87893520 ± 26653677, $p = 0.0210$). (****$p < 0.0001$, *$p < 0.05$).

$p = 0.0210$). When comparing the sham groups based on sex, the mean integrated density value of the male sham group was marginally higher, but this difference did not reach statistical significance (female sham mean = $1.20 \times 10^7 \pm 1.08 \times 10^6$, male sham mean = $2.25 \times 10^7 \pm 4.05 \times 10^6$, p=0.7730).

To determine if the size of rats was a confounding factor in this difference, we used a Spearman's correlation test comparing the integrated density values from each individual rat in relation to their body and brain weight. This analysis was performed to determine if there was a correlation between BBB permeability after injury and weight. In an unpaired t-test, male rats recorded a significantly heavier body weight compared to female rats (Male mean weight = 252.0 ± 2.58, Female rats = 216.9 ± 2.06; **Supplemental Figure 1**). While female CCI rats were significantly smaller in body weight compared to male rats (**Supplemental Figure 2**), there was no statistically significant correlation of body weight with a lower integrated density in all CCI rats (**Supplemental Table 1**). In an unpaired t-test, there was no significant difference in brain weight between male and female rats overall or male and female rats in their respective sham or CCI groups (male brain weight mean = 1.31, female = 1.327, $p = 0.64$; **Supplemental Figures 3**, **4**). When plotting integrated density values against brain weights of CCI rats, there was no significant correlation observed either (**Supplemental Table 1**).

Microglia Activation

Brain and spleen samples were obtained 7 days after injury for cell analysis with flow cytometry. Microglia markers included CD45, CD11b/c, P2Y12, CD32, and CD163. On cell counts, CD45, P2Y12, and CD11b cell surface markers were elevated in both the male and female injury groups when compared to their respective sham groups (CD45 male CCI vs male sham mean difference = 237.4 ± 72.8, $p = 0.0102$; female CCI vs female sham mean difference = 228.6 ± 35.6, $p = 0.0135$; P2Y12 male CCI vs male sham mean difference = 38.50 ± 2.26, $p = 0.0006$; female CCI vs female sham mean difference = 38.50 ± 3.07, $p < 0.0001$; CD11b male CCI vs male sham mean difference = 145.1 ± 33.776, $p = 0.0012$; female CCI vs female sham mean difference = 150.4 ± 9.91, $p = 0.0009$). When comparing injury groups based on sex, no significant difference in the mean counts was observed for CD32 (58.92 ± 15.3, $p = 0.76$), CD45 (0.000 ± 40.1, $p > 0.9999$), P2Y12 (3.600 ± 2.4, $p = 0.9702$), CD163 (0.50 ± 2.4), and CD11b (1.400 ± 24.9, $p > 0.9999$). Statistically significant differences were not observed when comparing the male and female sham groups (**Supplemental Figures 5A, F**).

Microglia flow data was further characterized using t-distributed stochastic neighbor embedding (t-SNE). t-SNE uses all data points obtained from flow cytometry for each specific injury group, forming a map of the different cell populations based on cell surface markers (supplemental figure 6A and 6B) and cell morphometry (**Supplemental Figure 6C**). We then coded these data plots to distinguish individual microglia on the basis of sex and injury (**Figure 3A**). This data was then used to make a density plot (**Figure 3B**), which distinguished where the cells from each group localize to on the plot. These plots were then compared it with the cell surface marker and the morphometric analysis plots. In the CCI groups, there is increased density of cell counts in regions corresponding with activated microglia, whereas in the sham groups there is a lower density in these areas. When comparing the female CCI with the male CCI groups, the density plots are similar; some differences exist in that the female CCI group may feature a larger population of typical non-activated microglia compared to the male CCI group.

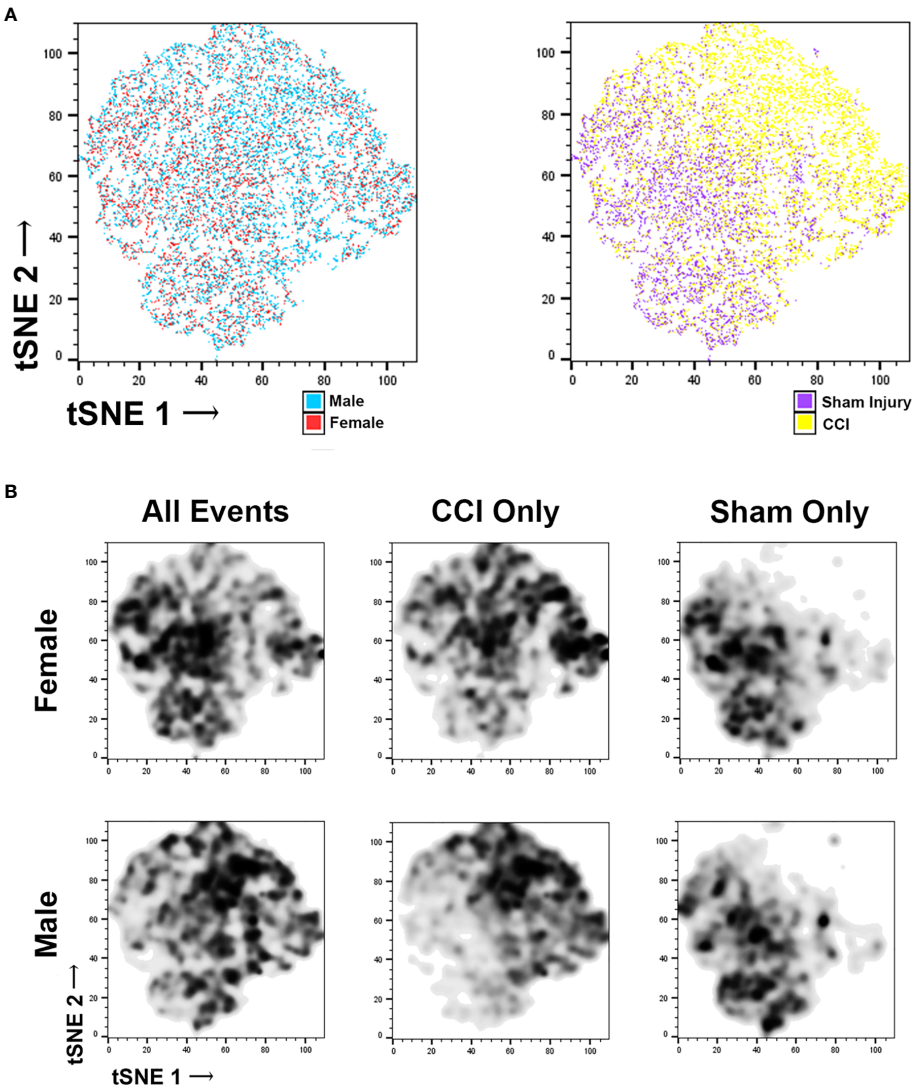

FIGURE 3 | t-Distributed stochastic neighbor embedding (t-SNE) of microglia flow cytometry data. Each individual data point in the tSNE plot represents individual microglia counted through the flow cytometer. The colors applied to the plots **(A)** allow us to distinguish the microglia based upon their sex (male or female) or their injury (sham or CCI). The colorless plots **(B)** represent density plots of the same microglia data portrayed in the color tSNE plots. The density plots depict specific microglia populations by highlighting the microglia representative of specific experiment groups. When viewing all microglia (CCI and sham injury) separated on the basis of sex, there are specific populations that are clear to distinguish, as expected. When viewing each sex group of only CCI injury, some minor differences are noted, but the microglial populations occupy similar areas, indicating that there are likely more similarities. The density plots of sham groups similarities between the male and female groups as well.

Lymphoid and Myeloid Polarization

Spleens were obtained 7 days after injury for cell analysis with flow cytometry. Splenocyte panel included CD3, CD4, CD8, CD45RA, MHC-II, CD8, CD11b, and CD25. This panel allows us to observe changes in the adaptive immune response. When comparing groups, the panel helps us determine differences in T cell subtypes, such as cytotoxic, helper, and regulatory, and peripheral monocytes. The time interval of 7 days post-injury is a commonly used interval for splenocyte activation in our lab, and was selected in order to remain consistent with our prior experiments (41).

Flow cytometry analysis of splenocytes showed no statistically significant differences were observed for CD3, CD4, CD8, and Treg cells when comparing injury animals to sham, male injury to female injury, and male sham to female sham. MHC-II was recorded at higher counts for the male groups compared to the female groups, but these differences did not reach statistical significance **Supplemental Figures 7A–H**).

Cytokine Analysis

Brain tissue samples were obtained from rats 6 hours post-injury for cytokine analysis, to study for differences in inflammatory

cytokine expression in the acute phase after injury. Flow cytometry was utilized for a multiple cytokine assay (BioLegend Legendplex). To complement this, individual cytokine ELISAs were performed for TNF-alpha, IL-1β, and IL-17.

In the analysis of the multiple cytokine assay, none of the mean differences between the male CCI and the female CCI groups reached statistical significance for any cytokine (**Figures 4A–K**). In the individual ELISAs, we found no statistically significant differences between the male and female CCI groups. Male CCI rats recorded a significantly higher concentration of IL-1β than the male sham rats (**Figure 5**; mean difference = 0.09070 ± 0.01, p = 0.0045).

DISCUSSION

Our data found little difference in neuroinflammatory markers of injury when comparing males and females in a CCI model in the acute time period. These data are important as many have called for evaluating sex as a biological variable, by testing both males and females in experimental protocols. In this study, we sought to compare multiple properties of neuroinflammation between male and female sex, in a TBI rodent model. The properties compared were blood-brain barrier permeability, splenocyte activation, microglial activation, and inflammatory cytokine production. Each study was conducted at certain time intervals after injury (**Figure 1**) to account for the progression of neuroinflammation after a TBI. Time points for each study were selected based on our prior experience with each test.

Splenocyte activation markers did not differ significantly between sexes at 7 days post-injury. Microglial activation markers 7-day post injury had some differences noted on t-SNE, as there may have been more typical non-activated microglia in the female CCI group. However, their density plots did show that this group also had a nearly equivalent number of activated microglia; morphometry of the microglia in male and female CCI groups was largely similar. Our multiple cytokine assay and traditional ELISA showed some minor differences between the male and female groups at the 6-hour time point post injury, but none that reached statistical significance. Of all the studies, only a subset of the BBB permeability analysis showed a significant difference between sexes.

In the analysis of BBB permeability at 72 hours post-injury, the female CCI group recorded a higher mean integrated density compared to the male CCI group, but not a higher mean intensity. This result does suggest that there may be some sex-based difference in how a traumatic injury affects BBB permeability. We considered that the significantly smaller body weights of the female rats may have factored into this, thus the reason for our correlation analysis between weights and integrated density values. However, there was not a statistically significant correlation of body weight with a lower integrated density in all CCI rats. We also looked for correlations between brain weights and BBB permeability after injury, but there were no trends or significant correlations observed. These lack of correlations between brain and body weight and BBB permeability make the potential for weight as a confounding factor unlikely. The result does suggest that BBB permeability may be greater in female rats after CCI, but this was only shown in one specific subset of the BBB permeability analysis, and only after a certain range of fluorescence intensities was set prior to the analysis. One could argue that if this was a true difference on the basis of sex, BBB permeability would be a consistent finding across all analyses using common threshold parameters. In contrast to our results, Julienne et al. used a similar CCI injury model, and did not find a difference in BBB alteration (27). Interestingly, they did find that there may be a difference in vascular repair, as male rats had an increased activation and expression of β-catenin, a marker of angiogenesis and vascular repair, as well as an increase in cortical vascular density (27). A recent study by Bharadwaj et al. found results regarding BBB permeability similar to ours. In a CCI-model of mice, they found that females had a greater extravasation of a macromolecular HRP tracer at 3 hours and 24 hours post injury, compared to males; they did not observe a difference in neuroglial response (28). An earlier study by Duvdevani et al. used a CCI rodent model as well, using Evans Blue dye to evaluate BBB permeability; they found no significant sex difference in BBB permeability, and no effect of hormonal status at 1 day post injury (29). O'Connor et al. found that BBB permeability may be elevated in male rats after a diffuse injury. In a frontal impact diffuse TBI injury model, O'Connor et al. found an increase in Evans Blue dye extravasation in male rats compared to female rats. Interestingly, administration of exogenous estrogen or progesterone reduced BBB permeability in males, and also attenuated the increase in cerebral edema post-TBI in males and ovariectomized females (21).

Our study does have limitations. We utilized a sham-injury model that does not include a craniectomy, as studies have found that craniectomy procedures create a risk of confounding injury to the brain and cerebral blood vessels and our study largely focuses on the difference between male and female CCI-injured groups (37, 38). In each individual experiment, we studied changes in neuroinflammation and TBI pathology at specified time points. These time points were selected based on prior studies using these tests in our lab and upon our interpretation of discussions in the literature of the importance of certain time points in the progression of neuroinflammation in TBI. It is possible however, that differences may be seen at different time points in each individual test. While the male and female rats were of equal age, we did not coordinate the injury to occur at a specific stage of estrous cycle of female rats. There was some variability in the female rat cytokine analysis, and it is possible that this variability may be due to individual rats being at various stages of the estrous cycle. However, it is worth noting that this variability was not limited to the female rats, as it was also noted in certain other neuroinflammatory markers for the male rat groups. Some markers of flow cytometry, such as CD45 and CD11b appear to have larger variability in the male CCI groups compared to the female groups. For this study, we selected time points used in previous studies of neuroinflammation following CCI in rats, however, there is a reasonable possibility that our assays and time points were not sufficient to capture dynamic or temporal differences between male and females in TBI.

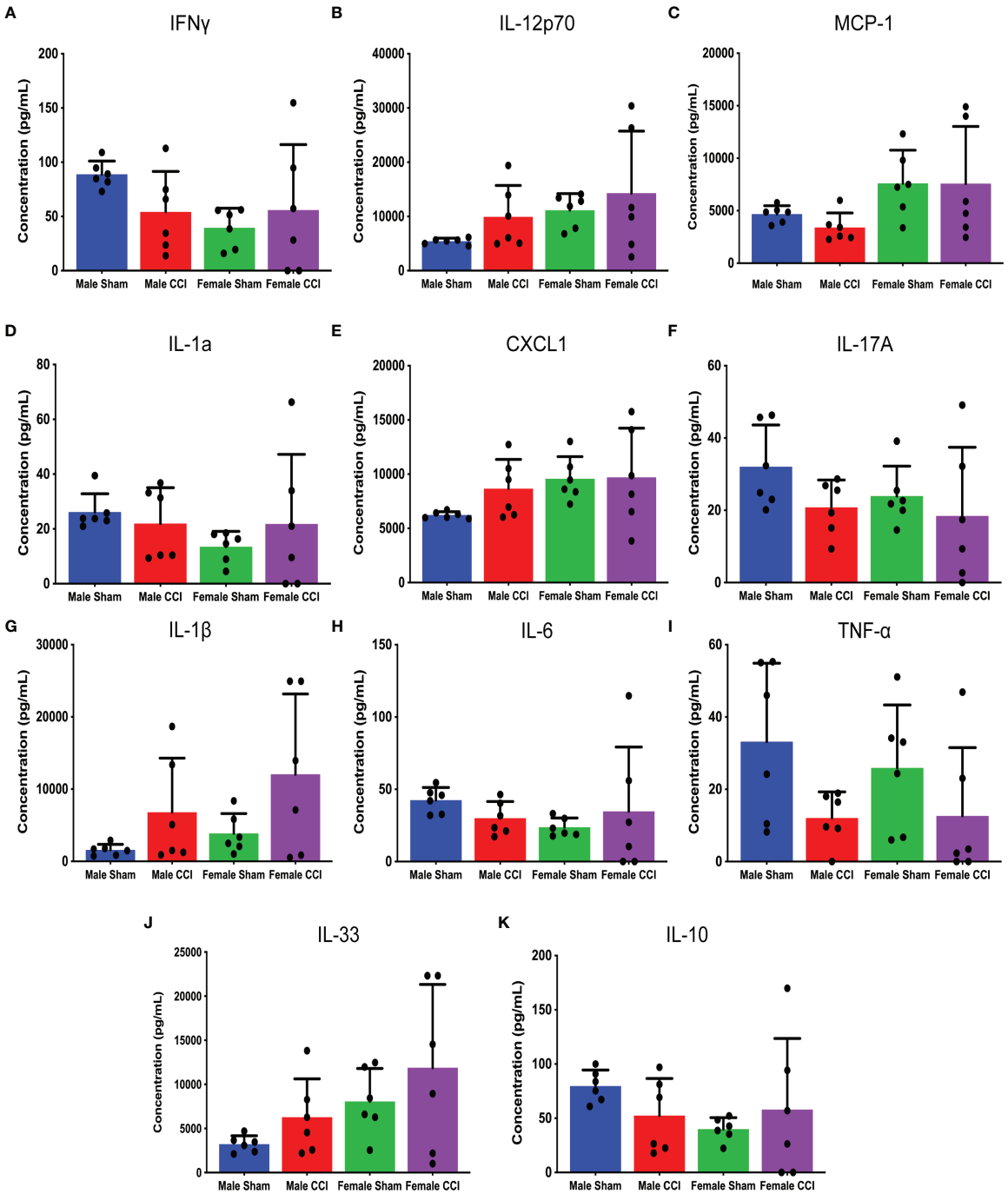

FIGURE 4 | Multiple inflammatory cytokine analysis of cerebral tissue culture. The above charts depict the mean concentrations of individual cytokines in cerebral tissue cultures detected by the cytokine assay. The following cytokines were analyzed in this assay: IFN-γ **(A)**, IL-12p70 **(B)**, MCP-1 **(C)**, IL-1A **(D)**, CXCL-1 **(E)**, IL-17A **(F)**, IL-1β **(G)**, IL-6 **(H)**, TNF-α **(I)**, IL-33 **(J)**, IL-10 **(K)**. While there were multiple differences detected between the male CCI and female CCI groups regarding specific cytokine levels, none of these differences reached statistical significance.

Our study sought to elucidate whether there were clear and significant differences in outcomes of neuroinflammation in our CCI-model between male and female rats. As stated above, the only significant difference detected was a potential increase in BBB permeability. In other pre-clinical studies, it seems that potential sex-based differences are varied. These differences can extend to multiple different features of neuroinflammation, and may vary with different models of TBI (**Table 2**). Similar to the

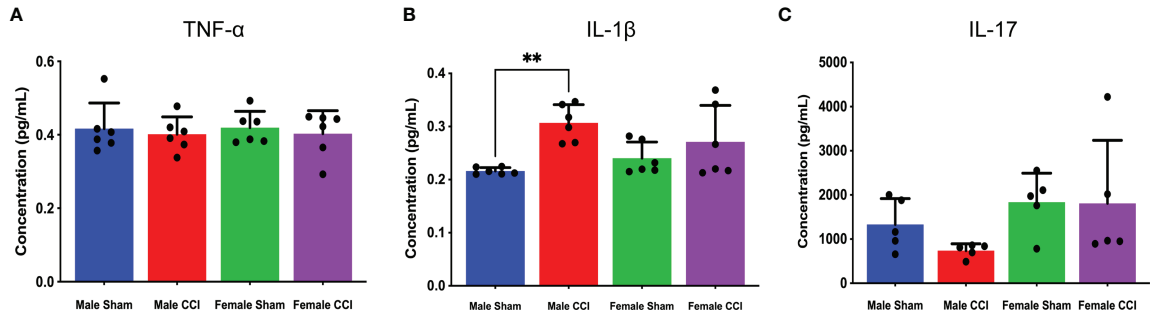

FIGURE 5 | Cytokine ELISAs. The above charts depict the average concentration of TNF-α **(A)**, IL-17 **(B)**, and IL-1β **(C)** detected in cerebral tissue culture from individual ELISAs. TNF-α concentrations were mostly equivalent across all groups. The female groups recorded higher IL-17 concentrations than the male groups, but there was no significant difference between the female CCI group and the female sham group **(B)**. IL-1β concentrations were elevated in the CCI groups, but there was no significant difference between the female CCI or male CCI groups **(C)**. **$p < 0.005$.

human studies, pre-clinical animal studies have yielded varying results. Neurodegeneration in rats after TBI may evolve at different rates in each sex, but it does not appear that one sex incurs a higher level of neurodegeneration (30, 31). In a series of experiments with piglets, Armstead et al. found that cerebral autoregulation males may be impaired after FPI, which is thought to be due to an upregulation of endothelin-1 (15–17). In contrast to our findings regarding microglia, Doran et al. found some functional differences between sexes in rats sustaining CCI injury. After CCI injury, microglia from female rats had decreased phagocytic activity and production of reactive oxygen species; however, they found no sex-based difference in the expression of pro-inflammatory cytokine expression by microglia (14). Villapol et al. found results that were similar; in their rodent CCI-model, there was a faster influx and activation of microglia in males compared to females, but the differences became indistinguishable after 1-week post-injury (32). Some studies have also indicated that it may be females with a more rapid pro-inflammatory response. In a meta-analysis of their data from a closed head injury of mice, Späni et al. found that females had a greater increase in pro-inflammatory markers like IL-1β, IL-6, and TNF-α in the 6-hour window post-injury; they also found that the anti-inflammatory cytokine IL-10 was elevated in males, but not females (51). In a CCI rat model, Taylor et al. found that ovariectomized females had an increased expression of IL-1β and IL-6 in the ipsilateral hippocampus, but males had a higher expression of TNF-α (22).

As mentioned previously, one limitation of our study is that we did not control for the stage of estrous cycle in female rats. However, the literature on the relationship between hormones and pathology presents various findings. Certain levels of circulating endogenous hormones have been linked to improved outcomes in TBI. Roof et al. found that pro-estrous female rats recorded a lower brain edema content at injury site compared to males. This difference was even greater in female rats with induced pseudopregnancy. They found that the decrease in cerebral edema after injury was more likely due to progesterone and not estrogen, as this decrease was not seen in ovariectomized rats until progesterone supplementation was administered (23). In a diffuse TBI model, Roof et al. found that cortical blood flow (CBF) decreased in male rats at a greater rate than females after injury; CBF was lower in ovariectomized females compared to intact females. However, the decrease of CBF in ovariectomized rats was not corrected with the administration of estrogen replacement (24). As mentioned above, Duvdevani et al. did not find an effect by hormonal status on BBB permeability (29), whereas O'Connor et al. found a treatment effect of estrogen and progesterone on reducing BBB permeability in male rats (21).

In another TBI model using ovariectomized rats, Bramlett et al. showed that intact female rats had smaller contusion volumes compared to male rats and ovariectomized female rats. Neuroprotection in this specific study was not limited to a specific stage of the estrous cycle, as the average contusion volume was similar between pro-estrous and non-pro-estrous female rats (25). Wagner et al. found that female rats achieved better functional outcomes, but the difference did not depend on estrous cycle stage. Using the CCI-injury model, Wagner et al. found that regardless of estrous phase, female rats performed better than male rats in motor performance tests. However, that difference in behavioral performance was not influenced by serum estrogen and progesterone levels at the time of injury (26).

Interestingly, there is some literature that supports the notion that it is actually the injury itself that induces disruption of hormonal signaling. In an observational human study, Wagner et al. found that progesterone levels increased in male patients after injury, and in female patients the progesterone level was equivalent to control patients in the luteal or follicular phase of the menstrual cycle. They found that differences in hormone levels between the male and female groups were small and did not reach significance. In their combined study sample of male and female patients, high progesterone levels post-injury were associated with an increase in acute care mortality (18).. This does raise the question of whether the endogenous hormones alone or the disruptions in the hormone signaling due to cerebral injury create these potential differences in potential outcomes. Follow-up studies with TBI patients have found that injury is associated with chronic hypopituitarism (19, 20). Amongst all of this, testosterone may also need to be considered, as it can be an estradiol metabolite. In the previously mentioned study by

Wagner et al., they reported that testosterone levels in male patients were lower after TBI compared to their controls, but in females, testosterone was elevated after injury. As with progesterone, they found a similar association with increased mortality in the patients that recorded elevated testosterone levels after injury (18). What our study does not evaluate is the potential response to any given treatment or intervention which may be influenced by sex of the animal.

Retrospective studies of sex-based differences in TBI have produced various findings of mortality rates after TBI (**Table 1**). A large retrospective clinical study of patients in the Chinese Head Trauma Data Bank of TBI was conducted by the Chinese Head Trauma Study Collaborators. In their study of 7145 acute TBI patients, the mortality rate in males and females was similar. When the study group stratified for severe TBI, they again found similar mortality rates (12). In another large retrospective study of 72,294 patients with moderate to severe TBI, males were compared to females. There was a lower risk in mortality in female patients overall, but when the female patients were stratified, it was found that this difference was present in perimenopausal or post-menopausal females. There was no difference in mortality when comparing pre-menopausal female patients to males, downplaying the influence of endogenous hormones on TBI outcomes (5).

Our results fit with the current literature discussing the potential influence of sex on TBI (**Tables 1**, **2**). Some sex-based differences have been noted in numerous studies utilizing various injury models. However, a distinct and common difference that is consistent across pre-clinical models has not been established in the most current literature. As our lab and others have discussed in prior reviews, the sex differences that may occur in TBI in the pre-clinical and clinical settings are wide, varied, and appear to have some dependence on the model or mechanism of TBI (51–53). TBI outcomes based on sex can also be dependent on the specific outcomes studied. In relation to our own model of TBI, our results indicate that the difference in outcomes between male and female rats may be minor, and difficult to control. In relating this to our review of the literature, further controlling our model on the basis of the hormonal balance or sex of rats may only yield small differences. These small differences may not reflect a significant or true variation in the progression of neuroinflammation based upon our preferred markers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Committee University of Texas Health Science Center at Houston, TX, USA.

AUTHOR CONTRIBUTIONS

MS and KP wrote the main manuscript text and prepared figures. MS and SO prepared **Figures 1**–**5**. AW prepared **Tables 1** and **2**. The manuscripts was reviewed by all authors. All authors contributed to the article and approved the submitted version.

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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.2022.753570/full#supplementary-material

Supplementary Figure 1 | Mean body weights of male and female rat groups in BBB permeability study combined with statistical analysis.

Supplementary Figure 2 | Subgroup analysis of male and female body weights in BBB permeability study with statistical analysis.

Supplementary Figure 3 | Subgroup analysis of male and female brain weights in BBB permeability study with statistical analysis.

Supplementary Figure 4 | Mean brain weights of male and female rat groups combined in BBB permeability study with statistical analysis.

Supplementary Figure 5 | Microglial traditional flow cytometry. The above charts depict the mean value of counted microglial cells expressing CD45 **(A)**, CD11b **(B)**, P2Y12 **(C)**, CD32 **(D)**, CD163 **(E)**, and RT1b **(F)**. No significant changes were noted between sham and injury in the number of cells expressing CD32. Male and female CCI groups recorded higher mean counts of cells expressing CD45, CD11b, and P2Y12 compared to their respective sham groups. No significant differences were detected when comparing the female CCI group to the male CCI group for any of the microglial activation markers.

Supplementary Figure 6 | t-Distributed stochastic neighbor embedding (t-SNE) of microglia flow cytometry data. These specific plots are coded to identify cells from

the ipsilateral cerebral hemisphere, in particular microglia, on the basis of cell surface markers **(A)**, CD45 and P2Y12 expression **(B)**, and size by morphometric analysis **(C)**.

Supplementary Figure 7 | Splenocyte traditional flow cytometry. The above charts depict the mean counts of cells derived from splenic tissue with the following marker expression: CD3-CD45RA+ **(A)**, CD3+CD45RA- **(E)**, CD11b **(C)**, CD4 **(D)**, MHC-II **(F)**, CD3 **(G)**, CD8 **(H)**. Regulatory type T cells are also identified *via* flow cytometry **(B)**. While the male CCI group did record a higher mean count for each of these markers, none of the differences reached statistical significance.

Supplementary Table 2 | Raw data of rat body/brain weights and BBB permeability mean fluorescent intensity and integrated density.

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Sex-Specific Cell Types and Molecular Pathways Indicate Fibro-Calcific Aortic Valve Stenosis

Veronika A. Myasoedova[1†], Ilaria Massaiu[1,2†], Donato Moschetta[1,3], Mattia Chiesa[1,4], Paola Songia[1], Vincenza Valerio[1,5], Valentina Alfieri[1], Romain Capoulade[6], Daniela Trabattoni[1], Daniele Andreini[1,7], Elvira Mass[2], Valentina Parisi[8] and Paolo Poggio[1]*

[1] Centro Cardiologico Monzino, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, [2] Developmental Biology of the Immune System, Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany, [3] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milano, Italy, [4] Department of Electronics, Information and Biomedical Engineering, Politecnico di Milano, Milan, Italy, [5] Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli Federico II, Napoli, Italy, [6] L'institut du thorax, INSERM, CNRS, University of Nantes, CHU Nantes, Nantes, France, [7] Department of Biomedical and Clinical Sciences "Luigi Sacco", University of Milan, Milan, Italy, [8] Dipartimento di Scienze Mediche traslazionali, Università degli Studi di Napoli Federico II, Napoli, Italy

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***Correspondence:**
Paolo Poggio
Paolo.Poggio@ccfm.it

[†]These authors have contributed equally to this work

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Background: Aortic stenosis (AS) is the most common valve disorder characterized by fibro-calcific remodeling of leaflets. Recent evidence indicated that there is a sex-related difference in AS development and progression. Fibrotic remodeling is peculiar in women's aortic valves, while men's leaflets are more calcified. Our study aimed to assess aortic valve fibrosis (AVF) in a severe AS cohort using non-invasive diagnostic tools and determine whether sex-specific pathological pathways and cell types are associated with severe AS.

Materials and Methods: We have included 28 men and 28 women matched for age with severe AS who underwent echocardiography and cardiac contrast-enhanced computed tomography (CT) before intervention. The calcium and fibrosis volumes were assessed and quantified using the ImageJ thresholding method, indexed calcium and fibrosis volume were calculated by dividing the volume by the aortic annular area. For a deeper understanding of molecular mechanisms characterizing AS disorder, differentially expressed genes and functional inferences between women and men's aortic valves were carried out on a publicly available microarray-based gene expression dataset (GSE102249). Cell types enrichment analysis in stenotic aortic valve tissues was used to reconstruct the sex-specific cellular composition of stenotic aortic valves.

Results: In agreement with the literature, our CT quantifications showed that women had significantly lower aortic valve calcium content compared to men, while fibrotic tissue composition was significantly higher in women than men. The expression profiles of human stenotic aortic valves confirm sex-dependent processes. Pro-fibrotic processes

were prevalent in women, while pro-inflammatory ones, linked to the immune response system, were enhanced in men. Cell-type enrichment analysis showed that mesenchymal cells were over-represented in AS valves of women, whereas signatures for monocytes, macrophages, T and B cells were enriched men ones.

Conclusions: Our data provide the basis that the fibro-calcific process of the aortic valve is sex-specific, both at gene expression and cell type level. The quantification of aortic valve fibrosis by CT could make it possible to perform population-based studies and non-invasive assessment of novel therapies to reduce or halt sex-related calcific aortic valve stenosis (CAVS) progression, acting in an optimal window of opportunity early in the course of the disease.

Keywords: aortic valve stenoses, fibrosis, inflammation, immune system, sex-difference

INTRODUCTION

Aortic stenosis (AS) is the most common valve disorder characterized by fibro-calcific remodeling of valve leaflets (1). Progressive aortic valve calcification (AVC) occurs in both sexes and multiple pieces of evidence indicate a sex-related difference in aortic valve composition in AS (2, 3). Cardiac computed tomography (CT) is recognized as a high-quality technique for AVC evaluation. To date, sex-specific CT thresholds of AVC have been implemented in clinical practice since it is now recognized that women have less AVC burden than men (4, 5).

Recently, Voisine et al. (6) using aortic valve histology, confirmed that women have more fibrotic remodeling compared to men. In addition, it has been shown that the quantitative assessment of both calcific and non-calcific (*i.e.*, fibrotic) aortic valve tissue by contrast-enhanced CT correlated well with AS severity (7). In addition, this study showed that, even if the indexed non-calcific volume is slightly lower in women compared to men, the fibro-calcific ratio is significantly higher in women. However, the analysis was performed in patients with mild, moderate, and severe AS, and only 4 women were classified with severe AS.

Furthermore, profound differences in mineral composition and morphology between sexes were recently reported, suggesting that aortic valve calcification follows different mineralization pathways in men and women (8). Of note, recent *in vitro* studies highlighted that human valve interstitial cells (VICs) isolated from men had a significantly higher calcification potential under pro-osteogenic and pro-inflammatory stimuli than cells isolated from women (9, 10). However, no study has identified sex-specific pathways and/or cell types linked to severe AS.

Therefore, we sought i) to confirm whether aortic valve fibrosis (AVF) is a more important contributor to AS in women compared to men, implementing a severe AS cohort, and ii) to determine which sex-specific pathological pathways and cell types are associated with severe AS. Moreover, with the aim to explore the global involvement in AS of circulating inflammatory cells, interstitial and endothelial cells, and the different cell subtypes contributing to the onset of pathology, we focused our study on the whole tissue.

MATERIALS AND METHODS

Study Population

Eighty consecutive patients (48 men and 32 women) who underwent Doppler echocardiography and cardiac CT within 3 months before aortic valve surgery were enrolled in Centro Cardiologico Monzino IRCCS (2018-2019). Exclusion criteria were AS due to rheumatic disease or radiotherapy, previous infective endocarditis, previous severe adverse reactions to an iodinated contrast agent, body mass index (BMI) ≥38 kg/m^2, and severely impaired renal function (estimated glomerular filtration rate < 30 ml/min/1.73 m^2). In the final analysis, 56 age and sex-matched patients, with both bicuspid (BAV) and tricuspid (TAV) aortic valve morphology, were included. This study was approved by the Institutional Review Board of Centro Cardiologico Monzino IRCCS (n. R1348/20-CCM1418), and the participants gave written informed consent. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

CT Examination and Imaging Acquisition

Cardiac CT (Revolution CT, GE Healthcare, WI) was performed using the following parameters: section configuration 256x0.625 mm, voxel size 0.625 mm, spatial resolution along the X-Y planes 0.23 mm, gantry rotation time 280 msec, and iterative reconstruction. A BMI-adapted scanning protocol (tube voltage and current) was used (11). All patients received a 50-mL bolus of contrast (Iomeprol 400 mg/mL, Bracco, Milan, Italy). Images were post-processed using dedicated software for AVC assessment (3Mensio Valves™, 3Mensio Medical Imaging, Maastricht, The Netherlands) (12) with manual correction. Contrast attenuation values (Hounsfield Units, HU) and contrast-to-noise ratio were measured at the level of the ascending aorta. Total AVF was assessed based on HU ranging between 30 and 350, adjusting the upper threshold by increments of 25 HU in either direction until blood pool was not highlighted,

as previously published for non-calcific aortic valve tissue evaluation (2, 7). The calcium and fibrosis volumes were assessed and quantified using the ImageJ (Java-based image processing program developed at the National Institutes of Health) thresholding method (**Figures 1A–H**) (13). Indexed contrast-enhanced CT calcium volume and fibrosis volume were calculated by dividing the volume by the aortic annular area for each patient.

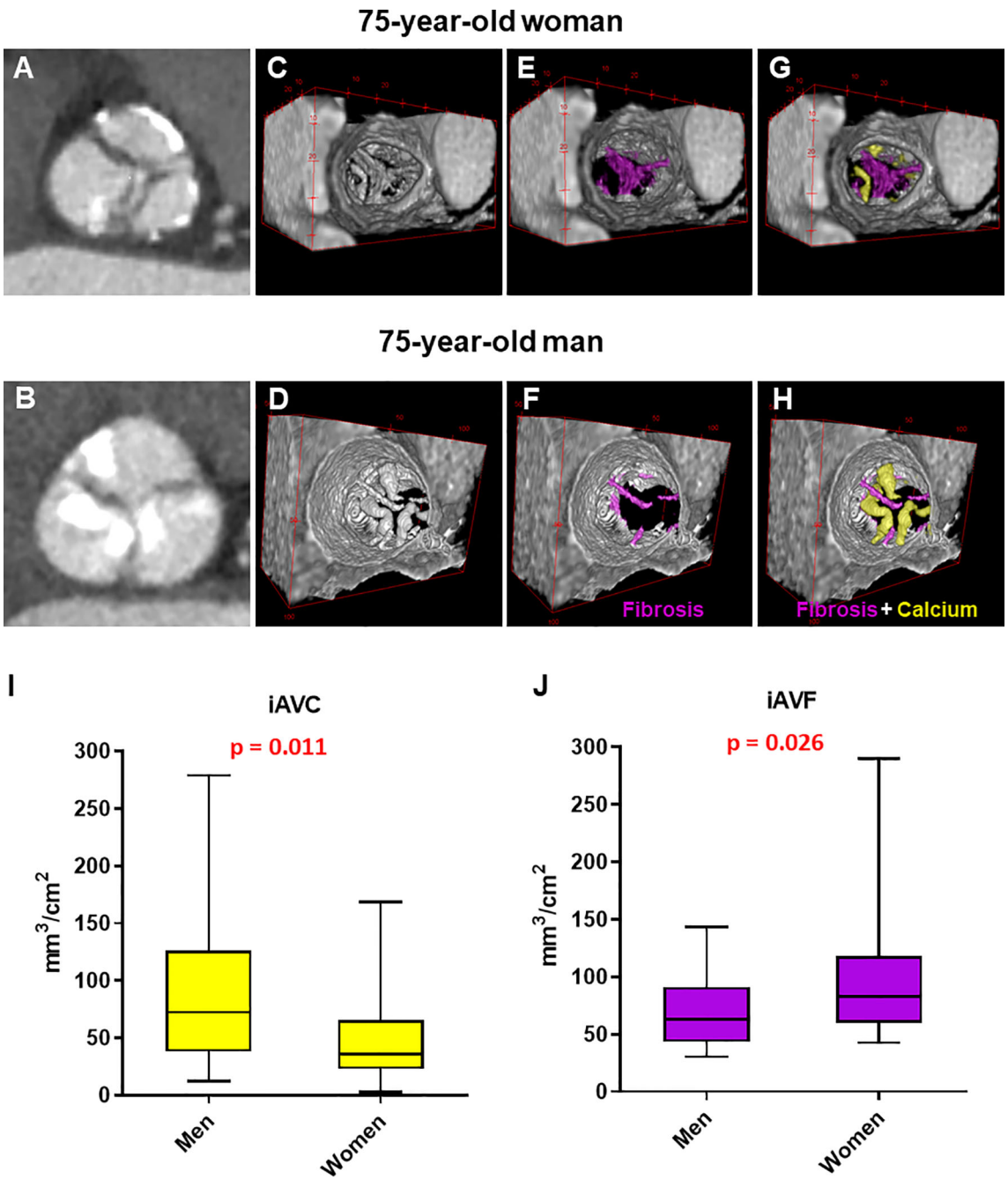

FIGURE 1 | Manual analysis of aortic valve calcium and fibrosis by contrast-enhanced computed tomography in men and women. **(A, B)** Computed tomography images reoriented into en face aortic valve view from a woman and a man with severe aortic stenosis. **(C–H)** Representative 3D contrast-enhanced volume-rendered views of aortic valve from a woman and a man with fibrotic tissue enhanced in violet and calcium in yellow. Box plots showing the difference in indexed aortic valve calcium **(I)** and fibrosis **(J)** volume between men (n = 28) and women (n = 28) with severe AS. Unpaired non-parametric Mann-Witney test was used to evaluate differences.

Deep Learning Workflow

The workflow diagram of the deep learning analysis is reported in **Supplementary Figure S1**. Each CT scan is composed of 256 or 224 slices (512 x 512 pixels) and stored in a DICOM object. In order to remove useless anatomical structures, such as aorta or ventricles, we selected the consecutive slices containing the aortic valve, which allows decreasing the computational burden and improving the overall network performances. Each selected slice was then used to perform a manual segmentation in order to generate the 'ground truth' images.

Thirty-eight out of 56 randomly selected samples were then used to train a U-Net model, with a default parameter, to segment the aortic valve. The remaining 20 samples served as the test set. The U-Net model was implemented with default hyperparameters, using the 'Keras' package; concerning the training step, we set the 'Root Mean Squared (RMS) propagation' optimizer to minimize the 'binary cross-entropy (BCE) dice' loss function, the number of epochs = 50 and the batch size = 10. The Dice Score was used to assess the segmentation accuracy, comparing the predicted mask and the ground truth.

Finally, the predicted masks were combined with the corresponding starting frames in order to extract the pixel intensity (Hounsfield Unit) of the aortic valve and, then, calculate the fibrosis and calcium volumes. The Pearson's correlation index (r_p) allowed assessing the consistency between the automatic predictions and the calculations of fibrosis and calcium volumes, made by human operators.

Gene Expression Data Processing

Gene expression data were retrieved from Gene Expression Omnibus (GEO) public repository [GEO accession number: GSE102249 (14)]; this dataset collects the transcriptomic analysis, performed by microarray technology, of 240 valve tissues, coming from 120 males and 120 females. We re-annotated the Illumina probe ID, using the Ensembl human gene annotation (GRCh38, version 97); then we removed outliers and normalized data by log2 transformation and quantile normalization. The analysis was performed by R (v. 3.6.3).

Pathways' Enrichment Analysis

Gene set enrichment analysis (GSEA) was conducted using GSEA pre-ranked tool (v 4.0.3) (15) and the biological functions were inferred by taking advantage of gene ontology biological processes (GO-BP), Reactome, WikiPathways, and BioCarta databases. To reduce redundancy and visually interpret GSEA results, the most significant GO-BP/Reactome/BioCarta pathway gene sets were identified by a false discovery rate (FDR) < 0.1. The network was drawn through the Enrichment Map software v.3.3.2 (16) implemented as a plug-in in Cytoscape v.3.8.2 (17).

Cell-Type Enrichment Analysis

Cellular composition of stenotic aortic valve tissue in women and men was inferred by the xCell R package (18), starting from the gene expression dataset of 240 samples. xCell raw scores were generated and transformed applying the spill-over compensation. Then, we assessed the association between the xCell scores and sex by Wilcoxon rank-sum test. The $\log_2$ fold-change (logFC) of each cell type scores between men and women was computed and the pValues were adjusted with FDR correction. It is important to note that xCell results are expressed in terms of enrichment scores. Therefore, any inference on the cell counts or proportion of cell types is not provided.

Statistical Analysis

The data were analyzed using IBM SPSS statistic 26 software. Continuous variables were expressed as mean ± standard deviation (SD) or, when variables had a skewed distribution, as median and interquartile range, while categorical ones were reported as frequency and percentage. Between-group differences were evaluated by Student t-test and by Pearson Chi-square ($\chi 2$) test. A pValue < 0.05 was considered to be statistically significant. Unpaired non-parametric Mann-Witney test was used to evaluate differences in indexed aortic calcification (iAVC) and fibrosis (iAVF).

Differential expression analysis between male and female samples was performed through the limma R/Bioconductor package (19). This statistical analysis was performed in the R environment v3.6.3. We deemed genes as significantly different at an FDR-adjusted pValue < 0.05. The robustness of the differential expression analysis results was assessed by exploring the histogram of the pValue distribution.

RESULTS

Contrast CT Evaluation of iAVC and iAVF

We studied 56 patients (28 men and 28 women) matched for age (71 ± 11 years) with severe AS (mean gradient 47.4 ± 13.6 mmHg, aortic valve area 0.82 ± 0.23 cm^2, and ejection fraction 64 ± 7%). **Table 1** summarizes the patients' characteristics. In brief, there was no difference between men and women in major cardiovascular risk factors, valve phenotype (17 BAV and 39 TAV in total), nor pharmacological treatment. As expected, men had higher body surface area and annulus diameter than women (1.89 ± 0.14 *vs.* 1.67 ± 0.17 m^2, respectively; p < 0.001) and (36.1 ± 3.0 *vs.* 31.9 ± 2.8 mm, respectively; p < 0.001), while women had lower aortic valve area than men (AVA; 0.74 ± 0.2 *vs.* 0.91 ± 0.2 cm^2, respectively; pValue = 0.007) but similar indexed AVA (0.44 ± 0.15 *vs.* 0.48 ± 0.12 cm^2/m^2, respectively; pValue = 0.262).

Our results confirmed that, for a same hemodynamic severity in both sex, women had significantly lower iAVC compared to men (36 [95% Confidence Interval (CI): 27-49] *vs.* 72 [95% CI: 48-114] mm^3/cm^2, respectively; pValue = 0.011). Furthermore, we showed that, in severe AS patients, the fibrotic tissue composition (*i.e.*, iAVF) of the valve leaflets was significantly higher in women compared to men (83 [95% CI: 65-111] *vs.* 63 [95% CI: 45-82] mm^3/cm^2, respectively; pValue = 0.026; **Figures 1I, J**). Finally, the fibro-calcific ratio, which indicates the predominance of valve fibrosis if > 1.0, was significantly higher in women compared to men (2.57 [95% CI: 1.16-3.62] *vs.*

TABLE 1 | Characteristics of sex and age-matched aortic stenosis (AS) patients that underwent contrast-enhanced cardiac computed tomography.

Variables	Men n = 28	Women n = 28	Total pValue
Age, years	71.4 ± 10.8	71.5 ± 11.1	0.961
Diabetes, n (%)	7 (25)	5 (18)	0.515
Hypertension, n (%)	22 (79)	21 (75)	0.752
Dyslipidaemia, n (%)	22 (79)	16 (57)	0.086
Smoking, n (%)	9 (32)	8 (27)	0.771
Body mass index, kg/m^2	26.9 ± 3.8	26.2 ± 6.0	0.588
eGFR, mL/min/1.73m^2	72.3 ± 17.6	80.1 ± 16.3	0.053
BSA, m^2	1.89 ± 0.14	1.67 ± 0.17	**<0.001**
LVEF, n (%)	64.0 ± 7.8	64.5 ± 7.2	0.798
Peak aortic jet velocity, m/s	4.4 ± 0.6	4.4 ± 0.7	0.916
Mean gradient (mmHg)	46.7 ± 11.8	48.0 ± 15.2	0.726
Aortic valve area, cm^2	0.91 ± 0.21	0.74 ± 0.23	**0.007**
Aortic valve area indexed (cm^2/m^2)	0.48 ± 0.12	0.45 ± 0.15	0.365
Annulus diameter, mm	36.1 ± 3.0	31.9 ± 2.8	**<0.001**
Antiplatelet agents, n (%)	17 (61)	13 (46)	0.284
ACE -inhibitors, n (%)	8 (27)	11 (39)	0.397
Angiotensin Receptor Blockers, n (%)	10 (36)	7 (25)	0.383
Beta-blockers, n (%)	17 (61)	18 (64)	0.783
Calcium channel blockers, n (%)	6 (21)	7 (25)	0.752
Diuretics, n (%)	11 (39)	7 (25)	0.252
Anti-diabetic drugs, n (%)	4 (14)	4 (14)	1.000
Lipid-lowering drugs, n (%)	18 (64)	11 (39)	0.061

ACE, angiotensin converting enzyme; eGFR, estimated glomerular filtration rate; LVEF, left ventricular ejection fraction. pValue < 0.05 are reported in bold.

0.78 [95% CI: 0.43-1.95], respectively; pValue = 0.0018). Interestingly, the significant difference in iAVF volume between men in women were observed only in TAV morphology (**Supplementary Figure S2**).

As the identification of valve in CT scans is a crucial step and could depend on the human operator experience, we validated our findings training a deep learning model (U-Net) to automatically recognize valve area in each frame without prior knowledge and calculate calcium and fibrosis volumes (**Figures 2A–C**). As shown in **Figure 2D**, we observed a high level of overlapping (Dice Score = 0.93+-0.09) between 400 predictions of valve area, performed by a human operator and the U-Net. Moreover, a Pearson's correlation index $r_p = 0.82$ in 10 females and $r_p = 0.93$ in 10 males was gained comparing the calcium volume calculated by human (x-axis) and obtained by the deep learning automatic framework on the test set (i.e., the dataset containing images never seen by U-net during training), while a $r_p = 0.96$ in 10 females and $r_p = 0.93$ in 10 males was gained comparing the fibrosis volume calculated by human (x-axis) and obtained by the deep learning automatic framework on the test set (**Figures 2E–H**).

Stenotic Aortic Valve Sex-Specific Gene Expression

The public microarray-based gene expression dataset of 240 human stenotic aortic valves was analyzed to identify the genes and the biological pathways differentially expressed between women and men with severe AS.

The sex classification for each sample was performed based on the expression levels of XY-linked genes. In particular, Ribosomal Protein S4 Y-Linked 1 (RPS4Y1) and X Inactive Specific Transcript (XIST) genes were considered sex-specific markers, to split 120 male and 120 female samples. During the differential expression analysis, the XY-linked genes and pseudogenes were filtered out.

We observed that 3105 genes were significantly regulated between samples of women and men (**Figure 3**). Setting a threshold of 0.2 $\log_2$ fold-change (logFC), 80 genes were up-regulated in women, while 173 genes were up-regulated in men (**Figure 3A** and **Supplementary Figure S3**). The heatmap (**Figure 3B**) shows that these 253 differentially expressed genes separate the specimens by sex. The top ten up-regulated genes in women and men are listed in **Tables 2** and **3**, respectively.

Functional Inferences Form Gene Expression Analysis of Women and Men Stenotic Aortic Valves

A total of 4979 pathways characterizing women and men's aortic valves were identified using GSEA (**Supplementary Tables S1**, **S2**). To enhance the interpretation of the results, we clustered the most significant pathways (FDR qValue < 0.1) into 33 macro-pathways (8 related to women and 25 related to men). The obtained enrichment network was drawn (**Figure 4**), considering the computed NES and the gene counts (**Supplementary Figure S4**).

The most representative pathways associated with women were related to the regulation of mRNA splicing, intraflagellar transport, post-translational modification (*i.e.*, mannosylation), elastin fibers formation, insulin-like growth factor 1 pathway, and regulation of tyrosine kinase receptor.

In contrast, the most representative pathways associated with men belonged to the immune system response (innate and adaptive immunity), cell migration, and chemotaxis, constituting the wider groups of highly interconnected gene sets. Other molecular pathways enriched in men are inflammation (interleukin 1, 2, 4, 6, 8, 10, and 12), bone

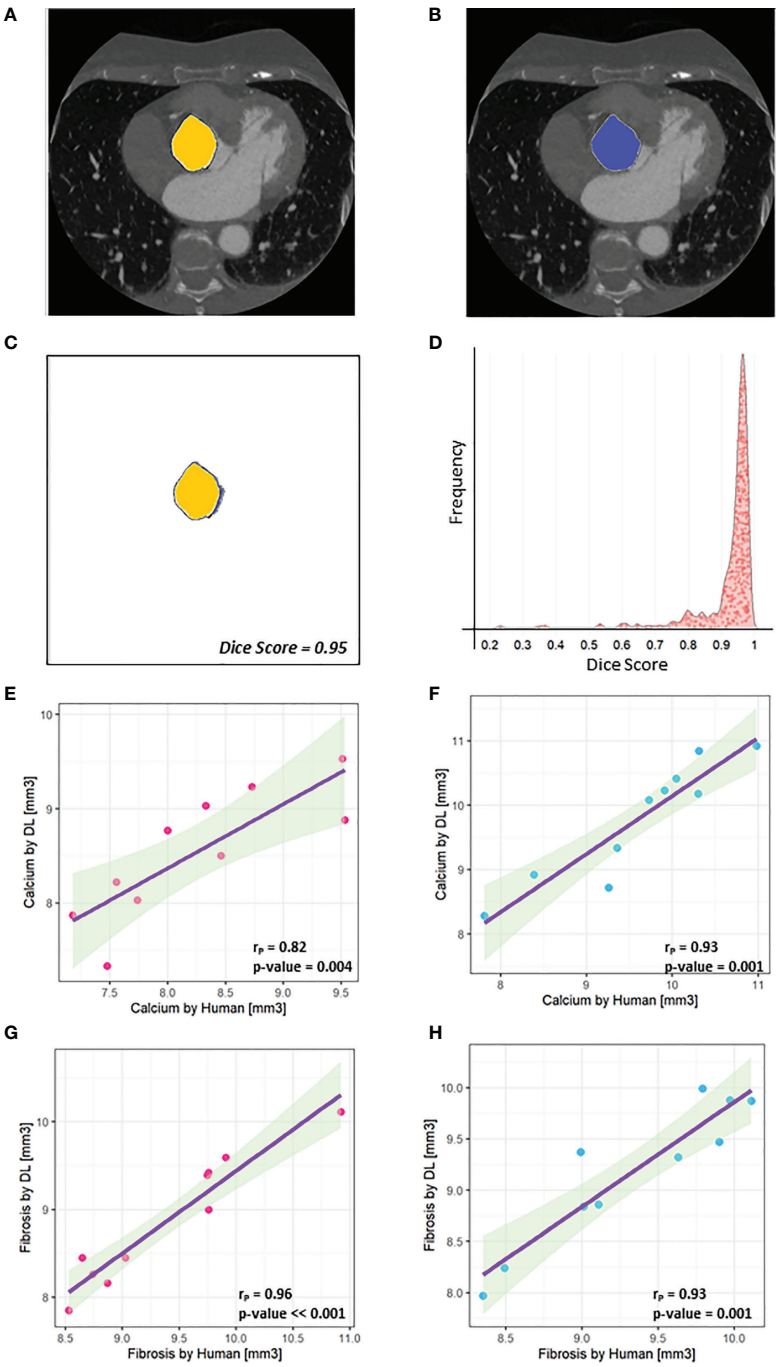

FIGURE 2 | U-net performances. **(A)** An example of mask designed by the human operator ('Ground truth') is highlighted in yellow and combined with the original frame. **(B)** An example of mask predicted by U-Net is highlighted in blue and combined with the original frame. **(C)** The high overlapping level between A and B is assessed by the Dice Score (0.95). **(D)** Distribution of Dice Scores shows that a higher level of overlapping (Dice score = 0.93 +- 0.09) was obtained between the learned U-Net model and the human operator. **(E)** A Pearson's correlation index rP = 0.82 was gained comparing the calcium volume in 10 female patients calculated by human (x-axis) and obtained by the deep learning automatic framework on the test set. **(F)** A Pearson's correlation index rP = 0.93 was gained comparing the calcium volume in 10 male patients calculated by human (x-axis) and obtained by the deep learning automatic framework on the test set. **(G)** A Pearson's correlation index rP = 0.96 was gained comparing the fibrosis volume in 10 female patients calculated by human (x-axis) and obtained by the deep learning automatic framework on the test. **(H)** A Pearson's correlation index rP = 0.93 was gained comparing the fibrosis volume in 10 male patients calculated by human (x-axis) and obtained by the deep learning automatic framework on the test set.

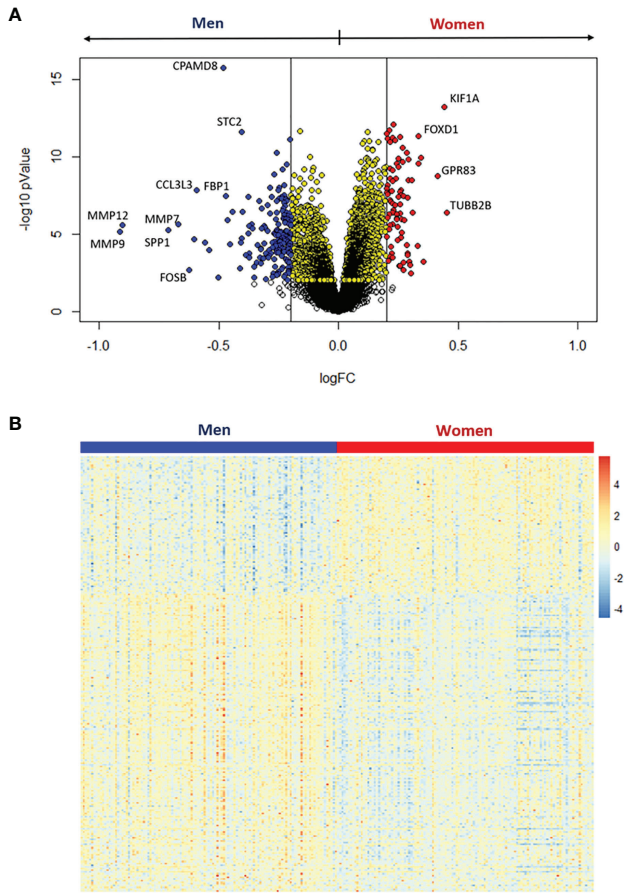

FIGURE 3 | Differential expression analysis between women and men stenotic aortic valves. **(A)** Volcano plot of the differentially expressed genes. Yellow dots represent significant differentially expressed genes at adjusted pValue < 0.05, whereas red and blue dots represent upregulated genes in women and men, respectively, with both the adjusted pValue < 0.05 and the absolute log2 fold change (logFC) > 0.2. **(B)** The heatmap shows the expression levels for the significantly upregulated genes with logFC > 0.2 in women (red) and men (blue).

remodeling, cell proliferation, endothelial cell differentiation, cellular senescence, lipoprotein metabolism, reactive oxygen species, and reactive nitrogen species production, and regulation of platelet aggregation.

Enrichment Analysis of Sex-Specific Cell Types in Stenotic Aortic Valve Tissues

To reconstruct the cellular composition of the stenotic aortic valve from bulk transcriptome, we employed the xCell method to convert the gene expression profile to enrichment scores of several immune and stromal cell types across specimens (18).

Aortic valve leaflets harbor a heterogeneous cell population (20). Thus, to deconvolute cell types within the bulk transcriptional data, we selected 28 cell-type-specific gene expression profiles (**Supplementary Table S3**).

The cell-type enrichment analysis showed that 14 cell types significantly differed between women and men (adjusted pValue < 0.05, **Figure 5A**). Of note, as shown in **Figure 5B**, 4 cell types were identified as over-represented in women (logFC > 0) and 10 in men (logFC < 0).

The most representative cell types in women's stenotic aortic valve leaflets were chondrocytes, fibroblasts, osteoblasts, and pericytes. Conversely, men's stenotic aortic valve leaflets were characterized by the presence of i) innate immune system cells (monocytes, macrophages, and dendritic cells), ii) adaptive immune system cells (B and T lymphocytes), and iii) mesenchymal stem cells.

DISCUSSION

Our study demonstrated a sex dimorphism in the fibrotic content of severe AS evaluated by contrast-enhanced CT and the expression profile of human stenotic aortic valve specimens confirm sex-dependent processes. Pro-fibrotic processes were prevalent in women, while pro-inflammatory ones, linked to the immune response system, were enhanced in men. Cell-type enrichment analysis revealed that mesenchymal cells were over-represented in women's AS valves, whereas transcriptional

TABLE 2 | Top 10 upregulated autosomal genes in women.

TOP 10 UPREGULATED GENES IN WOMEN			
Symbol	**Annotation**	**logFC**	**Adjusted pValue**
TUBB2B	Tubulin Beta 2B Class	0.45	2.31 e-5
KIF1A	kinesin family member 1A	0.44	4.37 e-10
GPR83	G Protein-Coupled Receptor 72/83	0.41	4.27 e-7
SLN	Sarcolipin	0.35	6.31 e-3
CHST6	Carbohydrate Sulfotransferase 6	0.34	3.13 e-8
FOXD1	Forkhead Box D1	0.33	7.81 e-9
DPT	Dermatopontin	0.33	1.10 e-7
COL6A6	Collagen Type VI Alpha 6 Chain	0.33	1.07 e-3
SOX8	SRY-Box Transcription Factor 8	0.31	2.49 e-5
RASL11B	RAS Like Family 11 Member B	0.31	6.68 e-7

TABLE 3 | Top 10 upregulated autosomal genes in men.

TOP10 UPREGULATED GENES IN MEN			
Symbol	**Annotation**	**logFC**	**Adjusted pValue**
MMP9	Matrix metallopeptidase 9	-0.91	2.03 e-4
MMP12	Matrix metallopeptidase 12	-0.90	9.40 e-5
SPP1	Secreted phosphoprotein 1	-0.71	1.62 e-4
MMP7	Matrix metallopeptidase 7	-0,67	8.99 e-5
FOSB	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit	-0,62	1.66 e-2
S100A8	S100 Calcium Binding Protein A8	-0.60	4.81 e-4
CCL3L3	Chemokine (C-C motif) ligand 3	-0.59	2.29 e-6
AQP9	Aquaporin 9	-0.56	6.88 e-4
HMOX1	Heme oxygenase (decycling) 1	-0.54	1.58 e-3
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	-0.50	3.86 e-2

signatures for immune cells such as monocytes, macrophages, T and B cells were enriched in men ones.

CAVS is the most frequent valvular heart disease in the Western world. No medical therapy is currently available (21) even if multiple studies have been performed to elucidate the molecular and cellular mechanisms underlining the progression of this debilitating and fatal disease (22, 23).

Recently, it has been shown, both at the clinical and experimental level, that fibro-calcification processes occurring during AS development and progression could be different in women and men (24, 25). Indeed, Voisine et al. (6) revealed that women had less calcification and more fibrotic remodeling than men, evaluated by CT and histological analysis. Our data, obtained from severe AS patients, confirmed these findings and demonstrated the possibility to quantify the fibrotic tissue present in the aortic valve in a semi-automated fashion. Of note, a recent study evaluating individual valve tissue components in AS showed that adding non-calcific parameters (*e.g.*, fibrosis) to the AVC score improved diagnostic and prognostic accuracy (2). Therefore, the routine use of CT could allow monitoring the valve remodeling process from its early stages (*i.e.*, aortic valve sclerosis) that are primarily due to fibrotic processes. Finally, the quantification of aortic valve fibrosis by CT could make it possible to perform population-based studies and non-invasive assessment of novel therapies aimed at reducing or halting sex-related AS progression, acting in an optimal window of opportunity that may be early in the course of the disease (26).

To date, sex-specific molecular pathways and cell types involved in AS progression at the tissue level remain elusive. *In vitro* experiments performed on human VICs stimulated with interferon-α and lipopolysaccharide showed that the Janus kinases (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays an important role in fibro-calcific processes, leading to a significant increased calcification in male VICs (9). Furthermore, the same group highlighted that STAT-1 was a potent inducer of hypoxia-inducible factor-1α (HIF-1α), resulting in increased sex-related VICs calcification (male VICs > female VICs) (10). Of note, a gene expression study performed on healthy porcine VICs showed that there is a sex-related propensity for CAVS (27). Our analysis discovered more than 250 genes differentially expressed between women and men stenotic aortic valves, for the first-time revealing a complex sex-specific molecular network at the end-stage of CAVS. We have found that genes predominantly expressed in women's stenotic aortic valves belong to pro-fibrotic pathways, while genes expressed in men belong to pro-inflammatory – mostly due to immune cell infiltrates – and pro-calcific ones. Remarkably, a recent study evaluating biomarkers of patients with AVC revealed that circulating proteins associated with fibrosis are mainly expressed in women, while, men had a positive association with biomarkers linked to inflammation and calcification (28).

CAVS is a chronic inflammatory disease involving innate and adaptive immune responses, characterized by extensive immune cell infiltration such as macrophages, dendritic cells, T cells, B cells, and mast cells (29). In addition, it has been shown that the crosstalk between these immune cell infiltrates and VICs plays a pivotal role in CAVS development and progression (29, 30). Surprisingly, up to 15% of cells present within healthy aortic valve leaflets are $CD45^+$, a marker of the hematopoietic lineage, and their number increases substantially when CAVS specimens are considered (31). Nevertheless, whether the influence of immune cells, in the CAVS contest, is sex-specific is still unknown.

Our enrichment analysis of sex-specific cell types in stenotic aortic valve tissues underlines that, in women, the immune cells' influence was mild compared to the contribution of chondrocytes, fibroblasts, osteoblasts, and pericytes. In contrast, the presence of macrophages, dendritic cells, B and T lymphocytes, in men's stenotic aortic valves, could indicate a sex-specific involvement of the immune system in CAVS.

Our study has some potential limitations. In particular, the public gene expression levels of 240 AS patients (14) were not corrected for the probable confounding factors, such as age, hypertension, diabetes mellitus, kidney disease, and AS severity, because of the unavailability of additional information. Indeed, previous studies showed that women present higher NYHA class and greater prevalence of chronic kidney disease, while men present a higher prevalence of CAD, peripheral artery disease (PAD), and diabetes (32, 33). However, the molecular results obtained from the gene expression analysis permitted to confirm the MDCT inferences on a validated publicly available dataset. However, the implication of immune response in men and women should be deeply investigated. Finally, our study was focused on the end-stage of CAVS disease. Certainly, the

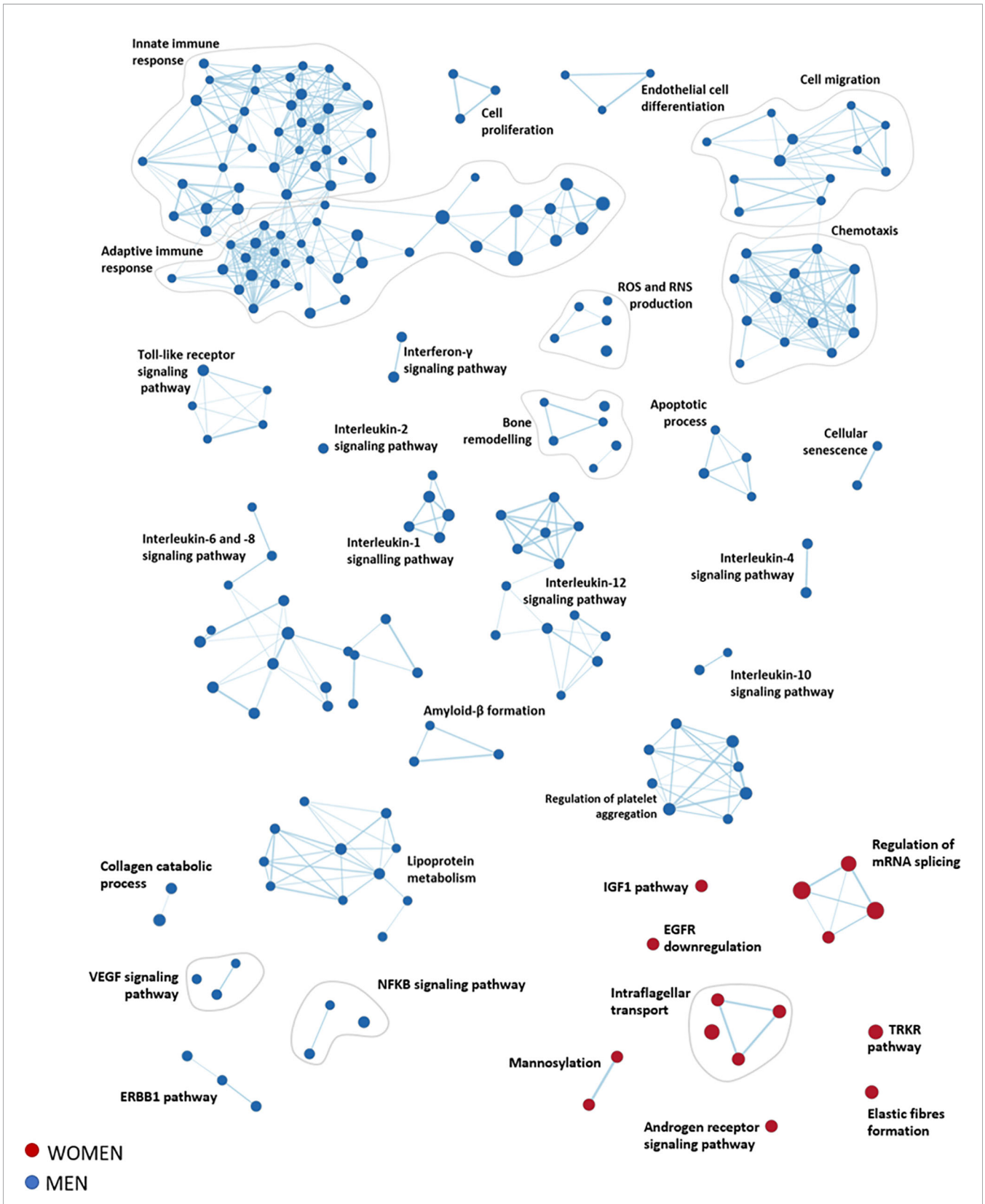

FIGURE 4 | Functional inferences from gene expression analysis of women and men stenotic aortic valves. The enrichment network shows the pathway sets (nodes) that are associated (FDR qValue < 0.1) with women (red) and men (blue), where the node size is proportional to the gene-set size.

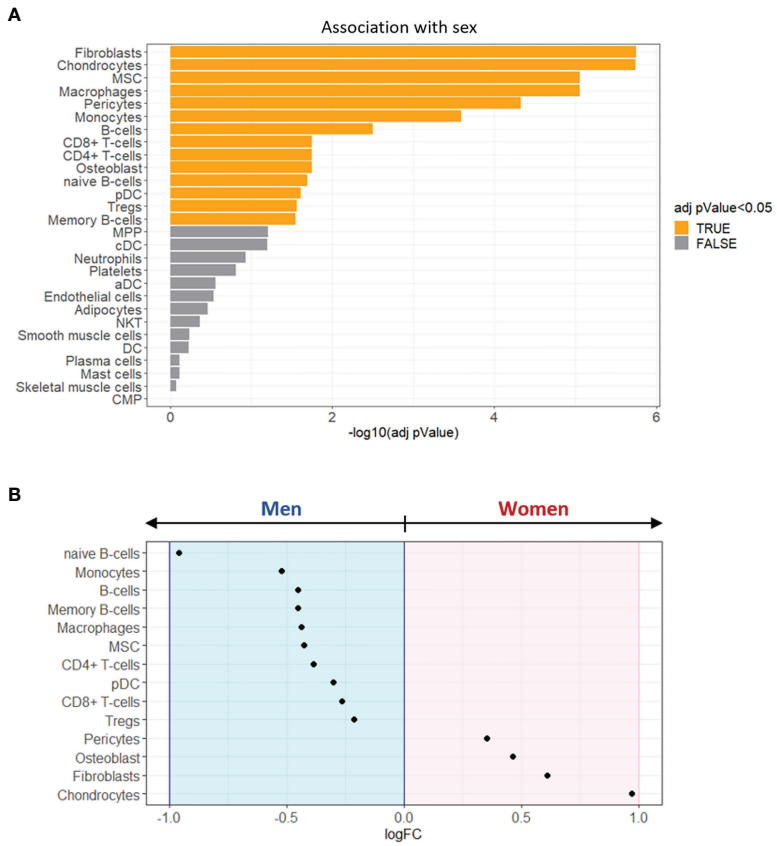

FIGURE 5 | Enrichment analysis of sex-specific cell types in stenotic aortic valve tissues. **(A)** Bar graph showing the sex-related association of xCell scores, performed by Wilcoxon rank-sum test. The cell types with FDR adjusted pValue < 0.05 (orange) were considered significantly associated with one of the two sexes. **(B)** The dot plot shows the log2 fold change (logFC) of xCell scores, being associated with women (logFC > 0; pink background) or with men (logFC < 0; light blue background).

inclusion of patients with early/mild stages of disease could allow deeper investigation related to molecular mechanisms underlying CAVS. Indeed, the results of our study suggested that routine use of non-invasive CT could allow monitoring valve remodeling progression from the earliest stage (*i.e.*, aortic valve sclerosis), which is mostly due to fibrotic processes. Finally, while there is evidence suggesting differences in pathology between men and women, a common pathological process manifesting clinically at different stages in men and women cannot be excluded.

CONCLUSION

Calcific aortic valve stenosis is a complex disease with many known molecular and cellular processes involved and, currently, there is no therapeutic option besides aortic valve replacement.

Our data suggest that the fibro-calcific process of the aortic valve is sex-specific, both at gene expression and cell type level. Thus, we should focus on sex-specific processes when looking for new CAVS pharmacological targets and novel prognostic and diagnostic approaches. The quantification of aortic valve fibrosis by CT could make it possible to perform population-based studies and non-invasive assessment of novel therapies aimed at reducing or halting sex-related CAVS progression, acting in an optimal window of opportunity that may be early in the course of the disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of Centro Cardiologico Monzino IRCCS (n. R1348/20-CCM1418). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The manuscript was mainly written by PP, VM, and IM with contributions from DM, MC, PS, VV, VA, RC, DT, DA, EM, and VP. VM, IM, and PP analyzed and interpreted data with contributions from MC, DT, and DA. VP and DT contributed to the design of the study. DM, MC, PS, VV, VA, RC, DT, DA, EM, and VP critically revised the manuscript. All authors reviewed and approved the 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.2022.747714/full#supplementary-material

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The Human Male Liver Is Predisposed to Inflammation *Via* Enhanced Myeloid Responses to Inflammatory Triggers

Adrian Kuipery[1,2†], *Deeqa Mahamed*[1,2†], *Shirin Nkongolo*[1], *June Ann D'Angelo*[3], *Alexandra Johnson Valiente*[1,2], *Aman Mehrotra*[1], *William C. Chapman*[4], *Peter Horton*[5,6,7], *Ian McGilvray*[8], *Harry L. A. Janssen*[1] *and Adam J. Gehring*[1,2*]

[1] Toronto Centre for Liver Disease, Toronto General Hospital Research Institute, University Health Network, Toronto, ON, Canada, [2] Department of Immunology, University of Toronto, Toronto, ON, Canada, [3] Molecular Microbiology and Immunology, Saint Louis University School of Medicine, St. Louis, MO, United States, [4] Division of Abdominal Transplant, Washington University School of Medicine, St. Louis, MO, United States, [5] Methodist University Hospital Transplant Institute, Memphis, TN, United States, [6] Division of Abdominal Transplant, Saint Louis University School of Medicine, St. Louis, MO, United States, [7] The University of Tennessee Health Science Center, Memphis, TN, United States, [8] Multi-Organ Transplant Program, Toronto General Hospital Research Institute, Toronto, ON, Canada

Background & Aim: Men have a higher prevalence of liver disease. Liver myeloid cells can regulate tissue inflammation, which drives progression of liver disease. We hypothesized that sex alters the responsiveness of liver myeloid cells, predisposing men to severe liver inflammation.

Methods: Luminex was done on plasma from Hepatitis B Virus infected patients undergoing nucleoside analogue cessation in 45 male and female patients. We collected immune cells from the sinusoids of uninfected livers of 53 male and female donors. Multiparametric flow cytometry was used to phenotype and characterize immune composition. Isolated monocytes were stimulated with TLR ligands to measure the inflammatory potential and the expression of regulators of TLR signaling.

Results: We confirmed that men experienced more frequent and severe liver damage upon Hepatitis B Virus reactivation, which was associated with inflammatory markers of myeloid activation. No differences were observed in the frequency or phenotype of sinusoidal myeloid cells between male and female livers. However, monocytes from male livers produced more inflammatory cytokines and chemokines in response to TLR stimulation than female monocytes. We investigated negative regulators of TLR signaling and found that TOLLIP was elevated in female liver-derived monocytes

Conclusions: Our data show that enhanced responsiveness of myeloid cells from the male liver predisposes men to inflammation, which was associated with altered expression of negative regulators of TLR signaling.

Keywords: sex, liver, monocytes, Hepatitis B virus, TLR signaling

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Luigia Trabace, University of Foggia, Italy

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***Correspondence:**
Adam J. Gehring
adam.gehring@uhnresearch.ca

†These authors have contributed equally to this work and share first authorship

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LAY SUMMARY

800 million people are at risk of developing cirrhosis as a result of chronic liver disease and this can put people at higher risk for liver cancer. The progression of chronic liver disease is caused by altered regulation of the immune response in the liver by viral infection, alcoholism or obesity. These external factors cause over-activation of immune cells in the liver, which kills liver cells, hepatocytes, which are then replaced by scar tissue. Epidemiological data clearly indicates that men are more likely to develop cirrhosis and liver cancer. We hypothesized that immune cells in the liver of men display enhanced activation to environmental triggers like viral infection, compared to women. We show here that specific immune cells in the liver, monocytes, look similar between men and women but show potential for enhanced inflammation upon activation. This enhanced inflammation could explain increased risk for men to develop cirrhosis and liver cancer.

INTRODUCTION

The liver is central to the overall health of the individual. The physiological function of the hepatocyte containing parenchyma contributes to nutrient transport, energy, fat storage, digestion, clotting factor production, and detoxification (1). However, the liver is also considered an immunological organ (2). The entire blood volume flows through the liver every 5 min, with 80% of that blood derived from the portal venous system (1). Portal blood is laden with bacterial and food antigens that are removed by immune cells lining the unique capillary bed of the liver. This system, when working properly, removes potentially toxic substances with minimal inflammation or damage to the normal physiological function. However, when dysregulated, the intrahepatic immune system causes chronic inflammatory disease that leads to fibrosis and cirrhosis, potentially ending in liver cancer (3).

Recent estimates put more than 800 million people at risk for the development of cirrhosis as a result of chronic liver inflammation (4). Epidemiological data highlights a clear disparity, based on sex, that underlies progression of liver disease. Men are more likely to develop cirrhosis than women (5), translating to the fact that men are three times more likely to develop liver cancer than women (6). Overall, men are more likely to die from chronic liver disease than women (7). Given the different outcomes of liver disease in men, compared to women, we hypothesized that the intrahepatic immune system would display differences in either composition or function between men and women.

Under normal conditions, the liver displays a tolerogenic environment that suppresses immune activation in the tissue (8). This environmental regulation is primarily controlled by liver and sinusoid resident myeloid cells, including macrophages and monocytes. Liver macrophages, or Kupffer cells, are a heterogeneous population of macrophages that produce IL-10 and TGF-β in response to LPS to maintain the suppressive liver environment (9). However, exposure to pathogen-associated molecular patterns (PAMPs) breaks the tolerogenic environment, triggering an inflammatory cascade that recruits inflammatory immune cells to the liver, causing non-specific hepatocyte killing (10). Resident within the liver and sinusoids, and recruited early in the inflammatory process, is a significant monocyte population with the plasticity to develop into inflammatory macrophages (11, 12). Given the central role of myeloid cells in the inflammatory process, we hypothesized the myeloid population within the liver may be an important source of the sexual dimorphism observed in the progression of chronic liver inflammation.

To investigate this hypothesis, we first validated the male-bias for hepatitis in a clinical cohort of chronic hepatitis B (CHB) patients that developed liver inflammation due to Hepatitis B virus (HBV) reactivation. Subsequently, we analyzed the composition and functional profile of immune cells isolated from uninfected donor livers prior to transplant. Our data indicate that biological sex did not significantly alter the composition or phenotype of myeloid cells derived from the human liver. However, exposure to Toll-like receptor (TLR) ligands unmasked a significant sex-bias in the inflammatory potential of intrahepatic monocytes. Finally, we suggest that males are at a higher risk for the development of liver disease due to lower expression of negative regulators of NF-κB signaling. Our data provides evidence in the human liver to explain male-biased progression of inflammatory liver disease.

MATERIALS AND METHODS

Ethics Statement

Peripheral blood and liver perfusion samples were collected from Saint Louis University Hospital and Barnes Jewish Hospital, in collaboration with Mid-America Transplant Services in Saint Louis, Missouri. Perfusion samples were also collected at the Toronto General Hospital, Toronto, Canada. Informed, written consent was obtained from next of kin. The study was approved by the Saint Louis University institutional review board and the University Health Network research ethics board.

Plasma Samples From Chronic Hepatitis B Patients

Plasma was collected from 45 patients who underwent a protocol-defined stop to their antiviral therapy for chronic HBV infection. The clinical characteristics of the patients and outcome of the study was previously published (13). Patient plasma was analyzed using a custom Luminex Bead Assay (R&D Systems) to measure CD14, soluble CD163 (sCD163), IL-1α, IL-1β, IL-6, TNF-α, FAS-L, TRAIL, and CXCL10 in serum of patients at baseline, 12 w and 18 w after stopping therapy. Each patient's baseline measurements were subtracted from the 12w and 18w samples to normalize change from baseline.

Blood Collection and Processing

Blood was collected by standard venipuncture. PBMC were collected by density gradient centrifugation and cryopreserved in liquid nitrogen in 90% knockout serum replacement (KO; Life Tech) + 10% DMSO (Sigma).

Collection and Characterization of Sinusoidal Resident Intrahepatic Mononuclear Cells

Non-adherent sinusoidal resident intrahepatic mononuclear cells (IHMC) were isolated from the livers of cadaveric donors. Liver donor characteristics can be found in **Supplementary Table 1** and the distribution of cause of death in **Supplementary Table 2**. Peripheral blood and liver perfusion samples were collected at the Mid-America Transplant Services for organs assigned for transplant at the Saint Louis University Hospital or Barnes-Jewish Hospital Abdominal Transplant Programs. Samples were also collected at the Toronto General Hospital. After blood collection, peripheral blood was removed from the liver by aortic flush and IHMC were collected by liver-specific, back table, portal vein perfusion with 1L of University of Wisconsin solution. Mononuclear cells were concentrated using centrifugation at 300xg for 10 min before being layered over ficoll (GE healthcare) to remove residual red blood cell contamination. Following density gradient separation, cells were cryopreserved as above.

Flow Cytometry

IHMC ($1x10^6$ cells) were stained with fixable viability dyes (ThermoFisher Scientific) and fluorochrome conjugated antibodies listed in **Supplementary Table 3**. Samples were acquired on BD flow cytometry instruments and analyzed with FlowJo V10 software (TreeStar Inc). Live singlet monocytes (CD14+CD3-CD7-CD19-CD20-CD56) were down-sampled to 5000 per sample and concatenated before tSNE dimensionality reduction analysis in FlowJo using the following markers for monocytes: CD32, CD64, HLA-DR, CD11b, CD11c, CD35, CD163, CD68, and CD88

Stimulation of IHMC With TLR Ligands

IHMC monocytes were purified using CD14+ Microbeads (Miltenyi) and cultured for 24h in the presence of TLR ligands (TLR2: 10^8/ml heat-killed *Listeria monocytogenes*, TLR3: 10 μg/ml polyI:C, TLR4: 1 μg/ml *E. coli* LPS, TLR5: 1 μg/ml *B. subtilis* flagellin, TLR8: 5 μg/ml ssRNA40; Invivogen). Supernatants were collected and measured for the accumulation of 12 different cytokines and chemokines using Cytometric Bead Arrays (BD Biosciences).

Sex Hormone Exposure and TLR Expression on Monocytes

Total peripheral blood mononuclear cells from 3 healthy donors were cultured in serum free, phenol red-free RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids, and 1mM sodium pyruvate. Cells were treated with 1 μM beta-estradiol (EST) or 5a-Dihydrotestosterone (DHT) for 18h and then stimulated with R848 (1 μg/ml) or TL8-506 (TLR8 agonist, 0.1 μg/ml) for 6h in the presence of 10 μg/ml Brefeldin A. Following the stimulation, PBMC were stained with viability dye and stained for CD14. Cells were permeabilized with cytofix/cytoperm (BD Biosciences) and stained for IL-6 or CCL4 for 30 min at 4°C, washed and fixed in 1% paraformaldehyde. Samples were acquired and analyzed using flow cytometry as described above.

To measure TLR expression, IHMC samples from 5 male and 5 female liver donors were stained for viability, CD14, and TLR5, followed by intracellular TLR8 staining in the method described above. Samples were acquired and analyzed using flow cytometry as described above.

Quantitative PCR of the TLR Pathway

IHMC monocytes from 6 patients were isolated as described above. Following isolation, RNA was isolated using a RNeasy micro kit (Qiagen) and quantified using a NanoDrop 2000. 350 ng of RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured with pre-designed TaqMan Gene Expression Assay (FAM) kits (Thermo Fisher). TaqMan qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) on a QuantStudio 6 thermocycler (Applied Biosystems) to investigate the expression of TLRs and regulators of TLR and expression was normalized to the expression of beta-2-microglobulin

TOLLIP Western Blot

CD14 monocytes from 10 PBMC and 12 IHMC donors were isolated as described above (PBMC: n = 5 male, 5 female; IHMC: n = 4 male, 8 female). Cells were lysed with RIPA Buffer with Protease Inhibitors (Thermo Scientific) according to manufacturer description. Cell lysate was run on a 12% reducing acrylamide gel and transferred to a PVDF membrane. The membrane was blocked for 90 minutes with MilliQ water with 5% skim milk. TOLLIP (Biolegend) and actin (BD Biosciences) staining was done 1:1000 and 1:10,000, respectively, in TBST with 2% BSA for 1 hour. Secondary staining with HRP conjugated anti-murine IgG at 1:2000 (Biolegend) in TBST with 2% skim milk was done for 1 hour. TOLLIP and actin staining was measured using ECL detection reagents (Bio-Rad) and imaged with a ChemiDoc Imaging System (Bio-Rad).

Data Analysis and Statistics

Statistical analysis was performed with GraphPad Prism 8 and R V3.5.3. T-sne analysis was performed in FlowJo (parameters: flt-tsne implementation, max iterations 2000, Theta 0.5, learning rate 200). The statistical analysis performed for each experiment is included in the figure legend. Chemiluminescence was measured using Image Lab V6.1.0 (Bio-Rad). Outlier analysis was performed using the Grubb's method (alpha = 0.05).

RESULTS

Enhanced Myeloid Inflammatory Responses in Men After HBV Reactivation

To validate clinical observations that males have worsened disease outcomes in the context of hepatitis, and to identify inflammatory cytokine profiles, we chose to investigate inflammatory cytokine production in males and females following HBV reactivation during nucleoside analogue cessation. HBV reactivation following nucleoside analogue cessation induces rapid rebound in HBV replication and induces liver inflammation and damage in a majority of chronic hepatitis B (CHB) patients (13, 14) However, HBV exclusively replicates within hepatocytes and naturally derived virus does not trigger inflammatory responses *in vivo* (15). The trigger for inflammation is currently unknown but HBV reactivation provides a predictable time frame to compare naturally induced, liver-specific inflammation between men and women.

We analyzed the kinetics, magnitude, and plasma immunological profile in 45 chronic hepatitis B (CHB) patients stopping antiviral therapy (13). The participants in this study were 43% female (n=19) and 57% male (n=26; **Figure 1A**). Patients were stratified by sex and the median ALT levels were plotted over a 48w follow-up period. ALT values were normalized for baseline differences between men and women, 25 U/L for females and 35 U/L for males and displayed as the upper limit of normal (ULN) (**Figure 1B**). The peak ALT for men was significantly higher than women (area under the curve (AUC) ± standard error of the mean; males: 240.6 ± 37.8, females: 134.7 ± 21.3), indicative of greater liver damage (**Figure 1B**). We then calculated the frequency of men or women who developed a clinical relapse from HBV reactivation (ALT>1.5xULN and HBV DNA > 2000 IU/ml).

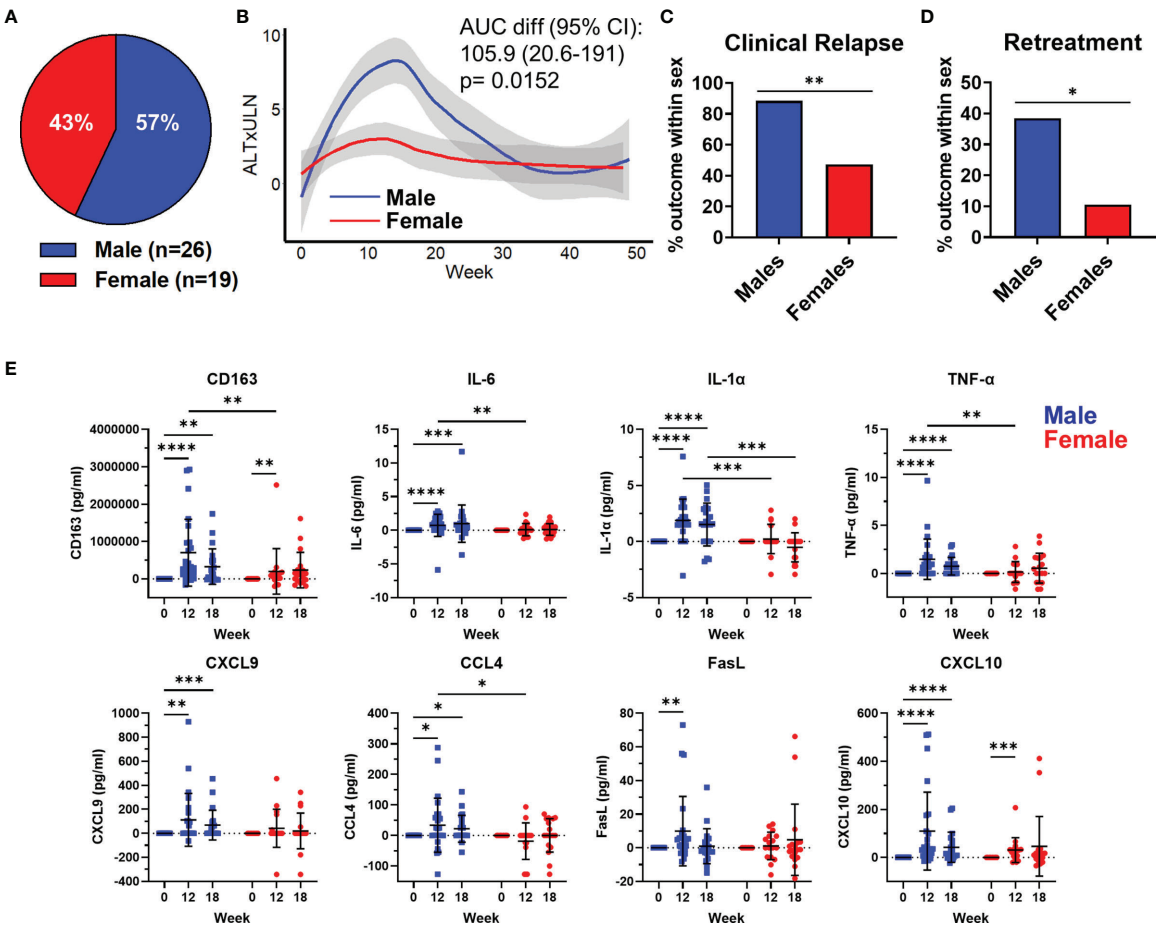

FIGURE 1 | HBV reactivation following treatment cessation induces a myeloid inflammatory profile in the plasma of chronic hepatitis B patients. A description of the cohort has been previously published (13). **(A)** Distribution of participants sex (n=26 males, 19 female) in the Toronto STOP study. **(B)** Loess regression curves of male and female ALT levels as a multiple of upper limit of normal (ALTxULN) during the 48 week follow up to stopping Nuc therapy. **(C)** Incidence of clinical relapse in males and females. **(D)** Proportion of male and female patients who required retreatment after stopping Nuc therapy. **(E)** Plasma levels of cytokines and chemokines at 12w and 18w after stopping Nuc therapy relative to baseline values at week 0. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney U tests. Figures depict mean +/- SD.

We found that 88% of men experienced clinical relapse, whereas only 47% of women relapsed (**Figure 1C**; relative risk of 1.9 (95% CI 1.23-3.28); p=0.0063). This translated clinically into more men having to restart antiviral therapy than women (**Figure 1D**; relative risk for retreatment for males = 3.65 (95% CI 1.07-13.98), p=0.047). These clinical data demonstrate that the male liver is primed for enhanced inflammatory responses with a pathological stimulus during natural infection.

We measured immune markers in the plasma and compared the change from baseline to immune marker concentrations at week 12 and 18, which corresponded to the average peak ALT. We observed a significant increase in sCD163, a monocyte/macrophage activation marker, at week 12 and 18. The significant increase in sCD163 was detectable in both men and women at week 12 but the magnitude of sCD163 increase was significantly greater in men and persisted until week 18 after stopping therapy (**Figure 1E**). Furthermore, we only observed significant increases in IL-6, IL-1α, TNF-α, CXCL-9, CCL4 and Fas ligand in men after stopping therapy (**Figure 1E**). CXCL-10 showed significant increases in both men and women at week 12 but, again, the magnitude of CXCL-10 was greater in men and remained significantly elevated at 18 weeks post stopping therapy (**Figure 1E**). Finally, we found that compared to females, men produced more sCD163, IL-6, IL-1α, TNF-α, and CCL4 at week 12. These data show that a liver-specific viral infection induces an enhanced myeloid inflammatory signature in men compared to women.

The Intrasinusoidal Myeloid Composition Does Not Differ by Sex, Age, or BMI

Having verified the predisposition for men in developing hepatitis and defining a link to biomarkers of myeloid activation in active hepatitis in hepatitis B patients, we next investigated if sex impacts human liver immune composition. To study the immune composition of the liver, we used portal vein liver perfusions collected prior to transplant. Liver perfusions flush non-adherent, intrasinusoidal immune cells from the liver and have become a recognized source to obtain immune cells from uninfected liver transplant donors (16–19). We analyzed the frequency of seven intrasinusoidal and peripheral blood myeloid populations from liver donors using multiparametric flow cytometry (**Figure 2A**). Because neutrophils and other granulocytes are lost during density centrifugation and do not survive cryopreservation, these populations have been excluded from our analysis. In addition, we did not find a significant frequency of Kupffer cells in the perfusions using cytospin or MARCO and TIMD4 staining by flow cytometry (not shown). Kupffer cells are tightly bound to liver endothelial cells and likely require tissue digestion for mobilization (20).

The majority of myeloid cells in the intrahepatic mononuclear cells (IHMC) were classical CD14+ monocytes. However, classical CD14 monocytes (CD14 MN) were significantly reduced in the IHMC compared to matched PBMC (67.2 ± 2.6% vs. 86.2 ± 1.9% of HLA-DR+ myeloid cells respectively). In comparison, the liver contained a significantly higher proportion of CD14+CD16+ intermediate monocytes compared to the blood (28.69% and 11.23% of HLA-DR+ myeloid cells respectively). CD16+ monocytes made up 1-2% of HLA-DR+ myeloid cells in both compartments (**Figure 2B**). We similarly observed significant differences in the frequency of plasmacytoid DCs between the PBMC and IHMC. The majority of dendritic cells (DC) in the liver were CD11c myeloid derived DC. Conventional dendritic cell populations were enriched in the liver when compared to matched blood (**Figure 2B** and **Supplementary Table 4**). However, CD1a+ DC were extremely rare within liver perfusions as they have been described as tissue DCs and, similar to macrophages, likely require tissue digestion for full mobilization (21). As stated above, we did not find evidence for liver macrophages in the liver perfusion. These data demonstrate that myeloid frequencies in the liver differ significantly from blood and displayed a composition consistent with the intrahepatic environment.

We then compared the frequency of myeloid populations between male and female donors. Sex had no influence on myeloid composition (**Figure 2C** and **Supplementary Table 5**). Age is associated with inflammation (22). Therefore, we compared myeloid composition by age and, similarly, found that the intrahepatic myeloid composition did not change with age (**Figure 2D** and **Supplementary Table 6**). Similarly, when the effect of BMI on myeloid population distribution was investigated, we did not identify any relationship at healthy, overweight, and obese BMI with any population (**Figure 2E** and **Supplementary Table 7**). Overall, our data indicated that biological sex, age, and BMI had a minimal impact on myeloid composition, suggesting that the functional profile of myeloid cells may be responsible for the sex-bias in liver disease progression.

TLR-Induced Cytokine Production Is Increased in Monocytes Isolated From Male Livers

With no differences in myeloid composition, we tested if the inflammatory potential of intrasinusoidal myeloid cells differed between males and females, as myeloid cells play key roles in liver inflammation and differentiate into inflammatory macrophages (11, 12). To investigate this, we tested the response of monocytes to inflammatory stimuli. Isolated monocytes were activated with agonists specific for TLR2, TLR3, TLR4, TLR5, and TLR8 for 24 h. After stimulation, we measured the production of 12 cytokines and chemokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, CXCL-10, G-CSF, GM-CSF, MCP-1 (CCL2), Mip-1α (CCL3), Mip-1β (CCL4), and TNF-α). Intrasinusoidal monocytes robustly produced cytokines and chemokines in response to stimulation *via* TLR2, TLR4, TLR5, and TLR8 (**Figure 3A**). TLR3 activation was the only condition that induced CXCL-10 production, although overall responses to TLR3 were low in monocytes (data not shown). We also tested the response of blood-derived monocytes to TLR agonist stimulation (**Figure 3B**).

We observed significant differences in cytokine/chemokine production between male and female liver monocytes after stimulation of TLR2, TLR4, TLR5, and TLR8. The immune markers showing the most significant differences were IL-6 and

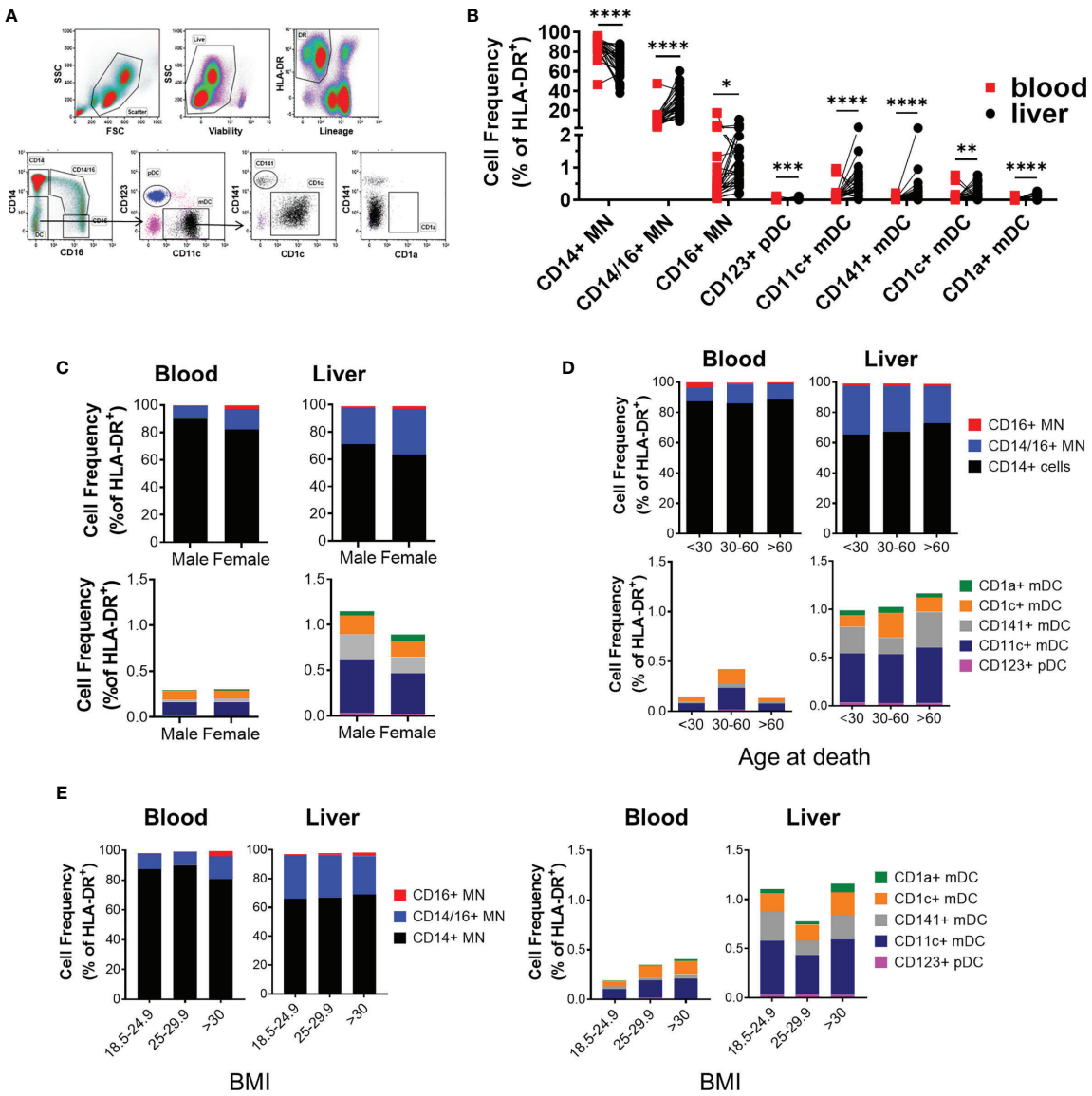

FIGURE 2 | Intrahepatic mononuclear cells from liver perfusions display a profile of organ-specific immune cells. **(A)** Flow cytometry panel to measure the frequency of IHMC and matched PBMC myeloid frequencies. **(B)** Summarized myeloid cell frequencies between blood and liver (blood n=27, liver n = 30). Data on frequency displayed as percent of HLA-DR+ cells. Percent of total HLA-DR+ cells for each myeloid population based on **(C)** Sex (n=18 males, 12 females), **(D)** Age (<30, n= 8; 30-60, n=15; >60, n=7), or **(E)** BMI (18.5-24.9 n=13; 25-29.9 n=8; >30 n=9). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Statistical analyses: Mann-Whitney U tests and Kruskal-Wallis tests followed by Dunn's multiple comparisons test when comparing cell frequencies by sex and across age groups.

IL-1α and the chemokines CCL3, CCL4, and CCL2 (**Figure 3A**). TNF-α, IL-10, IL-1β, and G-CSF also showed significant differences between men and women with select TLR stimulation (**Supplementary Figure 1**). CXCL-10, GM-CSF, and IL-12p70 did not show significant differences between men and women (**Supplementary Figure 1**). We did not find a significant correlation between spontaneous cytokine production and alanine aminotransferase (ALT) levels, the clinical marker of liver damage, confirming the differences between men and women were not due to liver damage at the time of organ transplant (**Supplementary Figure 2**). The most significant differences in cytokine/chemokine production were consistently observed after stimulation of TLR8, followed by TLR5, TLR2, and TLR4.

To determine if this differential response between male and female monocytes was restricted to the liver, we isolated monocytes from the peripheral blood of men and women and compared cytokine and chemokine production. In contrast to the intrasinusoidal data, significant difference between blood monocytes from men and women were much more restricted,

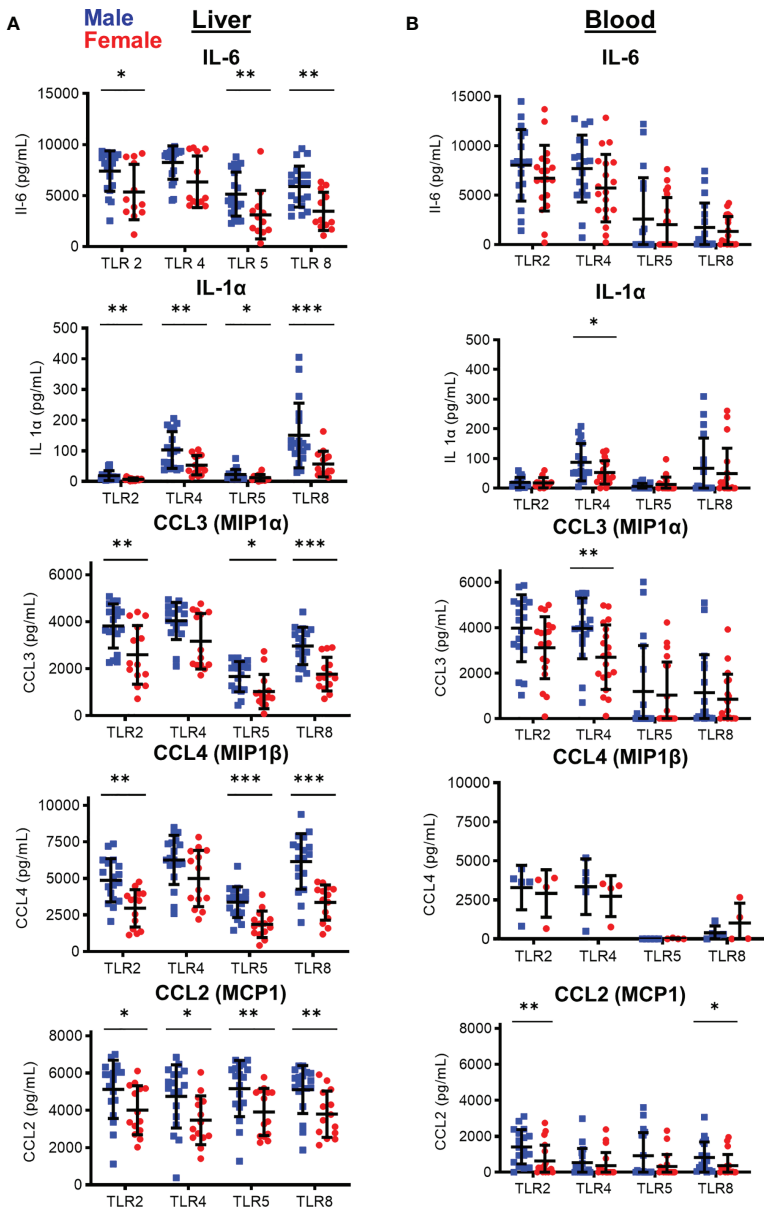

FIGURE 3 | Sex-based differences in cytokine and chemokine production by monocytes following *in vitro* TLR stimulation of enriched CD14$^+$ monocytes after 24 hours. Cytokine/chemokine production by **(A)** Liver and **(B)** peripheral blood isolated CD14+ monocytes after stimulation with TLR agonists. (IHMC males, n=18; females=14; PBMC: males, n=18; females, n=19; For CCL4 PBMC males, N=5; females=4) *p < 0.05, **p < 0.01, ***p < 0.001, Mann-Whitney U tests. Figures depict mean +/- SD.

with only IL-1α and CCL3 showing significant difference after TLR4 stimulation, and CCL2 after TLR2 and TLR8 stimulation (**Figure 3B**). All other responses did not show a significant difference between men and women in blood monocytes (**Figure 3B** and **Supplementary Figure 1**). These data show that, while statistically significant functional differences are detectable in the blood, functional differences between male and female monocytes are magnified in the liver and male liver monocytes respond to inflammatory stimuli with enhanced cytokine and chemokine production.

No Phenotypic Difference in Intrasinusoidal Monocytes Between Men and Women

With no difference observed in monocyte frequency, but a significant difference in function, we sought to determine if the liver environment altered monocyte phenotype between men and women, potentially enhancing their response to activating signals. Therefore, we performed deeper phenotypic analysis on monocytes, measuring the expression of scavenger receptors (CD68, CD163), complement receptors (CD35, CD88), Fc

receptors (CD16, CD32, CD64) and integrins (CD11c, CD11b) on CD14+ MN and CD14/CD16+ MN (**Figure 4A**). We did not observe any significant phenotypic differences between monocytes from male and female livers (**Figure 4B**). This data indicates that neither the composition nor phenotype of monocytes differs significantly by sex.

Regulation of TLR-Induced Cytokine Production

With no difference in frequency or phenotype of male liver monocytes to explain heightened inflammatory responses, we next investigated whether sex hormones, genetic expression, or the TLR signaling cascade showed differences between men and women. We focused our mechanistic analysis on TLR8 and TLR5 as these TLRs displayed the most significant functional differences between men and women. In addition, TLR8 is located on the X chromosome while TLR5 is not, allowing us to compare whether genetic expression plays a role. We first tested the effect of sex hormone exposure on TLR8-induced IL-6 and CCL4 production. Pre-incubation with either estrogen or testosterone had no impact on the frequency, or staining intensity, of IL-6 or CCL4 in CD14+ MN compared to untreated cells (**Figure 5A**). We then investigated whether TLR5 or TLR8 expression differed, at both the mRNA or protein level. We found no difference between male and female liver monocytes in terms of TLR5 and TLR8 mRNA expression, the percent of liver monocytes expressing the TLRs or the level of TLR expression based on mean fluorescence intensity (**Figure 5B**). Therefore, neither sex hormone exposure nor TLR expression could explain differences in cytokine/chemokine production between male and female liver monocytes.

Because we observed reduced cytokine and chemokine production in *ex vivo* stimulated monocytes from female livers, we chose to investigate negative regulators of TLR signaling. Specifically, we investigated the expression of both IRAK-M and TOLLIP. The expression of IRAK-M did not differ by sex. However, we observed that female liver monocytes expressed more TOLLIP when compared to male liver monocytes. When we investigated the expression of TOLLIP in blood monocytes,

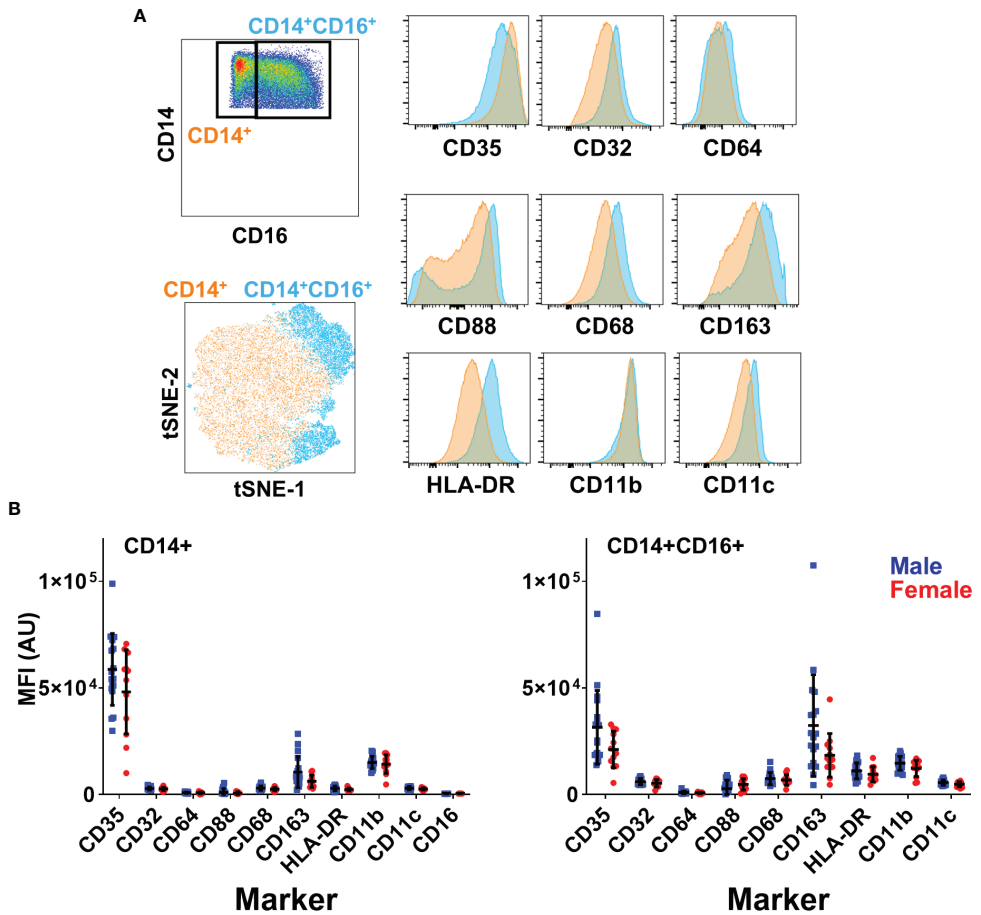

FIGURE 4 | No differences in monocyte phenotype based on sex. **(A)** Surface phenotype of liver CD14+ and CD14+CD16+ monocytes. T-SNE dimensionality reduction was performed on monocytes concatenated from all liver samples (5000 cells per sample). **(B)** Expression of surface markers in monocyte subsets by sex n=18 males, 12 females. Figures depict mean +/- SD.

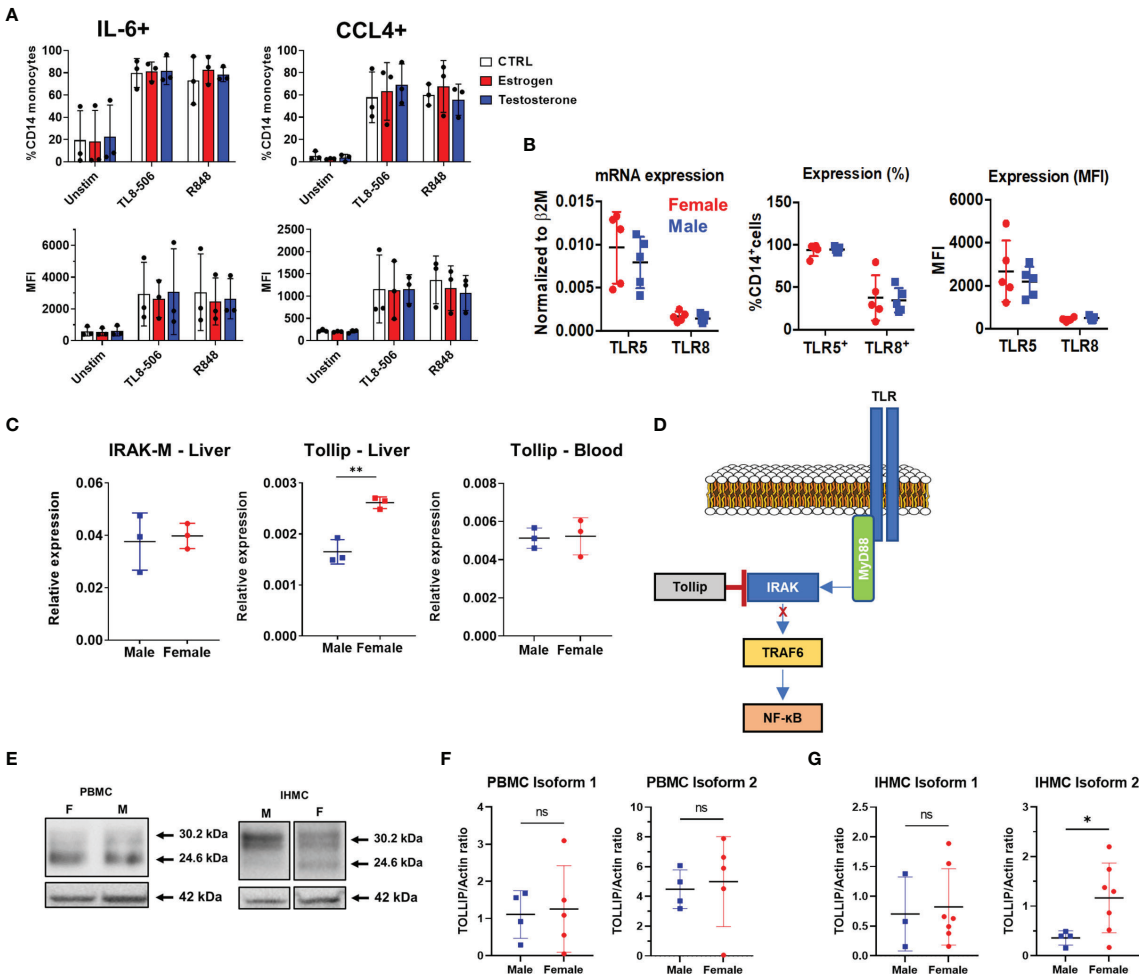

FIGURE 5 | Investigation of TLR expression and signaling. **(A)** Total HD PBMCs from exposed to estrogen or testosterone for 18 hours were stimulated with TLR8 agonists in the presence of Brefeldin A and IL-6 and CCL-4 production in CD14+ MN were measured by flow cytometry. Data displayed as percent of monocyte positive for each cytokine(top) or MFI (bottom) n=3 PBMC donors. **(B)** TLR expression in male and female liver monocytes. mRNA measured by qPCR (left) and protein expression measured by flow cytometry (right) n=5 males, 5 females. **(C)** Analysis of negative regulators of TLR signaling. TOLLIP expression was measured in both the liver (left) and blood (right). PBMC n=5 male, 5 female; IHMC n=3 male, 3 female. **(D)** Representation of TOLLIP's intersection of the TLR signaling cascade to demonstrate the potential to influence all TLRs. **(E)** Example western blot of TOLLIP protein (top) and Actin (bottom) in purified CD14+ monocytes. **(F)** TOLLIP isoform expression in blood monocytes does not show a significant relationship between sex and the expression of TOLLIP. **(G)** TOLLIP isoform 2 expression is elevated in female liver monocytes. Densitometry data was subject to outlier analysis with Grubb's method. PBMC n=5 male, 5 female; IHMC n=4 males, 8 female, *p<0.05, **p<0.01, ns, non-significant, Mann-Whitney U tests. Figures depict mean +/- SD.

we did not find that sex influenced TOLLIP expression (**Figure 5C**). We also investigated expression of key molecules in the TLR signaling cascade and terminal transcription factors for TLRs signaling, including MyD88, IRAK-4, IRAK-1, TRAF-6, RelA (NF-κB), and IRF-7 (**Supplementary Figures 3A, B**), but found no statistically significant differences between male and female liver monocytes. These data suggest liver-restricted regulation of TOLLIP, with increased expression of the negative regulator of TLR signaling in female liver monocytes.

TOLLIP regulates IRAK phosphorylation and is known to suppress TLR-mediated inflammatory cytokine production in human monocytes [**Figure 5D** (23)]. To determine if differences in mRNA expression translated to differences in TOLLIP protein expression, we used Western blot to confirm TOLLIP protein expression in liver monocytes from donors who underwent living donor liver transplants. Western blot data identified two known isoforms (isoform 1 and isoform 2; 30.2 and 24.6 kDa, respectively) of TOLLIP protein in both male and female monocytes (**Figure 5E** and **Supplementary Figures 4A, B**). Comparison of the expression of TOLLIP isoform 1 and 2 in monocytes from 10 PBMC donors revealed no statistically significant difference in expression between males and females (**Figure 5F**). Quantification of TOLLIP expression in liver monocytes revealed that while TOLLIP isoform 1 expression was similar between males and females, we found that the expression of TOLLIP isoform 2 was statistically significantly

elevated in female liver monocytes (**Figure 5G**). These data suggest that the MyD88 signaling cascade may be differentially regulated between the blood and liver and between men and women within the liver.

DISCUSSION

It is generally accepted that females display stronger cell mediated immune responses than males. Females have increased CD4 T cells (24), show stronger responses to vaccination (25–27) and have higher incidence of autoimmune disorders mediated by cells of the adaptive immune system (28). However, the inflammatory potential of innate immunity between men and women is less clearly defined and may be restricted to specific cell types or specific receptors. An extensive study performed by Human Functional Genomics Project (HGFP) found that monocyte-derived cytokines were higher in men vs. women (29). This study stimulated whole blood and PBMC from 500 healthy volunteers and found higher IL-6, IL-1β and TNF-α in males. The study was of low resolution in identifying relevant cell populations as they used whole blood and PBMC, instead of purified monocytes. Further, this study required a large patient cohort to demonstrate differences but are consistent with our data on purified monocytes. Similarly, a recent study also supported the observation of enhanced inflammatory potential of peripheral blood male monocytes, but analysis was focused on TNF-α, CXCL1, and CCL2 (30). Our data indicates that differences in cytokine production between male and female monocytes was magnified in the liver, suggesting regulation, at least in part, by a tissue-specific effect.

We first validated the clinical observations that men have worsened outcomes in hepatitis through a clinical cohort of patients with chronic HBV infection going through treatment withdrawal. We found that sCD163 was the most statistically significantly elevated marker in patients with HBV reactivation. Importantly, CD163 is expressed on monocytes and macrophages and is shed from their surface upon TLR activation (31, 32). Further, previous work has correlated sCD163 with HBV-related liver inflammation (33, 34). The increased inflammatory profile in male patient plasma was consistent with subsequent TLR activation on monocytes and macrophages. The myeloid cytokine/chemokine profile we observed in CHB patients matched closely with our *ex vivo* liver monocyte data, with higher IL-6, IL-1α, CCL-4, and TNF-α observed in male donors. Plus, additional chemokines, CXCL-9 and CXCL-10, were higher in men and have been associated with liver inflammation (35, 36). It is possible that the inflammatory cytokine profile we observed in CHB patients was not produced by liver-resident monocytes/macrophages, but this scenario is unlikely. HBV virions and antigens constantly circulate through the peripheral blood at high levels for the lifetime of the patients and do not engage innate inflammatory mechanisms (15, 37). In contrast, HBV reactivation triggers a poorly understood liver-specific danger signal, which induces liver inflammation in a majority of patients stopping antiviral therapy (38, 39). Therefore, we believe the immune profile we detected in the CHB patient cohort is derived from the liver and is indicative of monocyte activation. While we were unable to investigate the relationship between different myeloid populations in the blood and liver in this study cohort, it has been reported that HBV DNA and HBV antigens do not influence the distribution of myeloid population in blood (40, 41). However, investigating dynamic changes in these immune populations during active hepatitis may be key to understanding population and phenotype kinetics in *in vivo* inflammation.

Identification of specific mechanisms regulating sex-biased immune responses have been challenging due to the broad effects of genetics, environment and hormones (42). Sex hormone concentrations fluctuate and regulate the promoters of multiple immune-related genes. The X chromosome harbors numerous immune genes, including TLR7 and TLR8, and microRNAs with reported effects on immune function (43). We focused our mechanistic studies on TLR8 and TLR5, which allowed us to compare hormone and X chromosome dependent effects on protein expression. Our observation, that cytokine and chemokine production were similar in peripheral blood monocytes between men and women argues against direct regulation by sex hormones, as these hormones are systemic. Our *in vitro* mechanistic data involving sex-hormone exposure supported this conclusion, which was also found in the HFGP cohort (29). Similarly, our data did not show differences in mRNA or protein expression between TLR5 and TLR8, arguing against increased expression from the X chromosome.

Finding a significant difference in cytokine production after stimulation with all TLR agonists suggested a common mechanism in the signaling cascade. This would be similar to the regulation of IFN-α production after TLR7 stimulation in female plasmacytoid DC, where the terminal transcription factor, IRF-5 was increased (44). Our interrogation of the signaling cascade determined that the negative regulator of TLR signaling, TOLLIP, was expressed at higher levels in female liver monocytes compared to male monocytes, with no difference in the blood, suggesting liver-specific regulation of the TOLLIP promoter. TOLLIP interacts with the IRAK complex to reduce downstream phosphorylation events, reducing cytokine production in human monocytes (23, 45, 46). Recent studies have demonstrated that transduction of primary human monocytes with short hairpin RNA to knockdown TOLLIP enhanced cytokine production following TLR stimulation (23). Similarly, overexpression of TOLLIP in murine monocytes could dramatically suppress TLR signaling through NF-κB dependent pathways (47). Our Western blot data confirmed TOLLIP protein expression in both blood and liver monocytes from men and women. With our limited sample size for protein analysis, we observed a statistically significant increase in TOLLIP isoform 2 in female liver monocytes. The intersection of TOLLIP with the TLR signaling cascade is consistent with our functional data demonstrating that TLR2, -5, and -8, which signal through MyD88-IRAK, showed the most significant differences. This data is also consistent with the key role of

MyD88-dependent IL-6 production in the development of liver cancer in male mice (48). In contrast, TLR4 signaling can bypass MyD88-IRAK through the TRIF pathway and showed the fewest significant differences between male and female liver monocytes. Identification of key factors that can dampen inflammatory cytokine production in the human liver may yield targets to treat, and slow the progression of, multiple types of inflammatory liver disease.

We recognize there are caveats to our uninfected liver analysis. Liver perfusion samples are collected from deceased donors. All the livers used in our analysis were used for transplantation, verifying the quality of the organs. The cause of death or age at death could skew results. However, cause of death was distributed among all categories for both men and women. Age at death was not significantly different and *ex vivo* cytokine production did not correlate with the donor's APRI score (not shown) or ALT levels. Therefore, we are confident the differences we observed are physiologically accurate and not a consequence of the liver donors. Additionally, the cells available for analysis from the perfusion samples are those that can be flushed from the sinusoid upon portal vein perfusion. These were highly representative of liver derived cells but also do not capture tightly bound macrophages or parenchymal cells. Until the data are obtained, whether macrophages or parenchymal cells respond with heightened inflammatory potential remains an open question.

Our study characterizes the human liver, between sexes, in the absence of underlying liver disease. This data is vitally important to understanding the mechanisms driving liver inflammation that put hundreds of millions of individuals at risk of developing cirrhosis and liver cancer. We believe the sex-bias inflammatory potential is a general phenomenon of the intrasinusoidal environment. Elucidating liver-specific regulation of cytokine production between men and women, will further improve efforts to manage the pathogenesis of liver disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Saint Louis University institutional review board and the University Health Network research ethics board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AK, DM, SN, JAD, AM, AJV acquired and analyzed data; AK, DM, SN, JAD, WC, PH, IM, HLAJ, AG were responsible for study design and manuscript preparation and revision. All authors contributed to the article and approved the submitted version.

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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.2022.818612/full#supplementary-material

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Estrogen Acts Through Estrogen Receptor-β to Promote Mannan-Induced Psoriasis-Like Skin Inflammation

Huimei Wu[1], Longhui Zeng[2], Jiaxin Ou[1], Tingting Wang[1], Yong Chen[3†] and Kutty Selva Nandakumar[1†]*

[1] Southern Medical University - Karolinska Institute United Medical Inflammation Center, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China, [2] Department of Orthopedics, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [3] Department of Rheumatology and Immunology, Shenzhen People's Hospital, The Second Clinical Medical College of Jinan University, The First Affiliated Hospital of Southern University of Science and Technology, Shenzhen, China

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Antonietta Rossi,
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Zhanchuan Ma,
First Affiliated Hospital of Jilin University, China
Jianghong Zhong,
Beihang University, China

***Correspondence:**
Kutty Selva Nandakumar
nandakumar@smu.edu.cn

[†]These authors have contributed equally to this work

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Sex-bias is more obvious in several autoimmune disorders, but not in psoriasis. However, estrogen levels fluctuate during puberty, menstrual cycle, pregnancy, and menopause, which are related to variations in psoriasis symptoms observed in female patients. Estrogen has disease promoting or ameliorating functions based on the type of immune responses and tissues involved. To investigate the effects of estrogen on psoriasis, at first, we developed an innate immunity dependent mannan-induced psoriasis model, which showed a clear female preponderance in disease severity in several mouse strains. Next, we investigated the effects of endogenous and exogenous estrogen using ovariectomy and sham operated mice. 17-β-estradiol (E2) alone promoted the skin inflammation and it also significantly enhanced mannan-induced skin inflammation. We also observed a prominent estrogen receptor-β (ER-β) expression in the skin samples, especially on keratinocytes. Subsequently, we confirmed the effects of E2 on psoriasis using ER-β antagonist (PHTPP) and agonist (DPN). In addition, estrogen was found to affect the expression of certain genes (*vgll3* and *cebpb*), microRNAs (miR146a and miR21), and immune cells (DCs and γδ T cells) as well as chemokines (CCL5 and CXCL10) and cytokines (TNF-α, IL-6, IL-22, IL-23, and IL-17 family), which promoted the skin inflammation. Thus, we demonstrate a pathogenic role for 17-β-estradiol in promoting skin inflammation, which should be considered while designing new treatment strategies for psoriasis patients.

Keywords: psoriasis, estrogen, estrogen receptor β, γδ T cells, dendritic cells, IL-23/IL-17 axis

INTRODUCTION

The immune system in men and women differs significantly, especially after puberty (1) and a conservation in sex bias during expression of genes promotes phenotypic differences (2). Women have two X chromosomes containing many immune response related genes expressing Toll-like receptors, cytokine receptors, and contributing to T and B-cell immunity as well as their

regulation (3). Thus, women are comparatively better than men in having good health, longevity, and a stronger immune response to infections but this immunological advantage might contribute to susceptibility to autoimmune diseases (4). It is well known that the autoimmune diseases have gender bias, with female preponderance. During pregnancy, systemic lupus erythematosus worsens, while multiple sclerosis, rheumatoid arthritis, autoimmune thyroid diseases, and others improve (5) but psoriasis development in pregnant women is controversial. Therefore, how sex hormones affect the immune system and autoimmune disorders needs more experimental investigations.

Psoriasis affects about 25 million people in North America and Europe (6) in which genetic (7) and environmental factors (sex, emotional stress, smoking, etc.) are involved (8). The ratio of psoriasis incidence between women and men was reported to be 1.05 (9). However, the intensity and onset of psoriasis in men and women are different (10). Women undergo sweeping endocrinological changes in their lifetime (puberty, menstrual cycles, pregnancy, and menopause periods) and, show great variations in psoriasis development (11). During pregnancy, 55% of psoriasis patients had improvement, while 23% of them had an aggravated disease (12). Another study reported amelioration of psoriasis during pregnancy, though it relapsed in the puerperium period. Interestingly, psoriasis arthritis became highly severe in these patients after delivery (13). Of note, approximately 30% of Ps patients develop PsA and a strong association of different genes was reported for PsA and Ps (14). Women with irregular menstrual cycles had a higher psoriasis incidence with a ratio of 1.32 when 163,763 people were used in a study protocol (15).

Estrogen is one of the factors contributing to sex differences, which directly affects keratinocyte proliferation, IL-17-producing γδ T cells (16), and infiltration of macrophages (17) and dendritic cells (18). It also increases IL-23/IL-23R signaling and IL-17A production from Th17 cells (19). In psoriasis, estrogen might exert its functions by modulating the expression of certain genes like sex-biased transcription co-factor vestigial-like protein 3 (*Vgll3*) (20) and CCAAT enhancer binding protein beta (*cebpb*) (21), microRNAs (miR146a, 21 and 210) (22, 23), and chemokines (CCL5 and CXCL10) (24, 25). Estrogen mainly binds to nuclear receptors (ER-α and β) to mediate its functions but contribution of each receptor varies depending on the target tissue and disease conditions (26, 27). At the same time, estrogen could have disease promoting or ameliorating functions based on the type of induced immune responses and the target tissues (26, 27). Symptoms of psoriasis and psoriatic arthritis were reported to develop in reactive oxygen species (ROS)-deficient B10Q.Ncf1$^{m1j/m1j}$ mice after an intraperitoneal injection of mannan from *S. cerevisiae* (28). Here, we developed a modified mannan-induced psoriasis inflammation model, dependent mainly on innate immune cells, to study the effects of estrogen on psoriasis. This model showed a clear female preponderance in disease development and can be induced in different common mouse strains by epicutaneous applications of mannan, a natural route of antigen encounter in the skin.

At first, we compared psoriasis development between male and female mice and found an increased disease severity in females. Next, we investigated the role of endogenous and exogenous estrogen, as well as ER-β antagonist and agonist on experimental psoriasis. Interestingly, estrogen alone promoted the skin inflammation and increased mannan-induced psoriasis-like inflammation, which was mainly dependent on the IL-17 family of cytokines and keratinocyte proliferation, possibly by acting on ER-β.

Abbreviations: CFA, complete Freund's adjuvant; DCs, dendritic cells; DPN, diarylpropionitrile; E2, 17-b-estradiol; ER-α, estrogen receptor α; ER-β, estrogen receptor β; IFA, incomplete Freund's adjuvant; IMQ, Imiquimod; KCs, keratinocytes; MISI, mannan-induced skin inflammation; PASI, psoriasis area and severity index; PHTPP, 4-(2-phenyl-5,7-bis[trifluoromethyl]pyrazolo[1,5-a] pyrimidin-3-yl) phenol.

MATERIALS AND METHODS

Mice

Eight to twelve weeks old BALB/c, C57BL/6J, KM, DBA/1, C57BL/6NQ, ICR, and NIH male and female mice maintained in a pathogen-free animal house were purchased from Southern Medical University and Guangdong Medical Animal Experiment Center. All animal experiments were conducted in accordance with the guidelines of the National Institutes of Health (NIH Publication No. 8023) and approved by the ethics committee of Southern Medical University (l2018183). Mice were placed in cages, in a climate-controlled environment having 12-h light/dark cycles. All the procedures were approved and supervised by Southern Medical University Animal Care and Use Committee, Guangzhou, China.

Mannan-Induced Psoriasiform Inflammation

The area of 2 cm x 3.5 cm was shaved at the back of mice after injection of pentobarbital sodium (80 mg/kg, Hechun Guangzhou, China), and 5 mg mannan (100 μl/day, Sigma-Aldrich, Missouri) mixed with incomplete Freund's adjuvant (IFA, Sigma-Aldrich) in a ratio of 1:1 was applied on the back skin for 3 consecutive days and scored for 9 days. For the induction of disease relapse, at the end of initial psoriasis development (Day 9), a mixture of mannan and IFA was applied on the skin for another 3 days. Psoriasis area severity index (PASI) scores were given based on redness (0-4), scales (0-4), and thickness (0-4) with a total score of 12 by following the criteria: 0, none; 1, slight; 2, moderate; 3, severe and 4, very obvious signs. Increase in skin thickness was measured using an Ozaki digital caliper (Neill-Lavielle, Kentucky).

Ovariectomy and Estradiol Treatment

Ovariectomy (OVX) or sham operation was performed on 5-6 weeks old BALB/c mice (n = 8-12/group). After 2 weeks, psoriasis-like skin inflammation was induced using mannan and monitored up to 9 days in the endogenous estrogen depletion experiments. For estrogen treatment, after evaluating different routes (**Supplementary Figure S1**) and concentration

(**Supplementary Figure S1**), subcutaneous injection of 7.2 μg 17-β-estradiol (**Supplementary Figure S2**) was selected and used. In these experiments, two weeks after surgery, 17-β-estradiol (7.2 μg/day) or miglyol was given *s.c.* for 6 days and monitored for skin inflammation. Subsequently, mannan was applied from Days 7-9 with E2 or miglyol treatment and the disease development was monitored until Day 16. In ER-β antagonist treated group, PHTPP (1 mg/kg, MCE, New Jersey) was used in OVX mice for 9 days, and corn oil as well as E2 treated OVX mice were used as controls. DPN (12 mg/kg, MCE), an ER-β agonist was used in OVX mice for 9 days and corn oil was used as a control.

Blood Circulation Measurements

BALB/c mice (n = 10/group) were anesthetized by injecting phenobarbital on Day 5 after mannan application and Laser Speckle Contrast Imager (LASCA analyzer, Zanda, Shanghai, China) was used to scan the back of the mouse with infrared light for 60 s. Pictures were recorded using the Laser Speckle Contrast Analysis. A specific time (25 s-35 s) was chosen for collecting blood circulation data from the skin.

Histology and Immunohistochemistry

A group of mice was sacrificed on Day 6 and their skin samples were collected, fixed in 4% paraformaldehyde, embedded in paraffin, and stored at RT until used. Paraffin sections (8 μm) were cut using a paraffin slicer (Leica, Solmas, Germany) and stained with H&E (Beyotimes, Shanghai, China). Images of H&E staining were acquired using an eclipse upright optical microscope digital camera (Nikon, Tokyo, Japan). Two random positions from each mouse were selected to measure the epidermal thickness by Image J software (version 1.8.0, Maryland). Baker's scores (29, 29) were used to analyze the pathological severity of the skin. CD11c positive cells were stained with biotin-rat anti-mouse CD11c antibody (1:100, Biolegend, California) for 1 h, followed by streptavidin-HRP antibody (1:800, Yeasen, Shanghai, China) incubation for 40 min. Sections were developed with DAB (Vector Laboratories, California) and counter-stained with hematoxylin (Phygene, Shanghai, China) before visualization under the microscope (Nikon). CD11c positive cells (30) were counted in 5 microscopic fields under 200X magnification and expressed as cells/field, and mean ± SEM.

Immunofluorescence

For immunofluorescence staining, frozen skin sections were dehydrated, embedded, stained, and imaged using a confocal microscopy (A1HD25, Nikon). To evaluate the expression and location of ER-β, mouse skin tissues were incubated with the specific primary antibodies for rabbit anti-mouse ER-β (1:50, Abcam, Cambridge, UK), Alexa Fluor® 488 conjugated Pan-Keratin (1:100, Abcam) and CD11c (1:100, BioLegend) overnight. Alexa Fluor® 555 conjugated anti-rabbit IgG (1:800, Biyotimes) and Alexa Fluor® 488 conjugated streptavidin (1:800, Biyotimes) were used as secondary reagents. Nuclear DNA was detected by DAPI (5 mg/ml, Biyotimes) for 5 min at room temperature. Confocal images were acquired using a Nikon Laser Confocal Microscope and analyzed using NIS Elements Viewer Imaging Software (Nikon).

Flow Cytometry

Skin samples were separated into dermis and epidermis layers after digestion with dispase II (10 mg/ml, Solarbio, Beijing, China) for 2 h at 37°C. To obtain single cells, shredded dermal tissue was treated with collagenase II (3 mg/ml, Solarbio) and DNase I (5 mg/ml, Solarbio) at 37°C for 120 min. Single cells were also prepared from inguinal lymph nodes and spleen from mice by maceration. Surface staining was performed with fluorescent-labeled antibodies F4/80-PerCP-Cy 5.5, CD11c-PE, CD11b-APC, Ly6C/6G-FITC, CD45-PerCP-Cy 5.5, γδ T-PE (BD Biosciences, New Jersey) for 30 min at room temperature. FACS was performed using LSR II (BD Biosciences) and data were analyzed using Flow Jo version 7.0 (Tree Star, California). Gating strategy for different cell populations was given in **Supplementary Figure S3**.

RNA Isolation and RT-PCR

Total RNA was extracted from the skin with Trizol reagent (Invitrogen, California) and dissolved in RNAse-free DEPC water (Phygene, Shanghai, China) before analysis. The mRNA was reverse-transcribed to cDNA with PrimeScript RT reagent Kit (ThermoFisher, Massachusetts). Whereas MiR-146a, miR-21 and miR-210 were specifically reverse transcribed with the Ribobio Bulge-Loop miRNA RT-qPCR kit (Ribobio, Guangzhou, China). Each RT-PCR was performed with SYBR Premix Ex Taq II (Takara biotech, Osaka, Japan) using a LightCycler 96 thermocycler (Roche, Basel, Switzerland). U6 or β-actin was used as a general quantitative control. Transcript levels were calculated relative to controls and the relative fold expression was calculated using the 2– ΔΔCt algorithm. The primers of different genes were given in **Supplementary Table S1**. Primers for miR-146a, miR-210, miR-21, and U6 were purchased from RiboBio.

Statistical Analysis

The data were analyzed with GraphPad Prism 5 and are presented as mean ± SEM. Two-tailed unpaired Student's t test was used for comparison between the two groups. One-way analysis of variance (ANOVA) with Bonferroni or Newman–Keuls correction was used for multiple comparisons. Probability values < 0.05 were considered significant for 95% confidence interval.

RESULTS

Mannan-Induced Psoriasis-Like Skin Inflammation

We established a new psoriasis model in BALB/c mice with mannan from *Saccharomyces cerevisiae* (SC) cell wall. Plaque psoriasis was developed after a mannan mixture was applied epicutaneously for three consecutive days, and the disease symptoms reached the peak at Day 5 (**Figure 1A**). After initial mannan exposure, the peak of psoriasis was detected on Day 5,

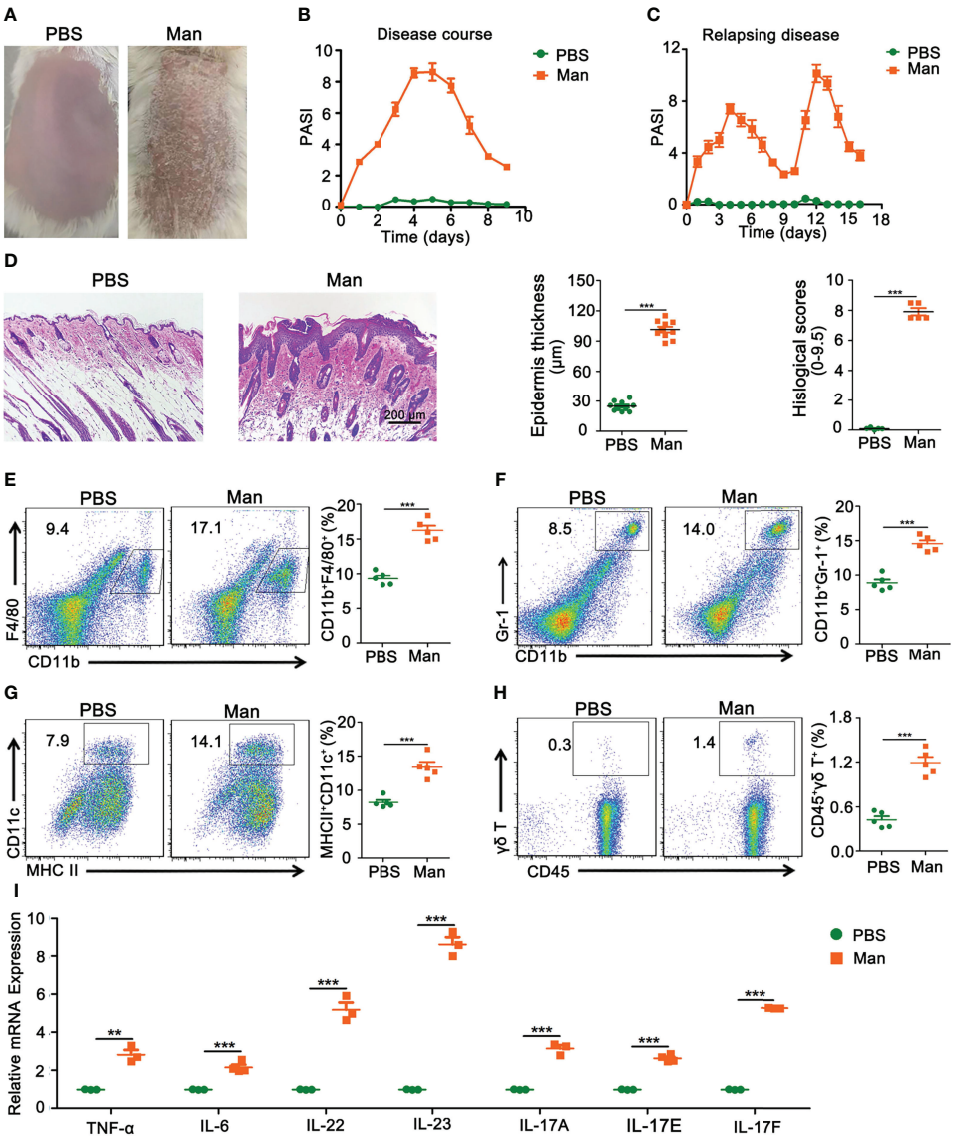

FIGURE 1 | Characterization of mannan-induced skin inflammation. **(A)** Representative pictures of mouse skin after PBS or mannan application on Day 5 (n = 10/group). **(B)** Disease course after mannan or PBS application mixed with IFA in BALB/c mice on three consecutive days starting from Day 0 (n = 10/group); **(C)** relapsing mannan-induced skin inflammation (n = 10/group); **(D)** representative pictures of HE staining of psoriatic skin and statistical results of epidermal thickness with histological scores at peak of psoriasis (Scale bars: 200 μm) (n = 5/group). Representative pictures and statistical results on the expression of **(E)** macrophages ($CD11b^+F4/80^+$), **(F)** neutrophils ($CD11b^+Gr\text{-}1^+$), and **(G)** dendritic cells ($MHCII^+CD11c^+$) expression in spleen in mannan-induced skin inflammation on Day 5 (n = 5/group). **(H)** Representative pictures and percentage of γδT cells ($CD45^+\gamma\delta T^+$) after PBS or mannan application on Day 5 in the draining lymph nodes (n = 5/group). **(I)** Expression of IL-23/IL-17 axis and its upstream cytokines (IL-6, TNF-α) (n = 3/group). Man, mannan. Statistical analyses were performed using an unpaired t test and n indicates number of mice in each group. The data represent mean ± SEM. $^{**}p < 0.01$. $^{***}p < 0.001$.

with a mean maximum psoriasis score of 9 and then the disease subsided (**Figure 1B**). Repeated mannan exposure at Day 9 caused a relapsing disease, with an increased severity (**Figure 1C**). On Day 5, inflamed skin had thickening of the outer layer of the skin (hyperkeratosis) as well as acanthosis with significantly increased histological scores (**Figure 1D**). There were fewer immune cells in the normal skin, while in the inflamed skin, cells were recruited from draining lymph nodes and spleen. A significant increase in the percentage of dendritic cells, neutrophils, and macrophages was found in the spleen after mannan stimulation (**Figures 1E–G**). A significantly higher expression of γδ T cells was found in the draining lymph nodes of inflamed mice (**Figure 1H**). The expression of TNF-α and IL-6 as well as IL-22, IL-23, and IL-17 family of cytokines (IL-17A, IL-17E, and IL-17F) was significantly increased in psoriasis skin lesions (**Figure 1I**).

Females Developed A More Severe Psoriasis-Like Skin Inflammation Than Males

In order to explore estrogen functions on mannan-induced skin inflammation, at first we detected differences in psoriasis scores between female and male mice. All the psoriasis area and severity index (PASI) parameters like redness, scales, and skin thickness were higher in female BALB/c mice (**Figure 2A**) as shown in the clinical pictures (**Figure 2B**) and H&E staining with an increased (twofold) epidermal thickness, infiltration of immune cells, and higher histological scores (**Figure 2C**). Similarly, females developed a more severe psoriasis in most of the tested mouse strains (BALB/c, C57BL/6J, C57BL/6NQ, KM, DBA/1, and ICR) (**Figure 2D**) except in NIH mouse strain. In addition, a higher level of blood flow in the skin (**Figure 2E**) and an increased vascular endothelial growth factor (VEGF) expression were observed in the diseased female mice (**Figure 2F**).

Endogenous and Exogenous Estrogen Promoted Psoriasis

Depletion of endogenous estrogen by ovariectomy (OVX) had significantly decreased the PASI scores compared to sham operated mice (**Figure 3A**). Interestingly, a single injection of 17-β-estradiol alone led to psoriasis-like dermatitis (**Figure 3B**) and these symptoms were enhanced after mannan application (**Figure 3C**). Among the three known receptors of estradiol, a higher level of ER-β expression was found in the skin of female mice, which was also increased by endogenous and exogenous estrogen in the inflamed mice, but 17-β-estradiol alone had no such effect (**Supplementary Figure S2**). Next, we used ER-β antagonist PHTPP and agonist DPN to treat the inflamed mice. Psoriasis scores were decreased by PHTPP treatment (**Figure 3D**) but increased after DPN injection (**Figure 3E**) during the disease course. Histological evaluation of the inflamed skin samples showed endogenous estrogen enhanced epidermal thickness (**Figure 3F**) and an increased level of keratinocyte proliferation in the epidermis after 17-β-estradiol injection, whereas mild keratinization of epidermis was found with the injection of 17-β-estradiol alone (**Figure 3G**). On the other hand, epidermal thickness decreased after PHTPP treatment but increased after DPN injection, which correlated well with the PASI scores (**Figure 3H**). Quantification of stained skin sections confirmed the increase in epidermal thickness

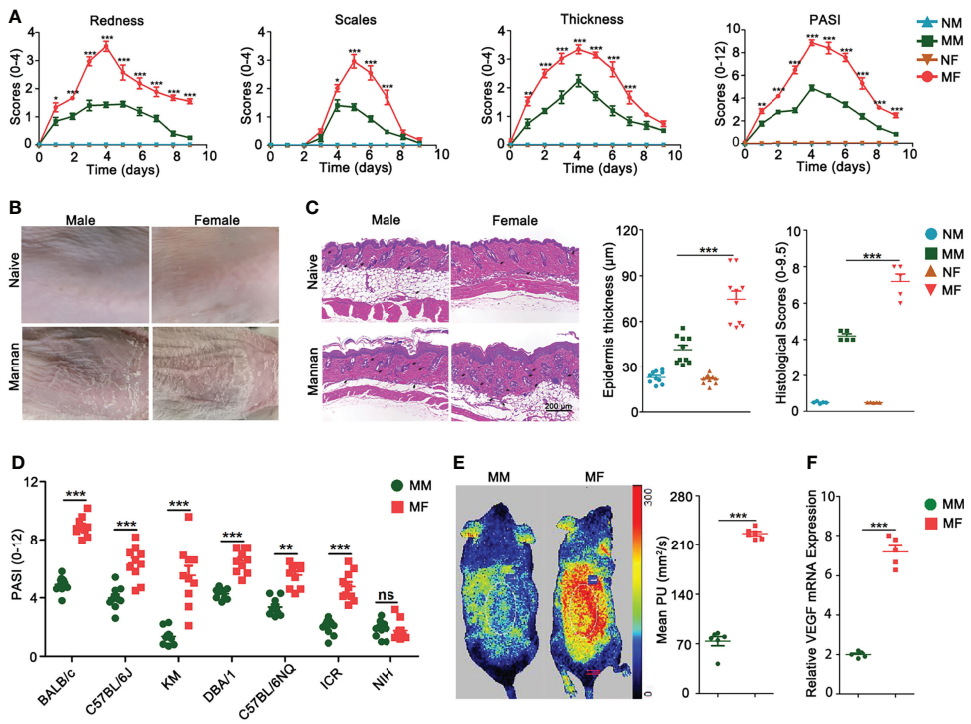

FIGURE 2 | Female mice developed more severe psoriasis. **(A)** Comparison of redness, scales, thickness, and total psoriasis area and severity index (PASI) between BALB/c female and male mice after mannan application (n = 15/group). **(B)** Representative pictures of psoriasis and naive skin from female and male mice on Day 5 (n = 12/group). **(C)** Comparison of H&E-stained skin sections, epidermal thickness, and histological scores between naive and psoriatic BALB/c female and male mice (n = 5/group). Scale bar: 200 μm. Infiltration of immune cells is marked by black arrows. **(D)** Comparison of total PASI at the peak of psoriasis in BALB/c, C57BL/6J, KM, DBA/1, C57BL/6NQ, ICR, and NIH female and male mice on Day 5 (n = 10/group). **(E)** Blood flow (n = 6/group) and **(F)** VEGF gene expression (n = 5/group) in the psoriatic skin of female and male mice on Day 5 after mannan application. Mean level of blood flow as well as VEGF gene expression in the PBS treated female and male mice was taken as one. Each experiment was repeated twice. Statistical analyses were performed using an unpaired t test and n indicates number of mice. The data represent mean ± SEM. NM, naive male; MM, Mannan + Male; NF, Naive Female; MF, Mannan + Female. ns, not significant; *p < 0.05; **p < 0.01. ***p < 0.001.

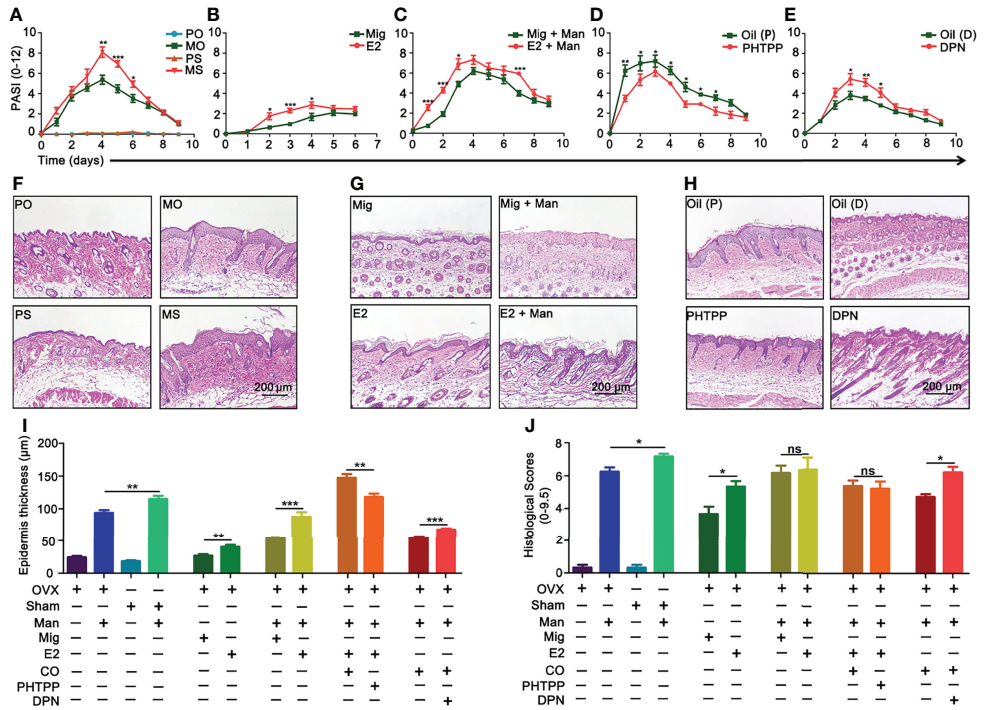

FIGURE 3 | Endogenous and exogenous estrogen promoted psoriasis. Comparison of psoriasis scores (PASI): **(A)** Ovariectomized (OVX) and sham operated mice with or without mannan application (n = 10/group); **(B)** single 17-β-estradiol or miglyol alone treated mice without any mannan application (n = 10/group); **(C)** 17-β-estradiol or miglyol alone injected mice with mannan application (n = 10/group); **(D)** ER-β antagonist (PHTPP) or corn oil alone treated mice (n = 5/group), **(E)** ER-β agonist (DPN) or corn oil alone treated mice (n = 5/group). Representative pictures of H&E-stained skin samples at peak of psoriasis from **(F)** OVX and sham operated mice, **(G)** 17-β-estradiol treated OVX mice, without and with mannan stimulation, and **(H)** after PHTPP and DPN treatment. **(I, J)** Epidermis thickness (n = 5/group) and histological scores of skin sections (n = 3/group) at Day 5 were calculated from Figures **(F–H)**. Scale bar: 200 μm. Each experiment was repeated twice. Statistical analyses were performed using an unpaired t test and n indicates number of mice. The data represent mean ± SEM. Man, mannan; PO, PBS + OVX; MO, Man + OVX; PS, PBS + sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn Oil + Man; PHTPP, E2 + PHTPP + Man; Oil (D), Corn Oil + Man; DPN, DPN + Man. The data represent mean ± SEM. ns, not significant. *p < 0.05; **p < 0.01. ***p < 0.001.

(**Figure 3I**) and histological scores induced by endogenous and exogeneous estrogen, treatment with 17-β-estradiol alone, or DPN (**Figure 3J**).

Increased Dendritic Cell Numbers in the Inflammatory Skin After Estrogen Treatment

Female mice had an increased expression of $CD11c^+$ cells in the dermis (**Figure 4A**). Treatment with 17-β-estradiol and ER-β agonist but not endogenous estrogen had upregulated the percentage of $CD11c^+$ cells, while ER-β antagonist decreased them in the inflamed skin (**Figures 4B–D**). Next, we confirmed our immunohistochemistry observations by immunofluorescence (**Supplemental Figure S4**). An increased number of $MHCII^+CD11c^+$ dendritic cells was found in the spleen from inflamed female mice (**Figure 4E**), with a negligible effect from endogenous estrogen (**Figure 4F**). Interestingly, treatment with17-β-estradiol increased the percentage of dendritic cells in the spleen both in naïve and inflamed mice (**Figure 4G**), while ER-β antagonist treatment significantly decreased their expression (**Figure 4H**).

Estrogen Promoted Mannan Induced Skin Inflammation Was Dependent on ER-β Expression

Binding of estrogens to specific receptors (ER-α, ER-β, and GPR30) activate transcriptional processes and/or signaling cascades in the cells, which results in the regulation of gene expression and cellular functions. Among these receptors, expression of ER-β in the inflamed skin cells was more prominently affected by estrogen (**Supplementary Figure S4**). Next, we investigated ER-β expression in the skin cells by immunofluorescence. ER-β was mainly expressed in the epithelial cells, where an extensive keratinocyte proliferation was observed. Female mice had a higher-level ER-β expression in the keratinocytes under inflammatory conditions (**Figures 5A, E**). Interestingly, endogenous (**Figures 5B, F**) and ER-β antagonist (**Figures 5D, H**) but not treatment with 17-β-estradiol (**Figures 5C, G**) or ER-β agonist (**Figures 5D, H**) enhanced ER-β expression in the keratinocytes present in the inflamed skin. Subsequently, we detected ER-β expression in $CD11c^+$ cells. Contrary to our expectations, more CD11c and ER-β double positive cells were detected in the male than female mice, though females had a significant increase in $CD11c^+$ cells in the

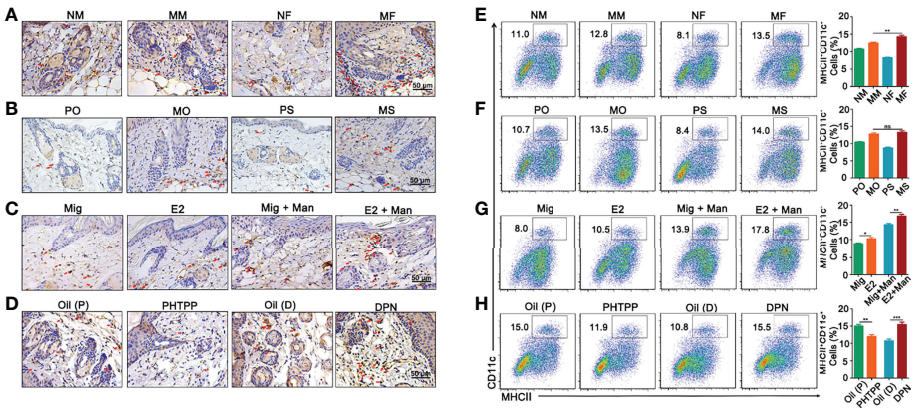

FIGURE 4 | Dendritic cells from dermis and spleen contributed to estrogen promoted psoriasis. **(A)** Immunohistochemistry staining of $CD11c^+$ cells in dermis (arrows mark representative cells) from female and male mice without or with mannan application (n = 5/group). Effect of **(B)** endogenous estrogen, **(C)** 17-β-estradiol, **(D)** PHTPP and DPN on $CD11c^+$ cells in the dermis under normal or inflammatory conditions (n = 5/group). Scale bar: 50 μm. **(E)** Presence of $MHCII^+CD11c^+$ cells in the spleen from female and male mice (n = 5/group). Effect of **(F)** endogenous estrogen, **(G)** 17-β-estradiol, **(H)** ER-β antagonist or agonist on $MHCII^+CD11c^+$ cells (n = 5/group). Each experiment was repeated twice. Man, mannan; NM, Naive Male; MM, Man + Male; NF, Naive Female; MF, Man + Female; PO, PBS + OVX; MO, Man + OVX; PS, PBS + Sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP +Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test and n indicates number of mice used in each group. The data represent mean ± SEM. ns, not significant. *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$.

inflamed skin (**Figures 6A, E**). However, 17-β-estradiol injection (**Figures 6C, G**) but not endogenous estrogen (**Figures 6B, F**) had significantly increased the expression of these double positive cells, while both ER-β agonist and antagonist decreased their expression (**Figures 6D, H**). It is of interest to note that both RNA and protein levels of estrogen receptors are autoregulated (31), which might depend on the level and nature of their ligands present in the tissue, possibly regulated by epigenetic pathways.

Estrogen Enhanced Mannan-Induced Skin Inflammation Was Promoted by γδ T Cells

Females had significantly more γδ T cells than the male mice. Similarly, both 17-β-estradiol and ER-β agonist treated mice after mannan application had increased levels of γδ T cells, while ER-β antagonist treatment decreased their numbers in the dermis (**Figure 7A**). Representative pictures of increased percentage of γδ T cells in the dermis of inflamed mice after 17-β-estradiol treatment and a gating strategy used was shown in **Figure 7B**. In the draining lymph nodes, an increased percentage of γδ T cells was found in the female mice (**Figure 7C**) and in endogenous estrogen-depleted mice (**Figure 7D**). A single injection of 17-β-estradiol alone enhanced the percentage of γδ T cells in the draining lymph nodes, which was further increased by mannan application (**Figure 7E**). On the other hand, ER-β antagonist decreased the percentage of γδ T cells, while the agonist increased their expression in the draining lymph nodes (**Figure 7F**).

Estrogen Promoted Psoriasis by Affecting Certain Genes, MicroRNAs, and Chemokines

Although male mice had a higher level of *vgll3* expression, 17-β-estradiol supplement in OVX female mice after mannan application has significantly increased its expression, which was reduced by PHTPP (**Figure 8A**). On the other hand, a higher expression level of *cebpb* was found in the female mice after mannan application, while both endogenous and exogenous estrogen have significantly increased its expression (**Figure 8B**). Estrogen related microRNAs (miR146a, 21, and 210) promote inflammation in several autoimmune diseases, and here 17-β-estradiol alone or mannan application in female mice has significantly up-regulated the expression of these miRNAs (**Figures 8C–E**). Keratinocytes promote immune cell recruitment to psoriasis skin by secreting the chemokines, CXCL10 and CCL5 (32, 33), and we observed a higher level expression of these chemokine genes in the female mice. Similarly, 17-β-estradiol treatment in the naïve or inflamed mice has significantly up-regulated their expression, while ER-β agonist increased CCL5 but not CXCL10 expression (**Figures 8F, G**).

Estrogen Promoted Psoriasis Inflammation Was Dependent on IL-23/IL-17 Axis

IL-6 secreted by macrophages promotes keratinocyte proliferation, which correlated with the expression of estrogen receptors (34). In this study, female mice had a higher-level expression of IL-6 and both endogenous and exogenous estrogen promoted its expression, which was confirmed by treatment with ER-β agonist or antagonist (**Figure 9A**). On the other hand, TNF-α expression did not show any clear pattern, though we observed its increase after DPN treatment, while treatment with PHTPP decreased its expression (**Figure 9B**). IL-22, produced in response to IL-6 and TNF-α, has a crucial function in the

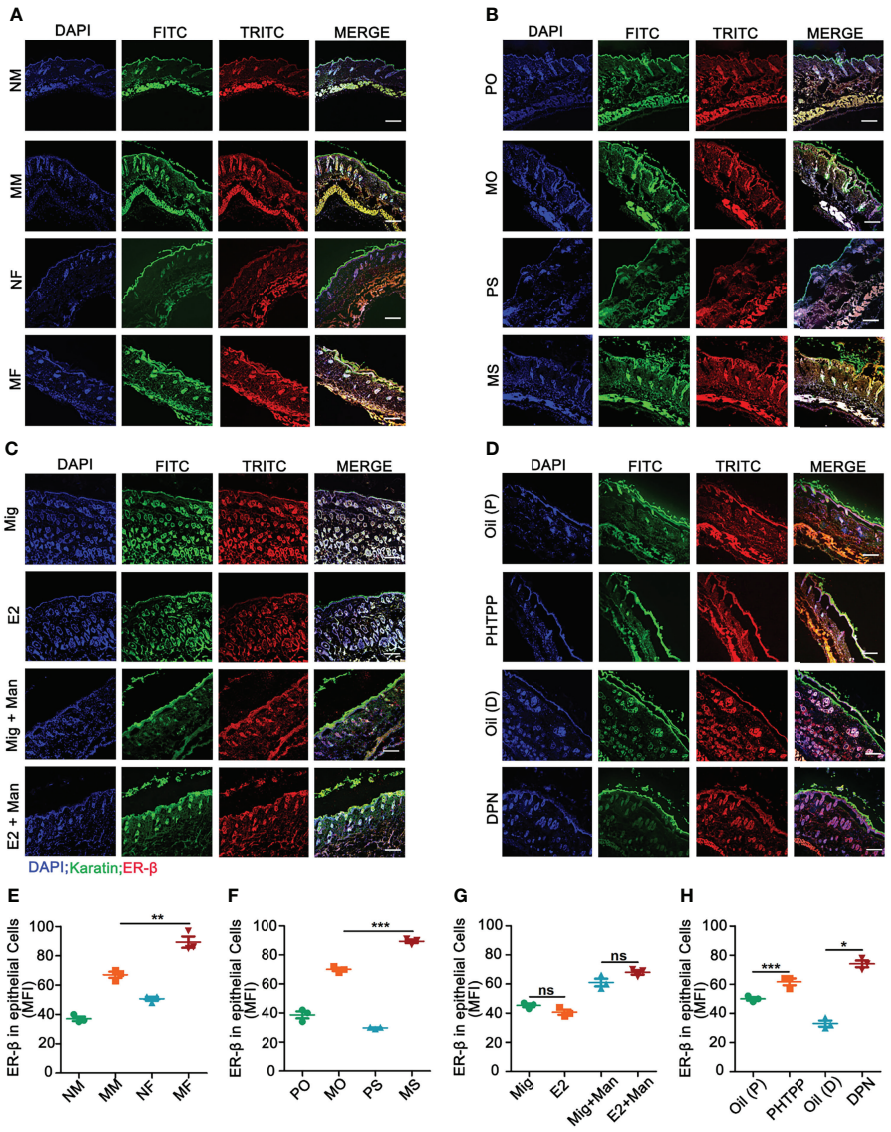

FIGURE 5 | Estrogen increased mannan-induced skin inflammation promoted ER-β expression in keratinocytes. **(A)** Immunofluorescence staining of ER-β (red) and keratinocytes (green) in male and female mice with or without mannan application (n = 3/group); Effect of **(B)** endogenous estrogen, **(C)** 17-β-estradiol, **(D)** ER-β antagonist or agonist on ER-β expression in keratinocytes under normal and inflammatory conditions (n = 3/group). Scale bar: 200 μm. Nuclei were counterstained with DAPI (blue). **(E-H)** For quantification of immunofluorescence staining, mean fluorescence intensity of ER-β stained keratinocytes was calculated using Image J software. Representative pictures are shown. Man, mannan; NM, Naive Male; MM, Man + Male; NF, Naive Female; MF, Man + Female; PO, PBS + OVX; MO, Man + OVX; PS, PBS + Sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP +Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test and n indicates number of mice used in each group. The data represent mean ± SEM. ns, not significant. *p < 0.05; **p < 0.01. ***p < 0.001.

development of dermal inflammation and epidermal acanthosis (35). Here, both endogenous and exogenous estrogen have increased IL-22 expression (**Figure 9C**). In MISI, a significant increase in IL-23 expression was found in the female mice, which was further promoted by both endogenous and exogenous estrogen, and confirmed by treatment with ER-β agonist and antagonist (**Figure 9D**). An earlier report showed that Th17 family of cytokines (IL-17A and IL-17F) secreted by skin contained infiltrating γδ T cells and RORγt$^+$ innate lymphocytes, which promoted the initiation of IMQ-induced psoriasis (36). In mannan-induced skin inflammation, an increased expression of the IL-17 family of cytokines (IL-17A, IL-17E, and IL-17F) was observed in the female mice. Similarly, endogenous and exogenous estrogen have also significantly enhanced their expression, while PHTTP treatment decreased it, though no effects were observed with DPN-treatment (**Figures 9E–G**).

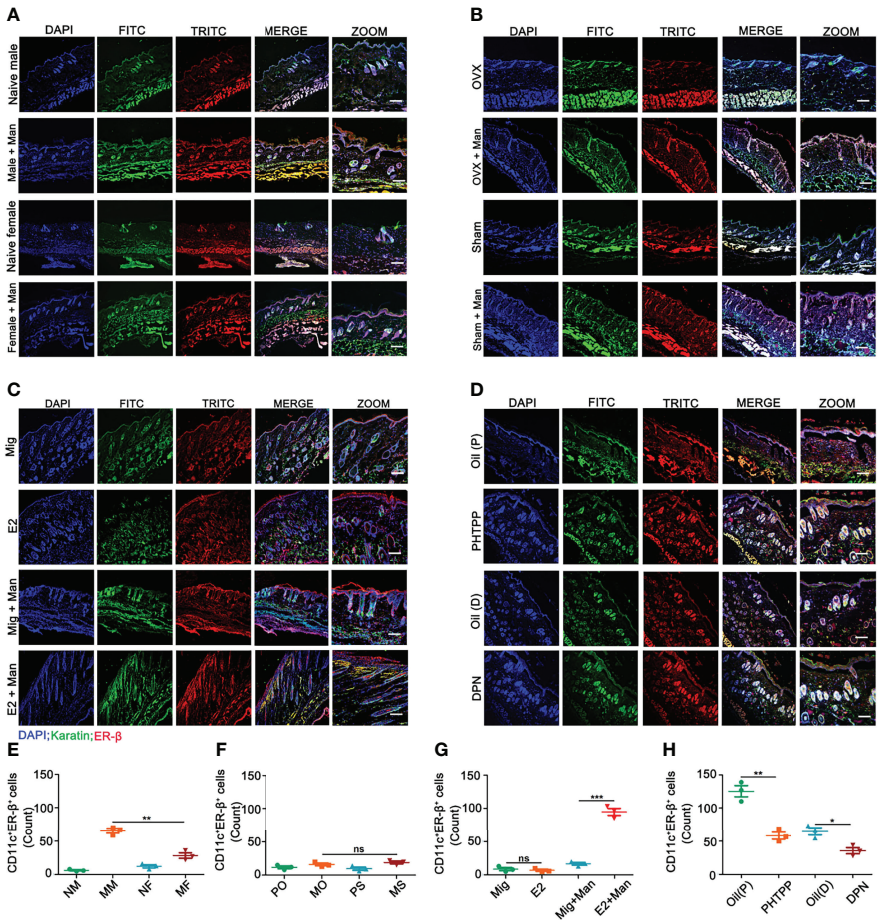

FIGURE 6 | Estrogen increased ER-β expression in CD11c$^+$ cells in the inflamed skin. **(A)** Immunofluorescence staining of ER-β (red) and CD11c (green) double positive cells in male and female mice under normal and inflammatory conditions (n = 3/group); effect of **(B)** endogenous estrogen, **(C)** 17-β-estradiol, **(D)** ER-β antagonist or agonist on the expression of ER-β and CD11c double positive cells (n = 3/group). **(E–H)** Quantification of CD11c$^+$ER-β$^+$ positive cells by immunofluorescence (n = 3/group). Scale bar: 200 μm. Nuclei were counterstained with DAPI (blue). Representative pictures are shown. Statistical analyses were performed using an unpaired t test. Man, Mannan; NM, Naive Male; MM, Man + Male; NF, Naive Female; MF, Man + Female; PO, PBS + OVX; MO, Man + OVX; PS, PBS + Sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP +Man; Oil (D), Corn oil + DPN; DPN, DPN + Man. n, number of mice. The data represent mean ± SEM. ns, not significant. *p < 0.05; **p < 0.01. ***p < 0.001.

DISCUSSION

The role of estrogen in psoriasis is often considered to be controversial and here we report a disease promoting role for estrogen using mannan-induced psoriasiform inflammation model. At first, we established a psoriasis model by epicutaneously applying 5 mg of mannan from *Saccharomyces cerevisiae* in BALB/c mice to explore the effects of estrogen in psoriasis. A significant increase in psoriasis scores, epidermal thickness, and blood circulation in the psoriatic skin of female mice was observed, possibly promoted by various genes and hormones. Both endogenous and exogenous estrogen have increased psoriasis scores, and ER-β expression was more pronounced in the skin, especially in keratinocytes. Next, we used ER-β antagonist and agonist to confirm the results. In addition, we observed significant effects of estrogen on keratinocytes, dendritic cells, and γδ T cells as well as cytokines and chemokines secreted by them under mannan-induced skin inflammation.

Sex bias was reported in many autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, mainly related to X chromosomes and sex hormones, especially estrogen. Although there are no significant differences in psoriasis incidence between men and women, estrogen fluctuations during puberty, menstrual cycle, pregnancy, and menopause could have a profound effect on the severity of psoriasis (37). At the same time, estrogen was shown to affect resident and infiltrating cells present in the skin as well as chemokines/cytokines secreted by them during psoriasis development (38, 39). Therefore, we explored the effects of estrogen on psoriasis.

Mannan-induced skin abnormalities resembled different aspects of human plaque psoriasis with a macroscopic increase

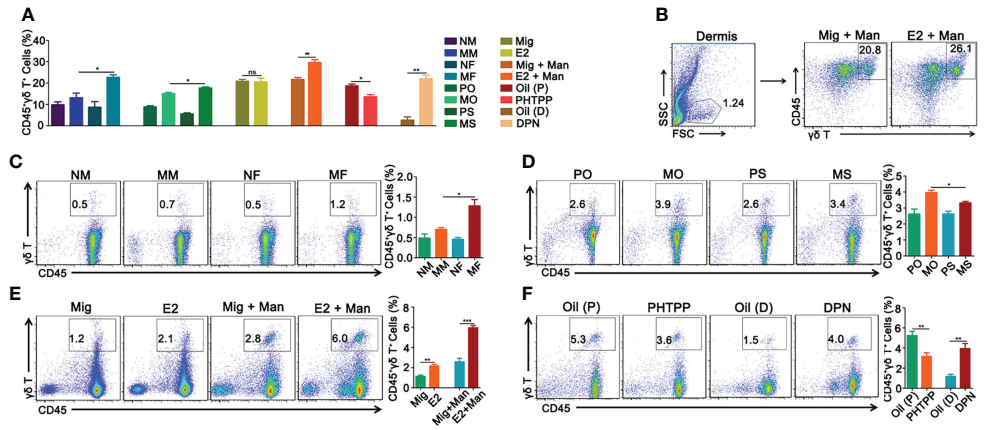

FIGURE 7 | Estrogen enhanced mannan-induced skin inflammation was promoted by γδ T cells. **(A)** Percentage of $CD45^{+}γδ\ T^{+}$ cells in the dermis. **(B)** Representative pictures of $CD45^{+}γδ\ T^{+}$ cell expression after 17-β-estradiol treatment in MISI (n = 5/group). **(C–F)** $γδ\ T^{+}$ cells in the draining lymph nodes from female and male mice, OVX and sham operated mice, 17-β-estradiol, miglyol, PHTPP or DPN treated mice with and without mannan application (n = 5/group). Each experiment was repeated twice. Man, mannan; NM, Naive Male; MM, Man + Male; NF, Naive Female; MF, Man + Female; PO, PBS + OVX; MO, Man + OVX; PS, PBS + Sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP + Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test and n indicates number of mice. The data represent mean ± SEM. ns, not significant. *p < 0.05; **p < 0.01. ***p < 0.001.

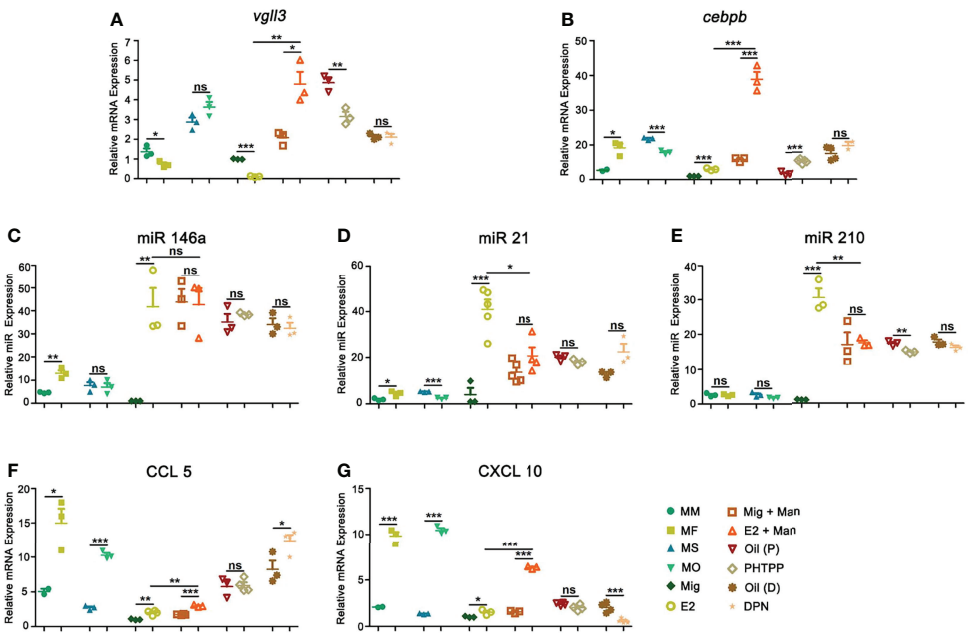

FIGURE 8 | Estrogen increased psoriasis by affecting certain genes, microRNAs, and chemokines. Expression of **(A)** *Vgll3*, **(B)** *cebpb*, **(C)** miR146a, **(D)** miR21, **(E)** miR210, **(F)** CCL5, and **(G)** CXCL10 in male, female, OVX and sham operated, E2 or miglyol, PHTPP or DPN treated mice with and without mannan application (n = 5/group) in the skin. Each experiment was repeated twice. Man, mannan; MM, Man + Male; MF, Man + Female; MO, Man + OVX; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP + Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test. n, number of mice. The data represent mean ± SEM. ns, not significant. *p < 0.05; **p < 0.01. ***p < 0.001.

in redness and scales. Hyperkeratosis, acanthosis, innate inflammatory cell infiltration (dendritic cells, macrophages, neutrophils, and γδ T cells) and an increased expression of IL-23/IL-17 family of cytokines were also observed in this model. Interestingly, sex preponderance was more obvious. Females developed a more severe psoriasis in most of the tested mouse strains after mannan application. Similarly, after mannan stimulation, an increase in epidermal thickness, infiltration of

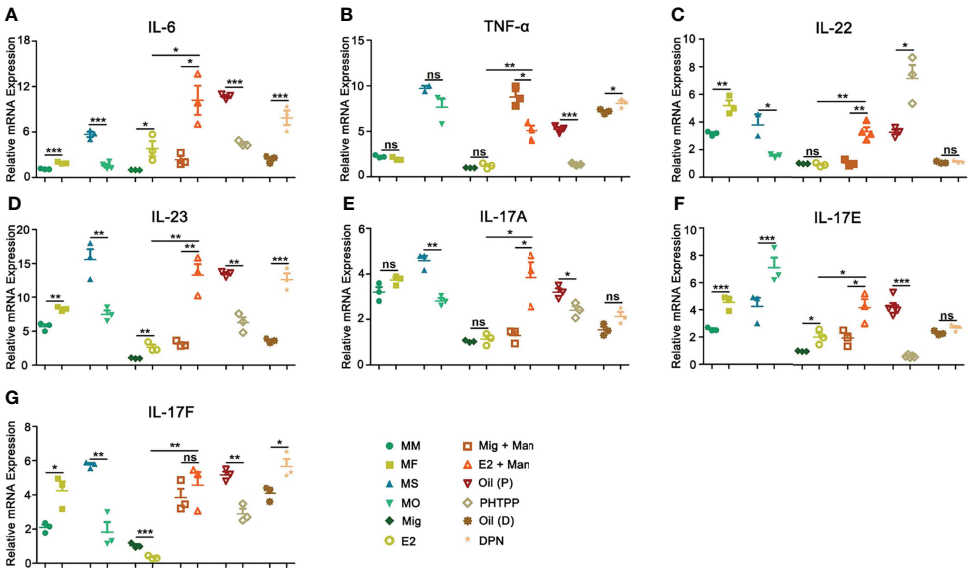

FIGURE 9 | Estrogen promoted psoriasis symptoms were dependent on IL23/IL-17 axis. Expression of **(A)** IL-6, **(B)** TNF-α, **(C)** IL-22, **(D)** IL-23, **(E)** IL-17A, **(F)** IL-17E, and **(G)** IL-17F cytokines in male, female, OVX and sham operated, E2 or miglyol, PHTPP or DPN treated mice with and without mannan stimulation (n = 5/group). Man, mannan; MM, Man + Male; MF, Man + Female; MO, Man + OVX; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP +Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test and n indicates number of mice. The data represent mean ± SEM. ns, not significant. *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$.

immune cells, blood circulation, and the expression ER-β and VEGF were more prominent in female than male mice. Earlier, topical application of estrogen was shown to increase keratinocyte proliferation and epidermal thickness in the aged human skin (39), and promote endometrial angiogenesis by increasing VEGF expression in non-human primates (40), which could possibly suggest a disease promoting role for estrogen in females.

Next, we explored endogenous and exogenous estrogen effects on psoriasis. Both endogenous and exogenous estrogen have enhanced psoriasis scores after mannan application by increasing the thickness of the epithelial layer and infiltration of immune cells. Interestingly, a single subcutaneous injection of 17-β-estradiol alone induced psoriasis-like lesions with an increased expression of γδ T cells and miRNAs (miR146a, miR21, and miR210). Interestingly, promoter analysis of genes expressed in the skin of psoriatic patients showed an enrichment of the ER-β gene (41). However, ER-α activation has enhanced IL-23 secretion by DCs in IMQ-induced psoriasis (42), which contributed to disease severity. Here we showed a significant increase in ER-β but not ER-α/GPR30 in the skin samples, thereafter, we further confirmed the role of ER-β using its specific antagonist (PHTPP) and agonist (DPN). Treatment with PHTPP decreased PASI and epidermal thickness but without having any profound effect on histological scores. Whereas DPN increased psoriasis scores which was further confirmed by an increase in the epidermal thickness and histological scores.

In women with low estrogen levels, skin thickness was reduced approximately 1% per year after menopause (43). On the other hand, a topical estrogen administration in elderly males and females has significantly increased keratinocyte proliferation and epidermal thickness after 2 weeks (44). In addition, 17β-estradiol was earlier shown to promote keratinocyte proliferation by enhancing the expression of activated form of transcription factors like p-Akt and p-Erk (45). In this study, sex difference, endogenous and exogenous estrogen levels, ER-β antagonist as well as agonist treatment have significantly affected keratinocyte proliferation in the epithelial cells of psoriasis skin. Importantly, estrogen promoted ER-β expression most prominently in the keratinocytes under inflammatory conditions contributing to skin inflammation. These results are in agreement with an earlier study, in which estrogen was shown to promote keratinocyte proliferation *via* ER-β in a cutaneous wound healing mouse model (46).

Estrogen has significantly increased the expression of *Vgll3* and *cebpb* genes, which were earlier shown to regulate keratinocyte secreted IL-17 family of cytokines (20), as well as other chemokines and cytokines (47, 48) suggesting a possible involvement of these genes in estrogen promoted psoriasis. Several miRNAs (miR146a, miR21, and miR210) regulated by estrogen contribute to psoriasis development by regulating cytokine synthesis from keratinocytes and controlling their proliferation (49, 50). In this study, all these three miRNAs were significantly increased after a single injection of 17-β-estradiol suggesting their direct involvement in estrogen-induced proliferation of keratinocytes. On the other hand, 17-β-estradiol can control the proinflammatory signals/pathways of the immune system (51). Of note, estradiol and ER activity show

clear dose- and context-dependent effects on immune signaling pathways and cell development (26).

IL-17A secretion from γδ T cells contributing to Ps-like inflammation was reported earlier by an intraperitoneal injection of mannan in ROS deficient BQ.Ncf1(m1j/m1j) mice (28, 52) and the direct relevance of γδ T cells was later demonstrated using γδ T knockout mice crossed to this mouse strain (53). Interestingly, 17-β-estradiol treatment in C57BL/6 OVX mice was shown to modulate γδ T cells, a major source of IL-17 (54) and in the imiquimod-induced psoriasis model, estrogen aggravated the disease by enhancing IL-23 secretion from dendritic cells (42). In this study, estrogen promoted mannan-induced skin inflammation by increasing the number of dendritic cells and γδT cells in the lymph nodes and skin, in addition to enhancing the expression of ER-β in keratinocytes and dendritic cells. Estrogen receptors are found in almost all cells of the immune system (26) and estrogen regulates several genes present in the cells of both innate and adaptive immune system (55). Interestingly, both ER-α and GPR30 are commonly associated with anti-inflammatory phenotypes, while ER-β, was shown to be associated with proinflammatory functions as well as an anti-inflammatory role. The differences in the effect of estrogen on cells could possibly be due to variations in receptor expression in cell types and prevailing different physiological states (56). However, the mechanisms behind sex differences in the expression of ERs in immune cells are not clear. In addition, how various concentrations of estrogens in men and women cause sex differences in ER expression in some cell types, but others are unknown but plausibly can be explained by epigenetic regulatory pathways (26).

Estrogen exhibits pro-inflammatory as well as anti-inflammatory effects through regulation of cytokine and chemokine synthesis, which depend on cell types, estrogen levels, and the inflammatory stimuli (57, 58). For example, chronic stimulation of murine macrophages with estrogen increased the production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) (38) and it can also stimulate the activity of neutrophils (59). Earlier it was shown that estrogen can increase IL-17 production by splenocytes, which was further enhanced with an exposure to IL-23 (60). IL-17 in turn promotes inflammation by recruiting various inflammatory cells like neutrophils, monocytes, and macrophages to the site of inflammation and stimulating the target cells to secrete several inflammatory molecules. In this study, we observed a significant increase in the expression of IL-6, TNF-α, IL-22, IL-23, and IL-17 family of cytokines in the estrogen promoted mannan stimulated skin cells.

In conclusion, estrogen can affect psoriasis during various physiological periods of women, but few reports thus far focused on estrogen effects on psoriasis. Therefore, we established a mouse model in which we found a disease promoting role for estrogen. Both endogenous and exogenous estrogen increased mannan-induced psoriasis-like skin inflammation possibly by acting on ER-β. An increased *cebpb* expression, epidermal thickness, infiltration of dendritic cells, and γδ T cells as well as the expression of pro-inflammatory cytokines (IL-6, IL-23, and IL-17) and chemokines (CCL5 and CXCL10) could have contributed to this increase in psoriasis severity. These findings highlight the possible underlying mechanisms involved in estrogen promoted psoriasis in female mice. However, more studies are needed to address estrogen promoted cellular interactions and signaling pathways in the inflamed skin using individual cell populations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee, Southern Medical University, Guangzhou, China (Approval no. l2018183).

AUTHOR CONTRIBUTIONS

HW did most of the experiments, analyzed data, prepared figures, and manuscript draft with contributions from KSN, who conceived the idea, designed experiments, supervised, interpreted the data, and modified the manuscript. LZ was involved in FACS experiments, and discussion for preparing figures and manuscript. JO contributed to FACS experiments. TW helped with the preparation of figures and analyzing results. YC modified the manuscript and helped with reagents. All the authors approved the content of this 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.2022.818173/full#supplementary-material

Supplementary Figure 1 | Estradiol *via* subcutaneous route induced more severe disease. **(A)** Single subcutaneous injection of 17-β-estradiol induced more severe psoriasis-like inflammation than oral, intraperitoneal, and topical treatment

(n = 5/group), **(B)** 17-β-estradiol promoted more severe mannan-induced skin inflammation through subcutaneous route. Mig, miglyol; E2, 17-β-estradiol; man, mannan (n = 5/group). Each experiment was repeated twice. Statistical analyses were performed using an unpaired t test. n indicates number of mice. The data represent mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$. $^{***}p < 0.001$.

Supplementary Figure 2 | Effect of estradiol concentration on psoriasis. PASI scores from ovariectomized BALB/c mice treated with **(A)** 1.0 μg, **(B)** 3.6 μg, or **(C)** 7.2 μg of 17-β-estradiol (n = 10/group). Each experiment was repeated twice. Statistical analyses were performed using an unpaired t test. n indicates number of mice. The data represent mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$. $^{***}p < 0.001$.

Supplementary Figure 3 | Gating strategy for $\gamma\delta^{+}$T cells and innate immune cells. **(A)** Gating strategy for $CD45^{+}\gamma\delta^{+}$T from draining lymph nodes. Gating strategy for innate immune cells including **(B)** neutrophils ($CD11b^{+}Ly6C/6G^{+}$), **(C)** dendritic cells ($MHCII^{+}CD11c^{+}$), and **(D)** macrophages ($CD11b^{+}F4/80^{+}$) from the spleen. All the immune cells were stained and detected individually.

Supplementary Figure 4 | Expression of estrogen receptors in the skin. Expression of **(A)** ER-α, **(B)** ER-β, and **(C)** G-protein coupled receptor 30 (GPR30) in naive, male, female, OVX, and sham operated as well as 17-β-estradiol treated mice with or without mannan application (n = 5/group). Man, mannan; MM, Man + Male; MF, Man + Female; MO, Man + OVX; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol. Statistical analyses were performed using an unpaired t test. n indicates number of mice. The data represent mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$. $^{***}p < 0.001$.

Supplementary Figure 5 | Immunofluorescence staining of $CD11c^{+}$ dendritic cells in the skin. **(A)** Immunofluorescence staining of $CD11c^{+}$ cells (green) in female and male mice in MISI (n = 3/group). Effect of **(B)** endogenous estrogen, **(C)** 17-β-estradiol, and **(D)** PHTPP or DPN on $CD11c^{+}$ dendritic cells (n = 3/group). Scale bar: 200 μm. Nuclei were counterstained with DAPI (blue). For quantification of immunofluorescence staining, mean fluorescence intensity (MFI) of CD11c in the skin was calculated using Image J software in the above groups and the number of CD11c+ cells were counted manually. Man, mannan; NM, Naive Male; MM, Man + Male; NF, Naive Female; MF, Man + Female; PO, PBS + OVX; MO, Man + OVX; PS, PBS + Sham; MS, Man + Sham; Mig, miglyol; E2, 17-β-estradiol; Oil (P), E2 + Corn oil + Man; PHTPP, E2 + PHTPP +Man; Oil (D), Corn oil + Man; DPN, DPN + Man. Statistical analyses were performed using an unpaired t test and n indicates the number of mice used in each group. The data represent mean ± SEM. ns, not significant. $^{*}p < 0.05$; $^{**}p < 0.01$. $^{***}p < 0.001$.

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